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# Transport of CaM Kinase Along Processes Elicited by Neuronal Contact Evokes an Inhibition of Arborization and Outgrowth in *D. melanogaster* Cultured Neurons

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***Abstract*** *Transgenic Drosophila* strains expressing an inhibitory peptide of $Ca^{2+}$/calmodulin dependent protein kinase II (CaM Kinase), or a constitutively activated CaM kinase, show altered neuronal process morphology compared to wild type in scanning electron microscopy (SEM) of cultured mature neurons from embryonic neuroblasts. We observed significantly enhanced process growth in cells with inhibited enzyme, and reduced process growth in cells with activated enzyme, suggesting that active CaM kinase is involved in the inhibition of neurite growth during development. The subcellular distribution of CaM kinase in wild type neuronal cultures was determined using a gold particle labeling procedure which allowed the mapping of the enzyme directly in the scanning electron microscope (SEM). Before neuronal contact there was little labeling of processes, but after connections had been made the processes were heavily labeled. Our results suggest that the major transport of CaM kinase to the terminals does not occur until after or during the formation of neuronal connections when a functional synapse might be formed. Taken together, these results suggest a target-dependent transport of the enzyme along processes and an inhibitory role for CaM kinase on neurite branching. 

**Key words:** *D. melanogaster,* neuronal contact, CaM kinase

The role of CaM kinase in the functionality of synapses, as well as the enzyme's biochemical characteristics (including its regulation by calcium/calmodulin and its autophosphorylation) are well documented [Hanson and Schulman, 1992; Colbran and Solderling, 1990]. Briefly, CaM kinase is a major protein component of the synapse which regulates many functions in the nerve terminal, including neurotransmitter release. The enzyme has also been shown to be involved in *D. melanogaster* behavioral plasticity in an associative conditioning test [Griffith et al., 1993], the physiological plasticity of *Drosophila* larval neuromuscular junction synapses [Wang et al., 1994] and associative spatial learning in the mouse [Silva et al., 1992].

In order to test the idea that molecules that are present in, and required for the functioning of, the mature synapse (neurosecretory granules, CaM kinase, receptors etc.), migrate to the terminus in response to a contact signal from the target cell, and also to gain insight into the events that occur prior to synaptogenesis (between the initial contact of the process with the target and the development of the fully functional synapse) we carried out a determination of the subcellular distribution of CaM kinase.

In an effort to understand a possible role of CaM kinase during neurite elongation and targeting, we used transgenic animals which express a CaM kinase inhibitory peptide based on the modified autophosphorylation site sequence under the control of the heat shock promoter, hsp70 [Griffith et al., 1993]. Two lines, *ala1* and *ala2,* were tested to distinguish any artefactual effects due to the site of insertion. To test the opposite effect of activating the enzyme, we again used two transgenic lines, *RQED1* and *RQED8,* bearing the coding sequence for the rat α CaM kinase that has been constitutively activated by an amino-acid substitution T (Threonine) to D (Aspartate) in position 286 [Waldman et al., 1990]. These transgenes are also under the control of heat shock promoter hsp70. This substituted amino acid is the auto-phosphorylation

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site involved in the activation of the enzyme [Fong et al., 1989]. The carboxyl group of the aspartic acid is thought to mimic the spatial and charge characteristics of the threonine-phosphate and causes the kinase activity to be partially independent of $Ca^{++}$. We designed these experiments using transgenically expressed polypeptides in order to avoid using chemical inhibitors which have the potential for non-specific interactions. For example, the CaM Kinase inhibitor KN 62 interacts with the calmodulin binding site [Tokumitsu et al., 1990] and, therefore, presents an untested risk of toxicity or interference with other functions of calmodulin. Other inhibitors of CaM Kinase, including K-252a and H7 are non-selective [Hidaka et al., 1984]. With regard to increasing kinase activity, we again decided not to use chemicals such as the calcium ionophore, 4 bromo A23187 [Debono et al., 1981] because modification of the physical properties of membranes could occur during long term exposure. Also, because the prolonged elevation of calcium is toxic, the procedure is risky. The biphasic action of $Ca^{++}$ during growth cone navigation has been described in the snail *Helisoma* [Mills and Kater, 1990; Rehder and Kater, 1992] where a transitory elevation of $[Ca^{++}]_i$ increases growth while a sustained high concentration promotes retraction of processes. Furthermore, it has been suggested that regulation of calcium levels has consequences for the regulation of neuronal architecture and cell viability, and a calcium load following a stimulus can have different effects at different stages of development [Mills and Kater, 1990]. More recently, it has been reported that the neurite growth inhibitor NI-35 mediates the collapse of growth cones by releasing calcium from intracellular stores [Bandlow et al., 1993]. Furthermore, calcineurin, a phosphatase that is dependent on calcium and calmodulin, has been described as being instrumental in the molecular events involved in neurite extension and growth cone motility [Chang et al., 1995]. Finally, a recent report provides evidence that neurite outgrowth is regulated by spontaneous waves of calcium, and that information is encoded in the frequency of these calcium transients [Gu et al., 1995]. When cells are stimulated with steady frequencies of calcium of 8–9 per h in calcium-free medium, which mimicks natural calcium wave frequencies, neurite outgrowth is regulated to that occurring naturally in calcium-containing cultures. It is interesting that decreasing the frequency of these applied calcium stimuli in calcium-free medium increases the lengths of neurites.

We adapted an existing cell culture procedure which uses embryos entering the early gastrula stage when a set of neuroblasts delaminate from the ectoderm layer [Prokop and Technau, 1991]. The technique we used allows for the attachment of only myoblasts and neuroblasts to the surface of plastic Petri dishes, and the growth of neuronal cell clusters containing mature neurons from the original neuroblasts. Besides this growth and differentiation, we are able to evaluate the connections that develop from cluster to cluster. The precursor cells in our system undergo maturation and division, providing an excellent model for studying the process of neuronal differentiation in vitro.

## EXPERIMENTAL PROCEDURES

The methods were those of the preceding study [Broughton et al., 1996] with the following exceptions.

### Neuronal Cell Culture

Eggs were collected after 2 h and were allowed to age undisturbed for 3 h, then heat shocked for 30 min at 37°C, and allowed to recover for 2 more h. Non-heat shock controls used embryos that were aged for 5 h 30 min after collection. Optimal growth of cultures occurred at 24 h, at which time the processes were stable and after which time there was a steady, gradual retraction and/or degradation of processes. Using 100 embryos in this protocol gave an average of 45 ± 16.6 cell clusters and 51 ± 21.1 myotubes per $cm^2$ (averages are ± standard deviation). We presume that after homogenization of embryos, the resulting 'soup' contained already activated neuroblasts and/or all required growth factors, which explains the rapid cell division and maturation in the first 10 h. After approximately 24 h, the necessary growth stimulating molecules were not present or their concentration dropped possibly because they were not generated or were degraded in our in vitro system. Such a retraction of processes became more and more dominating after 24 h, obscuring the phenomenon that we were observing. For these reasons, we chose to compare cultures at a point when we assumed that necessary growth factors were still active.

### Scanning Electron Microscopy and Internal Labeling

For internal immuno-labeling, we designed a procedure to label the internal CaM kinase molecules using the same gold particle-labeled secondary antibody technique as described above. We first bridged the proteins using a low concentration of glutaraldehyde for 30 min, and then we delipidated the fixed cells using a mixture of 1% SDS and 2.5% Triton for 2 days at 25°C, 60% humidity. Cells were washed extensively, and incubated with a rabbit antibody raised against a peptide sequence of *Drosophila* CaM kinase (a generous gift from L. Griffith, Brandeis U., MA). A secondary incubation with the gold labeled antibody was carried out for 4 h, followed by extensive washes in PBS (five times), and finally treatment with 3% glutaraldehyde in PBS overnight at 4°C. At this stage, the specimens were silver enhanced for 30–40 min, then dehydrated and coated with carbon as described above. The particle size depended on the length of time the silver enhancement was carried out. X-ray analysis was used to map the silver particles in the SEM, and to confirm that the dark globules which could be seen randomly on the plastic surface contained no traces of gold or silver. A negative control was performed using an anti-synaptotagmin rabbit antibody which failed to recognize its epitope after the same fixation and delipidation protocol was followed.

**Morphological and statistical analysis in the SEM.** Data for each genotype were tested for normal distribution using a Shapiro-Wilk W test. Statistical analysis using Student's $t$ test ($P = 0.05$) was performed to compare each genotype to the $w^z$ control.

**Fly stocks and maintenance.** Fly stocks were grown on standard cornmeal-molasses-agar medium at 25°C in a humidified incubator. The $w^z$ control is a white eyed Canton-S strain. The RQED site directed mutant plasmid was a generous gift from Dr. H. Schulman, the hsp70 RQED construct in P element vector CasPer-SV40, and the transgenic animals were made and generously given by L. Griffith (Brandies U., MA). For *ala1* and *2,* the vector used, the description of constructs and embryo injections, and the detection of the peptide synthesis in the adult animals and larvae are described in Griffith et al. [1993].

**In vivo expression of inhibitory CaM kinase II peptide and control of activity of partially $Ca^{++}$-independent CaM kinase from the transgenic animals.** Before using *ala* and *RQED* flies we tested CaM kinase activity with and without heat shock. Due to the dilution of the *ala* inhibitory peptide during the extraction process and its Ki of only 5 μM, it is difficult to assess the decrease of CaM kinase activity in *ala* flies. The transgenic flies and the synthesis of the peptide under heat shock control have been described elsewhere [Griffith et al., 1993]. However, we carried out immunoprecipitation using a *Drosophila* CaM kinase antibody (a gift from Dr. Griffith) on head extracts from flies previously fed with 32-P inorganic phosphate in sucrose solution. Flies were kept in sealed vials, into which radioactivity was injected using a syringe through the caps, and flies were killed by freezing at −70°C. After heat shock, one of the two bands corresponding to the two subunits of CaM kinase II (at 60 KDa) presents significantly reduced incorporation of radioactivity and the other slightly decrease of incorporation, suggesting an efficient competition by the inhibitory peptide for the enzyme, and an inhibition of autophosphorylation. This effect was observed in transgenic adult animals and transgenic embryos as well [Broughton et al., in press].

In addition, CaM kinase assays were carried out with *RQED1, RQED 8,* and wildtype, as described by Hanson et al. [1989, using a substrate peptide KKRQET. This assay was carried out with the extract of head, ground in a glass homogenizer in tris 40 mM, 0.05 Triton X-100, EDTA 1 mM, and inhibitors of proteases (PMSF, Aprotinin, leupeptin, 1 μg/ml). Without heat shock, we observed a moderate increase of activity for *RQED1* and *8* compared to wildtype (85 and 92 pmol/mg/min respectively versus 62 pmol/mg/min for wild type). After heat shock, *RQED1* and *8* showed a substantial increase of $Ca^{++}$/calmodulin independent activity of CaM kinase (55 and 58 pmol/mg/min for *RQED1* and *8* versus 38 pmol/mg/min for the $w^z$ control strain). The actual component of the activity that is calcium/calmodulin-independent may be more that the numbers suggest because of the likely interference by the numerous potential kinases which may inefficiently phosphorylate the substrate peptide KKRQET. The biochemis-

try of amino acid-substituted CaM kinase mutants is described elsewhere [Fong et al., 1989].

## RESULTS

**Up or down regulation of CaM kinase II does not effect the differentiation of neuronal cell types in vitro.** The neuronal cell culture system that we developed, based on previous work [Salvaterra et al., 1987], gives cell clusters consisting of an original neuroblast, intermediate cells (ganglion mother cells or GMCs) and maturing neurons, as first described in Broughton et al. [in press]. To address the question of the identity of the neuronal cell types in vitro, we used a surface antigen recognized by anti-horseradish peroxidase [Jan and Jan, 1982] and a secondary gold particle-labeled antibody directly visible on the SEM (Fig. 1). This surface marker appears to be specific for processes and the cell body of mature neurons. GMCs and neuroblasts are usually not labeled, as previously reported [Jan, 1992]. The mature neurons are roundish cells of a few μm in diameter.

As we consistently observed that the proportion of connected clusters was strictly related to the vigor of the growth of type I and II processes in each culture, we do not report data related to cluster targeting. The CaM kinase activated or inhibited strains showed no significant difference in the neuroblast/GMC/mature neuron ratio before or after heat shock compared to the control strain suggesting that the up or down regulation of the enzyme interferes little with the process of cell division (Table I and Fig. 4 for statistical analysis). The mean GMC:mature neuron ratios by genotype were 0.62, 0.55, 0.35, 0.42, and 0.45 for $w^z$, *ala1, ala2, RQED8,* and *RQED1,* respectively. Furthermore, we attempted to evaluate the possible toxic effects of the transgenes. The internal controls that were constant for each culture were the maturation of myoblasts into myotubes and the maturation/differentiation of neuroblasts into GMCs and finally mature neurons, suggesting that the transgenes had no obvious toxic effect on these two events. The effects of the transgenes were, therefore, specific to process outgrowth. Furthermore, partially inhibiting CaM kinase (*ala1* and *ala2*) gave slightly more processes than the control (the opposite morphotype of *RQED*) with again no effect on the internal controls. Therefore, we believe that we have the appropriate controls to at least minimize any toxic effects of the transgene. In addition, neither transgene affected viability of larvae or adults subjected to the same heat shock treatments.

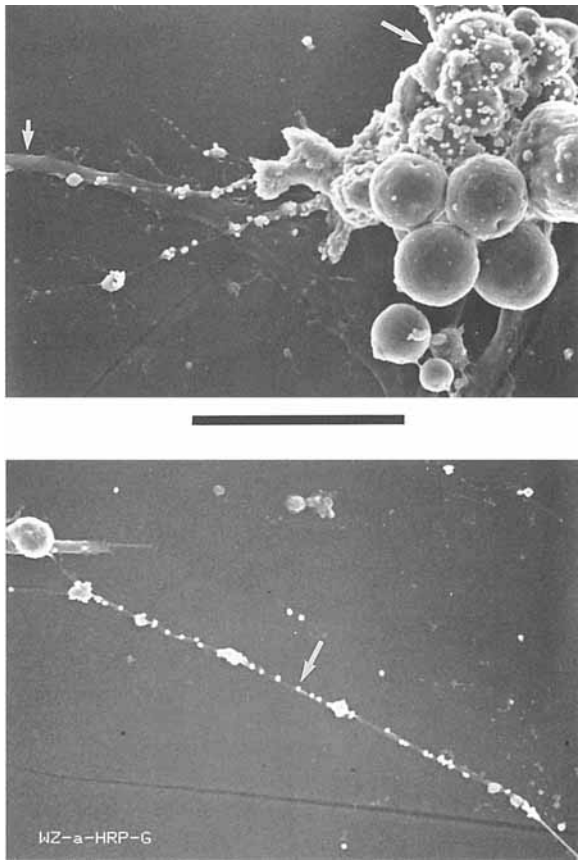



**Fig. 1.** Scanning electron micrographs of *Drosophila* neuronal cultures after external labeling with anti-HRP antibody. External labeling using a surface antigen recognized by anti-HRP primary antibody, plus a secondary 10 nm gold particle-labeled anti-rabbit antibody, followed by a few minutes of silver enhancement. Only processes and mature neurons are 'beaded' (*large arrows*). Myotubes (*small arrow*) are unlabeled. Black bars represent 10 μm.

**A partial activation or inhibition of CaM Kinase II effects the outgrowth of processes of neuronal cell clusters.** Typical morphologies of cell clusters from *ala, RQED,* and control ($w^z$) strains are shown in Figure 2. There were differences between the morphotypes of the mature neurons from heat shocked *ala1, ala2, RQED1, RQED8* embryos, which are summarized in Table II (see Figs. 4 and 5 for statistical analysis).

Without heat shock, we observed that the product of the constitutively activated CaM ki-

**TABLE I. Morphological Characteristics of Neuronal Cells After Heat Shock***

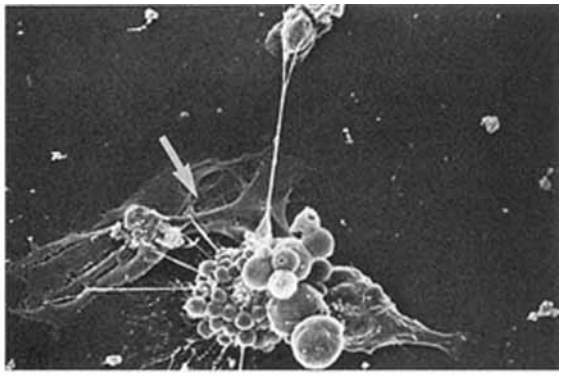
| | Presence of neuroblast/ cluster | Mean number of GMCs/ cluster | Mean number of mature neurons/cluster |
|---|---|---|---|
| *RQED1* | + | 4 ± 2 | 12 ± 4 |
| *RQED8* | + | 5 ± 2 | 14 ± 6 |
| *ala1* | + | 4 ± 2 | 12 ± 5 |
| *ala2* | + | 4 ± 2 | 13 ± 5 |
| $w^z$ | + | 5 ± 2 | 11 ± 3 |

*Neuronal cells were identified by expression of the HRP-like antigen, and visualized on the SEM using gold particle labeling. For each genotype, 15 clusters from three different individual cultures were analyzed and the numbers are the average per cluster.

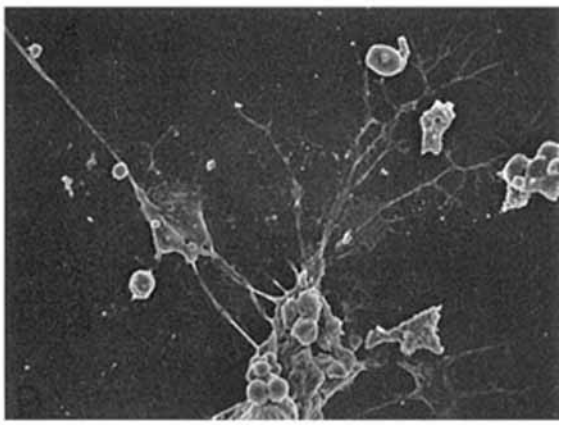
nase transgene in strains *RQED1* and *8* decreased the number of type I processes significantly compared to the control strain ($w^z$) probably due to the ectopic transcription of the transgene [heat shock promoters are known to be leaky, as reported by Wang et al., 1994]. Similarly, the number of growth cones was significantly reduced in both strains. The leaky expression of this constitutively active enzyme appears to be sufficient to obtain a substantial effect on processes and growth cones. Following heat shock, these effects were more pronounced.

Without heat shock, *ala2,* which expresses the inhibitory peptide of CaM kinase due to the leaky heat shock promoter, showed the opposite morphotype to *RQED,* with significantly more type I processes and growth cones than the control strain wz. The effect on growth cones was slightly accentuated following heat shock. A similar effect on type I processes was observed in *ala1* without heat shock. The effect on growth cones, however, was not significant which may be due to the lower level of expression of the transgene in this strain [Wang et al., 1994]. Following heat shock, however, the effect on type I processes in *ala1* was less than before heat shock, and in *ala2* was not accentuated following heat shock. Although we did not see any toxic effect of heat shock on the control, the combination of heat shock and the increased expression of the transgene may be slightly deleterious to the health of the cells to differing degrees in these two lines (See Figs. 4 and 5 for statistical analysis).

Due to these opposing effects of activated and inhibited CaM kinase, we conclude that transgenic CaM kinase has a down regulatory role during neurite elongation.



A



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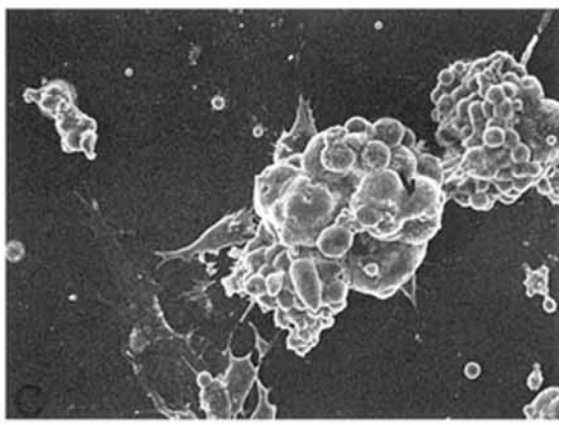

C

**Fig. 2.** Scanning electron micrographs of *Drosophila* neuronal cultures from control and transgenic embryonic neuroblasts after 24 h. This method was based on Salvatera with substantial modifications [Salvaterra et al., 1987]. Briefly, 100 embryos at the early gastrula stage were heat shocked for 30 min at 37°C, allowed to recover for 3 h, and then homogenized and seeded on a Primaria Petri dish. The detailed analysis of these clusters is presented in Tables I and II. Black bars represent 10 μm. **A:** Typical neuronal $w^z$ (control) cluster. We see the original neuroblasts (*a*), the intermediate ganglion mother cells (GMCs) (*b*), and the mature neurons (*c*) which are identified based on the size. **B:** Typical neuronal ala cluster. **C:** Typical neuronal *RQED* cluster.

**TABLE II. Analysis of Processes From Different Strains: A, After Heat Shock; B, Without Heat Shock***

| A | *RQED1* | *RQED8* | *ala1* | *ala2* | $w^z$ |
|---|---|---|---|---|---|
| Type I processes/cluster | 3 ± 1 | 2 ± 1 | 13 ± 4 | 15 ± 4 | 10 ± 5 |
| Type II processes/cluster | 1 ± 1 | 2 ± 1 | 5 ± 3 | 3 ± 2 | 5 ± 2 |
| Growth cone/cluster | 1 ± 1 | 1 ± 1 | 5 ± 2 | 6 ± 2 | 3 ± 2 |
| Terminal and lateral arborization of processes | +/− | +/− | +++ | +++ | ++ |
| **B** | ***RQED1*** | ***RQED8*** | ***ala1*** | ***ala2*** | $w^z$ |
| Type I processes/cluster | 5 ± 2 | 5 ± 2 | 15 ± 3 | 14.5 ± 3 | 10.5 ± 3 |
| Type II processes/cluster | 2 ± 1 | 2 ± 1 | 5 ± 2 | 6 ± 4 | 5 ± 2 |
| Growth cones/cluster | 1 ± 1 | 1 ± 1 | 4 ± 2 | 5 ± 2 | 3 ± 2 |
| Terminal and lateral arborization of processes | +/− | +/− | +++ | +++ | ++ |

*For each genotype, 15 cell clusters were quantified and the numbers in the tables represent mean ± standard deviation. The structures were identified by gold particle labeling after anti-HRP incubation. Briefly, two types of processes are distinguished by morphology. Type I are thick with few branches and large varicosities. Type II are thin with broad arborisation and heavy pearling.

### Subcellular Distribution of CaM Kinase II

Given that CaM kinase appeared to be regulating elongation, the subcellular localization of the enzyme is important particularly with regard to the growth cone. To examine this issue, we developed an internal labeling technique to visualize the molecules directly in the SEM. After delipidation, the processes are still visible in the SEM as nude filaments of protein, which had been previously cross-linked by treatment with a low concentration of glutaraldehyde. We saw that untargeted and navigating filopodia, as well as the body of the growth cones, showed few beads, whereas processes that appeared to have reached a target were heavily labeled suggesting that CaM kinase may be transported to the terminus after a target has been reached (Fig. 3). Although we cannot be certain that these connections are functional, the original system which inspired the work reported here demonstrates that functional neuromuscular junctions do differentiate in such an in vitro system [Seecof et al., 1972, 1973]. The authors show using electron microscopy that at synaptic clefts the axon terminal contains synaptic vesicles. Clear synaptic vesicles of about 620 Å in diameter are seen clustered against the presynaptic membranes. Moreover, when an axon is attached to a myocyte and is stimulated electrophysiologically, the myocite contracts. The authors report that 85% of the axons tested were functional [Seecof et al., 1972]. This indicates that most, if not all, of the synapses in our culture are functional, and "connected" processes will eventually make a functional synapse. We cannot determine whether or not any of the 'unconnected' neurons that we see in culture are predetermined to synapse with particular neurons or muscle cells that are not present in the culture environment. However, this does not detract from the biological relevance of our data on the differences in the subcellular distribution of CaM kinase in "connected" and "unconnected" neurons. The connections that we see may or may not be functional, mature synapses, but from a structural point of view, we only see labeling in connected processes and never in processes that are, for whatever reason, unconnected to any target. However, very rare unlabeled but connected processes were observed, possibly because such "contacts" are not functional. Alternatively, the CaM kinase could be at levels that are below those that are detectable by our antibody suggesting that the transport may occur transiently once connections have been made. This observation makes sense because, once formed, the synapse may require only a low level of synthesis of CaM kinase for consolidation or replacement due to the turnover of the enzyme. The heavy labeling of connected processes occurred as chains of beads along the length the processes. We observed that these beads observed after internal labeling were larger and more heterogeneous in size than those seen after external labeling. We believe that this may be due to the clustering of gold labeled antibodies together with residual lipids and/or detergent molecules that following silver enhancement appear as single beads. Myotubes never showed such labeling. The method works in vitro because even though the cell clusters are fused and chaotic, the processes are easily visible. Also, although

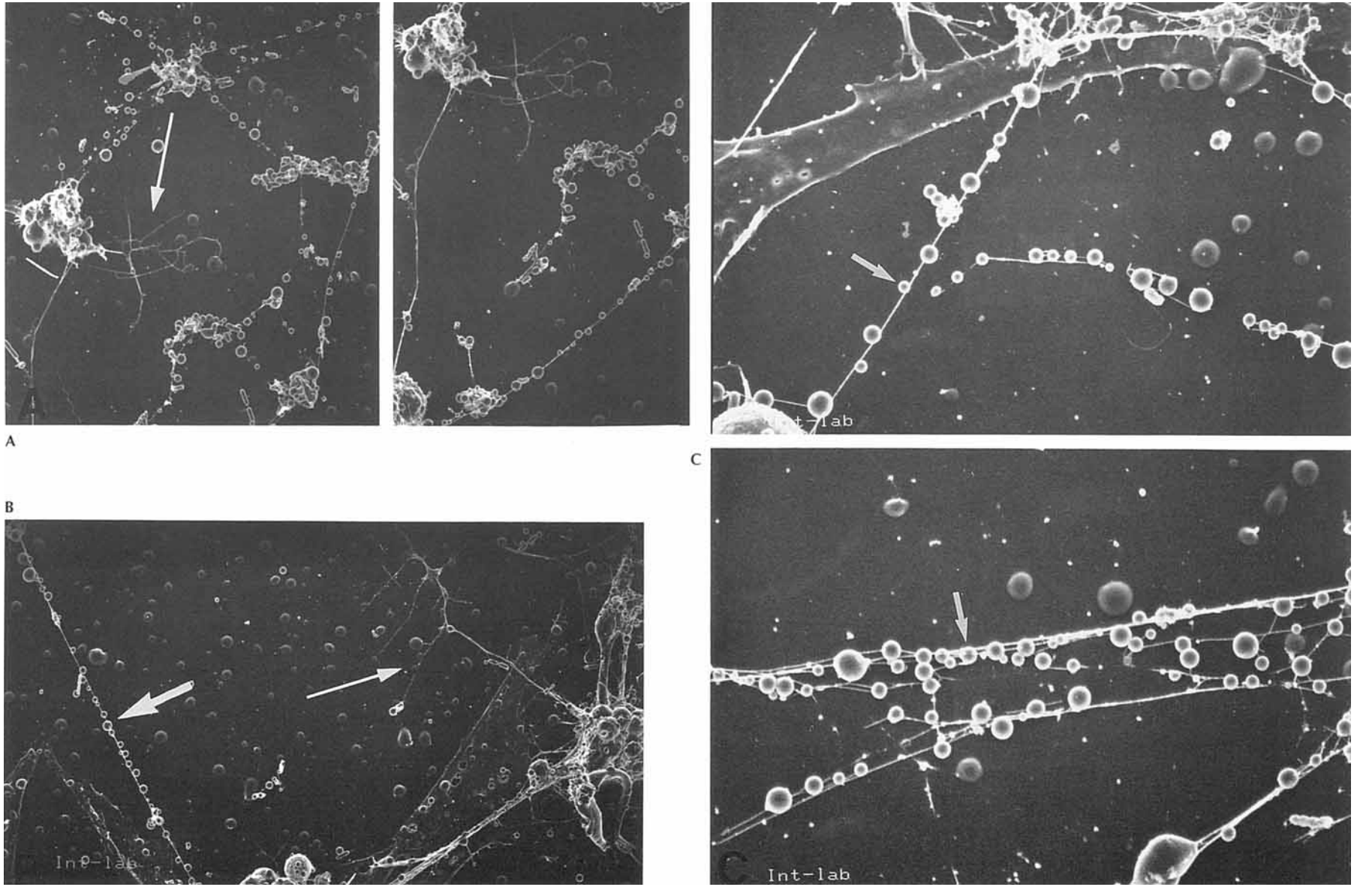


**Figure 3.**

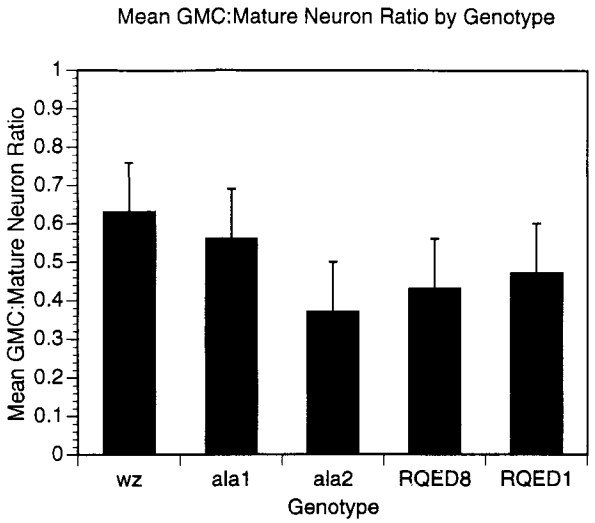


**Fig. 4.** Mean GMC:mature neuron ratios after heat shock. The Shapiro-Wilk W Test ($P = 0.05$) was used to confirm that data for all genotypes were normally distributed, and comparisons with the $w^z$ control performed using Student's $t$ Test ($P = 0.05$). All genotypes were not significantly different from the control. Error bars represent SEM.

the detergent treatment was not sufficient to gain access to CaM kinase in the cell bodies, it was sufficient in the much smaller and delicate processes. It was, however, impractical to address the question of the presence of CaM kinase in the terminals connecting the cell bodies of neurons in the clusters.

## DISCUSSION

The fact that CaM kinase appears to be abundant only when connections are established is consistent with our data showing an inhibitory role on navigating processes. Such data do not preclude low levels of the enzyme in unconnected growth cones, possibly in spatially restricted regions, at levels that are not sufficient to be detected in our system. It has been suggested that in addition to targeted protein transport, there exists another mechanism involving mRNA transport to spatially restricted regions of cells and the local synthesis of proteins. These studies have suggested that certain mRNAs are transported to the dendritic laminae where polyribosomes have been shown to exist. This localization of mRNA and protein synthesis appears to be restricted to post-synaptic sites. Interestingly, mRNA encoding MAP2 and the α subunit of CaM kinase II have been localized to this region [Steward and Banker, 1992, for review]. Although our data on the temporally restricted transport of CaM kinase along processes suggests that such an in situ protein synthesis may not be responsible for the abundance of CaM kinase II in the emerging synapse of the presynaptic cell, such a mechanism may exist in the growth cone prior to synaptogenesis. Davis et al. [1992] show that cultured neuronal growth cones of *Helisoma* can locally synthesize protein, and suggest that this capability allows the growth cone to respond rapidly and specifically to local signals in the environment despite the large distances that may exist between the cell body and the growth cone. We hypothesize that before processes reach a target, growth cones bear the minimum molecular equipment necessary for elongation and pathfinding because many will retract or disappear. It is difficult to believe that all the molecules in the functional synapse preexist in the growth cone during development because this would be energetically wasteful to the cell due to the fact that many early connections fail to produce functional synapses. Our data also point to a retrograde message from the target site to the presynaptic cell body calling for the appropriate synthesis of molecules which will migrate to the corresponding maturing synapse.

One of the roles of CaM kinase in the mature synapse is the phosphorylation of synapsin I [Greengard et al., 1993] which is involved in the $Ca^{++}$ dependent release of neurotransmitter from neurosecretory vesicles. As we have shown here for CaM kinase, synapsin I has also been shown to be translocated to the terminus following contact with the postsynaptic target [Fletcher et al., 1991]. Furthermore, the down-regulatory role of CaM kinase contrasts with the apparent up-regulatory role of protein kinase C (PKC) in neurite elongation. Using transgenic flies expressing a pseudosubstrate peptide that par-

**Fig. 3.** Scanning electron micrographs of *Drosophila* neuronal culture after Internal labeling with anti-CaM kinase antibody after delipidation. $w^z$ fly neurons were cultured from early gastrula stage embryos and seeded on Primaria Petri dishes. Cells were fixed in 0.5% glutaraldehyde for 30 min, then delipidated with 1% SDS, 2.5% Triton X100 for 2 days at 25°C, 60% humidity. The cells were then washed five times with distilled water and incubated overnight in PBS, 0.1% Triton X100 and the anti-CaM kinase antibody. Plates were washed three times and incubated with the secondary gold particle-labeled anti-rabbit antibody. *Large arrows* indicate gold labeled processes after silver enhancement. *Small arrows* indicate untargetted growth cones and filopodia (unlabeled). Black bars represent 10 μm. **A, B, C,** are a representative selection.

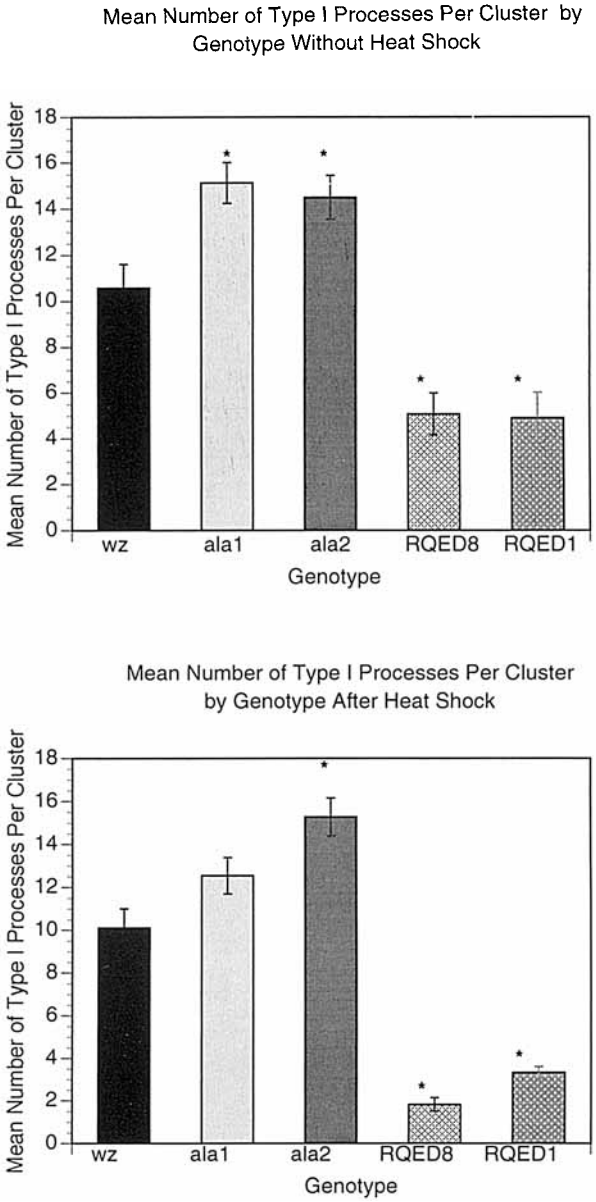


**Fig. 5.** Mean number of type I processes per cell cluster with and without heat shock. The Shapiro-Wilk W Test ($P = 0.05$) was used to confirm that data for all genotypes were normally distributed, and comparisons with the $w^z$ control were performed using Student's $t$ Test ($P = 0.05$). Genotypes that differed significantly from the control are marked with an *asterisk*, and error bars represent SEM.

tially inhibits PKC, we observed impaired development of processes [Broughton et al., in press], and therefore, during development the two enzymes seem to have opposing effects on the elongation and navigation of processes. Our data on the inhibitory effect of CaM kinase on the elongation of processes are consistent with the concept that the enzyme may play a role in stabilizing or immobilizing the synapse, in addition to its role in neurotransmitter release. In fact, when a specific filopodium elongates, under the attraction of guidance cues, reaches its target and makes a synapse, the corresponding growth cone and untargeted filopodia disappear [Davis and Murphey, 1994]. These two coincidental events could be related to the arrival of CaM kinase in the newly formed terminal from the cell body.

A second, possible, role of CaM kinase may be the physical consolidation of the synapse depending on its repeated activation by successive influxes of $Ca^{++}$. We hypothesize that the more

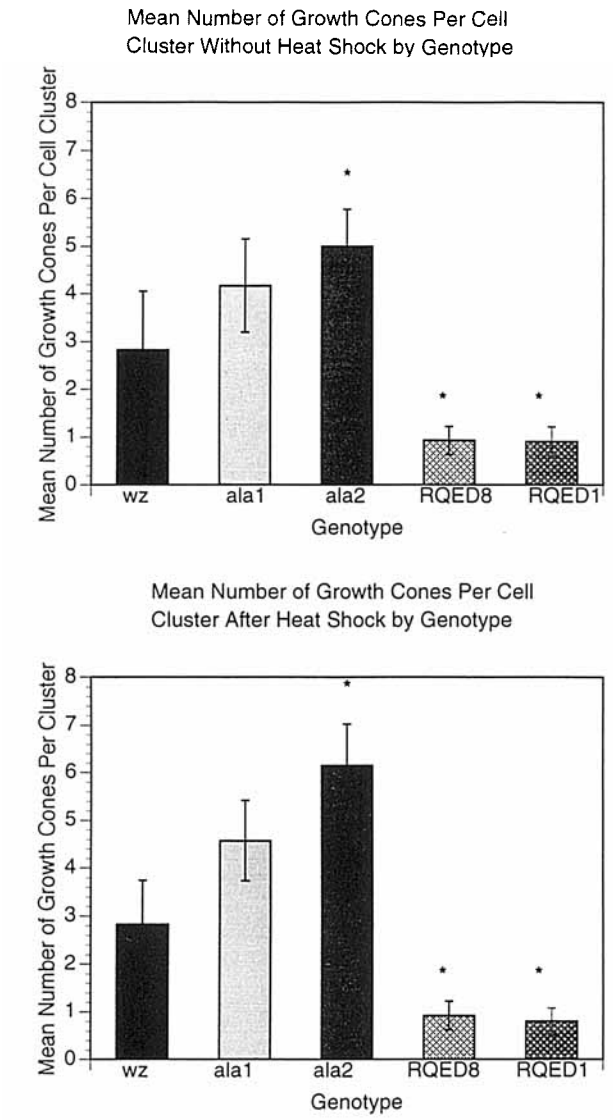


**Fig. 6.** Mean number of growth cones per cell cluster with and without heat shock. The Shapiro-Wilk W Test ($P = 0.05$) was used to confirm that data for all genotypes were normally distributed, and comparisons with the $w^z$ control were performed using Student's $t$ Test ($P = 0.05$). Genotypes that differed significantly from the control are marked with an *asterisk*, and error bars represent SEM.

frequently a synapse is stimulated, the more $Ca^{++}$ influx and degranulation occur, which consequently through the activation of CaM kinase confers physical stability to the pre-synaptic cleft. During development, two types of spontaneous $Ca^{++}$ transients, waves and spikes, regulate neurite extension and the development of neurotransmitter receptor expression, respectively [Gu and Spitzer, 1995]. These spontaneous transients appear to be necessary for neuronal differentiation. It is interesting that calcineurin, a phosphatase that is present in the growth cone, upregulates process growth, and, like CaM kinase, requires activation by $Ca^{++}$/calmodulin [Chang et al., 1995]. Although at present the two enzymes appear to have different intracellular substrates, it is possible that they are involved in maintaining a balance of phosphorylation/dephosphorylation of many substrates in the growth cone that regulate process growth and differentiation depending on the information encoded in spontaneous calcium transients. Furthermore, the mechanism of growth cone collapse following the release of calcium from intracellular stores elicited by the neurite growth inhibitor NI-35 is unknown [Bandtlow et al., 1993]. Our data are consistent with CaM kinase being one of the effectors of the response to elevations of $Ca^{++}$ that is responsible for either the immediate collapse of the growth cone or a reduction in neurite outgrowth.

We are interested in the timing of these "pre-synaptogenesis" events which occur between the initial contact of the process with the target and the emergence of the fully functional synapse. In vivo, synaptogenesis is a lengthy process that involves the loss of many early connections, and so in our system it may not be relevant to demonstrate whether or not the connections are in fact mature synapses. Clearly, we have a model in which neuronal clusters are able to differentiate and form processes that either make connections or run unconnected on the plastic surface. The unconnected, navigating processes are never seen to be substantially labeled for CaM kinase. Whatever the nature of the process/target interaction in our culture system, connections trigger transport of the enzyme in processes.

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