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Identification, Characterization, and Developmental Analyses of A Growth Cone Specific Epitope and a Synaptic Vesicle Protein

Stephen Henri Devoto

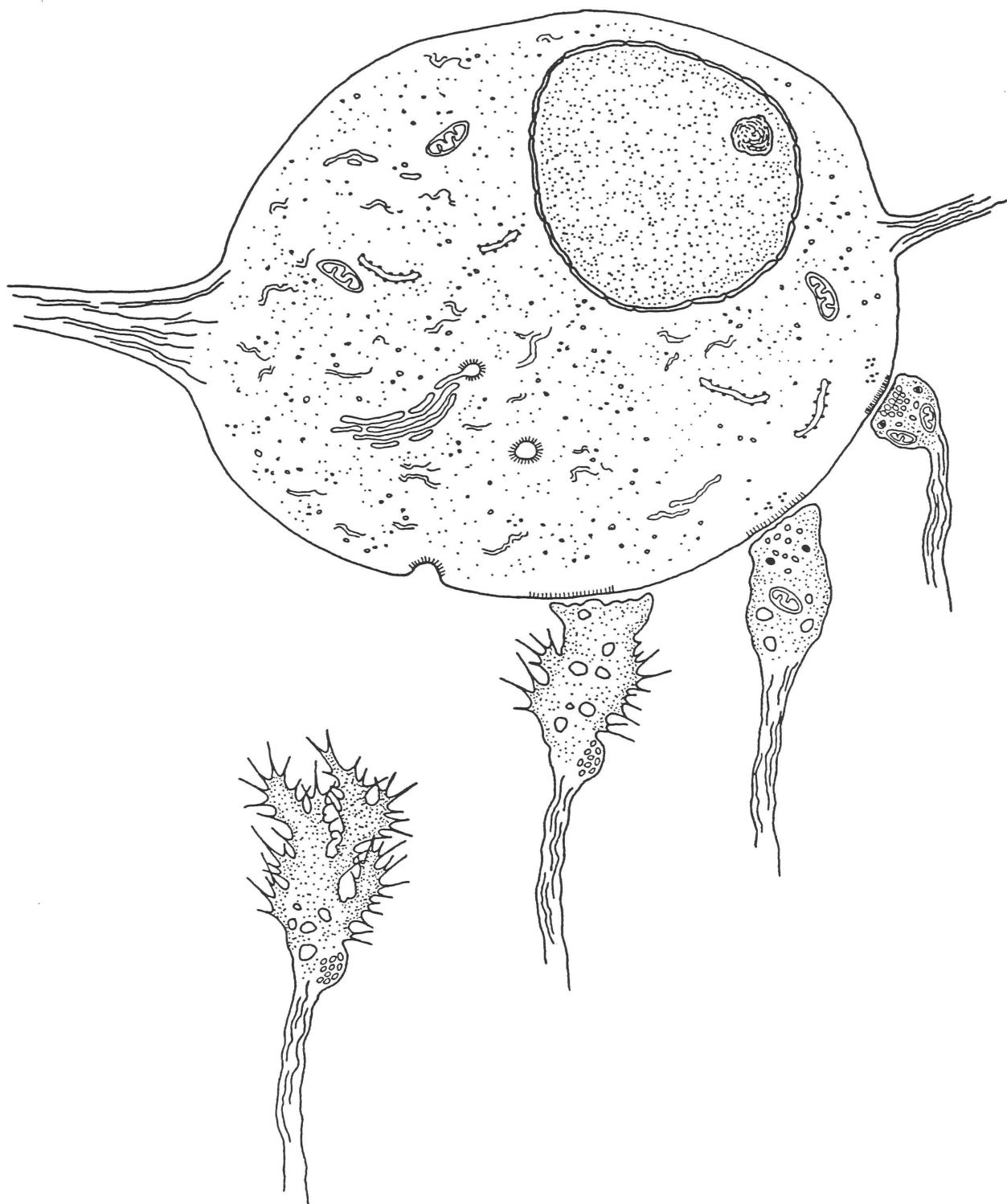
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**IDENTIFICATION, CHARACTERIZATION,
AND DEVELOPMENTAL ANALYSES OF
A GROWTH CONE SPECIFIC EPITOPE
AND A SYNAPTIC VESICLE PROTEIN**

Stephen Henri Devoto

The Rockefeller University
Laboratory of Neurobiology
New York, New York

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The Rockefeller University in partial fulfillment of
the requirements for the degree of Doctor of Philosophy

PREFACE

This thesis describes a growth cone specific epitope (CD'A 1), a synaptic vesicle antigen (SVP38), and the developmental expression of each in various tissues. Although CD'A 1 and SVP38 do not have any direct relationship to each other, I will show that the expression of both changes over the same time period. This provides a unifying theme for my work. I have therefore decided not to divide my thesis into independent sections, each with its own introduction, results, and discussion, but rather attempt to tie all of my work together into a single unit.

The thesis is divided into five chapters. The first chapter is the introduction to the thesis, and provides a selective review of growth cones, synapses, and synaptogenesis. The second, third, and fourth chapters present the results of my research on the growth cone epitope, the synaptic vesicle protein, and the developmental expression of both, respectively. The fifth and final chapter provides a discussion of all of the results. I decided to present all of the techniques in the second part of the appendix at the end of the thesis, for easy reference.

Our current understanding of growth cone function arises primarily from work carried out in the century between 1860 and 1960. The first appendix is a historical review of some of the most important of these experiments, and how they contributed to modern ideas of growth cone structure and function. Readers who are not familiar with the growth cone may prefer to read this section before reading the main introduction.

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My undergraduate professors at Haverford College taught me biology and inspired me to pursue it as a profession, they deserve much credit for this thesis. Finally, I would like to thank all the members of my family for the wonderful and generous support they have given me, which above all else has enabled me to complete this thesis.

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CHAPTER ONE

INTRODUCTION

1. The Growth cone

1.1. Early formation

Neuronal precursors generally assume a roughly spherical shape during mitosis, elongating their processes only after cytokinesis (Sauer, 1935). As neurons begin to migrate, their shape changes, and they elaborate a leading process and leave behind a trailing process. Neuronal migration has been most closely examined in the cerebral cortex and the cerebellum (Rakic, 1971, 1972). In the cortex, cells leave the mitotic cycle in the ventricular zone, and then migrate outwards in close apposition to radial glia. These cells have processes that extend from the ventricular surface to the pial surface, and function as a substrate and guide for the migration of immature neurons. During migration, the leading tip appears to be an active region of the cell, sharing many features with dendritic growth cones (Wise, et al., 1979; Rakic, 1972). The trailing process is initially simpler in form, and does not end in any specializations. However, before migration is complete, the trailing process is transformed into an elongating axon, tipped by a growth cone (Parnavelas and Uylings, 1980; Rakic, 1971, 1972). There are fundamental differences between the leading tip of a migrating cell and a growth cone. The leading tip tapers towards the end while the growth cone expands, and on average the distance between the tip of the leading process and the cell body does not change, while the growth cone steadily moves relative to the cell body. Very little is known about the transition of the trailing process of a migrating neuron into an axonal growth cone, or if the axon initiates elsewhere in the cell, the mechanisms of that initiation.

The formation of axons and dendrites by dissociated neurons can be readily observed *in vitro*, where it occurs in the absence of cell migration or any other cell

contacts (Collins, 1978; Dotti, et al., 1988). In hippocampal cell cultures, the spherical cells first elaborate lamellipodia in a ring around their cell body. A few of these lamellipodia become part of growth cones, extending away from the cell body with neurites behind them. During the second day in culture, one of the neurites begins to grow much faster than the others, and acquires the characteristics of an axon. A few days later, the other processes rapidly elongate and differentiate into dendrites. The axon always seems to develop from the longest process: if the axon is cut, then the longest of the minor processes becomes the axon (Dotti and Banker, 1987). *In vivo*, as mentioned above, cell polarity is often manifested during migration, and the counterpart to these *in vitro* results is not obvious. They may provide a model for systems in which axonal and dendritic differentiation is not coupled to neuronal migration. These cells also provide a good system in which to compare axonal and dendritic growth cones.

1.2. Structure and general behavior

Most work on the growth cone has been on the period of elongation two to three days after initial outgrowth. At the light microscope level, the growth cone appears to be an amoeboid expansion of the axonal membrane. The membrane has thin veils called lamellipodia, and fine spikes called filopodia, protruding from its main body. The lamellipodia and filopodia exhibit rapid motility, moving from side to side, extending and retracting as the growth cone moves forward.

Growth cone morphology can vary considerably, even within the lifetime of a single cell. In general, growth cones become less complex as cells get older. In cultures of rat sympathetic ganglia cells, the shape of the growth cone correlates with their rate of outgrowth. When the growth cone moves rapidly, it consists entirely of lamellipodia, conversely when only filopodia were present, the rate of advance is slowest (Argiro, et al., 1984; Roufa, et al., 1983). Changes in growth cone morphology are also observed *in vivo*. The growth cones of retinal ganglion cells are primarily lamellipodial in the optic nerve and tract, are very complex with both lamelli- and filo-

podia in the chiasm, and have only filopodia when they are within their target area (Bovolenta and Mason, 1987). This suggests that the shape of a growth cone becomes more complex at "decision points" in its trajectory. This is supported by data on chick spinal neurons: growth cones are larger, with more lamellipodia in regions where they frequently "make decisions", or turn (Tosney and Landmesser, 1985).

Electron microscopy of growth cones has been primarily on aldehyde fixed tissue. The structures seen using this technique differ from those seen using rapidly frozen and freeze-substituted tissue. Aldehyde fixed axonal growth cones have a number of very characteristic features: numerous electron-lucent, irregularly shaped vesicles, possibly indicative of a smooth endoplasmic reticulum, a few mitochondria, no ribosomes, very few microtubules or intermediate filaments, and numerous microfilaments (Yamada, et al., 1971). The plasma membrane is irregular in outline, and has a low density of intramembranous particles, compared to the neurite or the cell body (Pfenninger and Bunge, 1974). Not as many studies have been done on dendritic growth cones. In the chick spinal cord, dendritic growth cones are not as large and irregularly shaped as axonal growth cones, they have fewer microfilaments, fewer vesicles, and unlike axons have clusters of ribosomes (Skoff and Hamburger, 1974). No studies have been undertaken to examine whether the morphology of dendritic growth cones also varies during the time of outgrowth. It remains possible that some of the differences seen between axonal and dendritic growth cones are due to temporal variation in the properties of dendritic growth cones.

Chick retinal ganglion cell axonal growth cones have also been examined following tissue preservation by freeze-substitution. Using this technique, there is a multilamellar stack of membrane disks (MLS) in the palm of the growth cone, which may be an intermediate pool of membranes between the cell body source and the growth cone plasma membrane (Cheng and Reese, 1985). Single disks that appear to derive from the distal portion of the MLS are seen subjacent to filopodia, and may serve

as the immediate source of plasma membrane for an extending filopodium (Cheng and Reese, 1987). Aldehyde fixation probably transforms the MLS into irregularly shaped vesicles. The relationship of the MLS to vesicles involved in anterograde and retrograde transport remains unknown.

1.3. Motility and cytoskeleton

The motility of the growth cone has a number of components: filopodia and lamellipodia extend and retract, they move relative to the growth cone without changing their size, the growth cone body moves forward or back and changes shape, gradually becoming the axon or dendrite. All of these motions require movement of the cytosol, including the cytoskeleton, and the plasma membrane. The cytoskeleton is known to play an integral role in growth cone motility, I will consider this structure first.

The cytoskeleton of the mature axon is composed of microtubules (tubulin), intermediate filaments (neurofilaments), and a few microfilaments (actin), that lie immediately subjacent the plasma membrane. In many immature axons neurofilaments are not detectable morphologically or immunologically, and when expressed do not extend into the growth cone. Therefore they do not appear to be important for process outgrowth. Microtubules extend from the axon into the central part of the growth cone where the bundles splay out as a fan, and never extend into the lamellipodia or filopodia. The only known cytoskeletal component present in most of the growth cone and its lamelli- and filo-podia is actin. The actin filaments form a few bundles in the growth cone palm, and run the full length of filopodia, where they are surrounded by a network of individual filaments which are crosslinked to each other and to the inner face of the plasma membrane (Yamada, et al., 1971; Letourneau and Ressler, 1983). Actin is a polarized polymer, with a plus and a minus end that differ in their rate of polymerization. Monomers are added to the plus end at a rate that is ten times faster than to the minus end. In motile cells and in growth cones, the plus end is attached to the plasma membrane.

Actin can generate force by polymerization or depolymerization while remaining attached to a relatively stationary structure at one end and the motile structure at the other. Actin can also be involved in motility by generating force in combination with myosin, as it does in muscle cells. Myosin is present in growth cones, but it is not bundled into thick filaments (Letourneau, 1981). This means that if actin/myosin bridges are to generate force, both the actin and the myosin have to be anchored, one to a stationary structure, the other to the motile structure.

In growth cone whole mounts examined in the electron microscope, actin microfilaments often appear to be attached to microtubules at the base of the growth cone (Letourneau and Ressler, 1983). If the microtubules are relatively immobile to pushing and pulling, then this would allow the actin to exert force on the membrane. If the actin filaments are crosslinked to each other, then very few of them need to be linked to the axonal microtubules at the base of the growth cone. Two of the most prominent microtubule associated proteins, MAP2 and tau, are able to crosslink pure actin filaments *in vitro*, and furthermore this activity is inhibited by phosphorylation of the MAPs, for MAP2 the calmodulin dependent protein kinase II has the most effect (Selden and Pollard, 1983; Yamauchi and Fujisawa, 1988). However, the only direct evidence for crosslinking of actin to itself and to tubulin in the growth cone derives from electron micrographs of extracted growth cone wholemounts. The cytoskeleton may have suffered some artifactual crosslinking during preparation.

Drugs known to affect actin have a profound affect on growth cone motility. Cytochalasin B, which destabilizes actin filaments, causes the growth cone to round up, the filopodia to be withdrawn, and movement to cease in growth cones of embryonic chick dorsal root ganglion cells (Yamada, et al., 1971). Using video enhanced differential interference contrast microscopy on cultured adult *Aplysia* neurons, it is possible to see fibers under the plasma membrane in the lamellipodia that continuously move in the retrograde direction at a rate of 3.5 $\mu\text{m}/\text{min}$, even while the lamellipodia are moving

forward. The moving fibers are colocalized with phalloidin-stained actin filaments that can be found after fixation. Cytochalasin B reversibly destroys the filaments, blocks the retrograde movement, and eliminates most of the phalloidin-stained actin from the lamellipodia (Forscher and Smith, 1988). Although individual subunits within the filament are moving backwards, the filament can remain apposed to the membrane, and might even push the membrane outwards, because of constant addition of monomers to the plus end and depolymerization at the minus end.

The above results suggest that the actin-rich lamelli- and filo-podia are moving forward, exerting tension on the axon behind them. However, the importance of actin in axonal elongation seems to be inversely related to the adhesivity of the substrate. Chick embryo dorsal root ganglia cells, when plated on a highly adhesive substrate (polyornithine), elaborate extensive neurites in the presence of cytochalasin B, despite the absence of recognizable lamelli- or filo-podia (Marsh and Letourneau, 1984). The neurites are curiously looped and extensively branched, suggesting that one role of the growth cone in this situation is to steer elongation and that actin filaments are involved in the regulation of neurite branching.

In order for the axon to elongate, not only must the growth cone move forward, but the membrane and cytoskeleton of the growth cone must be transformed into axon. This rather self-evident observation must be considered when discussing the mechanisms of axonal pathfinding and the stopping of the growth cone at a target cell. It is likely that in addition to regulation of growth cone motility, there are cellular control mechanisms that regulate the rate and direction of the conversion of the trailing part of the growth cone to axon. At the base of the growth cone, there is a transition zone between the actin filament meshwork of the growth cone and the tubulin rich cytoskeleton of the axon. The ordered microtubule arrays of the axon must extend forward past this zone during axonal elongation.

Microtubule elongation could in principle take place anywhere along the axon, as axonal microtubules do not extend uninterruptedly from cell body to growth cone (Bray and Bunge, 1981). Four observations strongly suggest that microtubule elongation occurs at the growth cone, and that neither during development nor in the adult does the cytoskeleton translocate *en masse* down the axon. Firstly, tubulin in the axon (although not in the dendrite), like actin, is polar and the end to which monomers are rapidly added is distal to the cell body (Heidemann, et al., 1981). Secondly, the axon becomes narrower at the node of Ranvier, yet the cross-sectional density of microtubules and neurofilaments remains constant, if they were all moving down the axon, then they would have to accelerate at the nodes. Thirdly, when fluorescent tubulin is injected into a cell and then a portion of the growing neurite photobleached, the bleached area does not move relative to the cell body, even though the neurite is extending (Lim, et al., 1988). Finally, microtubule depolymerizing drugs block neurite extension when applied at the growth cone but show no effect when the same dose is applied at the cell body (Bamburg, et al., 1986). If the cell body and growth cone are equally permeable to the drugs, this result shows at a minimum that growth cone microtubules are much less stable than axonal or cell body microtubules. This instability can be utilized to closely regulate the direction of growth. However, it is not clear what form tubulin is in during transport. When newly synthesized tubulin is pulse-labelled, it is transported as a non-diffusing wave of polymerized tubulin (Lasek, 1982).

Branches in neurites are most frequently formed when the growth cone bifurcates, leaving two processes behind instead of one. Normal tubulin polymerization and depolymerization is required for branching: low doses of the microtubule stabilizing drug taxol inhibit the branching of embryonic chick sensory and sympathetic neurons (Letourneau and Ressler, 1984; Letourneau, et al., 1986). This raises the possibility that axonal and dendritic branching is regulated *in vivo* by endogenous agents that affect

tubulin polymerization. Cells treated with high concentrations of taxol do not extend neurites, having only very broad, short "meganeurites." However, if taxol and cytochalasin are added together, then long, thin neurites are extended that do not branch and are unattached to the substrate (Spero and Roisen, 1985; Letourneau, et al., 1987). It remains to be shown that the mode of elongation of these bizarre neurites is the same as for untreated neurites, i.e. whether membrane and cytoskeleton are added at the tips.

I would like to summarize the role of the cytoskeleton in growth cone movement with the following model. In normal, untreated neurites, the meshwork of actin filaments is in a state that may be considered to be a dynamic equilibrium. At any given moment, there is a mixture of actin filaments pushing and pulling on the lamellipodial membrane and the rear portion of the growth cone. Some filaments are pushing the membrane outward by the addition of monomers between the filament and the plasma membrane. Others are pulling on the membrane by depolymerizing from one end while remaining attached to the membrane at one end of the filament and to something anchored at the base of the growth cone at the other end. If some of the "pulling" actin filaments are anchored through the plasma membrane to an extracellular substrate, then the forward edge of the growth cone would be extended forward. If at the same time, the plasma membrane in the rear portion of the growth cone becomes detached from the substrate, then the growth cone as a whole will move forward. The movement of the growth cone actin filaments forward would contribute to the elongation of axon, either by actively pulling the microtubules forward, or by creating a space into which they can polymerize. Thus, the random polymerization and depolymerization of actin filaments, coupled with the adhesive properties of the plasma membrane, I think are sufficient to account for growth cone locomotion. Furthermore, if the adhesion is patterned or regulated, then this would provide a selective agent that could direct the movement of the growth cone. Under this theory, taxol blocks neurite elongation either by interfering with the actin/microtubule interaction, or otherwise disrupting microtubule dynamics.

Cytochalasin slows down the elongation by collapsing the space between the microtubules and the growth cone membrane. However, on adhesive substrates, exocytosis and the polymerization of microtubules are sufficient to elongate the neurite. When the two drugs are combined, the cytochalasin allows the taxol-treated microtubules to move forward, in a manner that I suspect is different from what occurs normally.

1.4. Membrane flux and neurotransmitter release

The membrane of the growth cone must also be in constant flux to support the rapid motility of the growth cone. When lamellipodia and filopodia extend and retract, there is a net flow of membrane into or out of these regions. As the growth cone advances forward, there must be an addition of membrane from outside the growth cone, equal to the increased length of the axon. Because the main body of the growth cone is irregular and relatively large, membrane for the axon or for extension of lamellipodia and filopodia can derive from there. When a growth cone is severed from its cell body, its activity continues for several hours, indicating that local membrane recycling is sufficient for growth cone motility in the short term (Shaw and Bray, 1977). However, over the long term, there must be a net flux of membrane into the growth cone, probably derived from anterograde transport of vesicles synthesized in the cell body. If this membrane were added at the cell body or along the axon, then all of the side branches would move away from the cell body at the same rate as the growth cone. Since this does not occur, membrane must be inserted at the growth cone (Harrison, 1910; Bray, 1973). In agreement with this is the observation that carmine particles located on the surface of the axon do not move relative to the cell body (Bray, 1970).

Another potential source for the membrane influx to growth cones is the uptake of lipids from neighboring cells or the extracellular fluid. This source would be especially abundant during the regeneration of axons through a degenerating myelin rich nerve. Apolipoprotein E (apo-E) is an extracellular protein that participates in the

transport of lipids into cells (reviewed in Brown and Goldstein, 1987). Apo-E is taken up by cells through receptor-mediated endocytosis, and the bound lipids are used by cells for a variety of metabolic pathways, including membrane biosynthesis. Apo E is present in the conditioned medium of cultures of injured sciatic nerves, where it is complexed with lipid, and is readily taken up by the growth cones of differentiated PC12 cells (Ignatius, et al., 1987). The vesicles and/or smooth endoplasmic reticulum present in growth cones could participate in the metabolism of these lipids prior to their insertion into the plasma membrane. This source could provide the growth cone with local control over the amount of available membrane.

In aldehyde-fixed growth cones most vesicles are in the palm of the growth cone, suggesting that membrane addition occurs in this region. Very few vesicles are found in lamellipodia and filopodia in fixed preparations, suggesting that either there is no exocytosis or endocytosis, or that vesicles are very rapidly transported or not preserved by fixation. Video enhanced contrast (VEC) microscopy of aplysia neurons suggest that the first explanation is true: although many vesicles can be seen in the core of the growth cone, only rarely do they move into the lamellipodia, and when they do, they invariably reverse their direction and return to the core (Goldberg and Burmeister, 1986; Forscher, et al., 1987). Since rapid, directed organelle movement uses a microtubule substrate, then the absence of vesicles in lamelli- and filo-podia could be a secondary effect of the absence of microtubules (Vale, et al., 1985a,b). Alternatively, the thinness of the lamellipodia may not allow vesicles to enter. The top lamellipodial membrane shows continuous retrograde movement, this may serve to translocate extracellular ligands towards the body of the growth cone. The source of the membrane for this retrograde movement is not clear. It is possible that the membrane of the substrate side of the lamellipodia moves anterogradely, or exocytosis occurs on the distal substrate side of the growth cone and endocytosis on the proximal surface side of the growth cone (Burmeister, et al., 1988). *In vivo*, some situations may resemble the tissue

culture situation, as when the growth cone is growing over a basement membrane. In other situations, all surfaces of the growth cone would be roughly equivalent, each contacting, and perhaps adhering to other cells. The plasma membrane movements in these cells might be quite different from what is seen *in vitro*.

The multilamellar stack of membrane disks (MLS) seen in the palm of the growth cone when it is preserved by freeze substitution may be an intermediate pool of membranes between the cell body source and the growth cone plasma membrane (Cheng and Reese, 1985). Following incubation with cationized ferritin, coated pits appear in the plasma membrane. The coated pits give rise to coated vesicles that ultimately fuse with the MLS (Cheng and Reese, 1987). Continual recycling of membrane would provide a convenient mechanism for retaining growth cone specific surface components of the growth cone.

Further evidence for exocytosis of growth cone vesicles will come from the determination of the contents of growth cone vesicles, and assaying for their release. Embryonic frog spinal cord neurons are able to synthesize and release acetylcholine in tissue culture (Hume, et al., 1983; Young and Poo, 1983). This release is sometimes in a burst, suggesting that it may be due to the exocytosis of an acetylcholine containing vesicle. However, elevated external calcium decreases burst release from growth cones, whereas the frequency of exocytosis at a mature synapse is increased (Young, 1986). Additionally, there is no evidence that these growth cones have acetylcholine in their vesicles, the release may be due to a cytoplasmic leak.

During neurite extension of *Aplysia* buccal ganglion neurons *in vitro*, there is a characteristic sequence of membrane movements that occur as lamelli- and filo-podia of growth cones are converted to neurite. Numerous filopodia extend from the growth cone, usually adherent to the substrate. A lamellipodium then rolls out, bounded by two of the filopodia. Vesicles moving randomly and by directed transport move into the lamellipodium from the body of the growth cone. The body of the growth cone then

assumes the cylindrical shape of the axon, with bidirectional organelle transport (Goldberg and Burmeister, 1986). This model of neurite extension suggests that the function of the filopodia is to guide lamellipodial extension, which in turn is responsible for neurite elongation. Consistent with the importance of lamellipodia is the observation that growth cones lacking lamellipodia grow very slowly. However, growth cones lacking filopodia extended at the highest rates (Argiro, et al., 1984). Video-enhanced differential interference contrast microscopy is necessary to understand the membrane movements responsible for growth cone extension in these apparently filopodia-free growth cones.

1.5. The effects of soluble factors on growth cone behavior and growth cone signal transduction

The presence and potentially regulated release of soluble factors (neurotransmitters and more traditional growth factors) provides a cellular mechanism for the regulation of neuronal form by extrinsic signals from neighboring cells. The neuron is most plastic during process outgrowth, and there is *in vitro* evidence that some growth cones can respond to exogenously applied soluble factors.

In identified neurons of adult *Helisoma in vitro*, serotonin causes the lamellipodia and filopodia to collapse and the growth cone to cease advancing (Haydon, et al., 1984, 1985). These effects are due to a local action of serotonin, as it has the same effect on growth cones severed from their cell body. Other identified neurons of the same ganglion show no response to serotonin.

One of the possible mediators of the effect of serotonin on growth cones is calcium, as growth cones have particularly abundant calcium channels (Grinvald and Farber, 1981; Anglister, et al., 1982). Serotonin increases intracellular calcium only in the affected growth cones, while other agents such as membrane depolarization and the Ca^{++} ionophore A23187, which elevate intracellular Ca^{++} have the same effect as serotonin, and Ca^{++} channel blockers suppress the response of the growth cone to

serotonin. Removal of all extracellular Ca^{++} , however, also suppresses growth cone activity (Cohan, et al., 1987; Mattson and Kater, 1987). These data suggest that the level of intracellular calcium modulates growth cone motility: a moving growth cone can be stopped by either an increase or a decrease in intracellular calcium. Mammalian central nervous system neurons also show a correlation between growth cone calcium levels and neurite extension (Connor, 1986). In dissociated embryonic hippocampal cells, the application of glutamate causes the regression of dendritic growth cones while not affecting the axons. Local application of glutamate to dendritic (but not axonal) growth cones is sufficient for this effect. The calcium channel blocker cobalt suppresses the effects of glutamate, suggesting that a glutamate-stimulated rise in cytosolic calcium is responsible for dendritic retraction (Mattson, et al., 1988). Calcium influx profoundly reduces the number of actin filaments in the growth cone, this may be the mechanism by which extracellular factors influence growth cone form and movement (Lankford and Letourneau, 1988).

Other intracellular second messengers also modulate neurite extension *in vitro*. Increases in cyclic AMP suppresses growth cone activity in a subset of *Helisoma* neurons. Within a subset of these, the effect is mediated by cyclic AMP stimulated calcium influx (Mattson, et al., 1988). In *Aplysia* bag cell neurons, increases in cyclic AMP cause the redistribution of cytoplasmic organelles from the central core of the growth cone into what were peripheral lamellipodia (Forscher, et al., 1987). The retrograde waves in the lamellipodial plasma membrane are also suppressed by cyclic AMP in these cells. Calcium affects the cyclic AMP induced spread of organelles in a subtle way: in the presence of calcium, the vesicles move out in a saltatory manner, in the absence of calcium, the spread appears to occur more directedly, as if on linear tracks. Cyclic AMP is probably promoting the polymerization of microtubules into the previously microtubule-free lamella (Vallano, et al., 1985; Jameson and Caplow, 1981).

This would provide a substrate on which the organelles move peripherally, and would thereby extend the palm of the growth cone forward.

Using a crudely purified growth cone preparation, it has been shown that growth cones have largely the same second messengers present in other cells. Besides the already mentioned abundant calcium channels, growth cones have calmodulin, receptor-linked adenylate cyclase, calcium and cyclic AMP dependant protein kinases, phosphoinositide kinase, phospholipase C, and protein kinase C (Hyman and Pfenninger, 1985; Lockerbie, et al., 1988; Ellis, et al., 1985; Garafolo and Pfenninger, 1986).

1.6. Effects of other cells and the ECM

Growth cone adhesion to other cells and the extracellular matrix (ECM) undoubtedly plays an important role in neuronal morphogenesis. The ability of cell surface and ECM molecules to promote neurite outgrowth, or the ability of antibodies against them to inhibit neurite outgrowth *in vitro*, is usually interpreted to mean that these molecules play an important regulatory role in neuronal development. Other explanations however must also be considered. The molecule may trigger the release of another factor from cells in the culture, or it may bind a growth factor, serving to concentrate it. Some molecules, such as NGF, may be able to act as adhesion molecules *in vitro*, even though there is no evidence that they do so *in vivo*. In most tissue culture situations, adhesion is necessary for neurite elongation, and any agent that affects adhesion, such as an antibody to a cell surface component (MacLeish, et al., 1983), a lectin (DeGeorge, et al., 1985), or cytoskeletal disrupting drugs (Yamada, et al., 1971), might modulate neurite outgrowth by affecting cell adhesion. Finally, with the exception of a few studies to be discussed below, there is no information on the site of action of extracellular neurite-promoting molecules, i.e. whether the effect is on the filopodia, lamellipodia, central growth cone, or axon.

The ECM molecules collagen, laminin, and fibronectin each promote neurite outgrowth from neurons *in vitro*, when the purified form is coated on the substrate (Lander, et al., 1985; Manthorpe, et al., 1983; Hawrot, 1980; Akers, et al., 1981; Baron-Van Evercooren, et al., 1982). A complementary approach is to analyze neurite elongation over cell surfaces in which many molecules are present and to use antibodies to determine which of them are required for neurite growth. This approach has suggested that the calcium dependent adhesion molecule N-cadherin, the calcium independent cell adhesion molecule NCAM, as well as the ECM components mentioned above, all play a role in neurite extension (Bixby, et al., 1987). Retinal ganglion cell neurites lose their responsiveness to both laminin and NCAM as they mature (Cohen, et al., 1987; Neugebauer, et al., 1988; Hall, et al., 1987). Polycations such as polylysine and polyornithine also greatly promote neurite outgrowth.

When a growth cone approaches a border between two different substrates, the filopodia continue to palpate randomly, but those that contact the more adhesive substrate remain adhered for longer times and seem to "pull" the growth cone along this substrate (Letourneau, 1975a,b). In cultured adult *Aplysia* buccal ganglion neurons, if growth cones contact a border between poly-lysine and untreated glass, they extend filopodia and lamellipodia onto both substrates. However, the membrane that has advanced onto the less adhesive substrate is resorbed, suggesting that selective "pruning" within the growth cone might lay a role in determining the direction of neurite elongation (Burmeister and Goldberg, 1988).

2. The Synapse

The ultimate purpose of growth cones is to establish specific connections between the cells of the nervous system and in some cases, cells outside the nervous system (muscles, glands). There are two types of connections in the nervous system: chemical synapses and electrical synapses. Since very little is known about electrical synapses, and all of my work is on chemical synapses, I will only discuss chemical

synapses. When I use the word synapse in this thesis, I am referring exclusively to chemical synapses.

2.1. Structure

Synapses in all regions of the nervous system, and indeed in all animals are remarkably similar. The plasma membranes of the pre- and post-synaptic cells are parallel, and separated by a 20-30 nm synaptic cleft (usually more than the surrounding membrane). There are numerous electron lucent spherical vesicles of uniform diameter (40-200 nm) along with numerous mitochondria in the presynaptic terminal. The plasma membrane of both cells usually shows some degree of specialization. Many presynaptic terminals, in addition to the electron lucent vesicles, have a number of larger vesicles with an electron dense core, which are thought to contain a neuromodulator or a neuropeptide.

There are several visible features that may play a role in neurotransmitter release. Especially when stained with phosphotungstic acid, the presynaptic plasma membrane typically has a number of regularly spaced electron-dense fuzzy projections. The site of these electron-dense projections has become operationally known as the active zone, because fusion of vesicles occurs in this region of the membrane. The spacing between the presynaptic dense projections is increased following neurotransmitter release (Triller and Korn, 1985). These tufts of material in the active zone might represent a fixation or staining artifact since in rapidly frozen tissue, these projections are not seen. Instead, there are filaments that seem to connect the vesicles to each other and to the plasma membrane. These filaments may serve to hold the synaptic vesicles near the active zone, and/or also regulate their availability for exocytosis. The identity of these filaments is not known, although some of them have a shape that is said to resemble the protein synapsin I, which is known to be present in these synapses surrounding the vesicles (Landis, et al., 1988). The possible functions of synapsin I will be discussed below. There are double rows of large intramembrane particles at the

active zone of the neuromuscular junction which have been proposed to be calcium channels, based on the binding of the calcium current blocker botulinum toxin to the presynaptic terminal (Hirokawa and Kitamura, 1979).

2.2. Function: Neurotransmitter release and vesicular recycling

One of the most well studied synapses is the neuromuscular junction. The clearest evidence that cells do not communicate by membrane fusion first came from this synapse, and the first neurotransmitter to be identified was acetylcholine (ACh), the transmitter of this synapse. When a nerve is stimulated, there is a large release of ACh. This is evidenced by the large membrane depolarization seen in the muscle, known as the endplate potential (EPP). In addition, small depolarizations, called miniature endplate potentials (MEPPs) are seen independently of nerve activity. By reducing the extracellular calcium and/or increasing the extracellular magnesium concentrations, the nerve-stimulated release is inhibited to the level of the MEPPs. Furthermore, fluctuations in MEPPs occur which have a magnitude equal to integer multiples of the smallest MEPPs. When ACh itself is applied, it produces a graded response, depending on the amount reaching the postsynaptic membrane. This indicates that the depolarization produced by each molecule of ACh is too small to be experimentally resolved and that therefore the MEPPs are due to the action of many thousands of ACh molecules (Fatt and Katz, 1952). In other words, ACh is released in quanta by the presynaptic terminal, and nerve stimulation increases the number of quanta by increasing the probability each one will release. The time between presynaptic depolarization and postsynaptic response in this synapse is about 0.75 msec; roughly one tenth of that is due to diffusion of the neurotransmitter across the synaptic cleft (Katz and Miledi, 1965). The size of each quantum is about 10,000 molecules of ACh, as determined by applying a known amount of ACh to the muscle and determining how much is necessary to mimic a MEPP (Kuffler and Yoshikami, 1975).

As indicated above, external calcium is necessary for ACh release. The requirement for calcium is after membrane depolarization, as demonstrated by iontophoretically applying calcium either before, during or after membrane depolarization (Katz and Miledi, 1967a). The squid (*Loligo vulgaris*) giant synapse has been another fruitful synapse in which to study neurotransmitter release because both the presynaptic terminal and the postsynaptic cell are large enough to insert several electrodes (Bullock and Hagiwara, 1957). Although the transmitter is not ACh and is unknown, the release is quantal, and shares all the features of neurotransmitter release at the neuromuscular junction (Katz and Miledi, 1967b). When calcium is injected into this terminal, neurotransmitter is released quantally, suggesting that calcium is the proximal stimulant for neurotransmitter release (Miledi, 1973). This synapse can be reliably voltage-clamped, so that the latency between calcium influx and neurotransmitter release can be determined very accurately, at this synapse it is about 200 μ s (Llinas, et al., 1981).

When electron microscopy revealed that synapses contain numerous uniformly sized vesicles, it was an obvious hypothesis that the vesicles contain neurotransmitter and that release occurs by vesicular exocytosis (del Castillo and Katz, 1956; Robertson, 1956). Unfortunately, the preparations that had been profitably exploited for the physiological demonstration of calcium triggered neurotransmitter release are not suitable for the isolation of synaptic vesicles. ACh is present in vesicles isolated from the *Torpedo* electric organ endplate, an organ that is ontogenetically similar to muscle. There are approximately 50,000 molecules of ACh per vesicle (Hebb, et al., 1958; Gray and Whittaker, 1962; Whittaker, et al., 1972), which is somewhat higher than the number of molecules estimated to be in a released quantum at the neuromuscular junction. The synaptic vesicles in *Torpedo* electric organ are larger than at the neuromuscular junction, this may account for the larger amount of ACh, compared to the size of a quantum at the neuromuscular junction.

If neurotransmitter is released by exocytosis then there must be a morphological correlate to this process. In presynaptic terminals there are occasionally "omega" figures (ω) at the active zone, confirming that exocytosis takes place there. In order for neurotransmitter release to occur by exocytosis, the exocytotic event must occur within the same time frame, and the number of events must correlate with the number of quanta released. This was demonstrated by rapidly freezing the nerve less than three milliseconds after electrical stimulation of the nerve. When the plasma membrane is fractured following stimulation in the presence of 4-amino pyridine, which augments release, a large number of pits in the membrane of the active zone are seen (Heuser, et al., 1979). These pits represent vesicular exocytosis and occur in the same time-frame as neurotransmitter release (Heuser and Reese, 1981). When a synaptic vesicle fuses with the plasma membrane, the intravesicular surface of the vesicle is exposed to the extracellular space. Following massive neurotransmitter release, an intravesicular epitope of a synaptic vesicle protein is exposed, again supporting exocytosis as the mode of release (von Wedel, et al., 1981).

Exocytosis should cause an increase in the area of the plasma membrane. Under normal conditions, following fusion with the plasma membrane, the synaptic vesicle membrane flattens out in a few milliseconds, too rapidly to be detected. When exocytosis is augmented by 4-aminopyridine, vesicle flattening is slowed down and occurs over a period of tens of milliseconds. The resulting increase in membrane surface area is then balanced by endocytosis in the seconds that follow. After exocytosis, there is an increase in the number of large intramembrane particles, presumably derived from vesicular membrane. These particles are concentrated into coated pits away from the active zone. The coated pits are internalized by endocytosis, and uncoated to form new synaptic vesicles. This occurs very rapidly, as there is no decrement of vesicles, even at a release rate of 140 quanta/second (Ceccarelli, et al., 1973). After stimulation in the presence of an extracellular tracer, nearly all of the synaptic vesicles are labelled,

further confirming that vesicles are recycled by endocytosis. It is not clear whether the endocytosed coated vesicles pass obligatorily through an endosome-like structure before becoming synaptic vesicles, or whether they are directly uncoated (Heuser and Reese, 1973). The rapidity of recycling suggests that if they do pass through an endosome stage, it occurs very quickly.

I will summarize the structure and function of the synapse by reviewing the events in the transmission of an impulse from one cell to another across the synapse. The invasion of the action potential from the axon causes the membrane of the presynaptic terminal to become depolarized. This depolarization opens voltage sensitive calcium channels, leading to the influx of calcium into the terminal. The increase in cytosolic calcium increases the likelihood of the vesicle membrane fusing with the plasma membrane, leading to the release of vesicle enclosed neurotransmitter into the extracellular space. The neurotransmitter diffuses across the synaptic cleft and binds to specific receptors on the postsynaptic membrane, causing a response in the postsynaptic cell.

2.3. A Brief Digression: Non-vesicular Release?

There are still gaps in our understanding of neurotransmitter release, and these gaps are large enough to leave a number of scientists unconvinced that neurotransmitter release occurs by exocytosis (for review, see Tauc, 1982). They propose instead the existence of transmembrane "gates", structures that open in response to a rise in intracellular calcium and release a quantum of ACh from the cytoplasm. The synaptic vesicles, if they are present, serve as a reserve to replenish cytoplasmic neurotransmitter. Exocytosis, if it occurs under normal conditions at all, serves to release calcium that was taken into vesicles and occurs after the neurotransmitter has been released. A high molecular weight protein has been isolated that is able to release ACh in a calcium dependent manner from proteoliposomes. However, there is no evidence that this

protein is in the plasma membrane, rather it may serve to transport acetylcholine into vesicles (Birman, et al., 1986).

Cone horizontal cells in the teleost retina, although contributing strongly to the overall electrical activity of the retina, form very few classical synapses. Many of the contacts made by these cells do not resemble synapses in any way (Sakai and Naka, 1986). In the catfish (*Ictalurus punctatus*) these cells are able to release γ -aminobutyric acid (GABA) in the absence of a rise in intracellular calcium, by a mechanism that resembles carrier mediated transport (Schwartz, 1987). Some release of glutamate from cone photoreceptors also appears to occur through a calcium- and synaptic vesicle-independent mechanism. This mechanism of transmitter release cannot be generalized to other synapses for at least two reasons. Firstly, horizontal cells in the frog (*Rana Temporaria*) release GABA by a calcium dependent mechanism (Cunningham and Neal, 1985). Secondly, these cells are unusual in that they tonically release transmitter in the dark. Flashes of light produce a hyperpolarization in the plasma membrane that can last several hundred milliseconds. This type of response to input is in stark contrast to the neuromuscular junction and many other synapses, where all or none action potentials trigger a rapid burst of neurotransmitter release.

The function of synaptic vesicles, in those synapses that have them, is to accumulate neurotransmitter for release by exocytosis. Some of the evidence for this was given above, in describing the experiments that led to this theory of neurotransmitter release. The strongest evidence for this mechanism is as follows. Firstly, neither the transmembrane voltage, nor the cytoplasmic concentration of ACh affects the amount released. If cytoplasmic ACh is released across the plasma membrane, both should affect the size and/or number of quanta released since ACh is a charged molecule (Fatt and Katz, 1952; Dunant, et al., 1977). Secondly, the compound 2-(4-phenylpiperidino) cyclohexanol (AH5183), which blocks ACh uptake into vesicles, blocks calcium dependent release of newly synthesized ACh from *Torpedo*

synaptosomes only when it is added during synthesis, and not if added after synthesis but before release (Bahr and Parsons, 1986; Michaelson, et al., 1986). It is very difficult to explain this result if the cytoplasm is the immediate source for released ACh. Finally, other forms of rapid, regulated release are both calcium dependent and vesicular (Satir, et al., 1973; Zimmerberg and Whittaker, 1985; Patzak and Winkler, 1986).

However, as discussed above, there is good evidence that growth cones release neurotransmitter. It is quite likely that growth cones do not have the same molecules that synapses have, some of which may be responsible for vesicular exocytosis. Neurotransmitter and growth factor release from growth cones could occur through a non-vesicular mechanism.

2.4. Synaptic Biochemistry

A molecular understanding of synaptic function depends upon the identification of the molecules present. With this in mind, procedures were developed to isolate synapses from homogenized brain or other synapse rich tissue. These procedures generally rely on a difference in size or density of synaptosomes when compared to other cellular fractions. In addition, Whittaker and his colleagues developed techniques for the purification of synaptic vesicles from synaptosomes, for the determination of vesicle components. This showed that vesicles from cholinergic synapses have a very abundant low molecular weight protein which they named vesiculin. Due to its high acidity, they proposed vesiculin to be the counter ion to acetylcholine inside the vesicle; although this is an attractive idea, there is no direct evidence that vesiculin is associated with acetylcholine or that a counter ion is necessary for acetylcholine accumulation in vesicles (Whittaker, et al., 1974).

Recently, vesicular purification procedures have been combined with immunological, biochemical, and molecular biological techniques to identify several proteins that are part of, or associated with synaptic vesicles. These include synapsin I, SV2, p65, VAMP1, TAP, and SVP38.

Synapsin I was identified as one of the major proteins in brain that is phosphorylated *in vitro* (Johnson, et al., 1972). It is a doublet of 80 and 86 kD that has a globular head region and a collagen-like tail region. Synapsin I is tightly bound to the cytoplasmic surface of small clear vesicles at almost all synapses in the body, the exception being ribbon synapses in the retina. The affinity of synapsin I for synaptic vesicles *in vitro* is decreased following phosphorylation by cAMP or Ca⁺⁺ dependent protein kinases (Huttner, et al., 1983). Injection of the dephosphorylated, but not the phosphorylated form into the presynaptic terminal of the squid giant synapse inhibits neurotransmitter release (Llinas, et al., 1985). Synapsin I may "tether" synaptic vesicles near the active zone and when phosphorylated, release them to be available for exocytosis.

Little is known about the functions of other synaptic vesicle proteins, which have been identified immunologically. P65 is a 65 kD glycoprotein that spans the membrane of synaptic vesicles in all synapses and neuroendocrine cells (Matthew, et al., 1981). SV2 is a 100 kD synaptic vesicle membrane glycoprotein also present in all synapses, endocrine, and neuroendocrine cells (Buckley and Kelly, 1985). TAP1 (terminal anchorage protein 1) is a high molecular weight proteoglycan that is present in synaptic vesicles as well as in the synaptic extracellular matrix of the electric organ (Carlson and Wight, 1987). VAMP1 is a synaptic vesicle associated protein from electric fish that was identified by using polyclonal antiserum against purified vesicles to screen a cDNA expression library (Trimble, et al., 1988). Finally, a synaptic vesicle glycoprotein of 38 kD was identified in a number of laboratories. This protein will be discussed further in the results and discussion sections.

3. Development

The transformation from growth cone to synapse is very difficult to analyze, as it takes several days and the histological and physiological techniques necessary to visualize each can only provide isolated snapshots.

In cultures of dissociated rat neonatal superior cervical ganglion (scg) cells and embryonic spinal cord, growth cones of the spinal cord contact the scg cells and form stable synapses. Usually a filopodium of the growth cone forms the first contact with the potential postsynaptic cell. In some contacts, the filopodium moves on the surface of the cell, then withdraws and the growth cone continues. Following other contacts, the filopodium remains, and the other filopodia cease to palpate and withdraw into the growth cone. These results indicate that while it is the filopodium which makes the first contact with a postsynaptic cell, the synapse arises from the body of the growth cone. Following the stabilization of this initial contact, the growth cone may extend new filopodia and continue to grow while maintaining the initial contact. During approximately the next 48 hours, a postsynaptic density appears, clear vesicles the size of synaptic vesicles begin to cluster near the postsynaptic density. The cleft between the cells widens, and the presynaptic dense projections (the active zone, discussed above) and synaptic cleft material appear. In the following several days, synaptic vesicles increase dramatically in number, cluster near the active zone, and the pre- and postsynaptic membrane specializations become more pronounced (Rees, et al., 1976).

In the inner plexiform layer of the rhesus monkey retina, the sequence of events in synapse formation have been proposed to be different. Before the growth cone contacts potential postsynaptic cells, there are electron dense membrane thickenings. Serial sections confirmed that these were not contacted by growth cones. The growth cones contact the cells, form presynaptic thickenings, accumulate vesicles, and the synaptic cleft widens (Nishimura and Rakic, 1985, 1987). The same sequence is seen in the rat visual cortex, although serial sectioning was not done to confirm that "unapposed" postsynaptic thickenings were truly not contacted by any part of a growth cone (Blue and Parnavelas, 1983a).

The development of the neuromuscular junction has been studied extensively, both *in vivo* and *in vitro*. Growth cones of embryonic motoneurons release acetyl-

choline spontaneously in the absence of any previous or concurrent contact with muscle cells (Hume and Fischbach, 1983; Young and Poo, 1983). This leads to depolarizations resembling endplate and miniature endplate potentials around the time of the first contact between growth cone and muscle. However, there is far greater variability in the amplitude and time course of the MEPP-like events than later in development, suggesting that the release mechanisms have not fully matured (see also section 1.4). Unlike the CNS synapses discussed above, there are no apparent postsynaptic membrane specializations prior to growth cone contact. The remainder of the ultrastructural maturation occurs similarly: at early stages there is a paucity of synaptic vesicles, then the pre- and post-synaptic membrane specializations appear, synaptic vesicles accumulate, and the synaptic cleft widens and acquires an electron-dense material (Bennett and Pettigrew, 1975; Kullberg, et al., 1977). Neurotransmitter release occurs before the accumulation of synaptic vesicles, suggesting the possibility that the release at immature neuromuscular junctions is non vesicular (see section 1.4). Neurotransmitter receptors are initially distributed randomly over the surface of the muscle, with a few "hot spots" of concentrated receptors (*in vitro*). After contact by the growth cone, clusters of receptors appear below the neuron due to movement of receptors and preferential insertion of newly synthesized receptors. Growth cones do not preferentially synapse onto pre-existing "hot spots".

Most of the studies of synapse formation in the central nervous system *in vivo* indicate that a postsynaptic specialization precedes the presence of the growth cone. This conclusion would be greatly strengthened by the demonstration that these pre-existing postsynaptic specializations are more than just membrane thickenings, for example, that they contained appropriate neurotransmitter or growth factor receptors. In addition, as is the case for the random, pre-existing clusters of acetylcholine receptors on muscles, it is possible that the membrane thickenings present before growth cone contact are unrelated to the thickenings present after contact.

CHAPTER TWO

CD'A 1

The growth cone is a specialized region of the neuron that has properties not expressed, or only weakly expressed, in other parts of the neuron. These include rapid exo- and endo-cytosis, rapid, directed membrane and cytoskeletal movement, and neurotransmitter release. These properties indicated to me that the growth cone would express molecules not found elsewhere in the cell, which would underlie these functions or provide specific regulation. I expected that some of these molecules would also be present in other cells, and some would be present in synapses.

1. The development of a growth cone specific marker

The neuronal growth cone preparation developed by Pfenninger and his colleagues provided the starting point for this aspect of my work (Pfenninger, et al., 1983). This preparation consists of a subcellular fraction from homogenized embryonic rat brain that has a fine structure resembling pinched-off growth cones. The plasticity of growth cone structure might make them very labile during homogenization, and the reliability of using morphological criteria for identification is unclear. However, without any molecular markers specific for growth cones, this is the only criteria that is available. I felt that the preparation was too crude to investigate using classical biochemical methods, and I therefore chose to use an immunological approach to search for specific molecules. This approach has been successful in a variety of similar situations (Barnstable, 1980; Arimatsu, et al., 1987). For this purpose, even if the "growth cone preparation" provided a two-fold enrichment for growth cones, it was an improvement over unfractionated membranes. I therefore immunized mice with this fraction and prepared monoclonal antibodies.

I screened two fusions biochemically, comparing the binding of antibodies to the growth cone fraction with that to an adult synaptosome fraction. This yielded eight

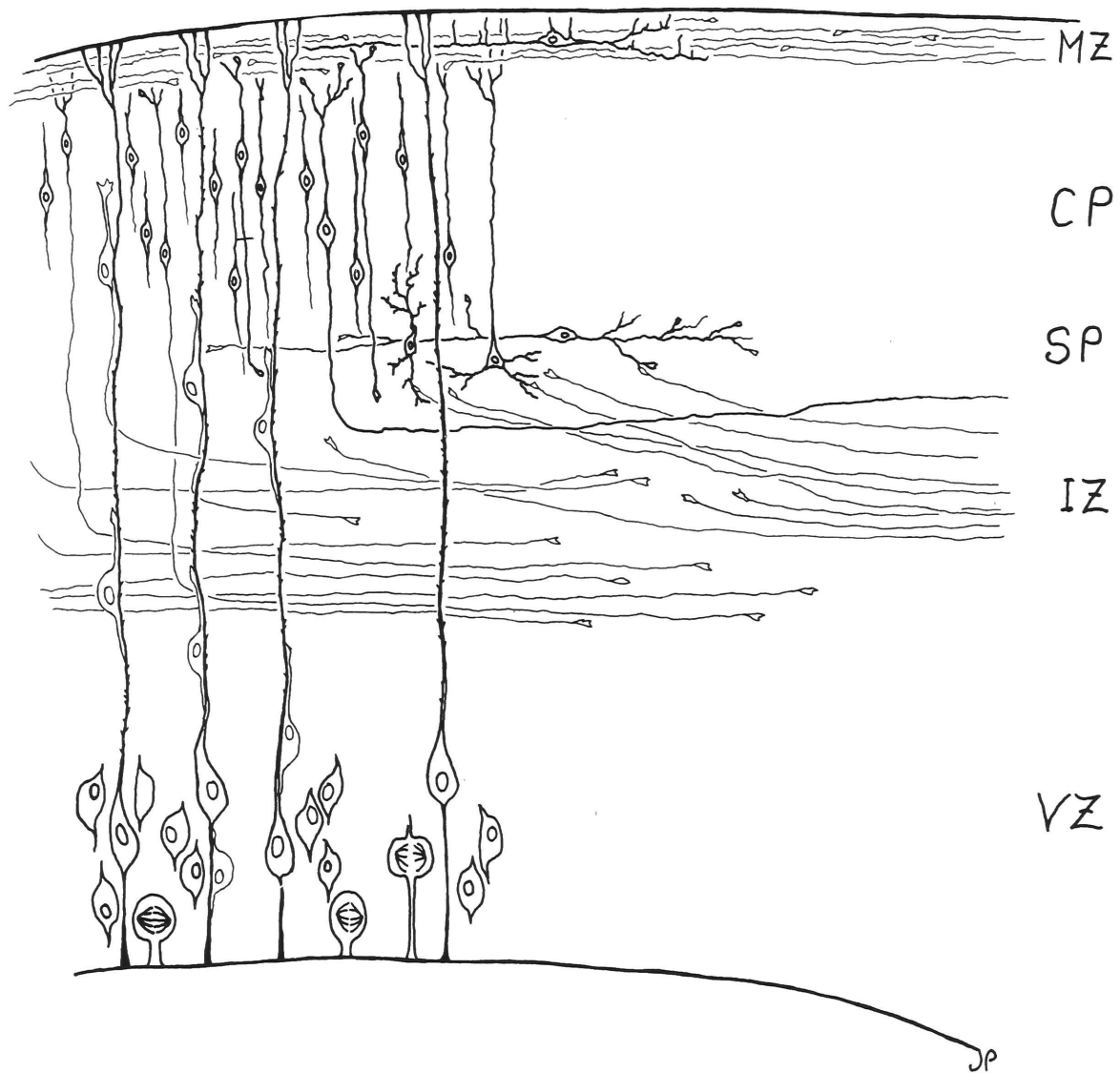
monoclonals that bound to "growth cones" but not to synaptosomes. Unfortunately, only two of these recognized any antigens in paraformaldehyde fixed tissue sections. One stained radial glia and the other stained long axons in the intermediate zone (not shown). Without immunohistochemical information about the other antigens, it was impossible to know if they were growth cone specific, given the uncertainties about the "growth cone preparation".

I therefore decided to screen the subsequent fusion immunohistochemically on embryonic brain, because this would immediately provide information on the distribution of the antigen. The positive antibodies were then tested on sections of adult brain, in order to identify developmentally regulated, and those that were present in both immature fibers and synapses. Finally, any antibodies that were interesting in any way I decided to screen on cultures of embryonic neurons, where the subcellular distribution of staining was readily interpretable.

The growth cone preparation that I immunized with was largely from E17 cerebral cortex, hence this was the tissue I used for screening. Its relatively large size and accessibility at all ages facilitated both the preparation of many tissue sections and a biochemical analysis. In addition, the E17 cerebral cortex is an informative tissue in which to immunohistochemically screen potential growth-related antibodies, since at young ages it is divided into a few relatively homogeneous zones, shown schematically in Figure 1. In the E17 rat cortex, the ventricular zone contains dividing cells that will migrate away from the ventricle after their final mitosis. These cells assume their final position in the cortical plate, where they begin to elaborate dendrites and axons. Their axons enter the intermediate zone, *en route* to extracortical targets. The intermediate zone and marginal zone are also full of fibers growing into the cortex, from the thalamus, the contralateral hemisphere, and other subcortical areas. The subplate and marginal zone are specialized regions of the developing cortex that contain the first neurons to migrate out of the ventricular zone, as well as a large population of incoming

Figure 1. Schematic drawing of the major elements of the cerebral cortex on day E17 in the rat.

The ventricle is at the bottom, and pia at the top of the figure. Neuroblasts are dividing in the ventricular zone (VZ). Postmitotic neurons become attached to radial glia and migrate outward to the cortical plate (CP), where they detach from radial glia and elaborate dendrites. Axonal growth probably begins prior to the completion of migration. The subplate (SP) and marginal zone (MZ) contain relatively mature cells that receive early synaptic connections (in the cat and monkey, this has not been investigated in the rat). The intermediate zone (IZ) contains incoming and outgoing axons. Growth cones can be found in the IZ, the MZ, and the SP. There seems to be a segregation of cortical afferents to the upper layers of the IZ and the SP, and cortical efferents to the lower layers of the IZ (personal observations).



fibers that have not yet grown into the cortical plate. These neurons are unusually mature, receiving synapses many days before other cortical cells (Boulder Committee, 1970). On E17, any antibody that selectively binds to growing axons should label the intermediate and marginal zones, and not the ventricular zone or the cortical plate.

TABLE I

	<u>General E</u> <u>General A</u>	<u>Selective E</u> <u>Selective A</u>	<u>Selective E</u> <u>General A</u>	<u>General E</u> <u>Selective A</u>	<u>Selective E</u> <u>Negative A</u>
Number	105	13	18	16	25

The results of the immunohistochemical screen are shown in Table I, where E refers to the staining in E17 cortex, and A refers to the staining in adult cortex. An antibody was classified as selective if it did not stain all regions of the cortex. The majority of antibodies stained all cells in E17 cortex, as well as all cells in adult cortex, these are presumably recognizing "housekeeping" molecules. The remainder gave selective or exclusive staining of either the embryonic or the adult cortex. The staining of cells in culture was very valuable for identifying the distribution of antigen. Neurons and glia could be readily differentiated, and cell bodies visually separated from neurites and growth cones. A number of antibodies seemed to selectively stain the intermediate and marginal zones of the embryo and the neuropil of the adult. However, in tissue culture it was clear that neuronal membrane was uniformly stained. The apparent selectivity *in situ* is probably due to the heavy concentration of membranes in those regions.

It is very difficult to use the results of an immunohistochemical screen to make any statements about the purity of the growth cone preparation I used to immunize. Even if the growth cone preparation were 99.9% pure growth cones, an immunodominant epitope in the 0.1% impurity would skew the proportion of antibodies. There was some indication that certain antigens were immunodominant. At least four of the

antibodies recognized NCAM, indicated by the characteristic change in molecular weight during development (not shown). There were also antibodies against the Jones epitope and VC1.1, based on a lipid and a protein immunoblot, respectively, and on their staining pattern. Jones is a ganglioside on migrating cells and fibers, and is also present in a gradient at certain stages of retinal development (Blum and Barnstable, 1987). VC1.1 is present throughout the developing cortical neuropil during the time of synapse formation, and then becomes restricted to a subset of GABAergic cells in the cortex (Naegelé, et al., 1988).

One of the antibodies that I produced stained in a pattern suggesting that only the growth cone region of cells was labelled. In one to two day old cultures of E16 cortical neurons, this antibody stained streaks that started near the tips of growth cones, and extended to varying lengths back into the neurites for different growth cones (figure 2). There was no detectable staining in neuronal cell bodies; it was rarely seen in neurites or flat cells, and only when a growth cone from another cell may have been growing alongside. I have named the antibody and the epitope that it recognizes CD'A 1, after *cone d'accroissement*, the first published name for growth cones (Ramon y Cajal, 1890a).

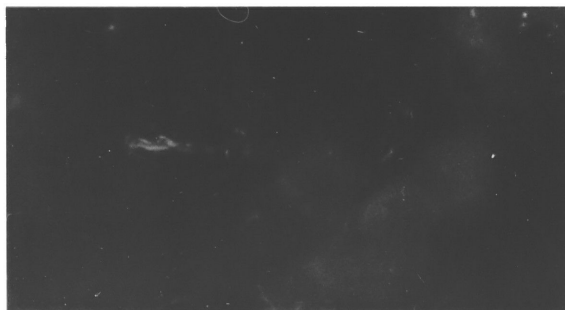
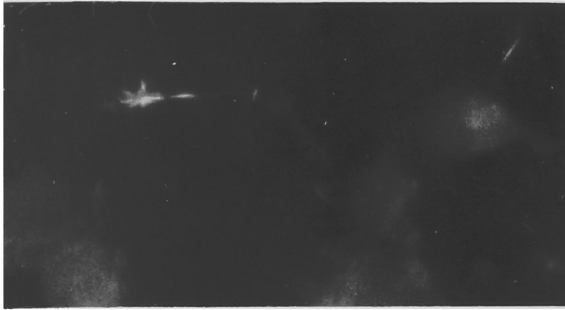
2. Distribution of CD'A 1 in situ

Figure 3A shows a low power view of E17 cortex, stained for CD'A 1. Staining was seen in the intermediate and marginal zones, where incoming and outgoing fibers are concentrated, while the ventricular zone and cortical plate had little labelling. A higher magnification of staining in the subplate region showed that CD'A 1 stained very narrow streaks, less than 1 μm wide and about 5 μm long (figure 3B). The streaks are not a consequence of the sectioning of longer labelled processes, most streaks could be traced to their termination within the section. The staining abruptly ended at the end of the streaks, making it impossible to know even which direction the growth cone was pointing. Many of the streaks appeared bifurcated or paired. These may have been part

Figure 2. CD'A 1 stains growth cones *in vitro*.

E16 cerebral cortices were dissociated and cultured for 1 to 3 days before fixation and staining. Phase (right) and CD'A 1 (left) photomicrographs are shown. CD'A 1 stains radial streaks that extend from approximately the tip of the growth cone back to varying lengths into the most distal portion of the neurite. Staining is confined to the growth cone and is not seen in neurites or cell bodies. Bar, 7.5 μm .

CD'A 1



Phase

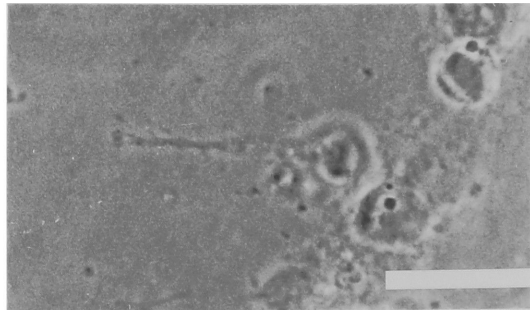
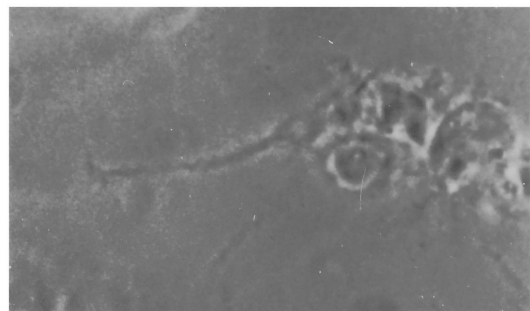
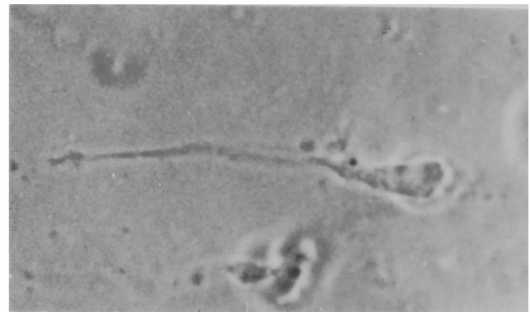
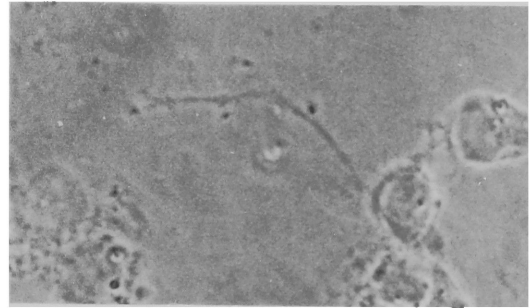
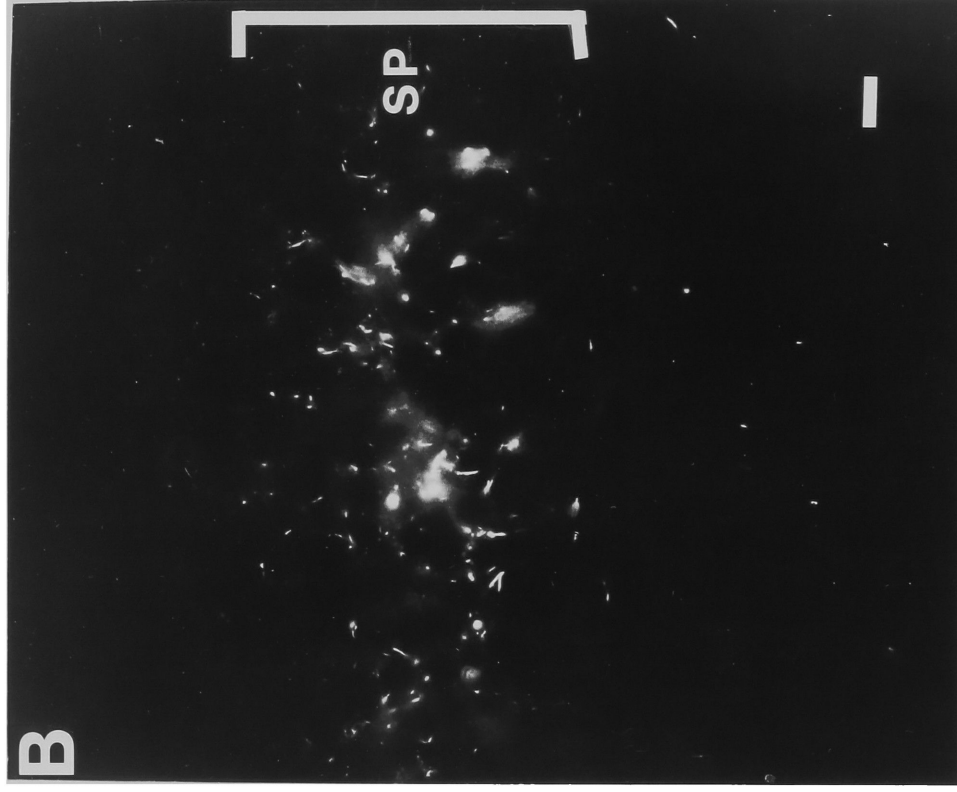
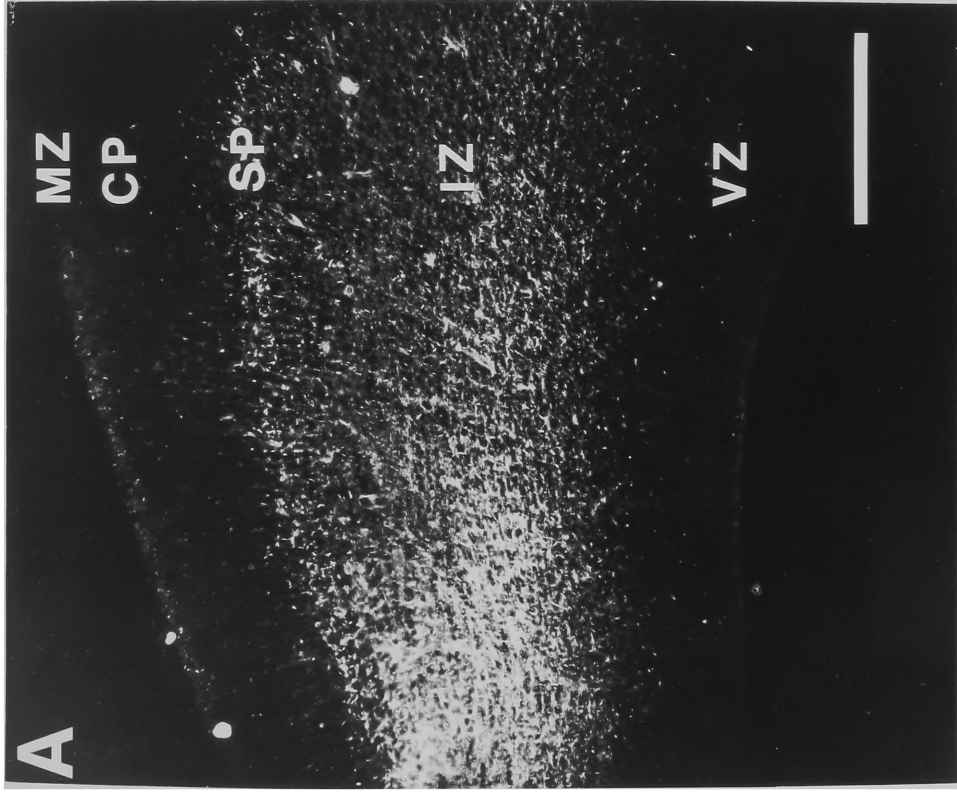


Figure 3. The distribution of CD'A 1 labelled growth cones in the embryonic cortex.

A. Frontal section, showing lateral cortex. Most of the staining is in the intermediate zone (IZ) and the subplate (SP), with a few labelled growth cones in the marginal zone (MZ). The ventricular zone (VZ) and cortical plate (CP) show very little labelling. For explanation of abbreviations, see Figure 1. Bar, 200 μm .

B. Higher magnification of labelled growth cones in the subplate. The growth cones appear as streaks, some of which appear paired or bifurcated. Most of the streaks end within the section and do not reflect the sectioning of a longer process. Bar, 10 μm .



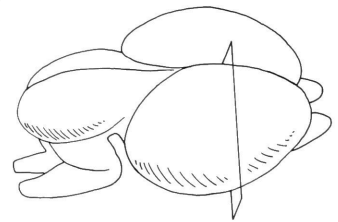
of the same growth cone, as this was also seen *in vitro* within single, well spread growth cones. The overall distribution and dimensions of these streaks are consistent with their identification as growth cones.

I examined staining in other areas of the brain to determine if all growth cones were stained by CD'A 1, or only a subpopulation of growth cones are stained. Labelled growth cones were found in most areas of the developing brain. A *camera lucida* drawing of a CD'A 1 stained section at the level of the developing caudate putamen is shown in Figure 4. The distribution of labelling matched the expected distribution of growth cones at this age. For example, in the developing cortex, more staining was seen laterally in the intermediate zone, while medially most of the staining was in the marginal zone. However, not all growth cones in the embryo are stained by the CD'A 1 antibody; at no stage in the development of the optic nerve or the optic nerve fiber layer in the retina have I been able to find labelling of the growth cones of retinal ganglion cells (data not shown).

The intensity of staining in the growth cone and the complete lack of immunoreactivity in the rest of the processes made it impossible to determine where the cell body of any of the growth cones was located. It was also difficult to demonstrate that all of the growth cones in the section were labelled, or even if certain subclasses were labelled at all. The distribution of labelled growth cones in the cortex matches very closely what is known of the distribution of immature axons, and it is therefore likely that many, if not all, of these growth cones are labelled. There are some dendritic growth cones in the cortical plate and marginal zone. Whether these are labelled, or the cortical plate staining represents a few incoming axons, is impossible to determine without a separate label that permits identification of either dendritic or axonal growth cones. Similarly, there are very rare labelled structures in the ventricular zone. These are probably growth cones of intermediate zone axons which have grown into the ventricular zone. They could be either "normal" axons which have not been previously

Figure 4. Growth cones throughout the developing brain contain CD'A 1.

Low power view of a section through E17 telencephelon. This is a camera lucida tracing of a labelled section at the level of the developing striatum. All of the streaks have been traced to their accurate length, however, they are drawn and photographed thicker than the scale would indicate. Medial (M), Lateral (L), dorsal (D), and ventral (V), are indicated. Bar, 500 μ m.



characterized, or intermediate zone axons that have become misrouted. Alternatively, the staining could be on the tips of migrating neurons or on a motile nonneuronal cell that has a structure resembling growth cones (see section 5).

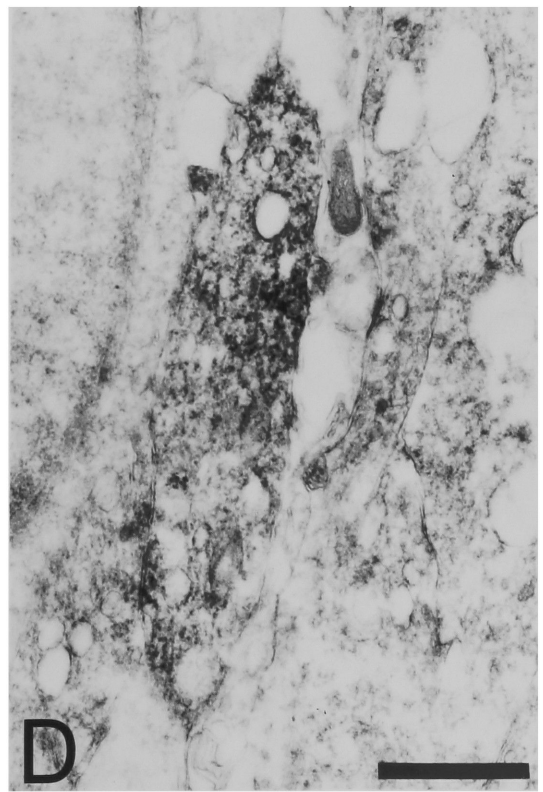
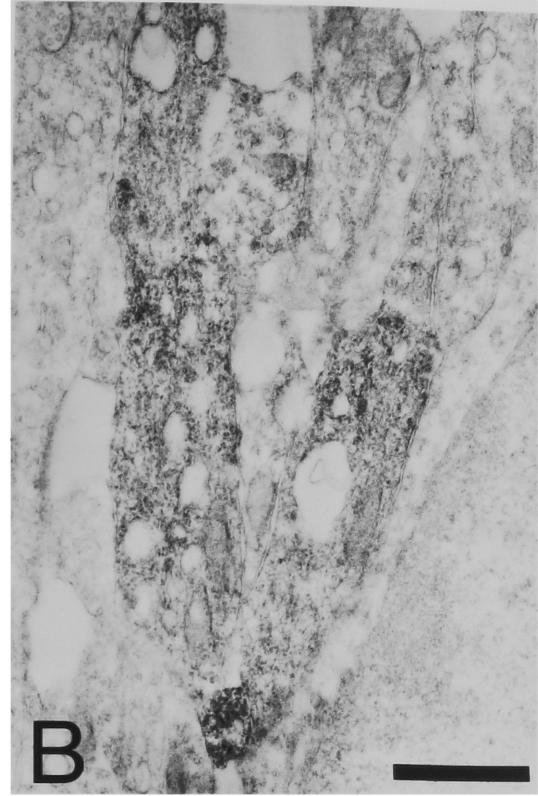
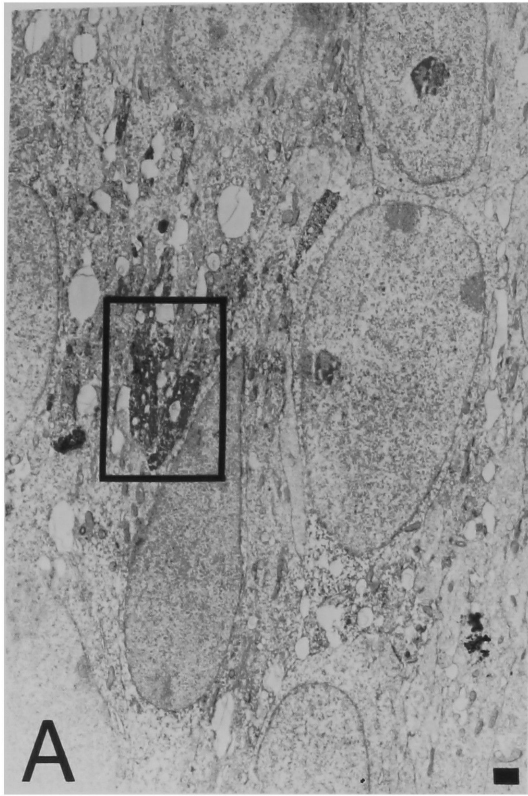
3. *CD'A 1 ultrastructural localization*

To determine more precisely the subcellular localization of the CD'A 1 antigen, I used electron microscope immunohistochemistry on newborn cerebral cortex. The indirect immunoperoxidase method on vibratome sections with CD'A 1 as the primary antibody gave a labelling pattern at the light level identical to that seen with immunofluorescence. There was no specific labelling when the primary antibody was omitted, or when the RetP1 antibody, which recognizes rhodopsin, was substituted for CD'A 1 (not shown). When CD'A 1 labelled sections were processed for electron microscopic observation, the peroxidase reaction product appeared to be exclusively cytosolic. Figure 5 shows stained structures from the cortical plate of P1 cortex. Unfortunately, CD'A 1 was sensitive to fixation in glutaraldehyde, and the ultrastructural preservation consequently was not precise enough to determine if the immunoreactivity was associated with any substructure in the cytoplasm.

Most of the labelled profiles were expanded at one end, were devoid of microtubules, and contained large clear vesicles from which the reaction product was excluded. The reaction product appeared to uniformly fill the cytoplasm, and was always excluded from vesicles. In some sections, the reaction product seemed to disappear rather abruptly within the labelled profile. There was no indication of a cellular structure that might be responsible for this apparent border between labelled and unlabelled cytoplasm. In general, the labelled profiles matched other published ultrastructural descriptions of aldehyde fixed, unambiguously identified growth cones *in vivo* (Bovolenta and Mason, 1987).

Figure 5. Ultrastructural immunohistochemistry of P1 cortex shows CD'A 1 to be cytosolic.

Electron dense reaction product is enclosed within amorphous membrane profiles, and excluded from large clear vesicles. These stained growth cones are from the cortical plate region of neonatal cortex. The labelled growth cone in figure A,B may correspond to a structure similar to the bifurcated growth cones seen in immunofluorescence (as in Figure 2B). Bar, 2 μm .



4. Subcellular fractionation.

CD'A 1 appeared to be most abundant in the cortex on E19. In order to probe the molecular nature of the CD'A 1 antigen, I examined this tissue by standard immunochemical methods. E19 cortex was homogenized and separated into cytoplasmic and a crude membrane fraction. When these fractions were separated on either a nonreducing or a reducing gel, blotted to nitrocellulose filters, and probed with the CD'A 1 antibody followed by an HRP-conjugated secondary, no signal was observed. This was also true when a rabbit anti-mouse antibody conjugated to sepharose beads was used with CD'A 1 in an immunoprecipitation experiment. Surprisingly, when crude membranes or cytosol were used in a dot immunobinding assay or a radioimmunoassay, no detectable signal was obtained. Results presented below suggest that the reason why standard immunochemical techniques have failed to provide information about CD'A 1 is that the epitope is strongly dependent on fixation and the conformation of the antigen.

In order to assay subcellular fractions for the presence of CD'A 1, I therefore used an immunocytochemical assay on agarose embedded membranes (De Camilli, et al., 1983). In this technique, membrane fractions from a subcellular fractionation of E19 cortex were fixed, then mixed with molten agarose. The agarose was pipetted into the frame shown below table II. Following cooling of this mixture, the result is a thin wafer of membranes embedded in agarose. This can be stained in the same manner as a tissue section. In the unseparated homogenate (H), the CD'A 1 labelled profiles appeared as dots and dashes among many unlabelled membranes. Following low speed centrifugation to pellet the nuclei and unbroken cells, a moderate speed centrifugation was used to prepare a crude membrane fraction (P2). CD'A 1 was present in this fraction, which contains plasma membrane vesicles that have resealed around cytoplasm following homogenization. Membrane and cytosol can be separated from this fraction by hypotonic lysis, followed by high speed centrifugation. When this was

done, CD'A 1 was lost from the membrane fraction, indicating that it is not a membrane associated molecule, and is likely cytosolic. Antibody 4F1 identifies a membrane antigen (S.D., unpublished observations), that was unaffected by hypotonic lysis. These experiments independently confirm the conclusions of the electron microscope immunocytochemistry. The results are shown in Table II.

The above experiments were all carried out on membranes that had been fixed prior to embedding. If the same technique was used on unfixed membranes, CD'A 1 immunoreactivity was not present. This was not due to leakage or modification of the antigen, since incubation of unfixed embedded membranes for the same length of time before subsequent fixation did not remove immunoreactivity (not shown).

The dependence of the CD'A 1 epitope on fixation was confirmed with immunohistochemistry of cryostat sections of E17 cortex. There was no CD'A 1 immunoreactivity in cryostat sections of unfixed E17 cortex, nor was there any when sections were fixed with glutaraldehyde, picric acid, cold methanol, cold acetone, and cold ethanol (not shown). No other antibody that we tested, including 4F1, showed such a dependence on fixation.

I believe that the CD'A 1 antibody is recognizing an epitope that is "created" by fixation. The epitope may include an aldehyde group from the paraformaldehyde reaction. Alternatively, the epitope may be exposed by paraformaldehyde induced denaturation. However, even if these explanations are true, the antibody is demonstrating that there is a unique feature in neuronal growth cones. This may be a specific protein that is restricted to growth cones.

5. CD'A 1 staining in fibroblasts.

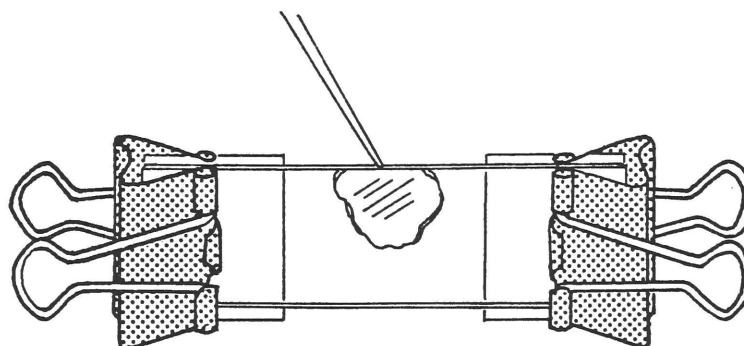
Neuronal growth cones have been frequently compared to other motile cells, ever since Ramon y Cajal described them as "amoeboid-like." Their behavior and structure have some parallels with fibroblasts and immune cells, which are highly locomotory *in vitro* and in certain conditions *in vivo*. To test whether these similarities

Table II. The CD'A 1 epitope is cytosolic and fixation dependent. Unless otherwise noted, each membrane fraction was fixed with 4% paraformaldehyde, mixed with molten agarose, and pipetted into the frame shown below. When the agarose had hardened, it was treated for immunocytochemistry in the same manner as a vibratome section.

	CD'A 1	4F1
H^a	+	+
S1	+	+
P2	+	+
L	-	+
LP	-	+
P2 unfixed^b	-	+

^aFor abbreviations, see Experimental Procedures. Each of these fractions was embedded in agarose as described above and in Materials and Methods.

^bImmunohistochemistry was carried out on an embedded, but unfixed P2 fraction. If an embedded, unfixed P2 fraction was incubated for the same time and under the same conditions as immunostaining, and then fixed and immunostained, CD'A 1 immunoreactivity was present.



extend to the CD'A 1 epitope, I examined whether CD'A 1 is also present in fibroblasts. I used the normal rat kidney cell line (NRK) for these experiments. This is an immortalized, but not transformed fibroblastic cell line (De Larco and Todaro, 1978). The same staining patterns were seen in rabbit embryo fibroblasts.

There were three types of staining in fibroblasts. Most mitotic cells were labelled, as shown in figure 6a-c. The chromosomes have been stained with DAPI and appear blue, and CD'A 1 has been visualized with a rhodamine conjugated secondary antibody and appears red. The staining was present as radial streaks at the poles of the dividing cells, and in cytokinesis often in the contractile ring at the equator (figure 6c). Cells in all stages of mitosis expressed CD'A 1. In addition, there were very rare cells that had bright rods of CD'A 1 in the cytoplasm or even the nucleus (figure 6d). There were no other features of these rare cells that differentiated them from unlabelled cells.

A third class of stained fibroblasts had the morphology of migrating cells (Figure 7). Often, both the leading edge, consisting of a broad lamellipodium, and the trailing process had CD'A 1. The leading lamellipodia of motile fibroblasts resemble growth cones in many ways, including the presence of CD'A 1. However, there were consistent qualitative differences in the distribution of CD'A 1 in growth cone and the leading edges of fibroblasts. In growth cones, CD'A 1 was invariably arranged in radial streaks (see figures 17-19), whereas in fibroblasts, CD'A 1 was more diffusely distributed, often in a more tangential manner.

When fibroblasts are confluent in culture, they cease to move and divide, a phenomenon known as contact inhibition. Their motility is rapidly re-expressed when new space becomes available, as at the edge of an *in vitro* "wound," where fibroblasts have been scraped off the dish. CD'A 1 is present primarily at the edge of the "wound," in the leading edge of the migrating cells (Figure 8).

Figure 6. CD'A 1 labels mitotic cells and occasionally stains nuclear rods.

The chromosomes have been labelled with DAPI and appear blue, while CD'A 1 has been visualized with a rhodamine-labelled secondary antibody and appears red.

A. The chromosomes have condensed during metaphase. CD'A 1 is present in radial streaks in the periphery of the cell. The staining in the cell to the left of the mitotic cell may be associated with cell motility, similar to the cells shown in Figure 7. Bar, 25 μm .

B. The chromosomes are separating in telophase. There is bright CD'A 1 immunoreactivity primarily at the poles of the cells. Bar, 25 μm .

C. These cells are at the end of cytokinesis, almost fully divided. CD'A 1 continues to be present at the poles of the cell (some of this polar labelling was in a different plane of focus). There is a punctate ring of staining at the contractile ring between the dividing cells. Bar, 25 μm .

D. A rather ominous example of CD'A 1 staining. This is an example of a very rare cell that has CD'A 1 actually enclosed within the nucleus. There were no other features of this cell that differentiated it from other unlabelled cells. Bar, 7 μm .

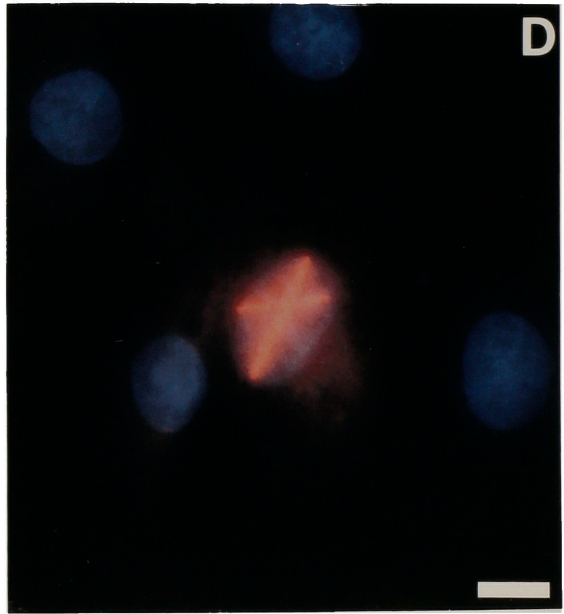
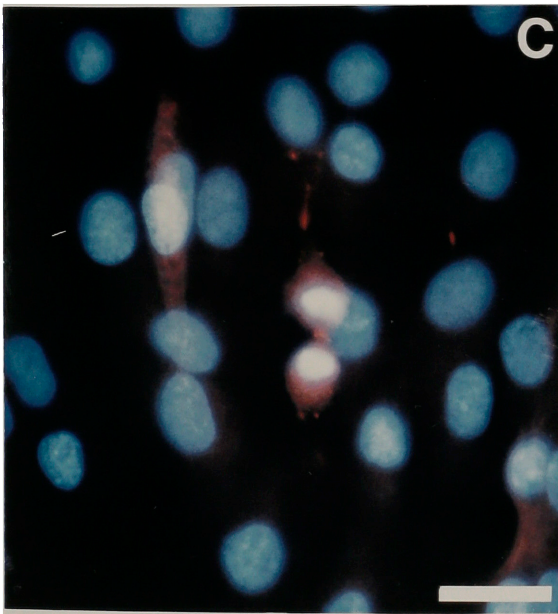
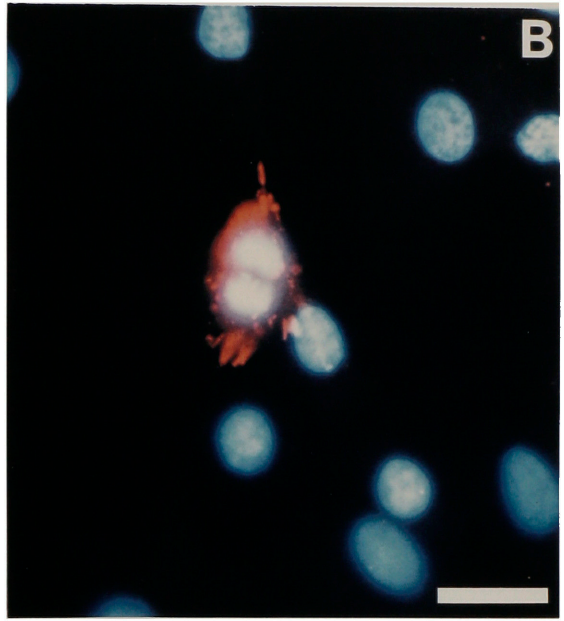
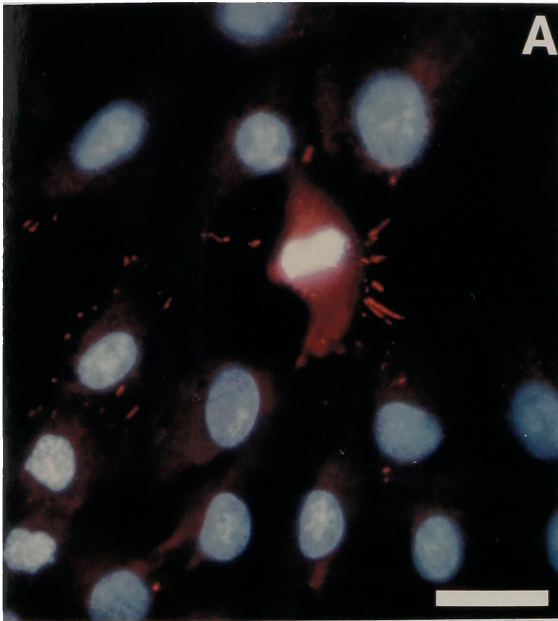


Figure 7. CD'A 1 stains motile fibroblasts.

Double labelled cells are shown, stained with phalloidin (A, C) or an actin antibody (E) on the left and CD'A 1 (B, D, F) on the right. When phalloidin staining was carried out, phalloidin was always added after the CD'A 1 incubation was complete.

A, B. The vast majority of the phalloidin labelled stress fibers are not immunoreactive for CD'A 1. CD'A 1 is at the periphery of the cell, in regions that do not contain stress fibers.

C, D. This cell is probably beginning mitosis, and is similar to the cell shown in Figure 6A. F-actin is present very diffusely throughout the cell.

E, F. These cells have the classic morphology of *in vitro* moving fibroblasts, in this case their direction of movement would be up. CD'A 1 is primarily in the leading lamellipodia and in the trailing process of the cell at the right. Although actin is in the same regions as CD'A 1, actin has a much more widespread distribution. Bar, 50 μm

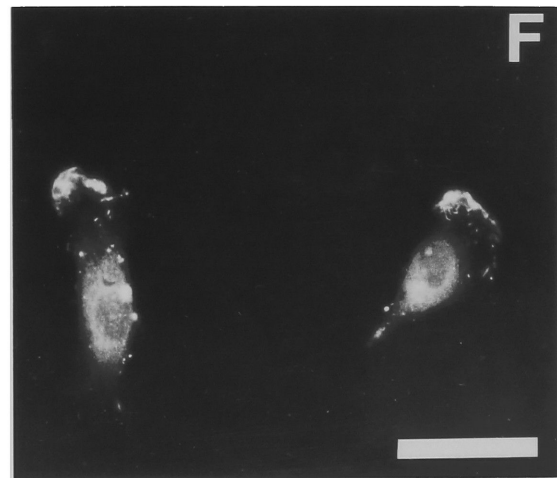
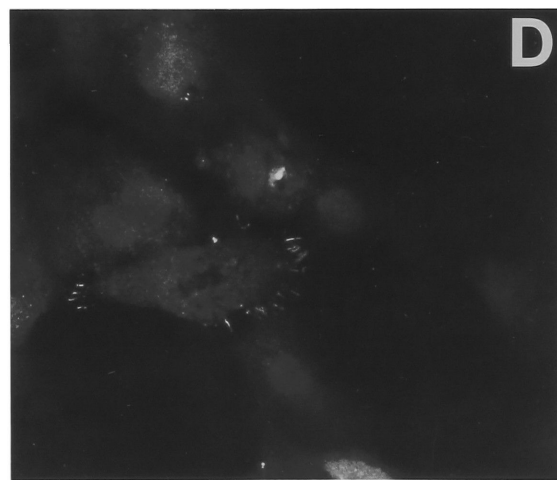
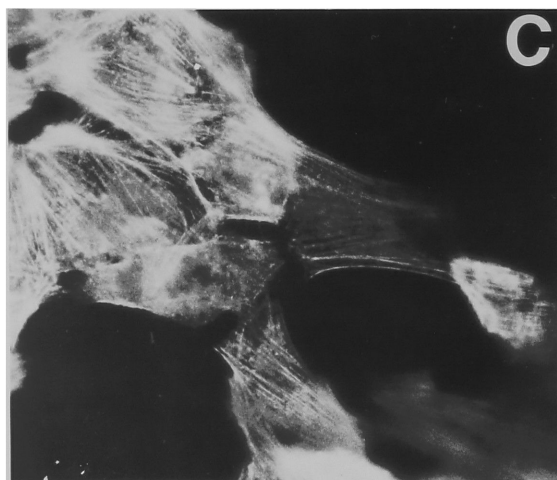
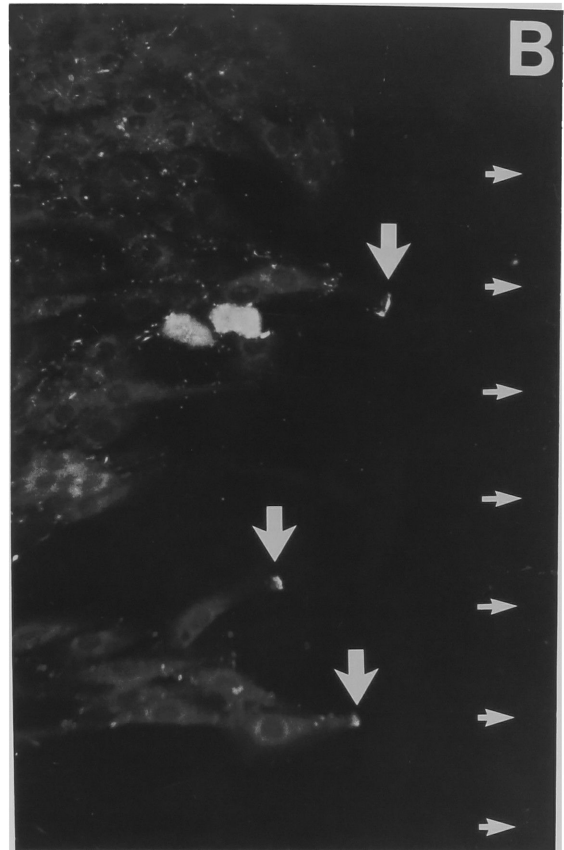
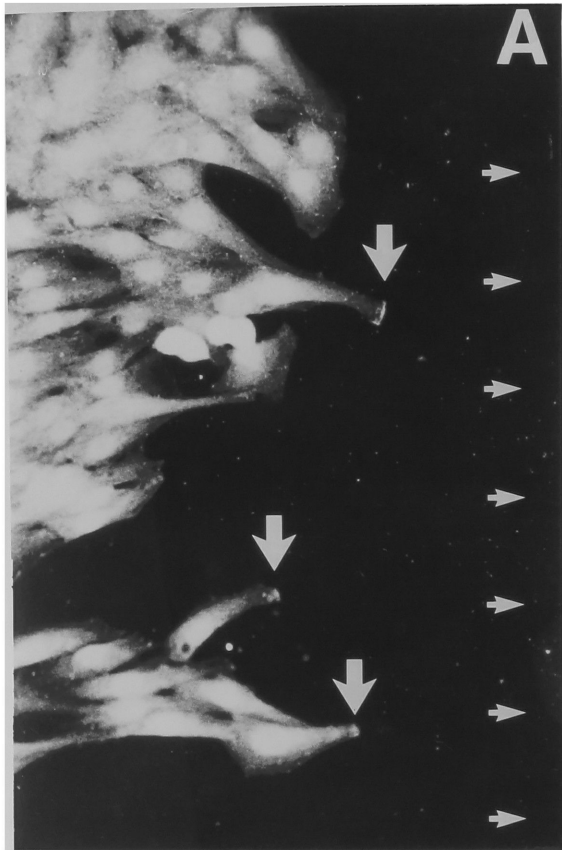


Figure 8. CD'A 1 stains the edges of migrating fibroblasts.

Double labelled cells are shown, stained on the left for actin (A) or phalloidin (C), and on the right for CD'A 1 (B, D). These cultures were "wounded" by drawing a polished pipette across the dish one day before staining was carried out. The row of arrows indicates the direction of movement. CD'A 1 is present at the leading tips of the motile fibroblasts (vertical arrows in A, B, and horizontal arrows in C, D). These regions of the cell also have actin (A), it seems to be arranged as F-actin (C), but not bundled into stress fibers. The brightly stained cells to the left of the upper vertical arrow in B are probably in mitosis. Bar, 100 μm



6. Actin association.

Both the leading edge of fibroblasts and growth cones have many actin filaments, arranged as a loose mesh apparently crosslinked to each other and to the plasma membrane. Phalloidin is a mushroom toxin that binds specifically to actin filaments. In cultured hippocampal neurons, phalloidin labelled F-actin and CD'A 1 have a remarkably similar distribution (see figures 17-19). In fibroblasts, the phalloidin stained filaments were far more extensive than CD'A 1 was (figures 7 and 8). Stress fibers, composed of many actin filaments bundled together, were stained with phalloidin, but were not labelled by CD'A 1.

Cytochalasin B (CB) disrupts actin filaments both *in vitro* and *in vivo*. The addition of CB to cultured hippocampal neurons abolished CD'A 1 immunoreactivity from the growth cones, indicating that CD'A 1 is tightly associated with actin microfilaments. In addition to eliminating the CD'A 1 epitope from the growth cone, CB treatment caused the appearance of CD'A 1 positive rods in the perinuclear region of the cell (Figure 9). The rods were approximately the same shape and size of the rods seen in the growth cones, of untreated cells. Due to the distance from growth cone to cell body, and the rapidity with which the cytoplasmic and nuclear rods appear, I believe it likely that the cell body rods were not the result of a translocation from the growth cones.

Cytoplasmic and nuclear actin containing rods have been seen in cytochalasin treated, DMSO treated, and heat shocked cells by other investigators (Rathke, et al., 1977; Fukui, 1978; Welch, et al., 1985; Fukuda, et al., 1987; Sanger, et al., 1980). In order to determine if these rods were the same as the CD'A 1 rods, I performed CD'A 1 and actin double labelling in cytochalasin and DMSO treated fibroblasts. All of the visible actin rods contained CD'A 1. The converse was probably also true, however the actin staining did not reveal rods in regions of the cells that also had high levels of soluble actin. These results are shown in Figure 10.

Figure 9. Cytochalasin B eliminates growth cone and lamellipodia staining and causes the appearance of nuclear and perinuclear CD'A 1 rods.

Phase contrast (A, C, E), and fluorescence micrographs of hippocampal cells showing CD'A 1 staining (B, D, F) are shown. Staining is completely lost from the lamellipodia (closed arrows) and growth cones (open arrow). Instead, brightly stained rods have appeared in the nucleus and perinuclear region. These cells were treated with 10 $\mu\text{g/ml}$ cytochalasin B for 30 minutes, then fixed and stained. Photos are at 560X magnification.

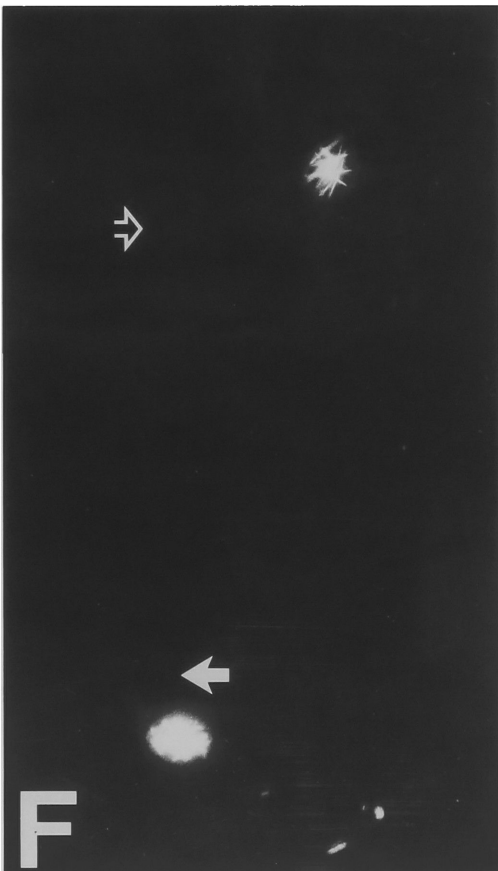
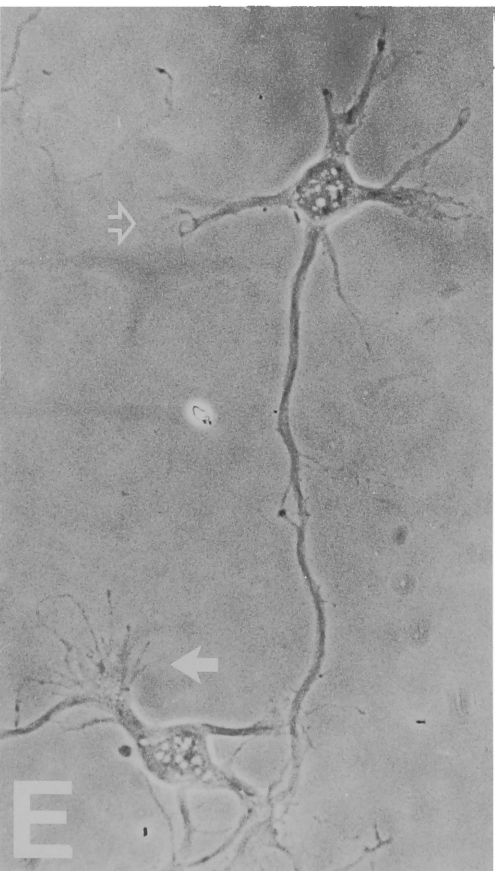
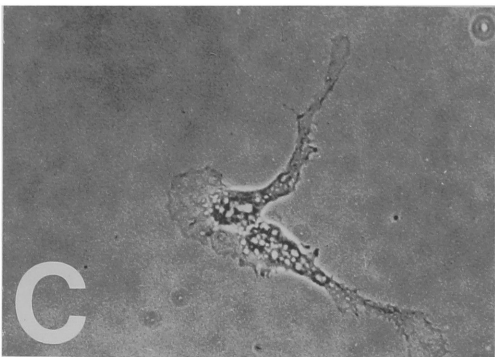
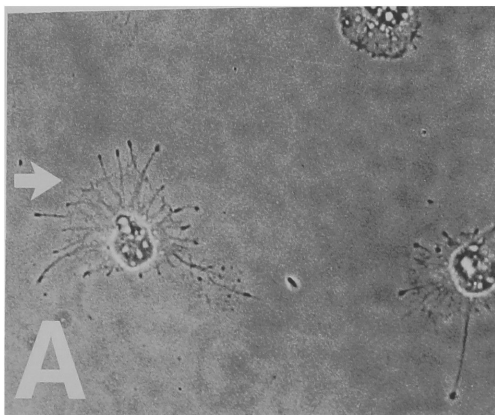
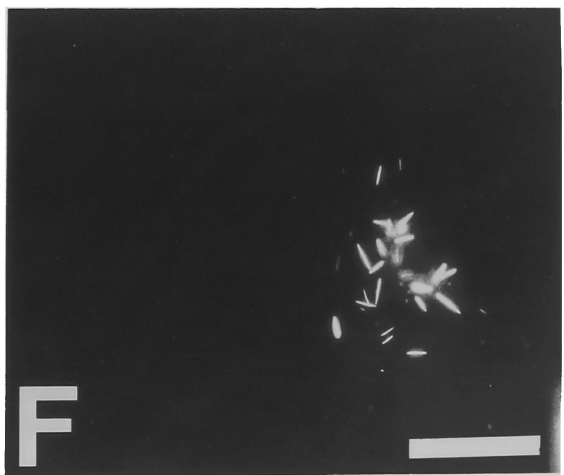
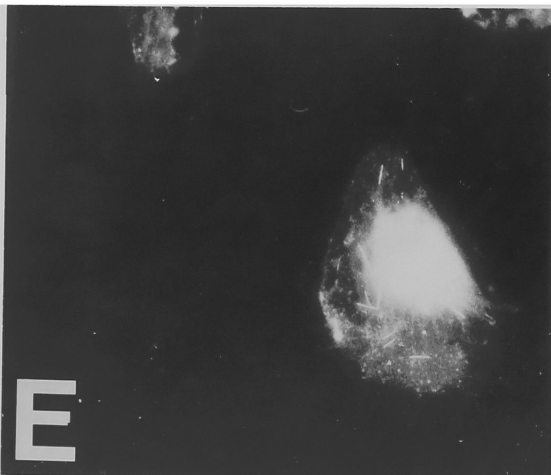
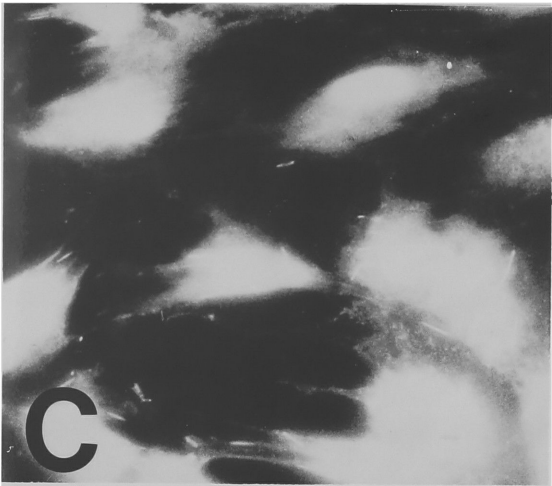
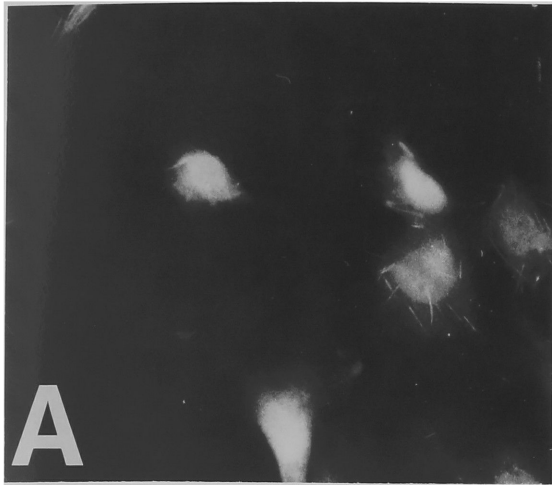


Figure 10. Cytochalasin induced CD'A 1 rods in fibroblasts contain actin.

Cells have been double labelled for actin (A, C, E) on the left and for CD'A 1 (B, D, F) on the right. In regions of the cell where there are low levels of cytosolic actin, it is possible to see that the CD'A 1 stained rods also stain for actin. The high levels of cytosolic actin make it impossible to determine if all CD'A 1 positive rods contain actin. Bar, 25 μm .



Cofilin is a 21kD actin binding protein isolated from adult brain that is also a striking component of DMSO and cytochalasin induced actin rods (Nishida, et al., 1987). Cofilin binds to F-actin in a 1:1 ratio of cofilin to monomer in the filament, and shortens the average length of the filament (Maekawa, et al., 1984). I tested whether cofilin and CD'A 1 were colocalized in NRK fibroblasts. In the untreated cells, cofilin was concentrated in lamellipodia, but it was broadly distributed in all cells. CD'A 1 was only present in fibroblasts that had the morphology of motile cells, and was much more heavily concentrated in specific regions of the cells, as shown above. There was no preferential association of cofilin with mitotic cells (data not shown).

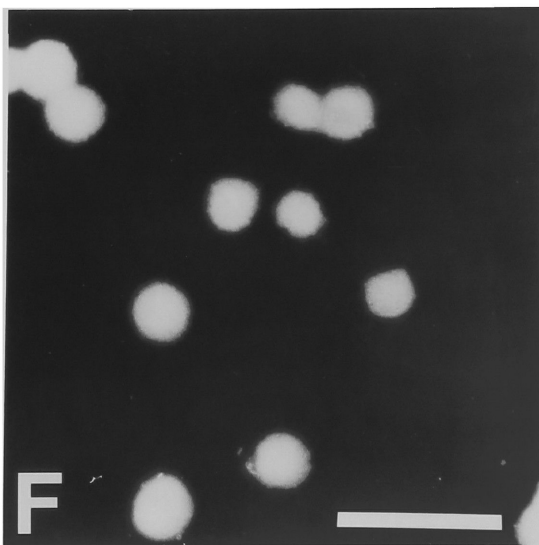
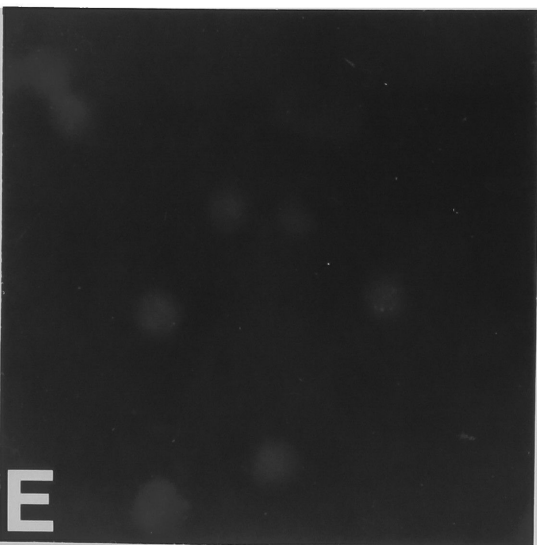
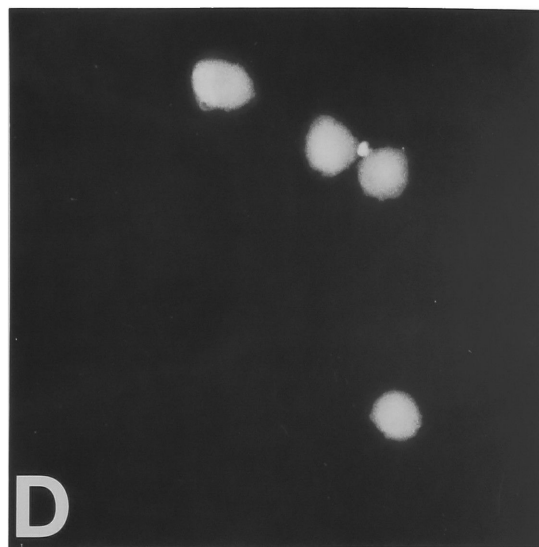
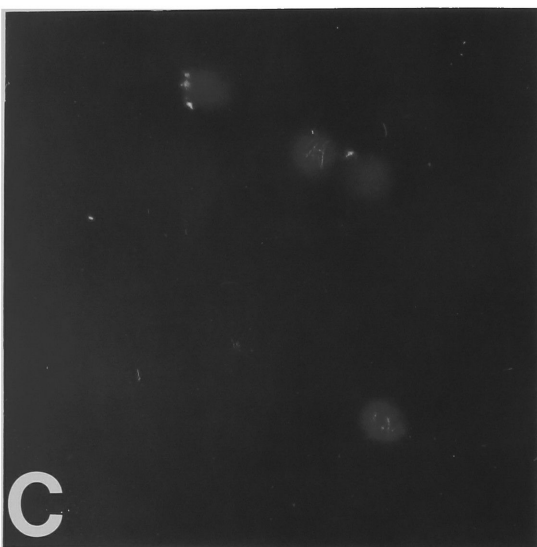
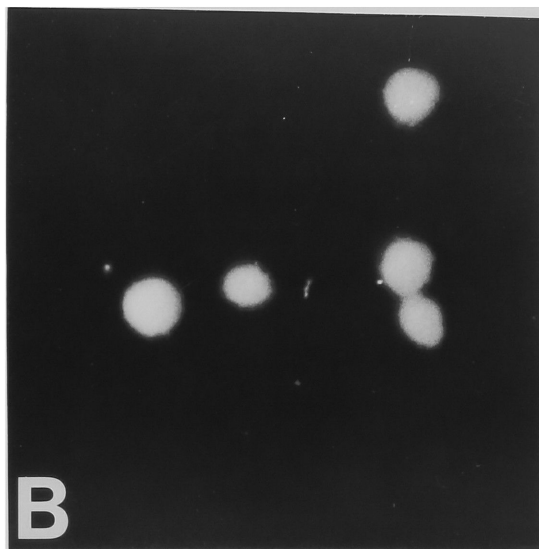
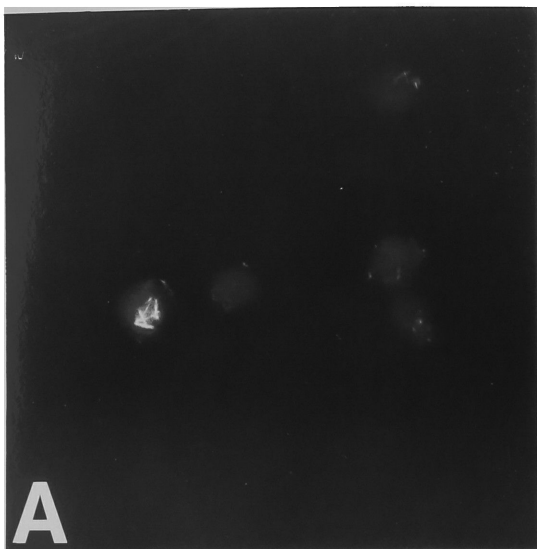
The above results suggest that CD'A 1 is closely associated with actin. I therefore tested whether any agents that bind to actin are able to interfere with the binding of the CD'A 1 antibody to its epitope. DNase I binds specifically to globular (G) actin, but not to F-actin, phalloidin binds to F-actin but not G-actin, and a polyclonal anti-serum should bind to all forms of actin. DNase I and the actin antiserum had no effect on CD'A 1 immunoreactivity, however, phalloidin markedly affected CD'A 1 binding. Phalloidin pretreatment completely eliminated all CD'A 1 staining from fibroblasts, both in untreated mitotic and motile cells, and in cytochalasin and DMSO treated cells. An example from a DMSO treated culture is shown in Figure 11. Pretreatment with the CD'A 1 antibody does not seem to affect phalloidin binding, although this observation is more tenuous. If CD'A 1 blocked only a small portion of phalloidin-labelling, it might not have been detected due to the widespread nature of phalloidin staining. I believe that the phalloidin binding site and CD'A 1 epitope are physically separated for the following reasons. 1) Phalloidin abolishes CD'A 1 binding while CD'A 1 has no effect on phalloidin binding, even though the CD'A 1 antibody is 150 times larger than phalloidin (about 150 kD versus 1 kD). 2) Phalloidin is known to cause a conformational change in actin as evidenced by its effects on actin polymerization and its effects

Figure 11. Phalloidin pretreatment eliminates CD'A 1 immunoreactivity.

NRK fibroblasts were treated with 5% DMSO for 20 minutes prior to fixation. Cells have been double labelled for CD'A 1 (A, C, E) on the left, and actin (B, D, F) on the right.

A-D. No blocking agent present before staining. DMSO treatment caused the cells to round up, and CD'A 1 stained rods to appear in the nuclear region of many of the cells.

E, F. Cells pretreated with 0.5 mg/ml phalloidin for 30 minutes after fixation. This pretreatment completely eliminated all CD'A 1 immunoreactivity. All photos were taken and printed identically. Bar, 50 μ m.



on biophysical assays of actin conformation. 3) If CD'A 1 recognized the phalloidin binding site, CD'A 1 would be expected to have a much more widespread distribution.

The above results lead me to hypothesize the following molecular model of the CD'A 1 epitope. Due to a difference in the structure of actin filaments existing in growth cones and motile fibroblasts, the paraformaldehyde reaction causes actin to assume the conformation that CD'A 1 recognizes. The epitope is not present following other fixations because they do not affect actin in the same way. Since unfixed membranes were the immunogen, the source of the antibody could have been a B cell that was not stimulated by my immunization of the mouse, or a B-cell that responded to a proteolytic fragment that conformationally resembles the conformation of the entire protein after paraformaldehyde fixation.

CHAPTER THREE

SVP38

1. Distribution of SVP38

Antibodies against adult retina were generated by Kimio Akagawa with the aim of identifying synapse-specific molecules that could be used in characterizing the extent of differentiation in retinal cultures. One of the antibodies generated gave punctate staining of both the inner and outer plexiform layers, while leaving the cell body layers and the optic nerve fiber layer unstained (Figure 12a, b). This labelling pattern suggested that the antigen was restricted to synaptic structures. I named the antigen SVP38, for reasons that will become clear below.

SVP38 was found in all synaptic regions of the brain and the periphery. In the cerebral cortex, there was staining in all cellular layers, while the white matter was unstained. The staining was punctate, outlining cell bodies and dendrites, shown in Figure 12c. SVP38 staining in the olfactory bulb was confined to the glomeruli, the inner plexiform layer and the granule cell layer, areas known to contain synapses, as shown in Figure 12d.

As well as this central nervous system distribution, SVP38 was found in a variety of structures in the periphery. Figure 13a shows a section of rat diaphragm muscle stained with rhodamine-labelled alpha-bungarotoxin to label acetylcholine receptors clustered at neuromuscular junctions. SVP38, visualized with a fluorescein secondary antibody in the same section, showed a similar pattern of labelling (Figure 13b). There was also punctate staining outlining the cells in the superior cervical ganglion, in a manner consistent with the known distribution of synapses on these cells (Figure 13c). The adrenal medulla, a neuroendocrine organ derived from the neural crest, was also stained for SVP38, whereas the cortex, an endocrine gland, was not stained (Figure 13d).

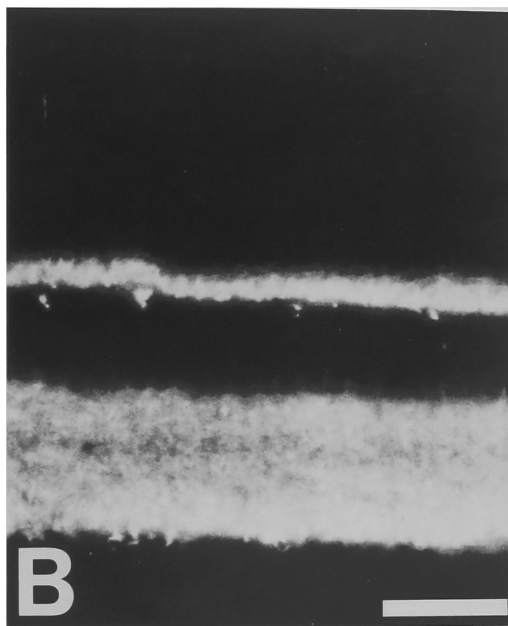
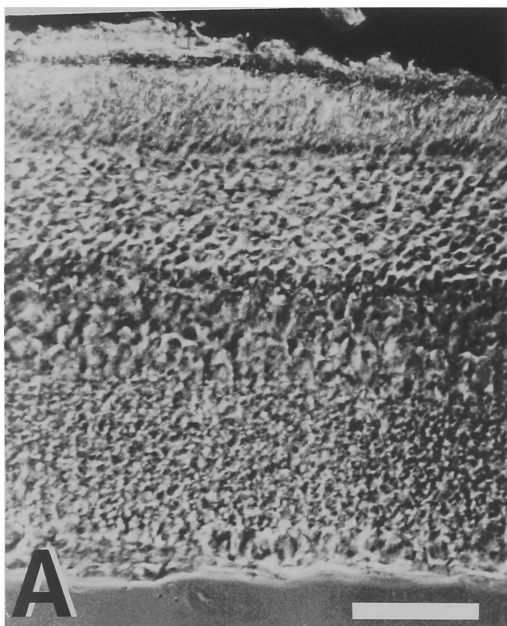
Figure 12. SVP38 staining in the central nervous system.

A. Phase contrast view of the adult retina. Bar, 50 μm .

B. SVP38 staining of adult retina. SVP38 is restricted to the inner and outer plexiform layers (IPL and OPL). There is no immunoreactivity in the outer segments (OS), the outer nuclear layer (ONL), the inner nuclear layer (INL), or the ganglion cells (GC). Bar, 50 μm .

C. High power view of layer 4 in the cerebral cortex, stained for SVP38. There is punctate staining throughout the neuropil, and outlining the cell bodies and dendrites. Bar, 50 μm .

D. Low power view of SVP38 staining in the olfactory bulb. There is bright, punctate staining within the glomeruli, and punctate staining in the external plexiform layer and the granule layer, but no staining in the nerve fiber layer. Bar, 250 μm .



OS
ONL
OPL
INL
IPL
GC

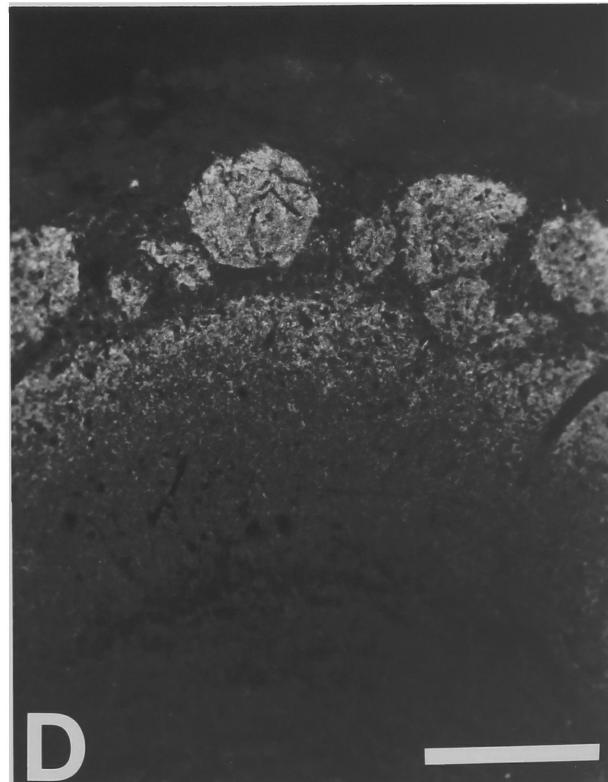
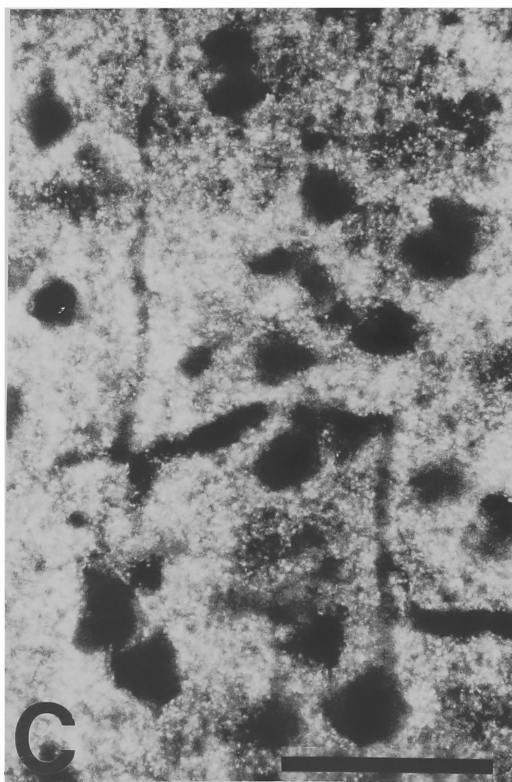
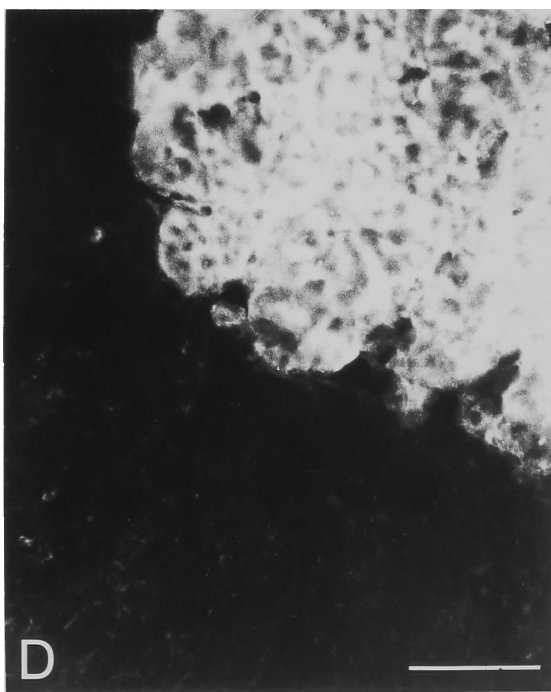
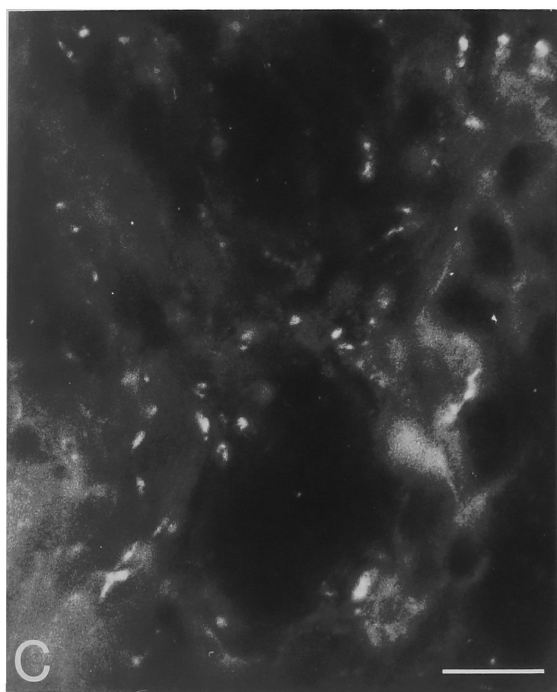


Figure 13. SVP38 staining in the periphery.

- A. Section of rat diaphragm muscle stained with rhodamine-labelled alpha-bungarotoxin to visualize the synaptic acetylcholine receptors. Bar, 25 μm .
- B. Same section stained for SVP38, and then a fluorescein-conjugated secondary antibody. Every synapse revealed by the bungarotoxin was also stained for SVP38. Bar, 25 μm .
- C. A section of superior cervical ganglion stained for SVP38. The punctate staining is consistent with the known distribution of synapses on these cells. Bar, 25 μm .
- D. Adrenal gland stained for SVP38. The medulla, a neuroendocrine gland, is brightly stained, whereas the cortex, an endocrine gland, is not stained. Bar, 150 μm .



2. Biochemistry and subcellular distribution.

To identify more precisely the localization of SVP38, adult rat cerebral cortex was fractionated by differential centrifugation. A dot immunobinding assay was used to determine the relative amounts of antigenic activity in each fraction. The results shown in Table III give the antigenic activities of the fractions relative to the initial homogenate. Enrichment was found in the synaptosome fraction (P2 and P2'), and the immunoreactivity was found in the supernatant (LS1) but not in the pellet (LP1) from the lysed synaptosomes. Since these results suggested localization to a synaptic vesicle fraction, the pellet, LP2 was resuspended and further fractionated by sucrose gradient centrifugation and column chromatography. The elution profile from the controlled pore glass column is shown in Figure 14. Most of the SVP38 antigenic activity eluted between the first peak, representing large membrane vesicles, and the second peak, representing cytosolic proteins (Huttner, et al., 1983). The known synaptic vesicle associated protein synapsin I was assayed for in these fractions by immunoblotting. Synapsin I eluted in the same fractions as SVP38. Pooled fractions containing SVP38 antigenic activity were enriched greater than 250 fold over the starting homogenate (Table III).

When a variety of central nervous system regions were analyzed by polyacrylamide gel electrophoresis, and the proteins probed by immunoblot with the antibody to SVP38, a single band with an apparent molecular weight of 38 kD was seen (Figure 15A, lane 1). To test whether SVP38 is glycosylated, membranes were treated with glycopeptidase F, which removes all N-linked oligosaccharides (Tarentino, et al., 1985). The apparent molecular weight of SVP38 was reduced by 7 kD, indicating that almost 20% of the apparent mass of SVP38 is in sugar residues (Figure 15A, lane 2). This experiment also demonstrates that the antibody which I am using recognizes the protein part of the molecule. This means that in the developmental studies to be presented in

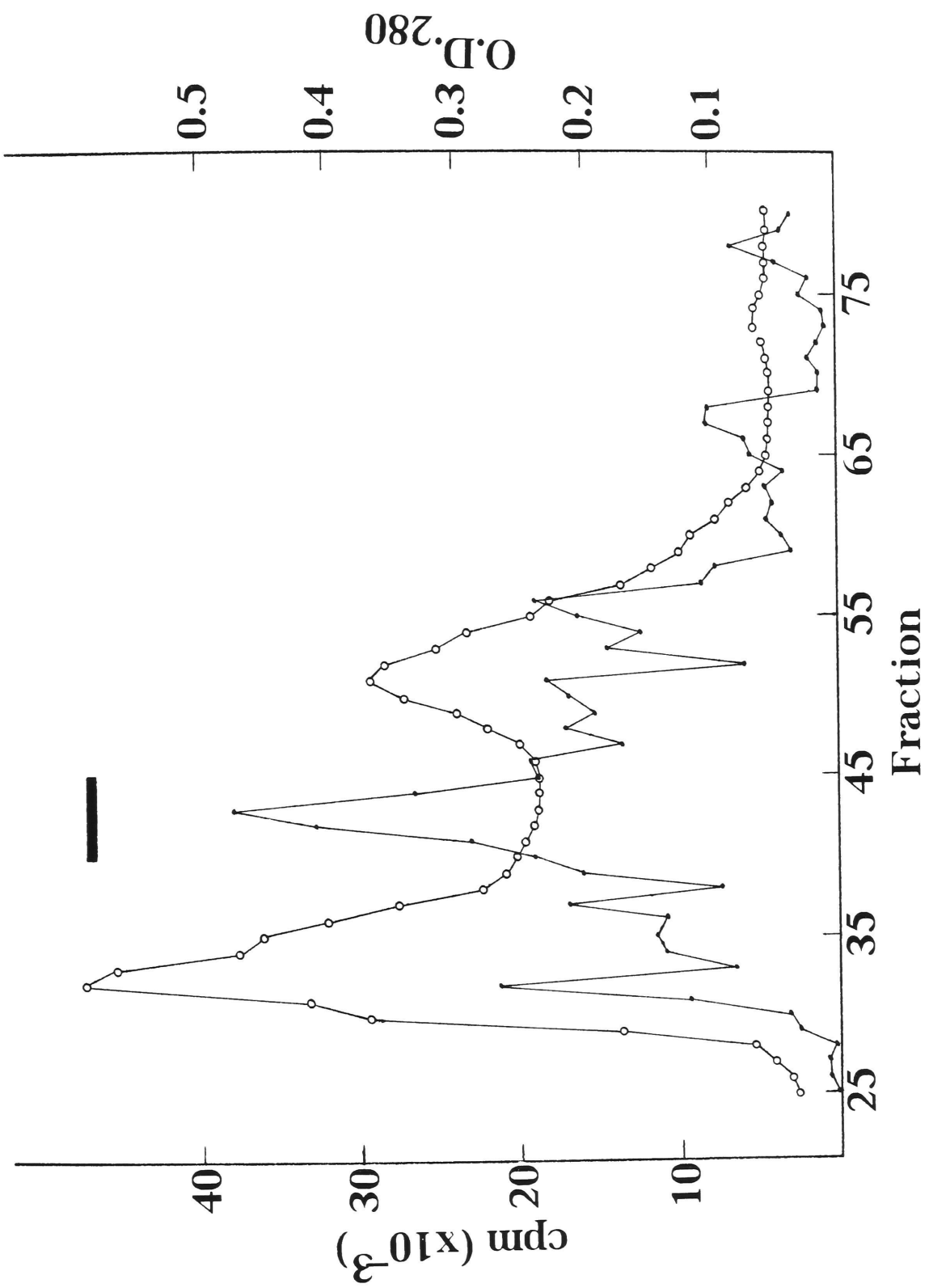
Table III. SVP38 is enriched in subcellular fractions enriched for synaptic vesicles.

The fractions in the left column (for abbreviations see methods section) were assayed in a dot immunobinding assay for SVP38 immunoreactivity. The enrichment relative to the starting homogenate is shown in the right column.

Fraction	Relative Enrichment
H	1.0
S1	4.0
P1	0.80
S2	0.58
P2	5.4
S2'	1.1
P2'	15
LS1	17
LP1	1.3
LS2	<0.01
LP2	18
CPG	252

Figure 14. SVP38 is a synaptic vesicle protein.

Elution profile from the last step in the purification of synaptic vesicles, controlled pore glass chromatography. The column was poured and run as described in Materials and Methods. The column was continuously monitored for protein by absorbance at 280 nm (open circles), and each fraction was assayed for SVP38 immunoreactivity (closed circles). Synapsin I immunoreactivity was found in the same fractions as SVP38 immunoreactivity, as shown by the bar over the elution profile.



chapter four, when there is an increase in SVP38 immunoreactivity, it is not due to a change in the glycosylation of the protein.

To determine whether SVP38 is an integral membrane protein, I treated purified synaptic vesicles with salt, which should remove peripheral proteins, and/or detergent, which should remove integral membrane proteins. The synaptic vesicles were then pelleted by centrifugation, and tested by western blotting to determine if SVP38 remained associated with the vesicle or was removed. 0.15 M KCl was unable to remove SVP38 from the vesicle, while 1% Triton completely removed it. In the same blot, I probed with an antibody to synapsin I, which showed that this concentration of Triton had no effect on the association synapsin I with the vesicles (Figure 15B).

Other investigators simultaneously identified a synaptic vesicle glycoprotein of 38 kD, which they named p38, synaptophysin, or also SVP38 (Jahn, et al., 1985; Wiedenmann and Franke, 1985; Buckley, et al., 1987; Obata, et al., 1987). Synaptophysin and p38 bind to DEAE sepharose columns at low salt concentrations, and are released as the salt concentration rises above about 100 mM NaCl. This experiment also demonstrated that there is another 38 kD protein in purified synaptic vesicles that does not bind to DEAE sepharose even in low salt concentrations. I repeated these experiments to determine which of these 38 kD proteins was SVP38. These results are shown in Figure 16. SVP38 bound to the DEAE sepharose column, and eluted at the same NaCl concentration that p38/synaptophysin did, indicating that p38/synaptophysin and SVP38 are identical. This was confirmed with a monoclonal antibody to p38 (not shown).

In summary, SVP38 is a 38 kD glycoprotein that spans the membrane of synaptic vesicles at all synapses, and is also found in neuroendocrine cells.

Figure 15. SVP38 is an integral membrane glycoprotein of 38 kD.

A. SVP38 is a 38 kD glycoprotein.

Lane 1: A crude synaptosome preparation was separated by SDS-PAGE and probed by immunoblotting with the antibody to SVP38. A single immunoreactive band with an apparent molecular weight of 38 kD was identified.

Lane 2: The crude synaptosome preparation was treated with glycopeptidase F and analyzed as above. Glycopeptidase treatment reduced the apparent molecular weight by about 7 kD. The molecular weight of standards are shown on the left.

B. SVP38 is an integral membrane protein. Crudely purified synaptic vesicles (LP2) were treated as described below, centrifuged, separated by SDS-PAGE, blotted, and probed simultaneously with antibodies to SVP38 (38 kD) and synapsin I (80 kD).

Lane 1: Untreated vesicles.

Lane 2: 0.15 M KCl treated vesicles. SVP38 was unaffected by this treatment, while synapsin I was slightly removed from the vesicles.

Lane 3: 1% Triton treated vesicles. This treatment completely eliminated SVP38 from the vesicle, while having no effect on synapsin I.

Lane 4: 0.15 M KCl and 1% Triton treated vesicles. This treatment completely eliminated both SVP38 and synapsin I from the vesicle.

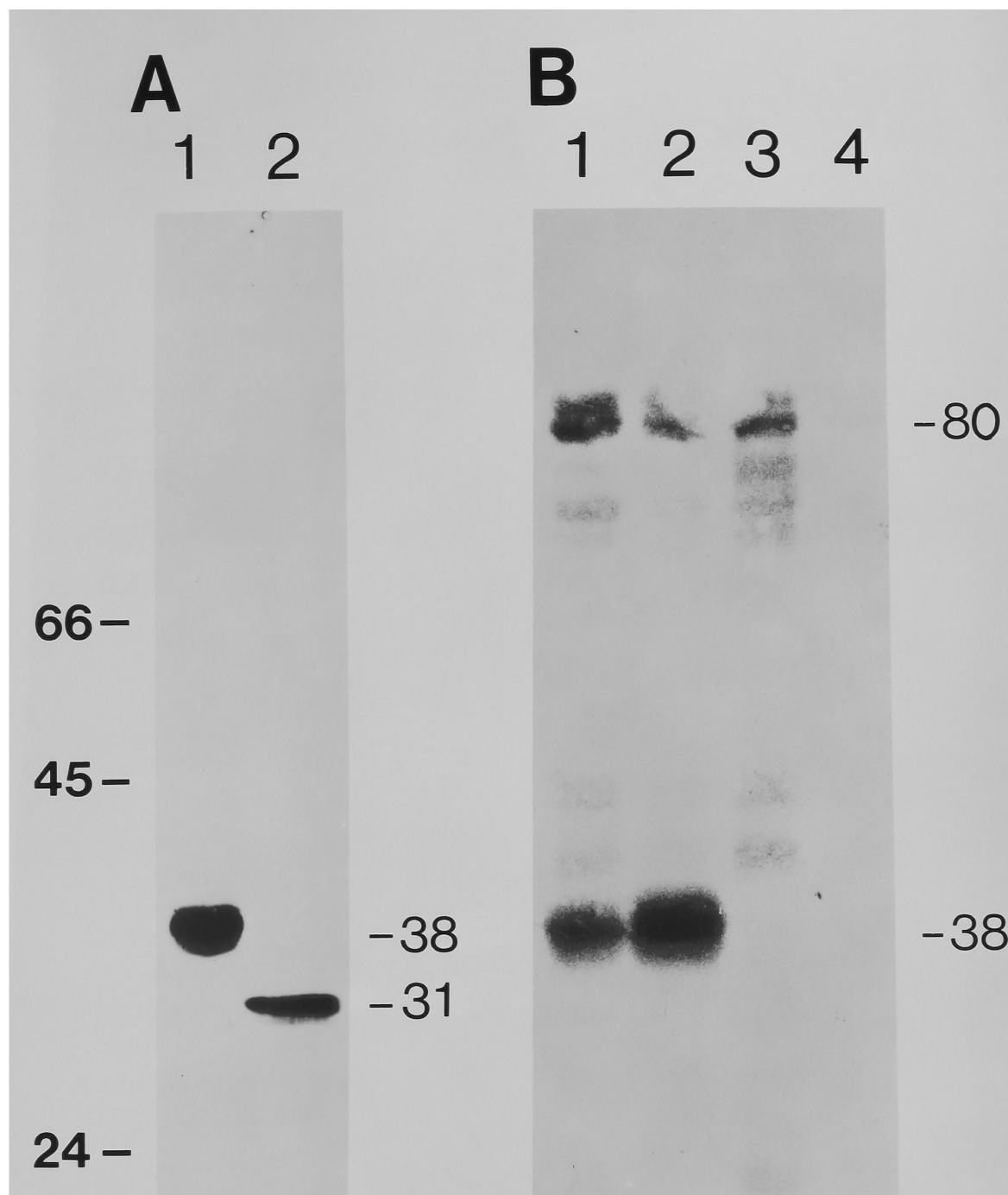
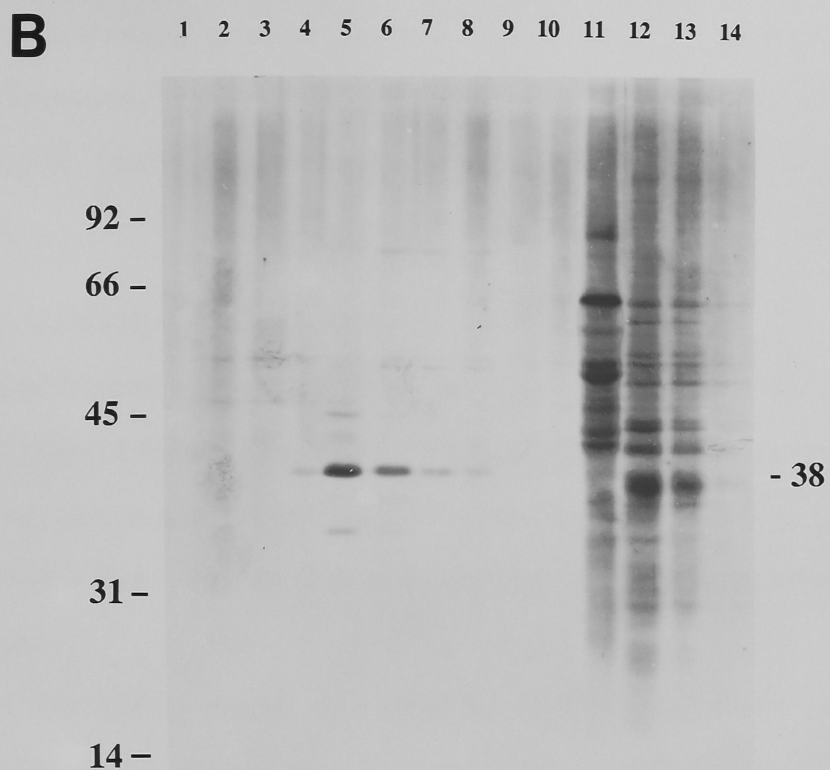


Figure 16. SVP38 behavior on DEAE Sepharose.

A. Crude synaptic vesicles were loaded onto a DEAE sepharose column and eluted with a step gradient of NaCl. The NaCl concentration changed after fractions 3 and 10, as shown.

B. Silver stained gel of the proteins eluting off of the column using the step gradient shown in A. Column fraction numbers are shown above the gel. The 50 mM concentration is sufficient to elute one major protein of about 38 kD. Several proteins were eluted as the salt concentration went to 125 mM.

C. Immunoblot of an identical gel to that shown in B. The SVP38 antibody bound to the 38 kD band present in the fractions that eluted after the salt concentration was raised to 125 mM.



CHAPTER FOUR

DEVELOPMENTAL EXPRESSION

The establishment of synapses between cells is dependent on four processes: growth cone formation, neurite elongation, growth cone target interactions, and synaptic maturation. I have examined the expression of CD'A 1 and SVP38 in some of these processes.

1. Growth cone maturation.

The initial formation of growth cones has been well characterized in cultured hippocampal neurons. I therefore examined the developmental expression of CD'A 1 in these cells as they initiate neurites and establish axonal/dendritic polarity *in vitro*. This work was a collaboration with Kim Goslin, in Dr. Gary Banker's laboratory at Albany Medical College.

CD'A 1 was initially expressed in the entire circumference of the lamellipodia that surrounded the freshly dissociated cells (stage 1 cells, shown in Figure 17A, B). As the lamellipodia transformed into growth cones and moved away from the cell body leaving a neurite behind, CD'A 1 remained in the growth cone (stage 2 cells, Figure 17C-F). In both the lamellipodia and the growth cone, CD'A 1 staining appeared as randomly spaced, fine radial streaks. During the second day *in vitro*, when one of the processes differentiated into the axon, CD'A 1 was present in both the axonal growth cone and growth cones of other processes, which would later become dendrites (stage 3 cells, Figure 18). Synaptophysin was not expressed in these early cultures (G. Banker, personal communication).

Following the initial outgrowth of axons in these cultures, the other processes begin to elongate rapidly and differentiate into dendrites. CD'A 1 continued to be present in the growth cones of these processes (stage 4 cells, Figure 19). However, within a day after dendritic outgrowth commenced, CD'A 1 staining began to

Figure 17. CD'A 1 staining in freshly plated hippocampal neurons *in vitro*. Bar, 25 μ m

Cells have been photographed in phase (A, D, G), and double labelled for CD'A 1 (B, E, H), and F-actin (C, F, I). The F-actin was visualized with rhodamine conjugated phalloidin. The regions of CD'A 1 labelling in these cells invariably have high levels of F-actin, although they are not precisely colocalized.

A, B, C. This is a cell fixed and stained shortly after plating, with only lamellipodia (stage 1). CD'A 1 staining is present as radial streaks in the lamellipodia that surrounds the cell.

D, E, F. This cell has begun to extend processes, but none of them are differentiated into axons yet (stage 2). CD'A 1 remains in the lamellipodia as they become part of growth cones and move away from the soma. This field also shows a nonneuronal cell in the upper left that has F-actin but is not stained for CD'A 1.

G, H, I. One of the processes of this cell is considerably longer than the others, and will differentiate into the axon (stage 2+). All of the growth cones remain heavily stained by CD'A 1.

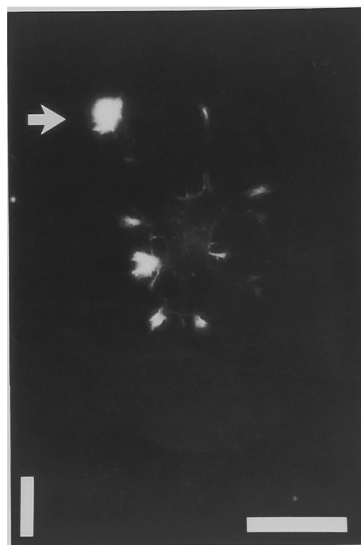
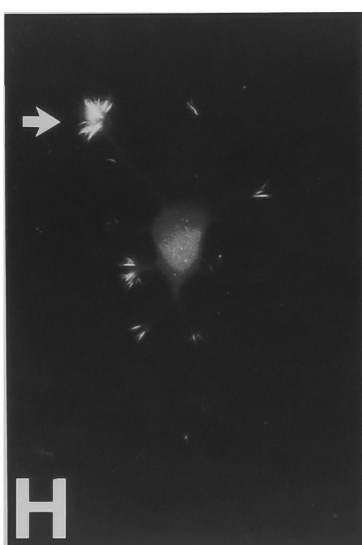
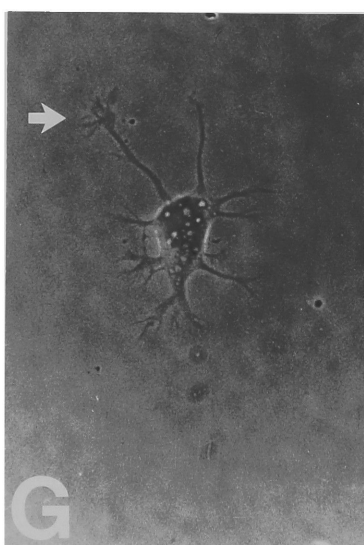
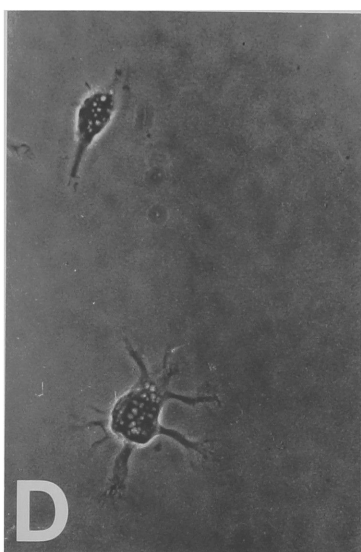
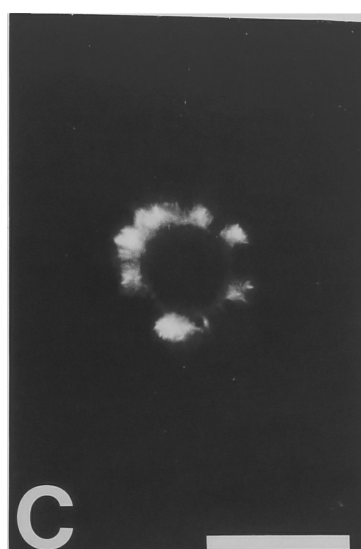
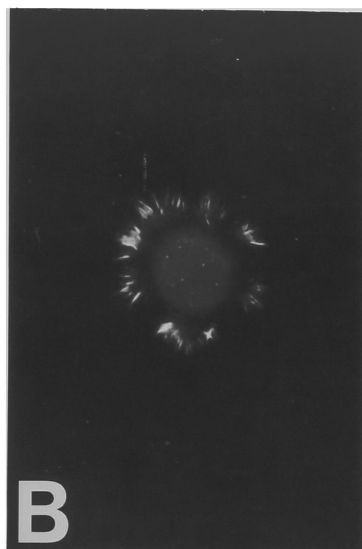
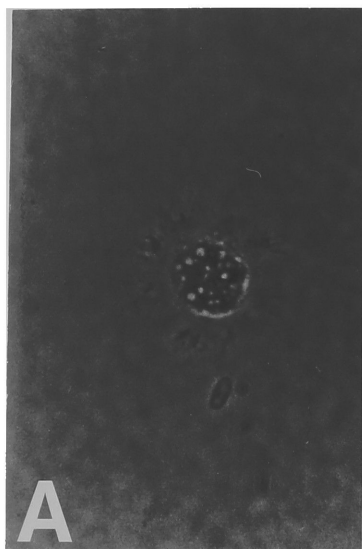


Figure 18. CD'A 1 staining in cells with axons.

After about a day in culture, neurons have elaborated one long axon and continue to have several minor processes (stage 3). All of the growth cones contain high levels of CD'A 1. The regions of CD'A 1 labelling in these cells invariably have high levels of F-actin, although they are not precisely colocalized. Bar, 25 μm

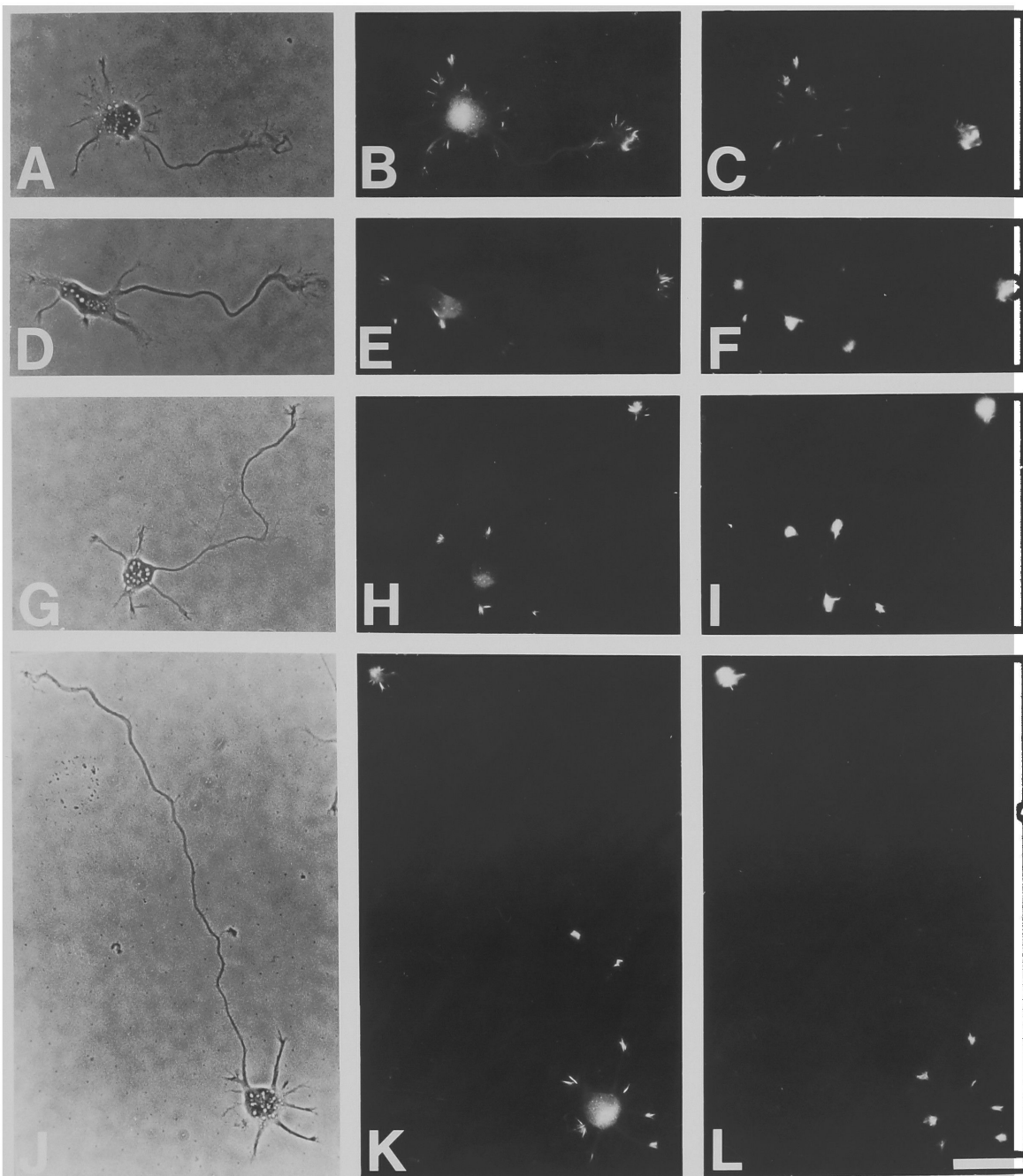


Figure 19. CD'A 1 is present in the growth cones of dendrites after they differentiate but is then lost from almost all growth cones.

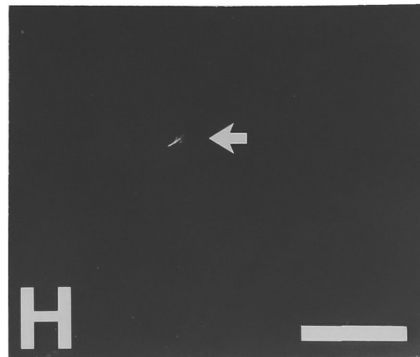
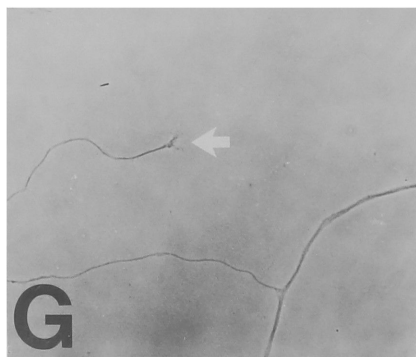
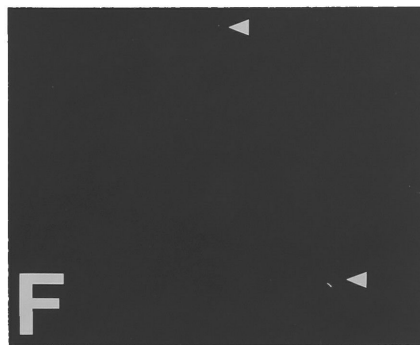
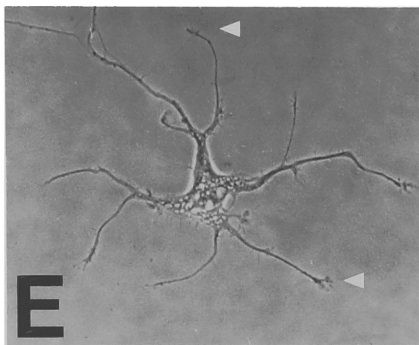
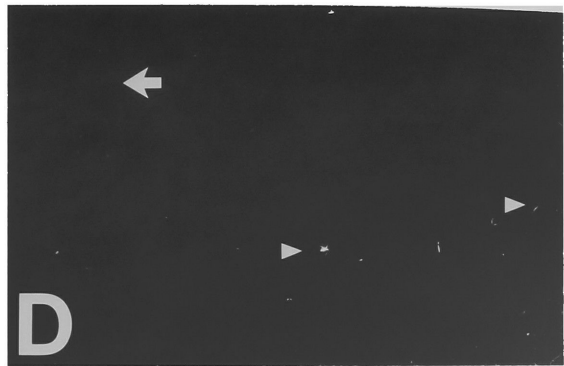
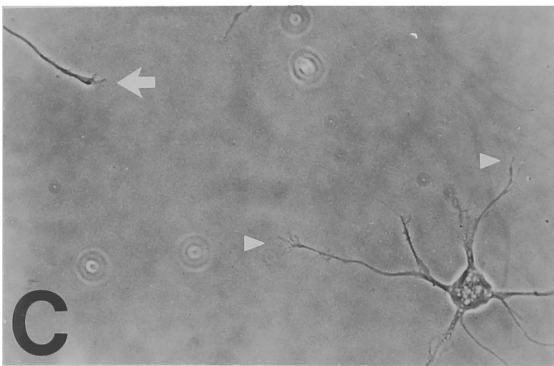
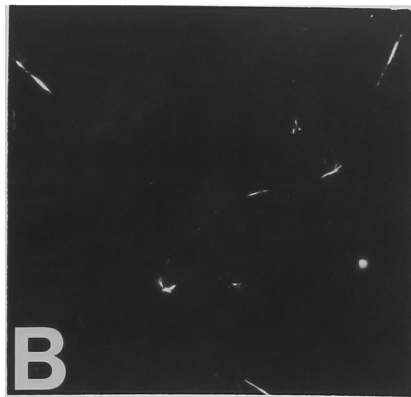
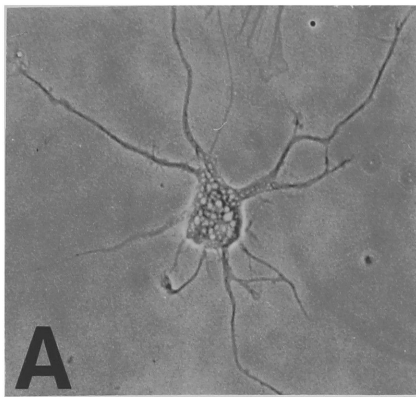
Cells have been photographed in phase (A, C, E, G), and stained for CD'A 1 (B, D, F, H). Bar, 25 μm

A, B. After two to three days *in vitro*, the minor processes begin to elongate more rapidly and acquire a dendritic phenotype. CD'A 1 is present at this stage in both dendritic growth cones and axonal growth cones.

C, D. Axonal growth cones begin to lose CD'A 1 in general shortly after dendrites differentiate. This field shows an axonal growth cone (arrow) that has lost CD'A 1, and dendritic growth cones that still express CD'A 1 (arrow heads).

E, F. Dendritic growth cones lose CD'A 1 shortly after the loss from axonal growth cones. In this field, one of the dendritic growth cones (arrow heads) has very faint CD'A 1 immunoreactivity, the others have all lost it.

G, H. Very rare axonal growth cones in older cultures retain CD'A 1 immunoreactivity. These growth cones tend to be more lamellipodial than the growth cones that are negative for CD'A 1 (compare growth cone in G with that in C, indicated by the arrows).



disappear. This occurred despite the fact that axonal and dendritic outgrowth will continue for weeks, until a network of fine axons and broad dendrites covers the substrate. Staining disappeared first from the axons and then from the dendrites. At intermediate stages, there was heterogeneity in CD'A 1 staining: some growth cones retained CD'A 1 while the rest were negative. The loss of CD'A 1 immunoreactivity was correlated with a change in the shape of the growth cones in these cultures. The growth cones of younger cultures were usually fan-shaped, with broad lamellipodia in which CD'A 1 was found. This contrasted with the majority of growth cones in older cultures, which were more bullet shaped, and CD'A 1 negative. The shape of the growth cone has been correlated in other systems with the rate of outgrowth, more lamellipodia indicating faster neurite elongation. However, preliminary observations suggest that this was probably not true during the range of times which we observed. Whether the change in shape of the growth cone correlates with other behavioral, structural, or biochemical changes remains to be determined.

I have examined the expression of SVP38 in the outgrowth phase of neuronal development in two systems *in vitro*: neonatal superior cervical ganglion (scg) cells , and E17 cerebral cortex. Cells from the neonatal scg expressed high levels of SVP38 immediately after plating. SVP38 was concentrated in the perinuclear region of the cell in the first day of culture, and was also present in the growth cone and neurite (Figure 20 and 21A, B). Over the next few days *in vitro*, the cell body and neurite staining disappeared, leaving only the end of the neurites with SVP38. Synapses are formed between scg cells in these cultures beginning after two or three days, SVP38 assumes a pericellular punctate distribution with the same time course. In addition, there were punctate accumulations of SVP38 at points of contact between two neurites.

In E13 cortical cells *in vitro*, after three days SVP38 was not expressed in the growth cones, in contrast to the scg cells discussed above (Figure 21C-E). The cortical growth cones had abundant CD'A 1 labelling in these cultures (see Figure 2). I believe

Figure 20. SVP38 expression in superior cervical ganglion cultures

1. One day after plating, SVP38 staining is primarily localized to the cell body and is barely detectable in the processes from this cell, which extend for many cell diameters (not shown).
2. Two days after plating, this neuron has been stained for SVP38 (A) and for neurofilament. In most cells, the staining has begun to appear in the processes.
3. Three days *in vitro*. There is now SVP38 in the processes of most cells (A, B), and in many cells the cell body staining has decreased. However, in some cells, the cell body staining is pronounced, and their processes have very little SVP38 staining. An example is shown in C and D. This cell has very little neuritic SVP38 staining (C), although it has developed extensively, as revealed with antibodies to neurofilament (D).
4. Twenty days *in vitro*. There is intense punctate staining for SVP38 along the neurites (A), Neurofilament staining revealed the likely presence of numerous processes within this bundle (B), and it is likely that the punctate SVP38 staining is at synapses between different individual fibers in the bundle. Most, if not all neurites were outlined by punctate SVP38 staining (C). The staining was always around, and never in cell bodies (D), the intracellular levels of SVP38 have decreased. The staining around the cell bodies resembles that seen in the adult scg, shown in 5.
5. Adult superior cervical ganglion, stained for SVP38.

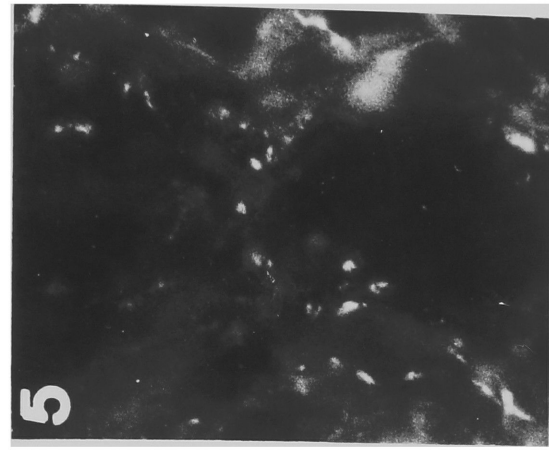
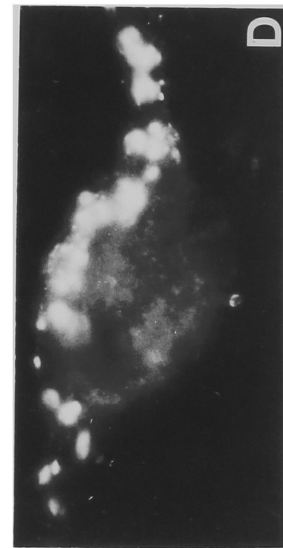
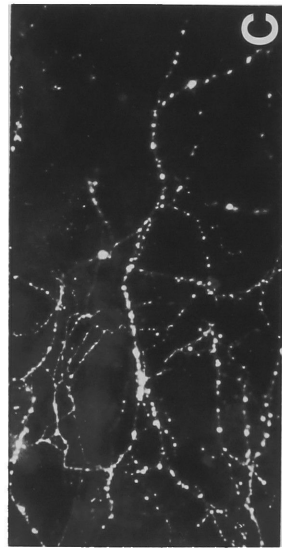
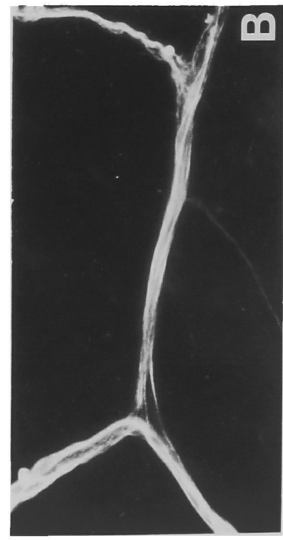
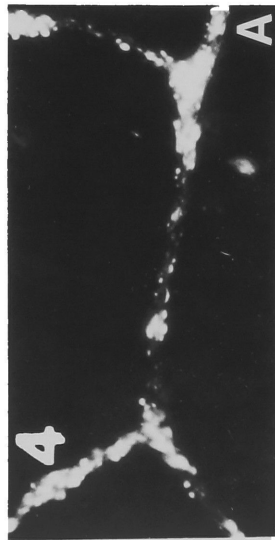
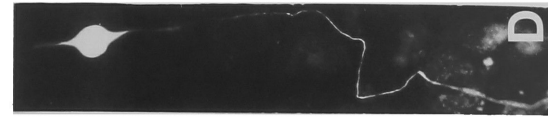
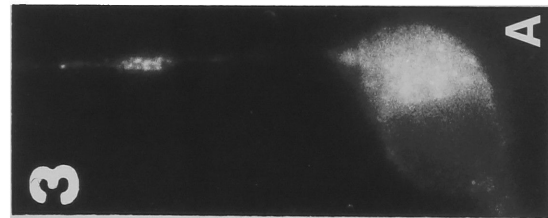
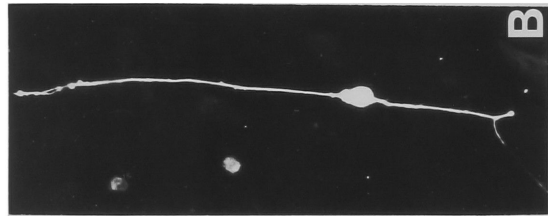
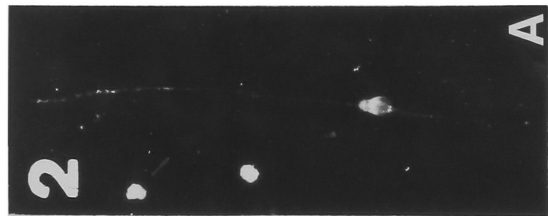
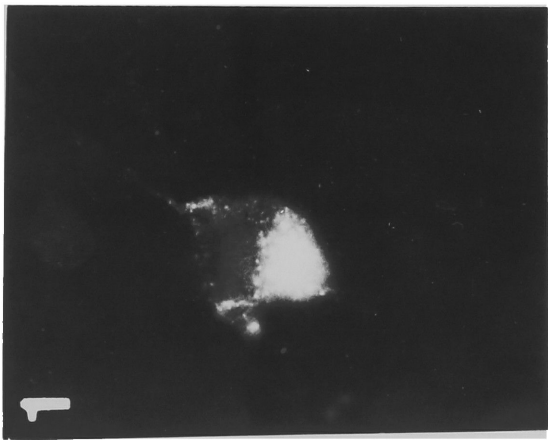


Figure 21. SVP38 is expressed in the growth cones of neonatal superior cervical ganglion cells *in vitro*, but not in the growth cones of embryonic cortical cells *in vitro*.

A, B. Two high power views of SVP38 labelled growth cones of scg cells three days after plating. The cell bodies of these growth cones are out of the field of view to the right.

C. Cell body of E13 cortical cell, three days after plating. SVP38 is present in the perinuclear region and within some of the processes.

D. Phase contrast view of a different region of the same culture, showing three growth cones (arrow heads).

E. SVP38 labelling of the field shown in D. None of the growth cones contain SVP38, even though some cells in the same culture have expressed SVP38 in their cell body (C).

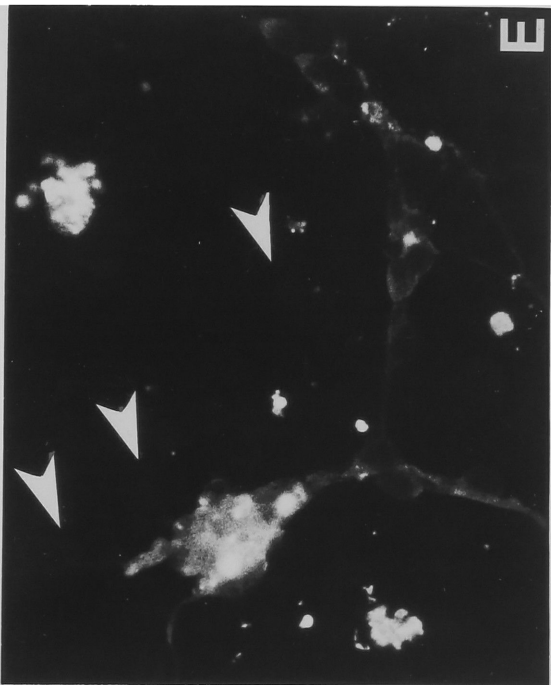
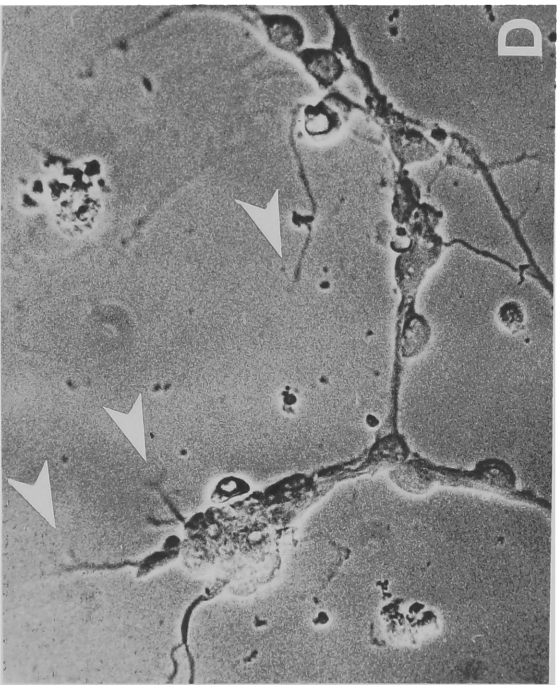
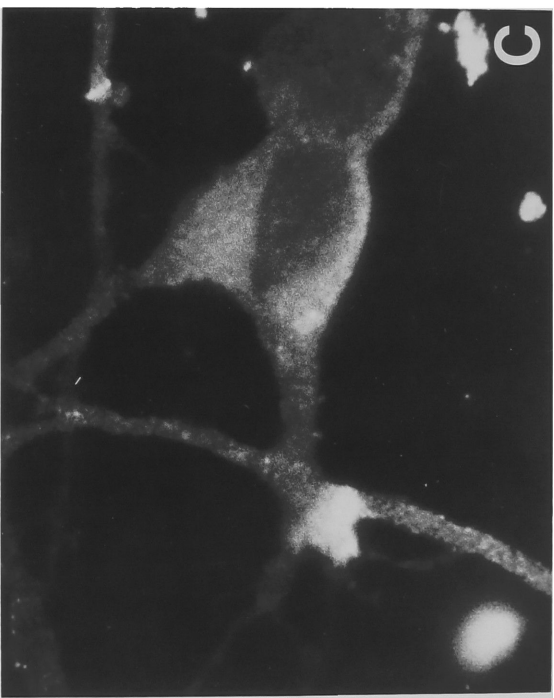
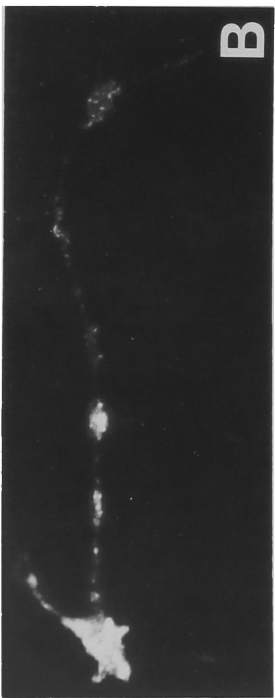
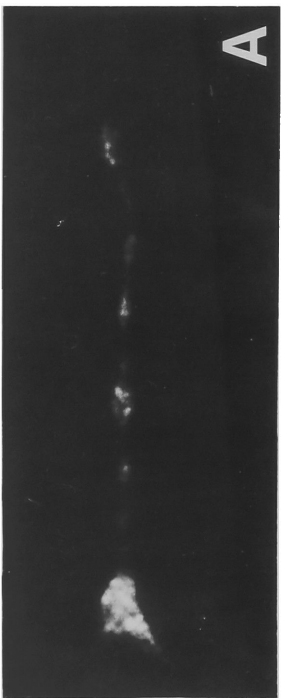
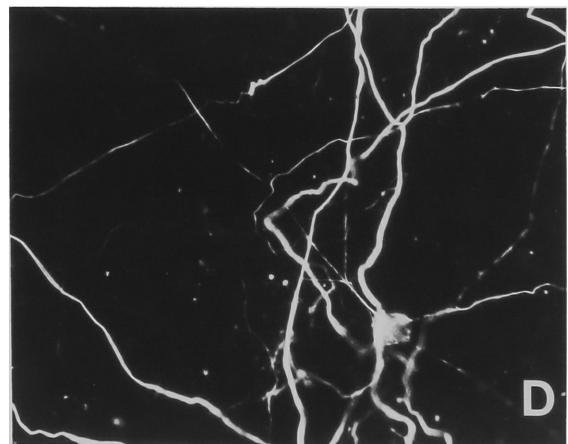
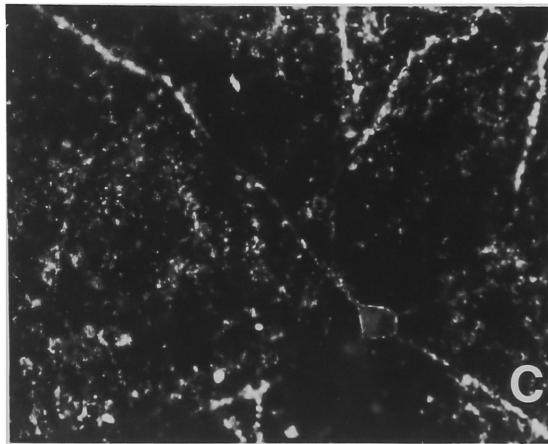
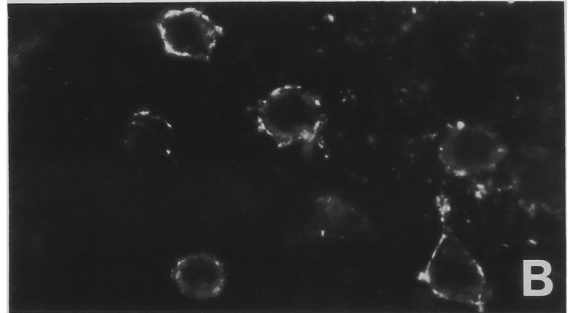
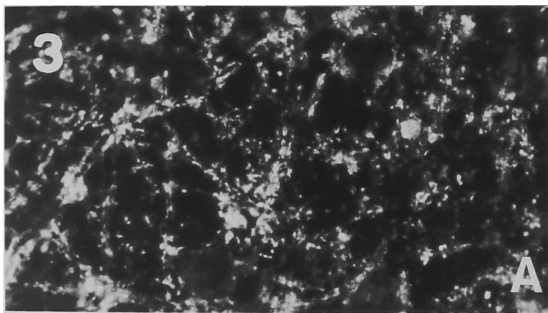
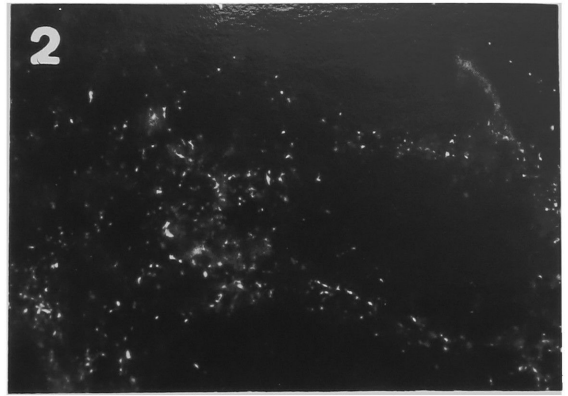
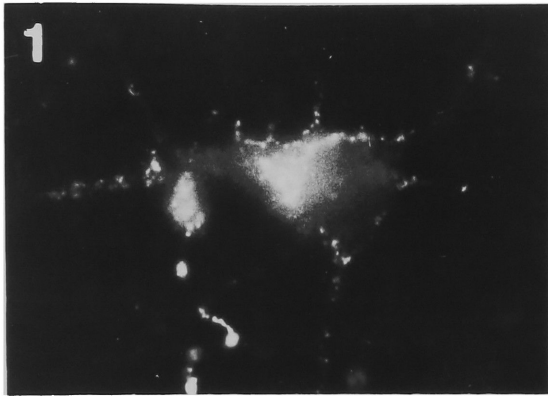


Figure 22. Embryonic rat cortical neurons express SVP38 soon after plating in dissociated cell culture. These cultures were established from E15 rat cortex.

1. Three days *in vitro*. After three days, there is already punctate staining between some neurites and cells, although most are not stained.
2. Twelve days *in vitro*. The intensity and number of punctate spots increases during the first few weeks in culture.
3. Fifty-four days *in vitro*. In mature cultures, punctate staining is widespread (A). Staining is especially prominent around the cell membranes of large cells (B). Most of the staining surrounds either these large cells or processes, as shown in cultures double labelled for SVP38 (C), and neurofilament (D). This staining pattern strongly suggests that SVP38 is being expressed at synapses in these cultures.



that the key difference is that the scg cells have expressed SVP38 *in situ*, while the cortical cells have not, as will be shown below. Once the gene has been activated, I suspect that it remains expressed *in vitro*, and is transported to the ends of the neurites. In the cortical cultures, SVP38 was not expressed until after the cells had begun to contact each other. SVP38 accumulated in punctate regions around neuronal cell bodies, until it strikingly resembled the distribution in the adult cerebral cortex (Figure 22-3, compare with Figure 12C). After three to four days in culture, the CD'A 1 labelling was difficult to assign to growth cones, since most growth cones were fasciculated with other processes. After about a week in culture, there were very few CD'A 1 labelled growth cones.

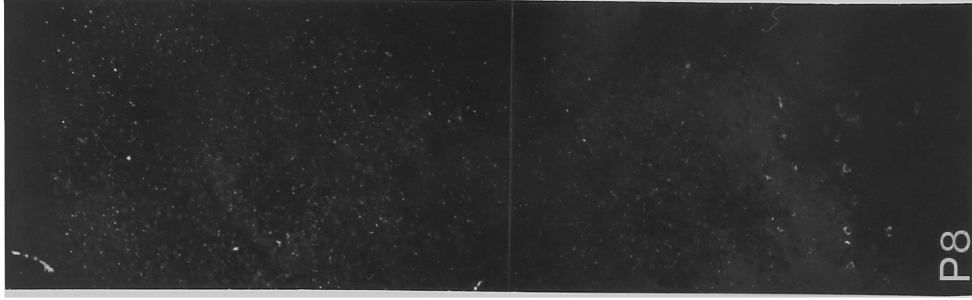
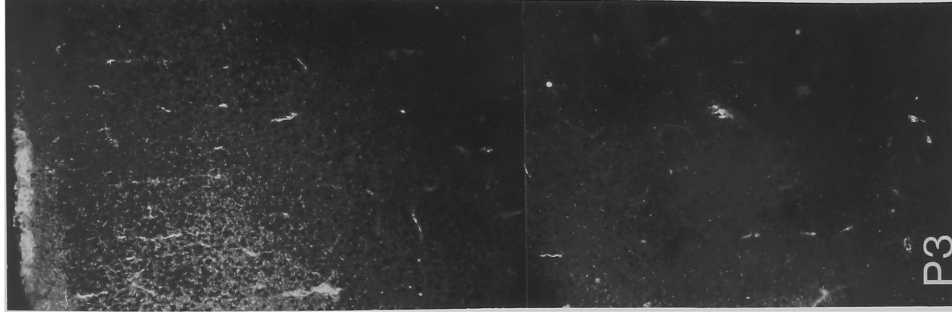
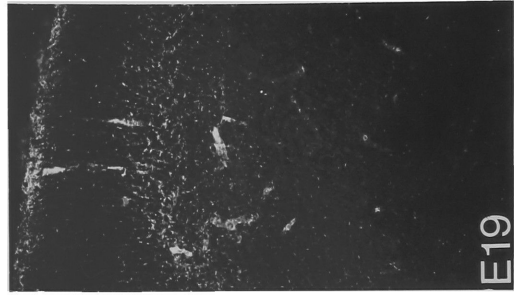
2. CD'A 1 and SVP38 expression in the developing cerebral cortex.

I have directly compared CD'A 1 and SVP38 expression in the developing cerebral cortex. The first detectable CD'A 1 staining appears on E14 as isolated growth cones in the intermediate/marginal zone (not shown). The first labelled growth cones were not as brightly labelled as those at later times. As the cortical plate forms, labelled growth cones are displaced to the intermediate and marginal zones. Both the number of growth cones, the length of the CD'A 1 stained portion, and their staining intensity increases during prenatal development, reaching a peak around E19. At this time, both the intermediate zone and the marginal zone were full of intensely labelled growth cones, and a few have begun to invade the cortical plate. As the cortex matures, the labelled growth cones appeared more frequently within the cortical plate, and then diminished in number, in intensity of staining, and in length. By postnatal day 12, no more staining was seen in the cortex. This sequence is shown in Figure 23.

CD'A 1 disappears from other areas of the brain with a time course that also reflects the time course of synapse formation in those areas. In the hippocampus, labelled growth cones disappear only a few days later than in the cortex. In the cerebellum, growth cone staining does not disappear until about P20. In the olfactory

Figure 23. CD'A 1 disappears as the cortex matures.

Staining first appears at E14 in the intermediate/marginal zone (not shown), and remains in these zones as the cortical plate separates them. As the cortex matures, labelled growth cones invade the cortical plate and then disappear completely by P12. Bar, 200 μ m.



bulb, there are still very heavily labelled growth cones in the fourth postnatal week, and some stained growth cones were present in all the adult ages I examined (not shown).

The olfactory bulb is the only structure that retains CD'A 1 immunoreactivity into adult ages, although the staining is not as intense as in the embryo. Figure 24 shows a *camera lucida* drawing of a CD'A 1 stained section from an adult olfactory bulb. There were less than one hundred labelled growth cones in the nerve fiber layer of this section, which is a representative section. I expect that there should be about 2500 growth cones in the nerve fiber layer per section.¹ This suggests that many of the receptor growth cones are not stained by CD'A 1. This may indicate that the structure of the growth cones is different from that of the growth cones in embryonic tissue that are stained by CD'A 1.

CD'A 1 labelled growth cones are found in all layers in the bulb. The labelled growth cones in the deeper layers of the bulb could derive from the axons of the mitral cells, the basal dendrites of granule cells, the axons of centripetal fibers, or other intra-bulb cells. Without other labels for these cells, it is very difficult to distinguish these possibilities. The presence of CD'A 1 in these regions of the adult bulb indicates that there is a greater plasticity in the deeper layers of the bulb than was hitherto suspected.

SVP38 expression in the developing cortex is shown in Figure 25. It has been reported that the synaptic vesicle proteins synapsin I and p65 are expressed at high levels in the fibers and synapses in the intermediate zone and subplate of the embryonic cat cortex. In E17 rat cortex, there is faint staining in these regions, SVP38, if present at this age, is apparently expressed at much lower levels than are p65 and synapsin I. SVP38 expression largely appeared postnatally, with the strongest staining initially in layer 1. The appearance of SVP38 in all of the other layers was roughly coincident.

¹There are approximately 20×10^6 receptor cells of the olfactory mucosa, each with a half life of about 30 days. The axonal growth cones of new receptor cells grow at the most at a rate of 2 mm/day, and there is about 20 mm between the receptor cell bodies and the bulb. It would therefore take each growth cone 10 days to reach its target. With a half life of 30 days, 1/6th of the receptors would have growth cones *en route* at any time. Thus, there should be $(1/6 \text{ growth cones/receptor}) \times (20 \times 10^6 \text{ receptors}) \times (15 \times 10^{-6} \text{ meters/section}) / (2 \times 10^{-2} \text{ meters total route}) = 2500 \text{ growth cones in a } 15 \mu\text{m section of bulb.}$

Figure 24. Distribution of CD'A 1 labelled growth cones in the adult olfactory bulb.

This is a camera lucida tracing of a labelled section of olfactory bulb. The glomeruli have been outlined with dotted lines, and the mitral cell layer schematically shown by the row of triangles. All of the streaks have been traced to their accurate length. The growth cones are not as intensely labelled as in the embryo (compare with figure 4). There are labelled growth cones in all laminae of the bulb, however, not as many growth cones are found in the nerve fiber layer as would be expected if all olfactory receptor growth cones were labelled. Medial (M), Lateral (L), dorsal (D), and ventral (V), are indicated. Bar, 500 μ m.

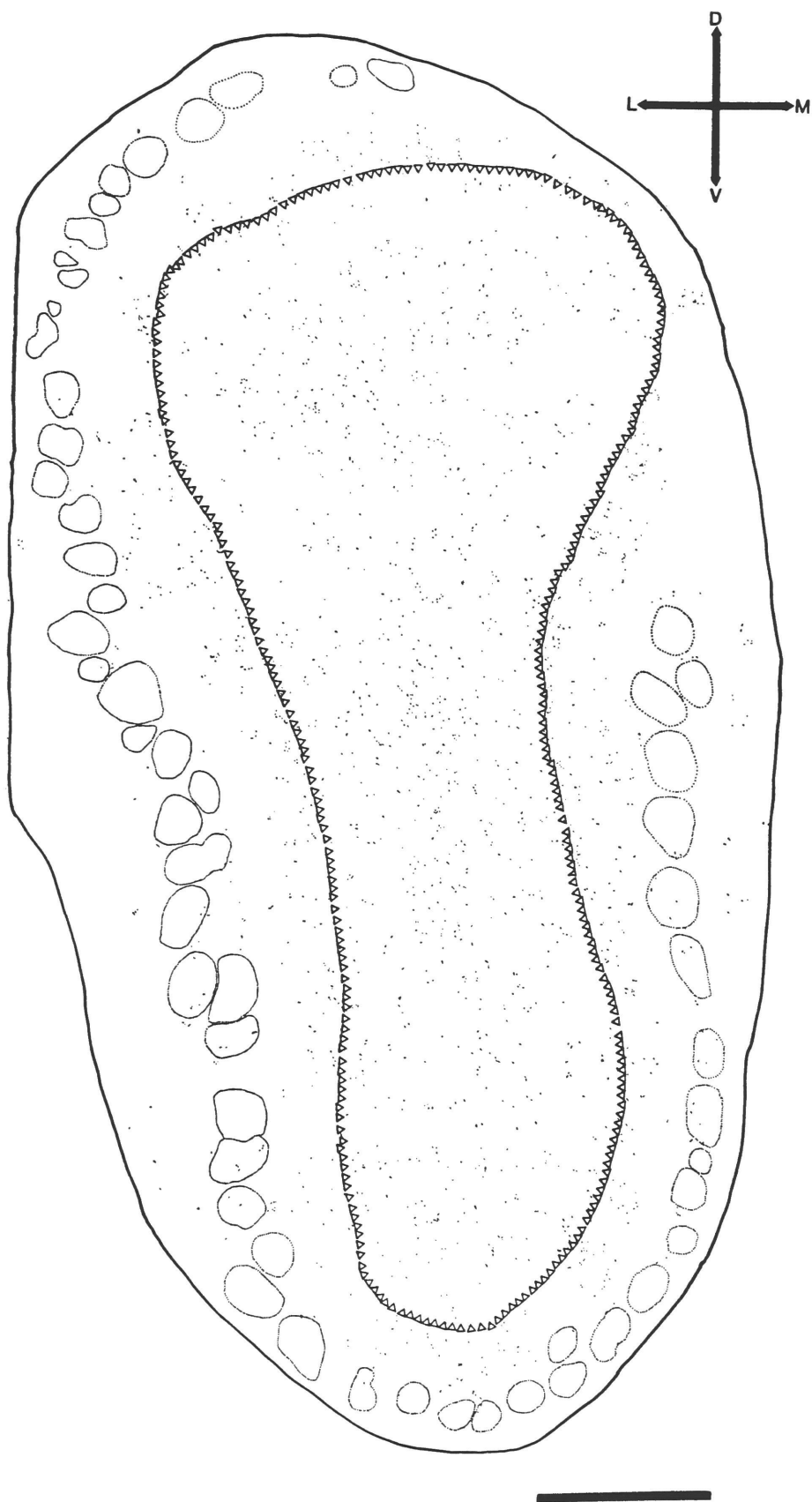
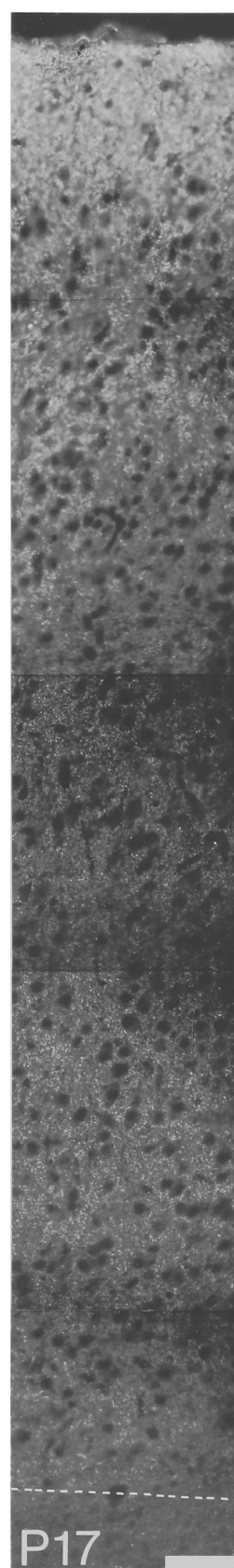
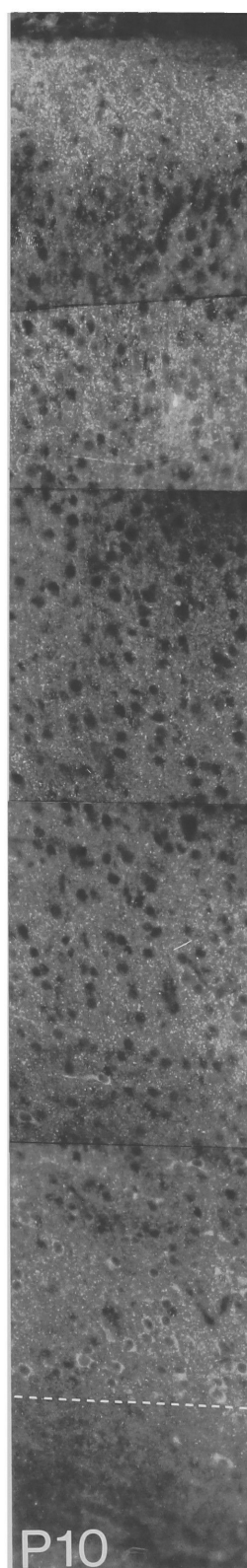
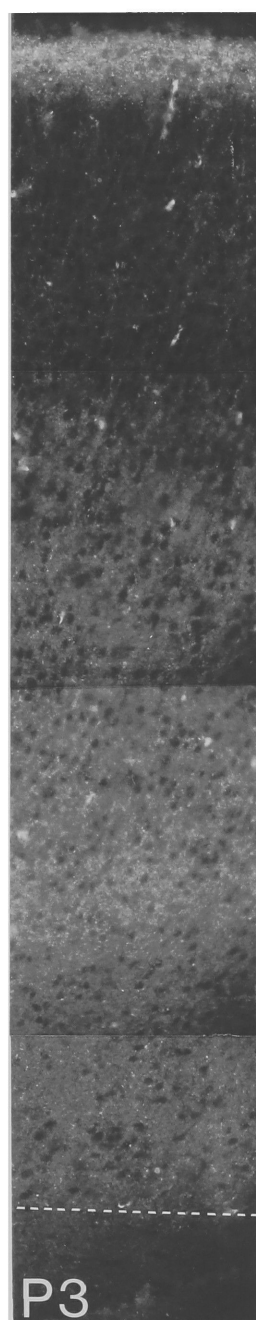
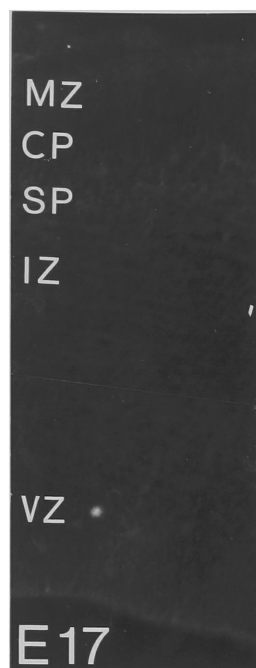


Figure 25. SVP38 expression in the cerebral cortex begins postnatally.

Abbreviations are as in figure 3; dotted line indicates the edge of the white matter. There is faint staining at E17 in the marginal zone and subplate, however, the major increase in SVP38 occurs postnatally. The perinuclear staining in the deep layers of P10 cortex was nonspecific, as it was seen with other, irrelevant antibodies as well. The adult pattern is qualitatively the same as on P17, though somewhat brighter (part of the adult cortex is shown at higher magnification in figure 12C). Bar, 100 μ m.



The intensity of labelling increased in all layers during the second and third postnatal weeks.

In order to provide quantitative data on the expression of SVP38, the S1 fractions from the cortex of rats of different ages were used in a dot immunobinding assay. This fraction is a post nuclear supernatant and contains greater than 80% of the SVP38 from a homogenized cortex. SVP38 was located in the membrane fractions at all ages (data not shown). The time course of SVP38 is shown quantitatively in Figure 26. To compare my data directly with previous data on synaptogenesis, in Figure 27, I have redrawn data from two electron microscope studies of synaptogenesis in different areas of the rat cortex (Aghajanian and Bloom, 1967; Blue and Parnavelas, 1983b).

To further verify the significance of the similarity between the curves shown in Figure 27, I used the same S1 fractions to monitor the increase in expression of Thy-1, a neuronal plasma membrane protein that is not concentrated in synaptic structures but whose appearance does reflect neuronal maturation. The results in Figure 28 show that the increase in Thy-1 expression precedes both morphological synapse formation and SVP38 expression.

Figure 26. SVP38 expression during cortical development, measured quantitatively.

Amount of SVP38 in the S1 fractions from different ages, as measured using a dot immunobinding assay and expressed as a percentage of adult levels. Standard errors are shown for each point. Curve was fitted by eye.

S V P 38

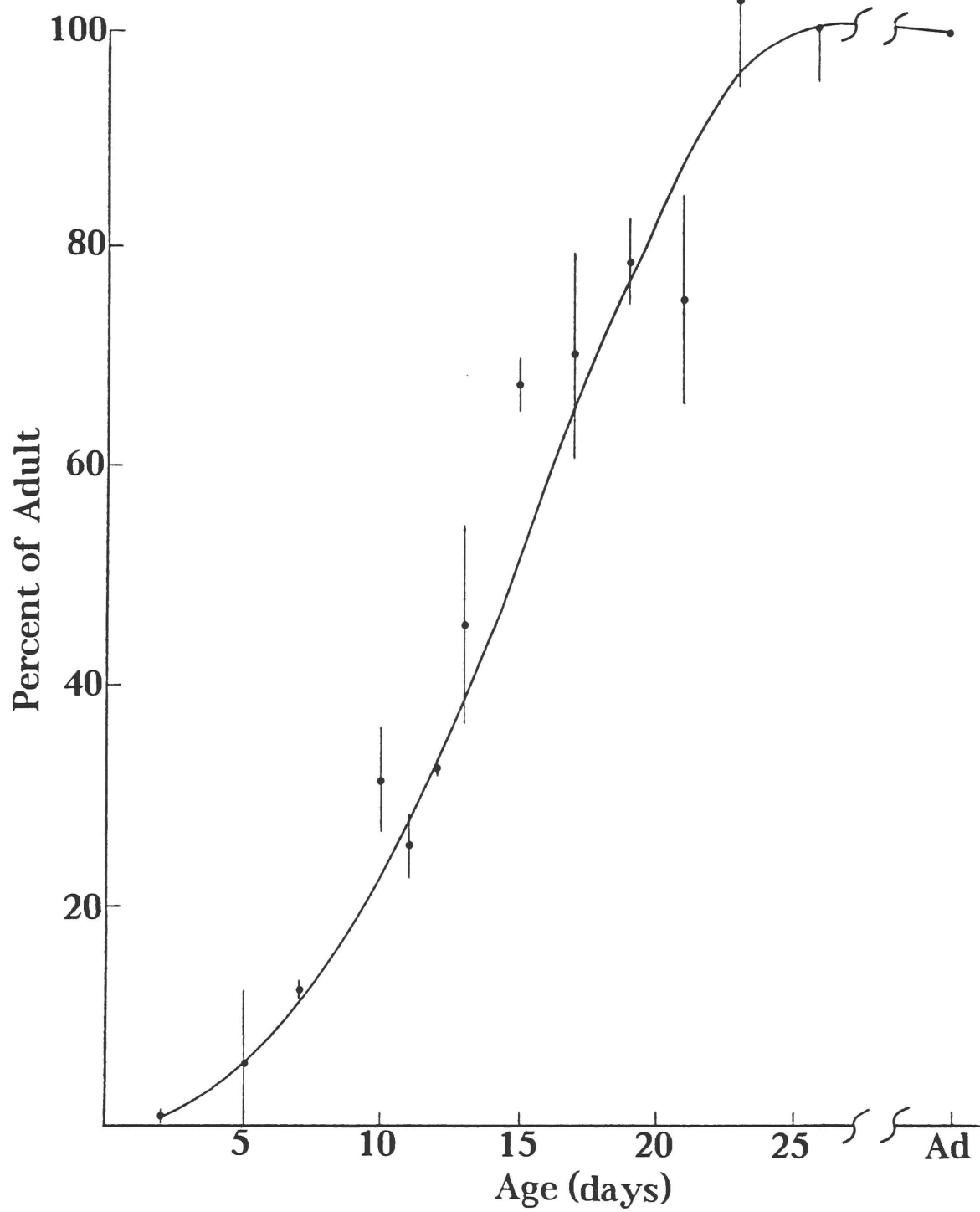


Figure 27. SVP38 expression quantitatively parallels synaptogenesis in the cerebral cortex.

Synaptogenesis in the visual cortex (o) (Blue and Parnavelas, 1983b), and in the parietal cortex (o) (Aghajanian and Bloom, 1967), as measured by electron microscope observations. Their data have been redrawn as a percentage of adult values in order to compare the curves directly with the data on SVP38 (dotted line, redrawn from fig. 26).

Synapses

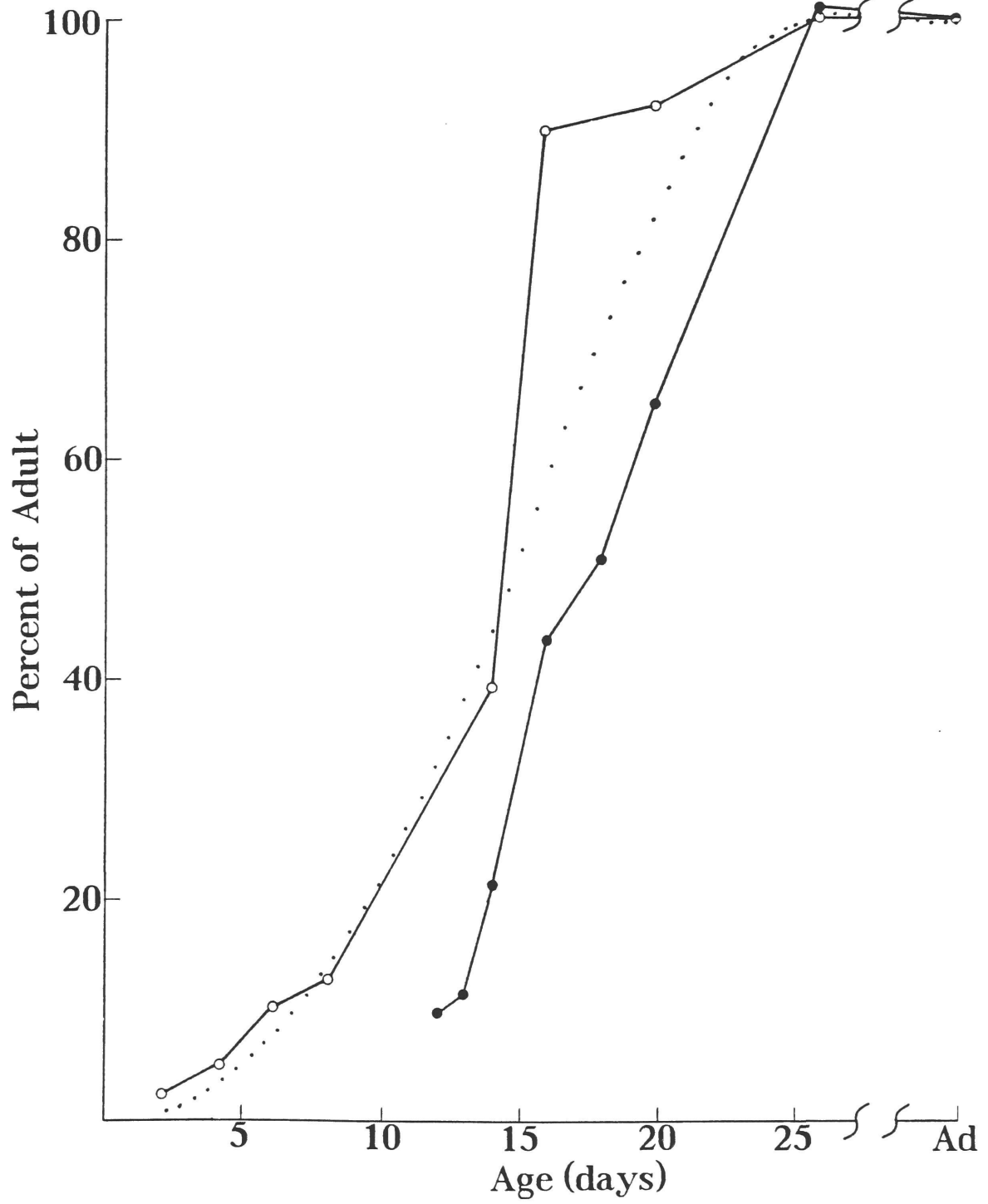
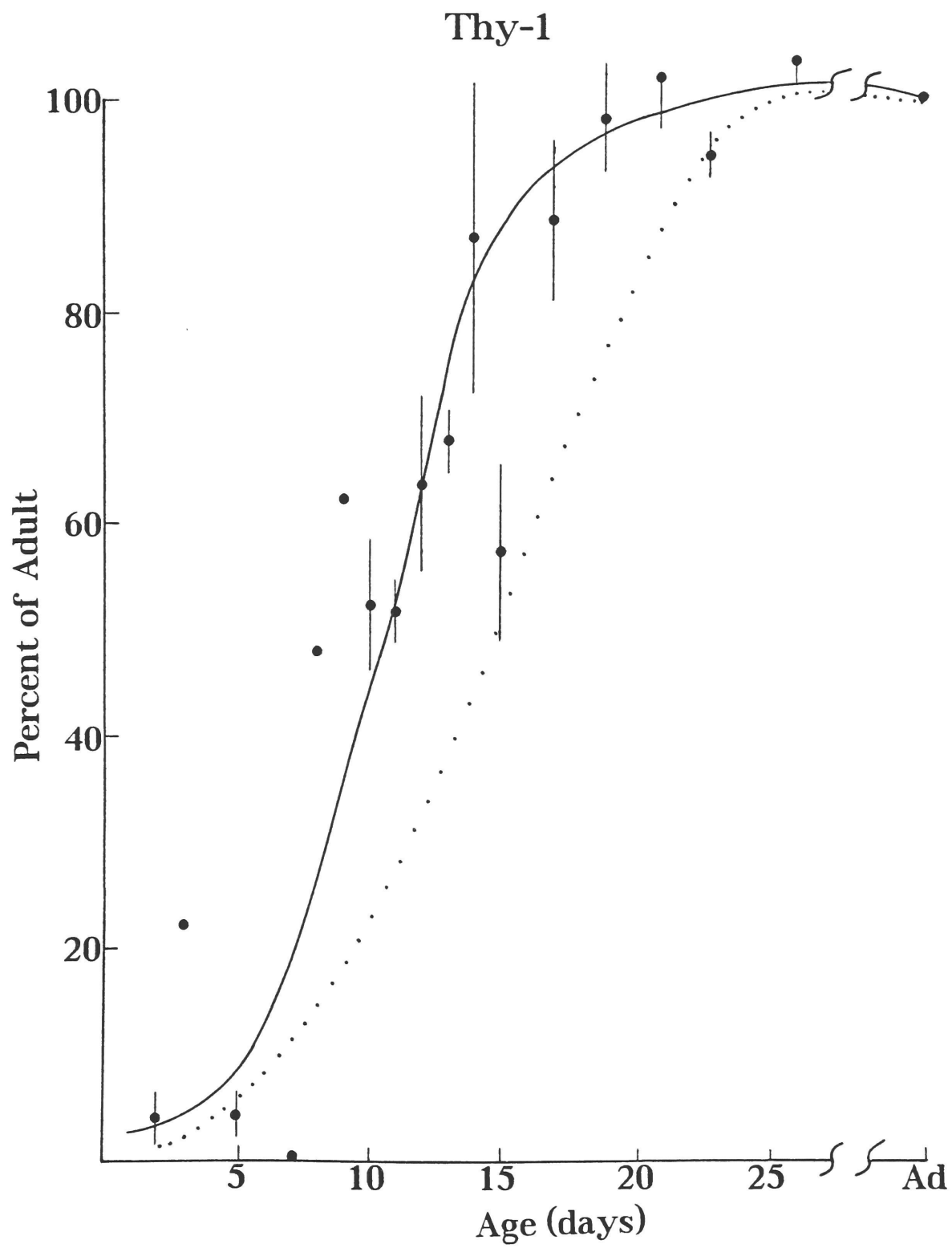


Figure 28. Thy 1 development in the cerebral cortex.

The amount of Thy 1 in the S1 fractions used for Figure 26 was determined using a monoclonal antibody and is expressed as a percentage of the adult values. The SVP38 curve from Figure 26 is redrawn (dotted line) for comparison. The standard error is shown for each point. Points without error bars represent single experiments in triplicate. Curve was fitted by eye.



CHAPTER FIVE

DISCUSSION

I have attempted to address two broad questions in this thesis. 1) Are there specific molecules that underlie the unique properties of growth cones and synapses? 2) If there are, how are these molecules regulated during the transition from growth cone to synapse?

1. CD'A 1

The CD'A 1 epitope is detectable *in vivo* solely in neuronal growth cones. *In vitro*, CD'A 1 is also found in motile and dividing fibroblasts. In both neurons and cultured fibroblasts, CD'A 1 is found in areas of the cell that have amorphous staining with phalloidin, which binds to F-actin. Cytochalasin causes a drastic redistribution of CD'A 1 immunoreactivity, and high concentrations of phalloidin completely eliminate immunoreactivity. These results suggest that the CD'A 1 antibody is recognizing an epitope on an actin associated protein, perhaps actin itself. The fixation dependence of CD'A 1 suggests that the epitope is not present in the unfixed cell. Thus, my working hypothesis is that because of proteins (either structural or enzymatic) present in the growth cone, the structure of actin is different from that in other parts of the cell or in mature neurons. The reaction with paraformaldehyde causes the actin to assume a growth cone specific conformation due to this structural difference. This conformation is recognized by the CD'A 1 antibody. Other fixatives may either preserve the native conformation of actin more precisely, or they may alter the conformation differently. The same explanation would hold for the CD'A 1 epitope in fibroblasts.

Given the above model of the CD'A 1 epitope, the effect of cytochalasin would be to eliminate the conformation of actin that results in the CD'A 1 epitope after fixation. This is probably a result of actin depolymerization. At the same time, cytochalasin increases the amount of actin in the cell body and nucleus that can give rise to the

CD'A 1 epitope. Cytochalasin may have different effects on this actin than it does on growth cone actin, and therefore induces the CD'A 1 epitope.

Cytoplasmic actin shares most of the properties of muscle actin. The conformation of muscle actin has been well studied because of its importance to the cell, its abundance, and its property of self-assembly. Phalloidin binding to actin induces a conformational change in the actin molecule, as manifested in a decrease in the disassembly of filaments, and in a change in the electron paramagnetic resonance of maleimide spin-labelled actin (Harwell, et al., 1980). This last technique has also been used to demonstrate a third conformation of the actin molecule, a paracrystalline conformation. This conformation occurs when purified actin is placed in a high concentration of divalent cations. When examined with the electron microscope, the actin appears as paracrystalline arrays, a structure distinct from F-actin bundles. The relationship of these actin paracrystals to the actin and CD'A 1 rods seen in DMSO or cytochalasin treated cells is unknown. In order to test whether CD'A 1 recognizes the paracrystalline conformation of actin, I plan to induce this conformation in purified muscle actin and test for CD'A 1 immunoreactivity. If CD'A 1 does not react with these paracrystals, it is possible that growth cone actin would form a slightly different paracrystalline conformation, or growth cone actin may form a hitherto unknown conformation, or the epitope may be also dependent on an actin binding protein.

There must be an underlying difference in the actin cytoskeleton of growth cones and motile fibroblasts that is responsible for the CD'A 1 epitope. This could be the presence of a specific actin binding protein, a specific post-translational modification of actin or an actin binding protein, or a specific isotype of actin, or a specific combination of these. Over twenty genes with homology to actin have been described, yet there are only six identified actin protein isotypes. It will be necessary to develop molecular probes for each of the actin genes to determine whether any of them are developmentally regulated in the nervous system. Recently, two new isotypes of

actin were found to be specific to the microvilli of intestinal brush border (Sawtell, et al., 1988). Adult brain synaptosomes contain several isoforms of actin (Marotta, et al., 1978), however, nothing is known about the biochemistry of growth cone actin.

Ultrastructural investigations have shown that growth cone actin is different from axonal and cell body actin. The microfilaments are not bundled, but are crosslinked into a meshwork that fills the growth cone. Although this state of actin assembly is probably related to the CD'A 1 conformation, without further information about CD'A 1, it is impossible to know whether the CD'A 1 conformation is responsible for the assembly state, or *vice versa*, or whether they both are caused by a separate factor.

The presence of the CD'A 1 epitope in cultured fibroblasts seemed inconsistent with its absence from any nonneural structures in the embryo or the adult, especially mesoderm derived structures, or the developing skin. However, fibroblasts under normal conditions *in vivo* do not express the same motility or structure as they express *in vitro*. Following the generation of a wound *in vivo*, fibroblasts do exhibit a high degree of motility, and they might then express the CD'A 1 epitope.

The staining of mitotic cells is probably not a general phenomenon, as the ventricular zone of the cortex, where there is abundant mitosis, does not in general stain with CD'A 1. It is likely that during mitosis *in vivo*, membrane contacts uniformly distributed around the cells provide the anchorage necessary for the cells to pull apart. *In vitro*, the cells remain attached to the substrate by only a few fingers at the periphery. These points of attachment might provide most of the force that separates the dividing cells. The presence of CD'A 1 suggests that these points of attachment are closely related to the structures used in cell motility.

The unifying principle underlying CD'A 1 staining therefore seems to be the generation of force by a system involving actin. In other words, CD'A 1 recognizes regions of the cell that are "pulling" on the rest of the cell.

2. Are there other growth cone specific molecules?

I have detailed the specific properties of the growth cone in the introduction. To date, all of the molecules described as components of the growth cone are also expressed at high levels elsewhere in neurons. All neuronal cell surface molecules are found on the surface of the growth cone. These include poorly characterized antibody binding sites from my own work (not shown), the ganglioside antigen Jones (unpublished observations), the cell adhesion molecule NCAM (van den Pol, et al., 1986), and one or more integrin receptors for extracellular matrix constituents (Bozyczko and Horwitz, 1986). The only known proteins that might be present in far greater numbers in the growth cone membrane than in the neurite membrane are calcium channels. This is based on the presence of larger calcium currents in the growth cone, which may reflect a difference in channel activity and not channel density. There are no other known cell surface molecules specific to or excluded from the growth cone.

There are a number of intracellular proteins that are enriched in the growth cone relative to the neurite, although all of them are also present at very high levels in the cell body. These include actin, actin depolymerizing factor, myosin, and probably most of the well characterized actin binding proteins. Growth cones also have high amounts of GAP43, relative to the neurite. This is a protein identified as one of a set of proteins whose expression is highly correlated with growing axons. In hippocampal cultures similar to those I have shown, from the time that the minor processes begin to acquire a dendritic phenotype, GAP43 is only found in axons, remaining strikingly concentrated in the growth cone, although detectable levels are also found in axons (Goslin, et al., 1988). GAP43 is a membrane bound phosphoprotein that is found at relatively high levels surrounding the Golgi apparatus and is probably transported on the surface of vesicles.

There has been speculation that there would be no growth cone specific molecules, partly because the growth cone becomes transformed into the axon and there would therefore be mixing of both cytosol and membrane components (Pfenninger, 1986). However, it is clear from the distribution of GAP43 and the cytoskeletal components discussed above that the cell can retain proteins in the growth cone even as the growth cone moves forward and part of it is transformed into the axon. It is also likely that some membrane molecules are specifically retained in the growth cone. Many membrane proteins, including receptors for extracellular matrix molecules, are linked to the actin cytoskeleton (Horwitz, et al., 1986). In addition, cycles of exo- and endocytosis could keep specific membrane components at the growth cone. There is substantial endocytosis at the growth cone. Therefore, even if the membrane lipids of the growth cone and axon are free to mix, some of the proteins might remain associated with the growth cone through cytoskeletal interactions or endocytosis and exocytosis. The spatial restriction of the active zone particles in the mature synapse, even while exocytosis occurs, also argue that neurons can restrict the movement of certain proteins in the plasma membrane. There is convincing evidence from polarized epithelia that cells can restrict the mobility of membrane proteins and retain them in specific areas of the cell.

Therefore, I feel that there are other growth cone specific molecules. These would include cytoskeletal associated proteins that help to explain growth cone motility. In addition, there might be vesicular and membrane proteins that underlie vesicular fusion and the movement of ions and small molecules across the vesicle and the plasma membrane. Finally, some receptors for extracellular ligands are probably restricted to the growth cone. It is likely that some of these growth cone specific molecules are currently unknown, while others are proteins whose distribution has not been examined in well-maintained neurons *in vitro*. One indication that the latter might be true comes from GAP43, which in standard cortical cultures is expressed at high

levels throughout the cell, but in carefully maintained hippocampal cultures is highly concentrated in the growth cone (Meiri, et al., 1986; Goslin, et al., 1988).

3. SVP38

SVP38 is a membrane glycoprotein of the synaptic vesicle. Other laboratories identified a protein with similar characteristics at the same time as I did, and named the protein p38, synaptophysin, or also SVP38. Based on the anatomical distribution, subcellular localization, molecular size, degree of glycosylation, and behavior on DEAE-Sepharose, these proteins are all identical to SVP38. SVP38 is a very immunogenic protein, many antisera raised against purified synaptic vesicles recognize nearly exclusively SVP38, this accounts for its independent discovery by so many laboratories. For the sake of clarity, I will consistently refer to this protein as SVP38, independently of whose laboratory worked on it.

The molecular nature of SVP38 has been investigated extensively. The native form is probably a disulphide-linked dimer. SVP38 is the major protein in purified synaptic vesicles that is capable of binding calcium after SDS-PAGE and western blotting (Rehm, et al., 1986). SVP38 is phosphorylated on a tyrosine *in vitro*, though there is no indication whether this is physiologically relevant, or what the stimulus to the tyrosine kinase might be. The SVP38 cDNA has been cloned and sequenced. This work indicates that there are four putative membrane-spanning domains, and a single likely N-glycosylation site (Buckley, et al., 1987; Sudhof, et al., 1987; Leube, et al., 1987). The availability of the cloned gene should make it possible to further examine the structure and function of SVP38, as well as probe the developmental expression in more detail. There is no published information that indicates the function of SVP38.

SVP38 is present on small clear synaptic vesicles and is undetectable on the large dense-core vesicles (Navone, et al., 1987). It is likely that SVP38, p65, and synapsin I are present on all of the small clear vesicles. The important exception to this is the ribbon synapses of photoreceptors, which apparently do not express synapsin I.

This latter result indicates that SVP38 and p65 may have functions more integral to vesicle targeting and release than synapsin I, which probably plays a regulatory role. The absence of all three of these synaptic vesicle proteins from dense core vesicles further confirms that these two populations of vesicles have different functions and are probably released under different regulation. The dense core vesicles are likely related to the vesicles in endocrine cells. The presence of SV2 in endocrine as well as neuronal cells suggests that this protein is also present on dense core vesicles.

4. Development

Information on the developmental expression of SVP38 and the CD'A 1 epitope is interesting in its own right, and in addition can restrict the possible functions that these molecules carry out. Molecules found in synapses but not in growth cones might be expected to carry out different functions than those found in both structures.

The loss of CD'A 1 immunoreactivity from the growth cones of hippocampal cells as they mature in culture was initially surprising, as the growth cones appear to be moving forward at approximately the same rate as those of younger cells. The heterogeneity in the loss of CD'A 1 staining suggests that there is greater variability in the growth cones than was previously suspected. This variability may result from differences between cells, or it may reflect differences in individual growth cones from one point in time to the next. Since other features of the cells indicate that they are a uniform population, the growth cones are probably varying over time. The growth of axons and dendrites in these cultures, although uniform over a period of hours, occurs in spurts, and it is possible that this underlies some of the variability. However, there is another factor responsible for the loss of CD'A 1 immunoreactivity, as the overall rate of growth remains approximately the same during the time that CD'A 1 disappears. This could be another behavioral, structural, or biochemical change in the growth cones as they mature.

It is very difficult to know if all growth cones in the cortex *in vivo* are stained by CD'A 1, since there is no independent way of labelling all of them. As the cortex matures and growth cones invade the cortical plate, the CD'A 1 labelled growth cones become fewer, shorter, and they stain less intensely. This occurs from about P3 to P12, when the staining has completely disappeared. Most of the loss of CD'A 1 occurs well before the major rise in the number of postsynaptic densities and SVP38 expression. This indicates that CD'A 1 might disappear very early in the process of synapse formation. The subsequent increase in the number of postsynaptic densities would then reflect the maturation of "pro-synapses". Alternatively, there is evidence that growth cone morphology changes as growth cones enter their target region. If this is true in the cerebral cortex, then CD'A 1 expression may correlate with this change.

The developmental expression of SVP38 in the rat cortex contrasts with that of synapsin I and p65 in the kitten cortex (Chun and Shatz, 1988). These two synaptic vesicle proteins appear to be present in growth cones and fibers of the intermediate and marginal zones, and in synapses of the subplate. My results suggest that SVP38 regulated differently. This may simply reflect a species difference, or SVP38 may have a function only utilized at synapses, while synapsin I and p65 play a role in growth cones. These results are consistent with other work on SVP38 (synaptophysin), which showed that expression in the mouse cerebrum begins after birth and peaks around postnatal day 20 (Knaus, et al., 1986). The developmental expression of SVP38 correlates very closely with the appearance of postsynaptic densities.

With the exception of the study on synapsin I and p65 in the cat cortex mentioned above, the expression of other synaptic vesicle proteins has not been analyzed. It will be of interest to determine whether some of the proteins are coordinately regulated. There are no other known proteins specific to synapses and present at all synapses. Thy 1 is a cell surface glycoprotein that is not concentrated at synapses, but has been proposed to be expressed during synaptogenesis (Zwerner, et al.,

1977). I have shown that Thy 1 is expressed in the cerebral cortex slightly prior to the appearance of mature synapses and the expression of SVP38. This finding is consistent with the hypothesis that Thy 1 plays a role in synaptogenesis. The developmental expression of the proteins involved in neurotransmitter synthesis, uptake, and binding, in general appear with the same time course as SVP38. These are summarized in Figure 30.

The time course of the expression of neuropeptides is in many cases different from the time course of SVP38 expression. Since these neuromodulators are present in dense core vesicles, it would be of interest to determine the time course of expression of other proteins specific to these vesicles.

5. Does synapse formation regulate gene expression?

I have shown that the expression of CD'A 1 and SVP38 immunoreactivity changes during the time of synapse formation in the cortex. The obvious question is whether the process of synapse formation itself is the underlying signal that controls the expression of these, or any other molecules. The developmental expression of SVP38 has been examined in two other central nervous system regions.

In the rodent retina, synapses appear in a centripetal sequence, the reverse of the sequence of layer formation (Weidman and Kuwabara, 1968). The outer plexiform layer (OPL) does not form until the fourth postnatal day, while the inner plexiform layer (IPL) is well developed at birth, yet synapses appear in the OPL first. Thus, on the fifth day after birth, many synapses are present in the OPL, yet not until the second week after birth do synapses appear in the IPL. SVP38 expression exactly mirrors this sequence, staining appears on day 2 in the OPL and on day 8 in the IPL (Barnstable, et al., 1988). As in the cortex, p65 and synapsin I are expressed prior to synaptogenesis in the IPL (Sarthý and Bacon, 1985; Gaur, et al., 1985). Both proteins are present in the IPL at birth, more than a week before the appearance of SVP38 and synapses.

SVP38 in the developing cerebellum appears to considerably precede synapse formation and parallels the expression of synapsin I (Leclerc, et al., 1989). At birth, there is strong SVP38 immunoreactivity in Purkinje cells, including their axons, cell bodies, and surprisingly even their apical dendrites. This latter staining, if real, may reflect massive synthesis and inefficient axonal transport of SVP38 at this age. SVP38 is also present in profiles that appear to be climbing fiber or mossy fiber growth cones and axons. There is also staining in axonal profiles of migrating granule cells. This distribution is generally in agreement with that of synapsin I (Mason, 1986). All of the growth cones that have been shown to have SVP38 are derived from cells that quite possibly have already formed synapses elsewhere, the granule cell axons (parallel fibers) form synapses and then continue to elongate, as is also true for the mossy and climbing fibers, which could have formed synapses in the deep cerebellar nuclei. However, SVP38 is expressed in the cerebellum before the bulk of synapse formation.

The growth cone and the synapse carry out different functions. Although neurotransmitter release can occur from growth cones, it has different properties than synaptic release, as discussed in the introduction. It is tempting to speculate that the initial stable contact between the growth cone and the synapse is responsible for the induction of synaptic components, and the repression of growth cone components. Alternatively, there could be other mechanisms that regulate the expression of these components. For example, the release of a soluble factor might trigger differentiation in a whole class of cells simultaneously, or cells may have endogenous "clocks" that determine when certain sets of genes are activated.

6. Future Directions

My work with the CD'A 1 antibody leaves a number of important questions unanswered. I have put forth a specific hypothesis about the nature of the CD'A 1 epitope, namely that it is on a specific conformation of actin or an actin associated protein. This can be tested by asking whether actin, when purified alone or with actin

binding proteins, can be made to assume a conformation that is recognized by the CD'A 1 antibody.

There is a hint from my work on the hippocampal cell cultures that the presence of the CD'A 1 epitope correlates with the shape of the growth cone. One *in vivo* test of this hypothesis is readily available in the optic system. The growth cones of retinal ganglion cells assume a variety of morphologies during their movement through the nerve, chiasm, tract, and target region. Only in the optic tract do they have large lamellipodia. There was no CD'A 1 staining in the optic nerve at the time when growth cones of the retinal ganglion cells were passing through. If this is because the growth cones lack lamellipodia, then there should be growth cone staining in the optic chiasm.

No studies have explicitly examined the disappearance of the growth cone as synapses form in the cerebral cortex. One intriguing aspect of this is the loss of CD'A 1 immunoreactivity. CD'A 1 immunoreactivity has almost disappeared from the cerebral cortex around postnatal day 8. Electron microscope immunocytochemistry of the cortex at this age would determine whether the staining that remains on P8 is present in growth cones or in immature synapses. This would shed some light on the morphological and biochemical transition of growth cones to synapses.

The CD'A 1 antibody can also be used to improve the growth cone preparation that I used as the immunogen. It offers the opportunity of using antibody binding instead of morphological criteria as an assay for growth cones in a subcellular fractionation protocol. An improved growth cone preparation might lead to the discovery of other growth cone specific molecules.

The availability of a simple histological probe for growth cones, like the CD'A 1 antibody, makes it easy to examine the distribution of growth cones in a wide area of tissue. Analyses of the distribution of growth cones during development and after injury or experimental perturbation should now be relatively easy.

One of the hypotheses that I had hoped to address in my thesis work is whether synapse formation is an inductive event that regulates the expression of genes associated with growth cone or synaptic function. This hypothesis proved to be very difficult to test experimentally. The key experiment is to isolate neurons from all contacts and examine whether all of the synapse specific proteins are expressed. Even contacts between a growth cone and other parts of the same cell can lead to stable synapses and should be avoided. If the cells nevertheless express synapse specific proteins, then it would be clear that the growth cone target interaction is not necessary for expression, although it might still provide quantitative regulation *in vivo*. An alternative experiment is to examine whether changing the time of contact between growth cone and target through heterochronic transplantation has any effect on the expression of these proteins.

7. Summary

I have identified CD'A 1 as a cytosolic epitope present in growth cones in most regions of the developing brain. CD'A 1 is likely a growth cone specific conformation of actin or a tightly actin associated protein. I have also identified SVP38 as a synaptic vesicle membrane glycoprotein present at synapses throughout the body and in neuroendocrine cells. These two markers are regulated oppositely as synapses form in the cerebral cortex: CD'A 1 disappears about the time that SVP38 expression begins.

APPENDIX A

HISTORY OF GROWTH CONE RESEARCH

The neuronal growth cone is the specialization at the tip of a growing nerve cell process and plays an integral role in guiding the growing nerve cell axon to the appropriate target cell. The growth cone therefore is intimately involved in the formation of the patterned cellular interconnections that underlie the function of the nervous system. Although it lies at the heart of developmental neurobiology, the growth cone was not discovered until after theories had been developed to explain the substructure of the adult nervous system.

The Cell Theory of Schwann and Schleiden provided a unifying principle underlying diverse tissues in all living organisms, however, it was not immediately applied to the nervous system. Initially, It was not obvious that nerves were anatomically part of neurons, and there was no theory to explain how cells could transmit signals if they were not fused to each other. In particular, it seemed preposterous that a 20 μm neuron could develop or support axons that were sometimes over a meter long.

Three theories have been formulated to explain the formation of nerves (Figure 29, taken from Ramon y Cajal, 1937). The first was proposed in 1839 by Theodor Schwann, one of the originators of the Cell Theory. Ironically, he proposed a cell-chain theory, namely that the axon in peripheral nerves is formed by the cells (now known as Schwann cells) that form the perineural sheath. This theory was made untenable when histological staining techniques had improved to the point where it was clear that in the adult the nerve is contiguous with the neuron and that neurons had the ability to at least elaborate short processes.

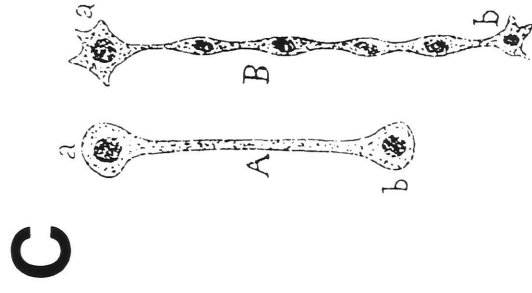
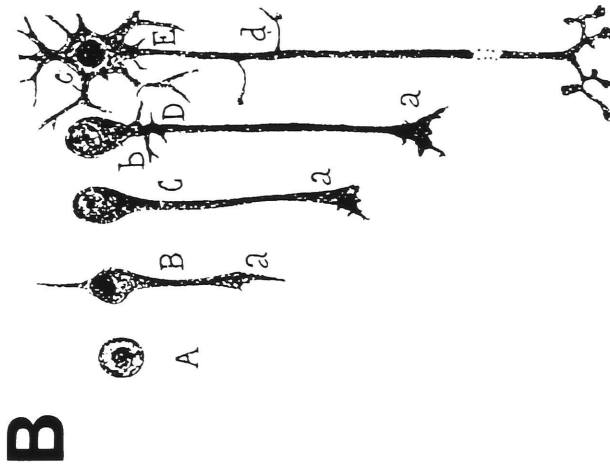
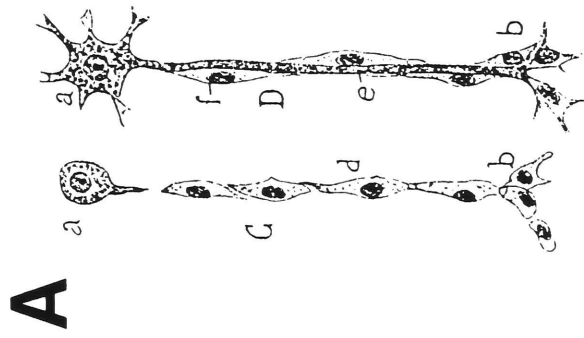
The second theory was the outgrowth theory articulated first by Friedrich Heinrich Bidder and C. Kupffer in 1857. They proposed that the nerve fiber is the outgrowth of a single nerve cell, however, they put forth only limited evidence. In

Figure 29. Three theories of nerve development, from three illustrations by Ramon y Cajal (Figures 1937).

A. Cell chain theory of Schwann. The neuron is not responsible for the generation of the nerve. The nerve (e) is formed by the fusion of the sheath cells (C) with each other and with the neuron (a).

B. Outgrowth theory of Bidder and Kupffer, and championed by His. The nerve is a product of the neuron, which is fully capable of generating and supporting it.

C. Plasmodesm theory of Hensen. The nerve is the product of the movement and incomplete cytokinesis of cells derived from the neuron.



1864, Victor Hensen proposed the plasmodesm theory, which was a modified version of the cell chain theory that took into account the importance of the neuron, and recognized that in the adult, the nerve and neuron were contiguous. He suggested that neurons generate daughter cells that migrate out into the future nerve path, and under the influence of neuronal activity, remain fused to each other and to the neuron to form the nerve:

In order to explain the commissural fibers up to now, one had to assume that the nerve bundles grow to the other side and then grow over their targets, near or far. I must assume that after the closure of the neural tube, cell growth and cell movement takes place from one side to the other. These assumptions themselves bring certainly no bigger difficulties than the former, and [the plasmodesm theory] is perhaps thus already preferred, while certain physiological observations on the crossing and backcrossing are explained through this... *An actual outgrowth, an actual stopping of the nerve at a particular spot of its later ending, nobody has seen* [italics mine].

I doubt that anywhere from the central nervous system or in the central nervous system nerves can grow out freely, to search out their physiological target and to bind with it, therefore the fact may be accepted that all nerves arise through the imperfect separation of the cells at the beginning and the end [of the nerve] (Hensen, 1864, pp. 71-72, translation by SHD).

For the next 50 years, there was often bellicose debate between the proponents of the plasmodesm theory and the outgrowth theory.

Wilhelm His used some of the newly discovered histological staining techniques to make a number of fundamental discoveries about neural development. He described the neural tube as a flat epithelium and first recognized the ventricular zone cells as neuronal precursors. Most importantly, in 1886 he observed the incipient axon forming as a protoplasmic outgrowth after the terminal mitosis of the neuroblast (His, 1886a). He surmised that the cells surrounding the growing axon neither nourished nor transformed the developing nerve:

I consider as a definitive principle the theorem that every nerve fiber originates as the outgrowth of a single cell. The latter is its genetic [embryonic], nutritive, and functional center. All other connections are either indirect or they originated secondarily (His, 1886b).

Despite his masterful work, His was unable to ascertain the mechanism of axonal elongation.

Santiago Ramon y Cajal discovered how axonal elongation occurs when he discovered the neuronal growth cone in 1890. Cajal used Camillo Golgi's technique of

silver impregnation on embryonic nervous tissue. This technique enabled him to see individual fibers standing out in black against a clear background. In an embryonic day 4 chicken spinal cord, he was able to follow the entire processes of immature neurons. When he saw that axons which were still growing had a specific structure at their tips, he was able to correctly infer the function of that structure. He named it the *cone de croissance*, also referring to it as *cone d'accroissement* (Figure 30, taken from Ramon y Cajal, 1890a). After looking at so many stained sections, Cajal must have developed an almost cinematographic image of neuronal development. He certainly wrote as if he did:

I had the good fortune to behold for the first time that fantastic ending of the growing axon. In my sections of the three-days chick embryo, this ending appeared as a concentration of protoplasm of conical form, endowed with amoeboid movements. It could be compared to a living battering ram, soft and flexible, which advances, pushing aside mechanically the obstacles which it finds in its way, until it reaches the area of its peripheral distribution. This curious terminal club, I christened the growth cone (Ramon y Cajal, 1937, pp. 368-369).

With the discovery of the growth cone, Cajal laid to rest Hensen's severest objection to the outgrowth theory, namely that no one had seen an actual outgrowth of a nerve. Cajal described growth cones in many other developing structures. In the development of the climbing fibers of the cerebellar cortex, he was able to demonstrate that the growth cone develops directly into a synaptic contact ("contactual connection") (Ramon y Cajal, 1890b). Thus, solely from histological investigations, Cajal discovered the growth cone and proposed that it is intimately involved in specific axonal elongation and synapse formation. All of Cajal's theories about growth cone function have been borne out by subsequent research.

Cajal foreshadowed much of current research in developmental neurobiology by articulating two ideas describing how the specificity of connections is established. At the same time that he analyzed the development of the climbing fibers, he noted that Purkinje cells initially have dendritic branches that they later lose:

I noticed that every ramification, dendritic or axonic, in the course of formation, passes through a chaotic period, so to speak, a period of trials, during which there are sent out at random experimental conductors most of which are destined to disappear. Like a miner who digs

Figure 30. The first discovery of the nerve growth cone, drawn by Ramon y Cajal.

This is a section of the embryonic day four chicken spinal cord, showing his drawing of growth cones (G).

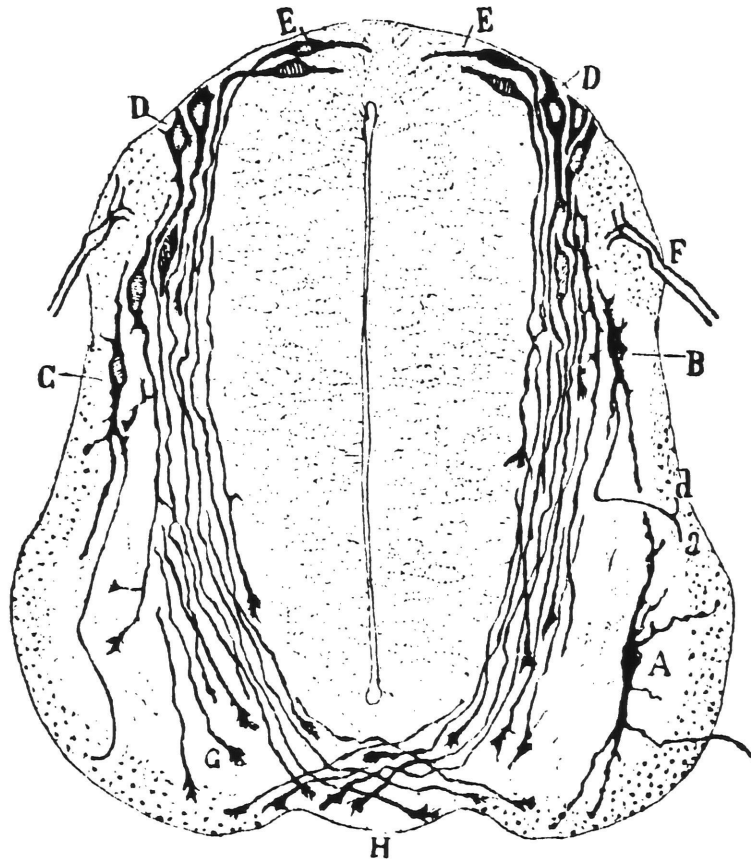


Fig. 1e. Coupe de la moëlle dorsale d'un embryon du poulet au 4^e jour de l'incubation. Méthode de GOLGI très rapide.

A cellule de la racine antérieure; *B* cellule du cordon antéro-latérale; *C* cellule du cordon antérieur; *D* névroblastes piriformes dont le cylindre-axe termine au niveau de la commissure antérieure par un cône d'accroissement; *E* cellules nerveuses primordiales qui conservent encore la forme des spongioblastes; *F* racine postérieure; *G* cônes de croissance; *H* commissure antérieure embryonnaire.

blindly in search of a lost vein, the protoplasmic buds try various roads to reach their proper end. Later, when the afferent nerve fibres have arrived, or when the neurons mould themselves and attain in due time functional solidarity, the useful expansions remain and become fixed and the useless or exploratory ones are reabsorbed. In this case nature proceeds like the gardener who guides for the well directed shoots and prunes off the defective or superfluous ones (Ramon y Cajal, 1937, pp. 370-371).

Another concept that Cajal had about the developmental mechanisms of synapse formation he articulated first in 1892 (Ramon y Cajal, 1892). This was the idea that nerves have chemical attractants that cause them to grow, and influence their direction of growth, which became known as neurotropism. In 1933, Cajal felt that the strongest evidence for this came from his studies of the degenerating and regenerating spinal cord, and from Tello's studies of regeneration in the cerebral cortex following the introduction of a piece of degenerated nerve:

The liberation in the [spinal cord] of neurotropic substances being initiated with the degeneration of the cells of Schwann, these substances diffuse into the columns of the cord, where the axons, formerly sluggish and apparently inert grow actively.

The same thing takes place in the cerebral hemispheres. As Tello has shown in his brilliant experiments, if a piece of degenerated nerve is introduced into a cerebral wound, the axons of the pyramidal cells, the most apathetic of nerve fibres and the most recalcitrant to any regenerative process, emerge from their inertia, swell up and send off long shoots which penetrate the nervous implant with the same activity and power of growth which characterize the regeneration of the severed sciatic nerve.

Such facts, of great biological significance, definitely refute the generally accepted dogma of the essential irregenerability of the central tracts. Moreover, these and many other facts demonstrated at the same time teach us that the morphology of the nerve cells is not governed by immanent and fatal factors which are passed on by inheritance, as certain authors have maintained, but depends entirely on the actual physical and chemical conditions in the surrounding medium (Ramon y Cajal, 1937, pp. 569-570).

Because of Cajal's vast experience with the histology of the developing nervous system, he considered the outgrowth theory to be fully proven with his discovery of the growth cone. However, many scientists remained unconvinced by Cajal's histological analyses, arguing that one could not really see everything that was there. There were perhaps protoplasmic bridges that could not be seen connecting the cells to each other, or perhaps there were unseen fibers already present in the future nerve path that the neuron recruited to form the nerve. The application of electron microscopy to biology in the 1950s was necessary to finally lay these ideas to rest.

In the first decade of this century, Ross Granville Harrison conceived of experiments that would directly test whether a neuron could elaborate a long axon on its own, or whether it obligatorily needed the surrounding tissue. He had already found that after removing the source of the sheath cells, motor neuron axons grew as naked cylinders, often reaching their target muscle (Harrison, 1904, 1906). However, it was still possible after these experiments to argue that although the neuron is necessary for nerve formation, enough sheath cells survived the removal of the neural crest to play an integral role in nerve formation, or possibly another component of the nerve pathway was able to substitute for the sheath cells. To address these possibilities, Harrison had a major conceptual breakthrough. He wrote:

The really crucial experiment remained to be performed, and that was to test the power of the nerve centers to form nerve fibers within some foreign medium, which could not by any possibility be suspected of contributing organized protoplasm to them....

[This] line of experimentation, which consisted in the isolation of pieces of living tissue in unorganized media, gave considerable difficulty at first, but in the spring of 1907 a method was finally devised, which satisfactorily accomplished the purpose (Harrison, 1910).

This approach was tremendously fruitful for the understanding of growth cones and how neurons develop their form and initiated the entire field of tissue culture. Harrison found that fragments of embryonic spinal cord would elaborate nerve fibers when placed on a cover slip in a drop of clotted lymph from an adult frog. By repeatedly observing the living cultures, he was able to view dynamic neuronal morphogenetic activity directly as it was happening. Harrison described the activity of the growth cone, and could unambiguously show that neurons were able to elaborate very long processes without any supporting cells.

One of the major criticisms of the outgrowth theory had been that the growth cone was not really the end of the developing nerve, that indistinguishable processes connected the tip with other cells. In a footnote, Harrison addressed that issue, which had been recently raised by Theodor Schaeppi regarding some of Harrison's preliminary reports (Schaeppi, 1908).

Turning now to the criticism of the present experiments, I feel confident that if Schaeppi had had before him the figures which I am able to present here, he would hardly have asked: "Wer in aller Welt will mich denn davon ueberzeugen, dass das, was unter dem Mikroskope als das Ende einer Faser erscheint, nun wirklich im Tat und Wahrheit das Ende ist?" ["Who in the world will then convince me that that which looks under the microscope like the end of a nerve is actually, truly the end?" (translation by SHD)]

In doubting that such ends, in which motion and extension can be observed with absolute certainty, are actual ends, it seems to me that the supporters of the protoplasmic bridge theory are pushing skepticism about one thing beyond the utmost limit, and at the same time are placing an equally unbounded faith in the invisible.... Could the botanist, if pressed for an absolutely rigorous proof that the roots of a plant grow out from the radicle and are not preformed in the soil, give an answer based on evidence of any different kind from that given here for the outgrowth theory of nerves (Harrison, 1910, pp. 827-828)?

Harrison's work also addressed a number of issues about the growth cone. He directly saw for the first time what Ramon y Cajal had surmised, namely that "embryonic cells ... manifest striking amoeboid activities which are especially pronounced in cells taken from the nervous system, and result in such cases in the formation of long threads of hyalin protoplasm (Harrison, 1910, p. 791)." Harrison's drawings documented the ability of the growth cone to change its shape rapidly and to initiate side branches that were subsequently withdrawn (Figure 31, taken from Harrison, 1910). By showing that branch points did not move relative to the cell body, while the distal fiber grew, he also showed that growth occurs at the growth cone and not in the fiber or cell body:

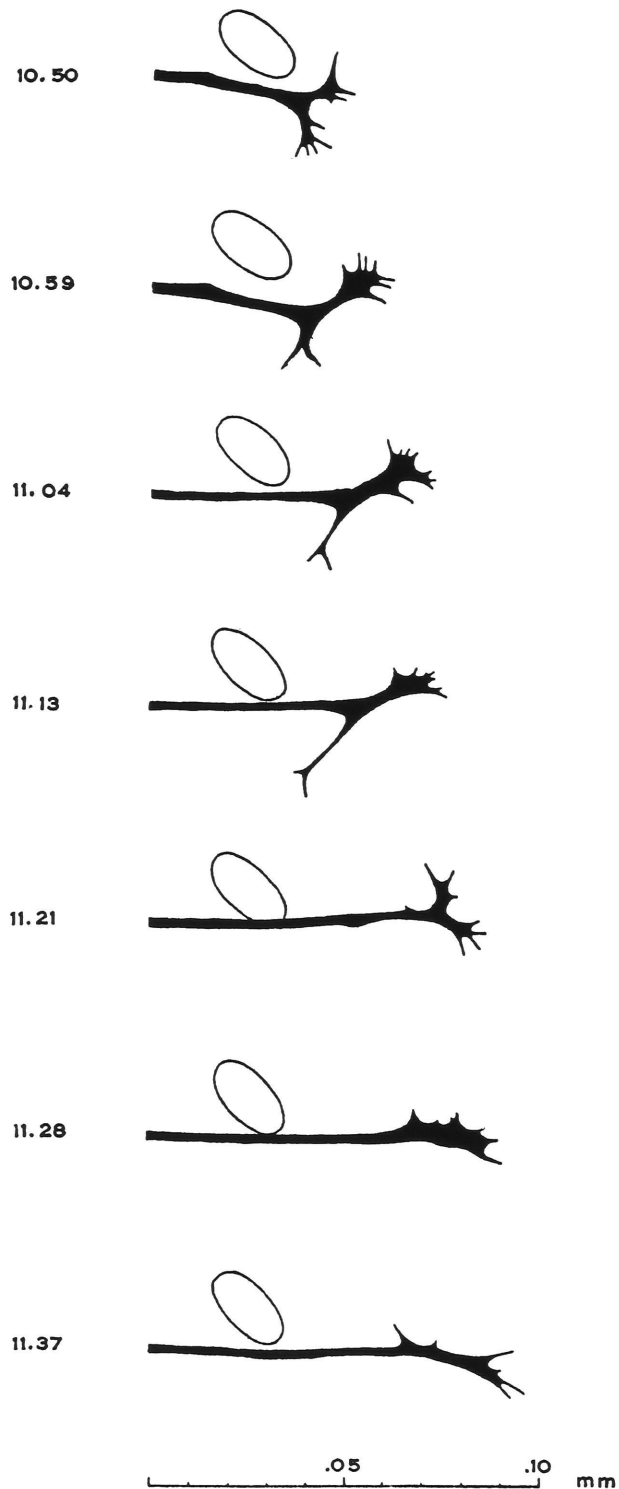
We cannot escape the conclusion that the extension of the fiber is due to the activity of the enlargement at its end (Harrison, 1910, p. 819).

Harrison invented tissue culture for a specific reason, namely to prove the outgrowth theory of nerve formation. Once he had done so, he abandoned tissue culture and returned to histology.

However, other workers continued to use tissue culture to examine the development of neurons in tissue culture. From the first introduction of tissue culture until the present day, many researchers hoped that the many diverse factors that might cause directional growth of axons *in vivo* could each be tested in isolation in tissue culture. The growth cone became far easier to study following the development of phase contrast microscopy and time-lapse cinematography, which C.M. Pomerat and his coworkers made pioneering use of to study developing neural tissue *in vitro*. Their

Figure 31. An example from Harrison's work on neurons in tissue culture, demonstrating that the growth cone changes shape rapidly and repeatedly, and moves forward, leaving a process behind it.

Note the movement of the growth cone, relative to the piece of debris (oval). This sequence also showed that neurites put out processes (lower branch at 10:59), that they later retract (11:21).



Seven successive views of the end of a growing nerve fiber showing its change of shape and progression. The sketches were made with the aid of a camera lucida at the time indicated on the left. The red blood corpuscle, shown in outline, marks a fixed point. The observations were made upon a living preparation of ectoderm from the branchial region, isolated in lymph, four days after isolation. The total length of fiber at that time was 800μ .

films revealed that the lamellipodia and filopodia of growth cones exhibited rapid extension, retraction, and "flag-like undulation of the membrane" (reviewed in Pomerat, 1967).

Junnosuke Nakai, using these techniques, laid the foundation for much of our current understanding of growth cone behavior and the mechanisms of pathway selection with his careful observations of growth cone behavior *in vitro*. In 1959 he described what happened when a growth cone contacted other objects in the dish. He concluded:

1. The filopodia of the outgrowing neurite touch or adhere to almost all kinds of obstacles, such as neuronal or non-neuronal cell elements, cell debris, glass, metal, starch, polyethylene, paraffin and cholesterol.

2. There is some difference in the manner of contact or adhesion of filopodia attributable to the nature of the object.

It is assumed that the growth cone "palpates" indiscriminately the structures placed in its course and reacts to them accordingly (Nakai and Kawasaki, 1959).

The idea that growth cones show relatively indiscriminate movements followed by responses that are specific to what they contact has been borne out by subsequent research.

The tail fin of the tadpole had been used by Hensen to examine the formation of nerves because it was a transparent structure that needed no sectioning. In the early 1930s, Carl Caskey Speidel returned to this preparation, but examined nerve development in the living tail, in an anaesthetized animal. Speidel was able to observe individual nerves from the very first elongating axon through to the process of myelination. Speidel used the term "pioneer axones" to describe the first axons to grow out from the spinal cord:

Just as there exist preterminal plexuses in the muscle and skin of the embryo which are important for "methodical and serial organization of nerve terminations later on" (Cajal, Harrison, Schultze, and Coghill), so also there seem to exist, in my opinion, preliminary nerves, an important function of which is the general orientation and guiding of advancing myelinated fibers.

An important point still to be determined is the ultimate fate of these early pioneer axones (Speidel, 1932, p. 312).

He showed that "myelin is formed as a result of the cooperative activity of myelin emergent sprout and primitive sheath cell (Speidel, 1932, p. 303)," and also showed that there was an intrinsic difference between naked axons that were myelinated at their base and those that were unmyelinated at their base: sheath cells never formed myelin on the latter. Many of Speidel's observations were on the growth cone. He fully confirmed Harrison's observations:

Little need be said concerning the progress and general behavior of the pioneer growth cones. Harrison's drawings of these as seen in tissue cultures are quite representative of the ones visible in the living animal. The mode of locomotion is typically ameboid (Speidel, 1933, p. 5).

Speidel looked for evidence that one or more factors directed or stimulated growth cones. He could find no clear answer because although he noted a "general tendency" for growth cones to move in one direction, individual growth cones could do just about anything. He described one example where two growth cones extended in "diametrically opposite directions along the same fiber at the same time." He wrote:

This remarkable example is significant for all theories of neurogenesis which attempt to elucidate the distribution and orientation of nerve fibers.

This case beautifully illustrates also the conflicting stimuli that must impinge on growing nerve sprouts. In regenerating zones after tail sections, sprouts can be found growing in a variety of directions (Speidel, 1933, p. 13).

Finally, Speidel noted that other cells in the developing tadpole tailfin have structures and behaviors remarkably similar to growth cones, "In brief, growth cones are not limited to nerve cells. They are essentially present in the case of endothelial cells, fibroblasts, and possibly other cells (Speidel, 1933, p. 26)." Many subsequent investigators have noted the similarity between growth cones and other cells, however Speidel remains alone in finding these similarities in the living animal.

The work of His, Ramon y Cajal, Harrison, Speidel, and Nakai constitutes the classical work on growth cones. His laid the foundation, while the others were responsible for generating most of the current growth cone concepts:

1. The growth cone is the structure responsible for axonal elongation (Ramon y Cajal, 1890a).
2. The growth cone is transformed into the synapse (Ramon y Cajal, 1890b).
3. The growth cone is an extremely motile structure that can be readily studied *in vitro* (Harrison, 1910).

4. Growth cones behave similarly *in vivo* and *in vitro* (Speidel, 1933).
5. Growth cones palpate their environment indiscriminately, then show specific responses dependent on what they contact (Nakai, 1959).

These early investigators formulated all of the basic questions that are currently being pursued. Most of the modern questions concerning growth cones are really specific versions of one of the questions they asked, best exemplified by the following question by Ramon y Cajal:

What mysterious forces precede the appearance of these processes, promote their growth and ramification, stimulate the corresponding migration of the cells and fibres in predetermined directions, as if in obedience to a skillfully arranged architectural plan, and finally establish those protoplasmic kisses, the intercellular articulations, which seem to constitute the final ecstasy of an epic love story (Ramon y Cajal, 1937, p. 373)?

APPENDIX B

MATERIALS AND METHODS

Animals

Sprague Dawley and Long Evans rats (*rattus norvegicus*) were used interchangeably. Pregnant rats were from an in-house breeding program and from Zivic-Miller; embryonic day 1 (E1) was the first day a vaginal plug was seen, birth was usually on E22. The stage of development was confirmed by comparing the crown to rump length to published figures (Christie, 1964). Postnatal animals used for histology were given an overdose of nembutal prior to cardiac perfusion, the young rats used for biochemistry were sacrificed by rapid decapitation, and older rats and pregnant rats were sacrificed by carbon dioxide exposure. Mice (*mus musculus*) were sacrificed by cervical dislocation.

Preparation of growth cones

Growth cone enriched membrane fractions were prepared as described by Pfenninger, et al. (1983). Forebrains of E17 rats were dissected and homogenized in 10 volumes of buffered sucrose (300mM sucrose, 1mM TES, 1mM MgCl₂, pH 7.3). The interface above 1.0 M sucrose was collected, diluted with buffered sucrose, and pelleted by centrifugation at 48,000 rpm for 2 hours. The pellet was resuspended by homogenization into buffered sucrose and stored at -20°C. This material will be referred to as the growth cone enriched membrane fraction.

Preparation of Monoclonal Antibodies

Mice were immunized subcutaneously with 0.4 mls of an equal mixture of about 500 µg of the growth cone enriched membrane fraction in complete Freund's adjuvant. Two weeks later, they were boosted by another subcutaneous administration of the same immunogen. After ten days, the serum response to the immunogen was tested,

and the best responding mice were selected for intravenous immunization. About 50 μ g of the growth cone fraction was injected into the tail vein two weeks after the most recent subcutaneous immunization.

Four days later, the spleen was removed, mechanically dissociated into single cells in RPMI (serum free) and pelleted by centrifugation. The pellet was resuspended into 10 mls RPMI, centrifuged, and the pellet resuspended into 5 mls RPMI. Exponentially growing NS1 cells were harvested, centrifuged, resuspended into 10 mls RPMI. This was again centrifuged and resuspended into 5 mls RPMI. The NS1 cells and the spleen cells were pooled and centrifuged. The supernatant was completely aspirated, and the tube gently vortexed. 1 ml of polyethylene glycol (PEG 1500 in 75 mM HEPES, Boehringer Mannheim) was added over 1 minute, and the cells let sit for 1 minute. 1 ml RPMI was added over 1 minute, then another 1 ml over 1 minute. 8 mls RPMI were then added over 4 minutes. This was gently centrifuged for 10 minutes, and the pellet resuspended into growth medium. (10% Fetal Calf Serum, in RPMI). Hypoxanthine, Aminopterin, and Thymidine were added to select for the hybridoma cells.

The resultant hybridomas were plated into ten 96 well plates that had had a feeder layer of macrophages added to them the day before. The cells were fed every three days. When colonies of hybridoma cells were visible to the naked eye, the supernatant was removed and tested by immunofluorescence on cryostat sections of 4% paraformaldehyde fixed tissue. Colonies that gave interesting staining patterns were grown up in 24 well plates and subcloned by plating 100 cells into a 96 well plate with a macrophage feeder layer. Cells were stored over the long term in freeze mix (FCS/DMSO), at liquid nitrogen temperatures.

Ascites fluid was used for most experiments. This was prepared by injecting 2-5 X 10⁶ hybridoma cells intraperitoneally into mice that had been primed i.p. with pristane (2,6,10,14 tetramethyl pentadecane) a few days previously. After ten days to

two weeks, when the abdomen was very swollen, mice were sacrificed by cervical dislocation, and the peritoneal cavity drained. This was centrifuged at 2,000 rpm to pellet red blood cells and junk, let sit overnight at 4°C, then centrifuged at 10,000 rpm (SS34), for 30 minutes, and stored at -80°C.

Antibodies

The hybridoma secreting the CD'A 1 antibody was the product of a fusion of the myeloma NS1 cell line with splenocytes from a mouse hyperimmunized with the growth cone enriched membrane fraction. The name CD'A 1 is derived from *cone d'accroissement*, the first published name for growth cones (Ramon y Cajal, 1890). The monoclonal antibody recognizing SVP38 was one of a series of antibodies produced in this laboratory against rat retinal membranes (Akagawa and Barnstable, 1984). Antibody CD'A 1 is an IgG1, the antibody to SVP38 is an IgG2b. The monoclonal antibody recognizing rat Thy-1 has been described previously (Barnstable and Drager, 1984). Monoclonal antibody RET-P1 binds to rhodopsin and has been described (Fekete and Barnstable, 1983). Rhodamine-labelled alpha-bungarotoxin was the generous gift of Dr. L. Rubin (Rockefeller University). Rabbit antiserum to cofilin was provided by Dr. Nishida. Antiserum to actin was purchased from Miles or from Sigma. Fluorescein, rhodamine, and peroxidase conjugated secondary antibodies were from Boehringer Mannheim or Miles Scientific.

Light microscope immunohistochemistry

All staining was carried out on cryostat sections of tissue fixed with 4% paraformaldehyde, either by immersion (prenatal), or cardiac perfusion (postnatal). Sections were blocked with 5% normal goat serum (NGS) in PBS for 15 minutes, followed by primary antibody for 60 minutes. The sections were washed in PBS, and then incubated in rhodamine conjugated goat anti mouse IgG for 60 minutes. Following another washing, sections were viewed with a fluorescent microscope.

Cell cultures

Embryonic day 16 rat forebrains were dissected into ice cold dissection medium (Eagle's MEM + 0.1% gentamycin + 34mM glucose). They were dissociated by gentle trituration in dissection medium with a 5 ml glass pipette. After three times up and down, the large pieces were allowed to settle, and the dissociated cell suspension was removed. This was repeated with the remaining pieces, the suspensions were pooled and collected by centrifugation. The cells were resuspended into growth medium (Eagle's MEM + 5% rat serum + 2mM glutamine + 34mM glucose + 0.1% gentamycin), counted, and plated at a density of 10^5 cells/well in 8 well chamber slides (Miles Scientific). After 1 or 2 days, cultures were fixed with 4% paraformaldehyde, and immunostained as were the cryostat sections, except that 0.1% Triton X100 was included in all steps after fixation to permeabilize the cells.

The rat fibroblastic cell line NRK (De Larco and Todaro, 1978) was the generous gift of Stephen Tatter and Dr. Allan Goldberg (Rockefeller University). Cells were grown in 10% Fetal Calf Serum in RPMI on untreated tissue culture plastic.

Neonatal superior cervical ganglia were dissected from rat and dissociated mechanically into single cells by tugging the sheath off and vibrating on the vortex. These cells were plated onto a heart cell substrate in growth medium (10% FCS, 2 mM glutamine, 0.3% glucose, 0.1% gentamycin, and 10 u/ml Nerve growth factor (kindly provided by Dr. I. Black, Cornell). The heart cell substrate was prepared 1-4 days previously by dissociating a heart from a 1-7 day old rat pup with 2 mg/ml collagenase, and plating onto collagen covered dishes.

Electron microscope immunohistochemistry

Neonatal rats were perfused with 4% paraformaldehyde. The cortex was dissected and postfixed overnight in the same fixative. 100 μ m vibratome sections were cut and collected into PBS. The sections were blocked with 5% NGS in PBS for 1 hour at 4°C, then incubated in primary antibody, diluted in 5% NGS, for 24 hours.

Following a 1 hour wash period with several changes of PBS, the sections were incubated for 2 hours at room temperature in HRP conjugated secondary antibody. They were then washed again for one hour, reacted with DAB, fixed with 2% glutaraldehyde for 1 hour, osmicated and embedded in Epon-Araldite resin. Silver to gold sections were cut on an ultramicrotome, collected on copper mesh grids, and viewed with a JEOL electron microscope. Some vibratome sections were stained *en bloc* with uranyl acetate before dehydration to enhance membrane contrast.

Agarose embedded membranes

The procedure of De Camilli, et al. (1982) was used. Neonatal rat cortex was homogenized in 10 volumes of buffered sucrose (H), and centrifuged at 1000xg for 10 minutes to pellet nuclei. The supernatant, S1, was centrifuged at 27,000xg for 20 minutes to yield a crude membrane fraction, P2. This pellet was resuspended in a small volume of buffered sucrose, and lysed by the addition of 10 volumes of ice cold distilled water and high speed homogenization. 1/10th volume of 1 M Hepes, pH 7.3 was then added and this was incubated on ice for 30 minutes. This lysate (L) was centrifuged at 170,000xg for 2 hours to yield lysed membranes (LP). Each of the above membrane fractions was fixed, mixed with molten agarose, and pipetted into a frame made of glass slides separated by cover slips. After cooling, these agarose embedded membranes were removed from the frame and treated for fluorescence immunocytochemistry in the same manner as a section of tissue. The same results were obtained when fixation followed embedding, as long as it preceded incubation with the primary antibody.

Preparation of Synaptic vesicles

Synaptic vesicles were isolated as in Huttner, et al. (1983). Rat brains were dissected into ice-cold 0.32 M sucrose, 4 mM HEPES, pH 7.3 (buffered sucrose). They were homogenized in 8 volumes of buffered sucrose at 1100 rpm with 7 up and down

strokes using a teflon/glass homogenizer. The homogenate (H) was centrifuged at 800xg in an SS34 rotor for 10 minutes to give a pellet (P1) and a supernatant (S1). S1 was centrifuged at 9200xg (10,000 rpm) for 20 minutes to give the supernatant (S2) and a pellet (P2) which was resuspended into buffered sucrose. P2 was centrifuged at 10200xg (10,500 rpm) for 20 minutes to give a supernatant (S2') and a pellet (P2'). P2' was resuspended into a total of 2-8 mls buffered sucrose and placed in the glass/teflon homogenizer; 9 times this volume of ice-cold water was added, and this was homogenized at 2200 rpm with 3 strokes. It was then added to 1/10th volume of 1 M HEPES-NaOH, pH 7.4 and incubated on ice for 30 minutes. This suspension, known as lysed synaptosomes, was centrifuged at 25,000xg (16,500 rpm) for 20 minutes to give a pellet (LP1) and a supernatant (LS1). LS1 was centrifuged at 165,000xg (48,000 rpm in Sorvall T875 bucket) for 2 hours to give a supernatant (LS2) and a pellet (LP2). LP2 was taken up into 40 mM sucrose and fully resuspended by homogenizing it at 2200 rpm in the glass/teflon homogenizer. This was layered onto a linear gradient of 50 - 800 mM sucrose and centrifuged (to equilibrium) for 5 hours at 65,000xg (19,000 rpm in Sorvall AH627 bucket). The region of the gradient corresponding to 200 - 400 mM sucrose was removed and subjected to column chromatography. For this purpose, a 450 x 12 mm column of controlled pore glass beads (mean pore size 3000 Å, 80/120 mesh) was equilibrated with 300 mM glycine, 10 mM HEPES, 0.02% azide, pH 7.3 (buffered glycine) and run at a flow rate of 1 ml/minute, with 2 ml fractions then collected. The fractions containing synaptic vesicles were pooled, centrifuged at 65,000xg and resuspended into buffered glycine (purified synaptic vesicles).

Assay for the membrane integrity of SVP38

Purified synaptic vesicles were diluted into 10 mM HEPES (pH 7.2). To this was added 1.5 M KCl and/or 10% Triton to a final concentration of 0.15 M KCl or 1% Triton or both. The vesicles were incubated in these solutions on ice for 30 minutes,

and then centrifuged at 165,000xg (48,000 rpm in T875) for 1 hour. The pellet was assayed for SVP38 and synapsin I by western blotting.

Glycopeptidase Treatment

Glycopeptidase F was purchased from Boehringer Mannheim, and was used as in Tarentino, et al. (1985). Crudely purified synaptic vesicles (fraction LP2) were suspended in buffer A (100 mM Sodium Phosphate, 10 mM EDTA, 0.2% Triton X-100, 0.1% SDS, pH 8.0), with 6 units/ml glycopeptidase F for 18 hours at 37°. After glycopeptidase treatment, the samples were separated by polyacrylamide gel electrophoresis and probed by immunoblotting. There was no effect on SVP38 when synaptic vesicles were incubated under the same conditions in buffer A without glycopeptidase. α -acid glycoprotein (Sigma) was used as a control to determine the efficacy of the glycopeptidase treatment.

Polyacrylamide gel electrophoresis and immunoblotting

SDS-PAGE was performed using the buffer system of Laemmli (1970), and was typically run at 3 volts/cm, constant voltage. Proteins in the gel were electrophoresed from the gel onto nitrocellulose paper according to the method of Towbin et al. (1979). The nitrocellulose was either stained with 0.1% Amido Black to visualize the molecular weight markers or blocked with 5% normal goat serum and probed with antibody, followed by peroxidase-labelled secondary antibody and 3,3'-diaminobenzidine as chromogen (Fekete and Barnstable, 1983).

Dot immunobinding assay

The quantitative dot-immunobinding assay was carried out using the procedure of Hawkes, et al. (1982), with some further modifications from those introduced by Jahn, et al., (1984). Samples were spotted onto sheets of nitrocellulose using a S & S minifold dot-binding filter system. The nitrocellulose was fixed with 10% Acetic Acid/25%isopropyl alcohol for 15 minutes, and rinsed with double-distilled water. It

was blocked for at least 2 hours with 5% BSA in PBS before incubation with the primary antibody for at least 8 hours. It was washed for 20 minutes with 3 changes of a large volume of PBS. The sheets were incubated for at least 8 hours with ^{125}I -labelled F(ab)₂ fragment of an affinity purified Rabbit anti-mouse IgG (20-40 mCi/mg, 6×10^6 cpm/ml) in 5% Normal Goat Serum with 0.1% Triton X100 present. The sheets were washed extensively in PBS with 0.1% Triton X100. They were then dried, and individual dots were cut out and counted using a gamma counter. Background was defined as the counts on a dot loaded only with buffer and was subtracted from all points.

DEAE-Sepharose characterization of SVP38

The LP2 fraction of the synaptic vesicles preparation (see above) was resuspended into 1.8% Triton in 5 mM Tris-Cl, pH 8.0. This was centrifuged at 18,000 in an SS34 rotor for 30 minutes. The supernatant was applied to a DEAE-Sepharose column equilibrated with 10 mM Tris-Cl, pH 7.4 with 0.5 mM EDTA, 0.1 mM DTT, and 0.1% Triton X-100 (column buffer). Protein elution was monitored with an in-line spectrophotometer coupled to a chart recorder. The column was washed with the column buffer until no more protein came through. A step gradient of 50 mM KCl and 125 mM KCl in column buffer was then applied to the column.

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