THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC

Supporting Biomedical Investigation

Research Grant Application

CURRENT DATE: 11/28/96

TITLE OF STUDY (*DO NOT EXCEED 70 CHARACTERS*)

Mechanisms of Activity-Dependent Neuronal Development

KEY WORDS (LIMIT TO FIVE)

PROPOSED START DATE	PROPOSED DURATION (YRS) 1, 2 OR 3	FUNDS REQUESTED (INCLUDING INDIRECT COSTS)	YEAR 1	YEAR 2	YEAR 3
7/1/97	3 years	TOTAL REQUEST (page 5):	90,382	105,990	110,230
		PERM. EQUIPMENT (page 5):	11,500		- 0 -

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Signature 11/27/96 Date

Hollis T. Cline
Cold Spring Harbor Laboratory
Mechanisms of Activity-Dependent Neuronal Development

Background

The focus of my research is to determine the cellular mechanisms by which neuronal activity modifies the development of structure and function in the CNS. The role of neuronal activity in shaping circuits within the brain has been apparent since the pioneering work of Hubel and Weisel, who demonstrated that visual experience governs the formation of synaptic connections within visual cortex. Despite the widespread recognition that the developing brain can be influenced by input activity, we have only begun to understand how neurons translate this "experience" into modified neuronal growth and the strengthening of synaptic connections. Work from my lab and others suggest that this is likely to be accomplished through changes in both neuronal structure and the strength of synaptic connections. I now propose to examine the role of several proteins which are turned on by neuronal activity to determine how they might control the development of CNS circuits.

We perform our studies using the developing visual system of the frog Xenopus, in which the retina projects directly to the optic tectum. This system is widely recognized for its morphological and synaptic plasticity in the developing and mature brain [2]. The optic tectum is the major site for processing visual information in non-mammalian vertebrates. The tadpole optic tectum is particularly advantageous for the study of developmental changes in synaptic connections because new neurons are constantly being generated in the caudal region of the tectum. Consequently, at any time in tadpole development, there exist neurons in the tectum at a wide range of developmental stages, arranged along the rostrocaudal (RC) axis of the tectum [4, 11]. We take electrophysiological recordings in the whole cell patch clamp mode from tectal neurons at different positions along the RC axis of the tectum, which represent different stages of neuronal maturation. The optic nerve is stimulated electrically to evoke retinotectal synaptic responses.

Retinotectal evoked synaptic currents recorded from neurons in rostral (mature) tectum show classical glutamatergic synaptic responses, indicating that both the NMDA and AMPA type glutamate receptors are present on the mature neurons. However, our recordings from younger neurons in caudal tectum suggest that the first glutamatergic retinotectal synapses are mediated purely by NMDA receptors and that synapses develop progressively from pure NMDA responses to gradually acquire mixed AMPA/NMDA responses. What cellular mechanism could control this maturation process? One possibility is that the calcium calmodulin-dependent kinase type II (CaMKII) plays a role in synaptic maturation. Calcium entry through the NMDA receptor activates CaMKII [6] and is hypothesized to trigger the stabilization of synaptic connections. CaMKII is a developmentally-regulated protein kinase implicated in control of neuronal growth, synaptogenesis and synaptic plasticity. The kinase has several substrates relevant to neuronal development, including cytoskeletal proteins and the AMPA receptor.

The albino Xenopus tadpole is also an excellent system in which to study the control of morphological development of neurons. The animal is transparent so the single neurons labeled with dye in the intact animal can be imaged over time. We have taken in vivo images of retinotectal axons [9] and tectal neurons [8, 10, 12, 14] in the living anesthetized animal to observe their morphological development for up to five days in vivo using time-lapse confocal microscopy. The resulting movies of neurons growing in their normal complex environment indicate that they grow in a counter-intuitive manner: many branches are added to the growing neuron, but the majority are rapidly retracted again. Only 10% of the newly added branches are retained and contribute to the net growth of the neurons. Over 8 h an average of 50% of the total branch length is remodeled. The dynamic rearrangements of these neurons would have been impossible to detect without time-lapse in vivo imaging we have used. This is one of the only experimental systems which permits direct recordings of synapse formation and maturation in the intact CNS, along with the ability to observe the morphological development of the neurons by in vivo timelapse imaging. In addition, we use viral vectors to deliver genes of interest into mature frog neurons. We are now using this range of techniques to test the hypothesized roles of activity-regulated proteins in the coordinated development of the structure and function of the CNS.

Specific Aims

We have tested whether CaMKII is involved in the signal transduction pathway controlling synaptic maturation by expressing a constitutively active truncated form of CaMKII (tCaMKII) in tectal neurons using a recombinant vaccinia virus [13]. Expression of tCaMKII in tectal neurons increases the calcium/calmodulin independent CaMKII activity by about 30% [16]. Patch clamp recordings indicate that expression of tCaMKII increases AMPA receptor function at retinotectal synapses [11] and thereby mimics the normal the maturational program seen in glutamatergic synapses. Increased CaMKII activity in tectal neurons also causes morphological changes in the dendritic arbor and presynaptic axon arbor morphology consistent with the idea that CaMKII mediates a stop growing signal in the CNS [10, 14, 16]. These exciting data indicate that increased CaMKII activity is pivotal for brain development. To strengthen this conclusion we now propose additional experiments probing the role of CaMKII in synaptic development.

Specific Aims:
1. To test the hypothesis that CaMKII activity in tectal neurons triggers the maturation of glutamatergic retinotectal synapses and coordinates the stabilization of pre- and postsynaptic neuronal structure. This aim includes the following experiments:
a. To test the hypothesis that excitatory synaptic activity and CaMKII cooperate to promote the maturation of retinotectal synaptic responses.
b. To determine whether tectal neurons display a developmental increase in AMPA receptors.
c. To test the hypothesis that decreased CaMKII activity in tectal neurons deters or prevents the maturation of glutamatergic retinotectal synapses.
d. To test the hypothesis that decreased CaMKII activity in tectal neurons alters the elaboration of the presynaptic retinal axon arbor and the tectal cell dendritic arbor.
2. To test the potential effect of additional activity-regulated genes on the development of neuronal morphology and synaptic function.

Experimental Design and Supporting Data

Specific Aim #1a. To test the hypothesis that excitatory synaptic activity and CaMKII cooperate to promote the maturation of retinotectal synaptic responses.

Our observations on the maturation of glutamatergic synapses indicate increased CaMKII activity increases AMPA responses. Our working model suggests that CaMKII activity is increased following synaptic activity and specifically NMDA receptor activity. To test this model further we propose to determine whether synaptic activity and increased CaMKII cooperate to increase AMPA receptor function.

Our preliminary data (Fig 1) suggest that pairing 1 Hz stimulation of the retinal afferents with tectal cell depolarization triggers the maturation of retinotectal synapses, assessed as an increase in the AMPA component of the excitatory postsynaptic current (EPSC) and that tCaMKII expression promotes pairing-induced increases in AMPA responses. The increase in the AMPA component following stimulation is similar to the conversion of NMDA response to mixed AMPA/NMDA responses seen with long-term potentiation [3, 5]. Therefore these experiments will determine whether the activity-dependent synapse maturation that we observe in the tectum has similar requirements for induction as does hippocampal LTP.

Does increased afferent stimulation increase the AMPA component of the EPSC?

Whole cell recordings will be taken from tectal cells in the caudal third of the tectum. Retinotectal synaptic responses in these cells are mediated primarily by NMDA receptors [11]. A baseline of synaptic currents will be recorded at -65 mV to record AMPA responses (if present) and at +55mV to record mixed NMDA and AMPA responses by stimulating the optic nerve at 0.2Hz. After collecting baseline data for 100 traces at each potential, the optic nerve will be stimulated at 1 Hz for 3 minutes, while depolarizing the tectal neuron to -20mV, in order to ensure NMDA receptor activation. The rate of optic nerve stimulation will be returned to 0.2 Hz and the AMPA and NMDA components of the synaptic currents will be assessed by recording at -65 mV and +55 mV as for the baseline data.

The data will be analyzed to determine the relative contribution of the NMDA and AMPA components to the total synaptic currents as described below.

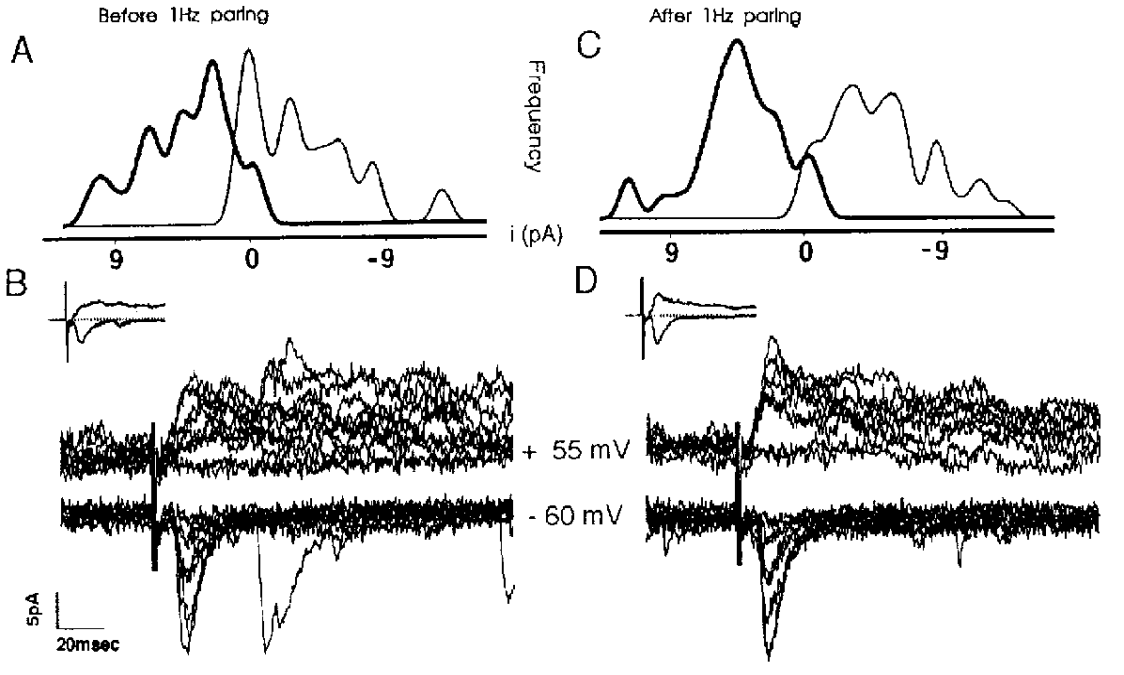

Fig 1. Activity-dependent increase in AMPA responses. Amplitude distribution histograms (A) were generated from baseline data collected from 100 sweeps each recorded at -60 (light trace) and +55 mV (dark trace) (B). Then the neuron was depolarized to -20mV while the optic nerve was stimulated at 1 Hz for 3 minutes, after which optic nerve stimulation was reduced to the test rate of 0.2 Hz, and 100 sweeps were collected again at -60 and + 55 mV (D). A second pair of amplitude distribution histograms was generated from the second data set (C). The principle difference between the histograms in A and C is that the peak at 0 pA designating failures at -60 mV is smaller after 1 Hz stimulation and is comparable to the failure rate at +55 mV. The insets in B and D show the averaged traces. This indicates that there are more AMPA responses after pairing.

Do NMDA or AMPA receptor blockade prevent the activity-dependent increase in the AMPA component?

To test whether the stimulation of NMDA or AMPA receptors on tectal cells is required to trigger the maturation of synaptic responses we will repeat the experiment described above, but include either 100 µM DL-APV or 10 µM CNQX in the bathing medium during the period of 1 Hz optic nerve stimulation. The relative contribution of the NMDA and AMPA components to the synaptic response will be determined and compared statistically to the relative contributions in neurons recorded in the absence of APV or CNQX.

Our preliminary experiments have also suggested that prolonged exposure to APV might result in neurons that have relatively small AMPA components. To address this possibility, animals (st 46/47) will be exposed for 24 h to 100 µM DL-APV, after which recordings will be taken from neurons in the middle third of the RC axis of the tectum. These neurons would normally have increased their AMPA/NMDA ratio over the last 24 h to about 1. The AMPA/NMDA ratio will be determined as described below and compared to that of neurons from untreated animals located within the corresponding region of the tectum.

Is a rise in intracellular calcium required for the activity-dependent increase in the AMPA component?

Our studies suggest that a rise in postsynaptic calcium and the subsequent activation of CaMKII may be part of the pathway that controls synaptic maturation. The NMDA receptor is permeable to calcium and calcium entry through the NMDA receptor is required to trigger the subsequent changes in synaptic physiology that result in the increase in the AMPA component.

BAPTA (10 mM in the pipette solution) will be added to the patch pipette to prevent a rise in intracellular calcium during the 1 Hz stimulation protocol. Recordings will be taken from neurons in caudal tectum which produce the most dramatic increase in AMPA component with the 1 Hz stimulation. The probability of increasing the AMPA component following stimulation in the presence or absence of BAPTA is the recording pipette will be determined.

Does expression of tCaMKII increase the AMPA component following afferent stimulation to a greater extent than in uninfected or β-gal-infected neurons?

Our preliminary data suggest that for the same afferent stimulation, the relative increase in the AMPA component is greater in tCaMKII-expressing neurons than in β-gal-infected or uninfected control neurons. To determine whether expression of CaMKII facilitates the addition of AMPA responses following afferent stimulation, animals will be infected with either the tCaMKII virus or the β-gal virus at stage 46/47. Three days later, whole cell recordings will be taken from tectal cells in caudal tectum, which show a large increase in AMPA responses following tCaMKII expression [11]. The same protocol pairing afferent stimulation at 1 Hz with tectal cell depolarization will be used here as described above. The AMPA and NMDA components of the synaptic currents will be assessed by recording at -65 mV and +55 mV as for the baseline data.

The data will be analyzed to determine the relative contribution of the NMDA and AMPA components to the total synaptic currents as described above. We anticipate that these experiments will show that increased CaMKII activity cooperates with synaptic activity to increase AMPA receptor function. As such they will define some of the mechanisms underlying activity-dependent synapse maturation. In addition, these experiments will demonstrate the degree of convergence of cellular mechanisms of neuronal plasticity and the initial formation of synaptic connections.

METHODS

Patch clamp recordings will be performed as described in [11]. Briefly, the dissected brain will be laid out in a recording chamber and a stimulating electrode will be placed in the optic chiasm. A patch pipette containing 80 mM cesium methanesulfate, 10 mM EGTA, 20 mM TEA, 5 mM $MgCl_2$, 2 mM ATP, 0.3 mM GTP and 20 mM HEPES, pHed to 7.2 with CsOH will be positioned with visual guidance over neurons with pear-shaped somata at a chosen location along the RC tectal axis. The optic nerve will be stimulated at 0.2 Hz. The strength of the stimulation will be adjusted to minimize polysynaptic responses. Recordings will be rejected if the latency of the response changes with stimulation strength or frequency. NMDA and AMPA components of the synaptic responses will be distinguished pharmacologically, using DL-APV (100 μM) and CNQX (10 μM), and according to their voltage-sensitivity. Drugs will be added to the bathing solution (115 mM NaCl, 4 mM KCl, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 5 mM HEPES, 10 μM glycine, 100 μM picrotoxin and 10 mM glucose), which is continuously perfused over the specimen. Because the picrotoxin blocks all outward currents at 0 mV, we conclude it blocks all $GABA_A$ responses. The internal solution in the patch pipette will include Lucifer Yellow, to allow reconstruction of the neuronal morphology at the end of the recording session. In addition, a low magnification brightfield image of the tectum will be taken to record the position of the cell body within the tectum.

Responses will be amplified with an Axopatch 1D, digitized (3-10 kHz) and stored on disk for analysis off-line. Data will be analyzed as described previously [5]. In the off-line analysis, sweeps with spontaneous events contaminating the evoked responses will be deleted from the analysis. To determine if neurons in caudal tectum have pure NMDA responses, EPSCs will be recorded from these neurons at -60 mV and +55 mV for 100 sweeps. Neurons that have EPSCs at +55 mV, but none at -60 mV have pure NMDA responses, because they are blocked by APV.

To generate the amplitude distribution histograms and estimate the rate of failures of synaptic transmission, the peak amplitude of the AMPA component will be determined by measuring the amplitude of the individual EPSCs over a 5-10 ms window that includes the peak response and subtracting the amplitude response during a baseline window, when there is no stimulus. The frequency of occurrence of responses of different amplitudes will be plotted as the amplitude distribution histograms. The peak at 0 amplitude represents failures of synaptic transmission. The rate of failures will be estimated from the amplitude distribution histograms by doubling the

fraction of responses with amplitudes less than zero. This minimizes contamination from the stimulus artifact.

The AMPA:NMDA is determined by measuring the peak amplitudes of the early and late components in averaged responses of the EPSCs recorded at -60 mV and +55 mV. The AMPA response will be measured in a 5-10 ms window encompassing the peak response at -60 mV. The NMDA response will be measured in a second 10 ms window, placed about 40 ms later than the first window, when the AMPA component contributes <5% of the total current [11].

Specific Aim #1b. To determine whether tectal neurons display a developmental increase in AMPA receptors.

Our recordings from younger neurons in caudal tectum suggest that the first glutamatergic retinotectal synapses are mediated purely by NMDA receptors and that synapses develop progressively from pure NMDA responses to gradually acquire mixed AMPA/NMDA responses. One interpretation of these data is that the maturation of glutamatergic synapses involves the addition of AMPA receptors to pre-existing synapses where synaptic transmission was initially mediated solely by NMDA receptors. Alternatively, the postsynaptic membrane may already contain AMPA receptors that are not detected in our assay of synaptic responses. These AMPA receptors could migrate laterally to contribute to synaptic responses with synapse maturation and CaMKII activity. The maturation process may be due to the development of presynaptic release machinery or changes in the removal of glutamate from the synaptic cleft rather than the appearance of synaptic AMPA receptors. Because NMDA receptors have a higher affinity for glutamate than AMPA receptors, low concentrations of glutamate that might be released from newly formed synapses would preferentially activate NMDA receptors even when AMPA receptors are present.

To determine whether the developmental increase in AMPA responses at synaptic sites reflects a developmental increase in AMPA receptors in tectal cell membrane, we will make use of caged glutamate. Glutamate will be uncaged with a brief flash of UV light. The response to the uncaged glutamate will be recorded from patch clamped neurons at different positions along the RC axis of the tectum. Our preliminary data indicate that we can record AMPA and NMDA responses to the uncaged glutamate from the mature neurons in rostral tectum. We propose to determine the AMPA/NMDA ratio in response to uncaged glutamate for neurons at different RC tectal locations and compare this to the AMPA/NMDA ratio of evoked synaptic currents. This will demonstrate whether the receptor distribution at synapses differs from that in extrasynaptic membrane. We will further test whether tCaMKII expression increases the AMPA/NMDA ratio in response to uncaged glutamate in a comparable fashion to the tCaMKII-induced increase in AMPA/NMDA ratio at synaptic sites. If AMPA receptors are present in young neurons, but not activated synaptically, this suggest the maturation of the evoked responses we record may due to a maturation of the presynaptic release process or lateral migration of AMPA receptors from extrasynaptic regions to synaptic sites. If we observe no AMPA response in young neurons in response to uncaged glutamate and an increase in AMPA responses as the neurons mature, then these data would support the hypothesis that the maturation of glutamatergic synapses includes the addition of functional postsynaptic AMPA receptors.

Specific Aim 1c. To test the hypothesis that decreased CaMKII activity in tectal neurons deters or prevents the maturation of glutamatergic retinotectal synapses.

Our current data indicate that CaMKII activity is pivotal for synaptic maturation in the CNS. We plan to strengthen this conclusion by decreasing endogenous CaMKII activity by expressing an inhibitory peptide in the tectal neurons using the vaccinia virus. We have generated recombinant vaccinia viruses containing the sequences for several different CaMKII inhibitory peptides including a new inhibitory peptide, AIP, which has a lower IC_{50} (0.3 μM) and greater specificity for CaMKII versus protein kinase C than other available CaMKII inhibitory peptides [15]. Control virus has an inactive form of the peptide (AIP_i). Our preliminary data indicate that these recombinant viruses inhibit endogenous CaMKII activity in the RK13 cell line. To determine whether they inhibit CaMKII in tadpoles brains, animals will be infected with virus encoding either active or the inactive peptide. Three days later homogenates of brain tissue will be assayed for CaMKII activity as described previously [16].

To test the effect of AIP on synaptic maturation, animals will be infected with the AIP virus or the AIP_i virus at stage 46/47. Three days later whole cell patch clamp recordings will be taken from neurons at different positions along the RC axis of the tectum. The AMPA and NMDA components of evoked retinotectal synaptic responses will be determined as described above. Data will be analyzed to determine whether decreased CaMKII activity prevents or slows the normal developmental increase in the AMPA/NMDA ratio and whether neurons expressing the AIP virus have a higher fraction of pure NMDA responses than neurons of comparable positions along the RC axis of control animals infected with the AIP_i virus or uninfected animals. The experimental protocol for these experiments is described in Specific Aim 1a and published [11].

Specific Aim 1d. To test the hypothesis that decreased CaMKII activity in tectal neurons alters the elaboration of the retinal axon arbor and the tectal cell dendritic arbor.

We have found that expression of tCaMKII in tectal neurons modified the elaboration of the presynaptic retinal ganglion cell axon arbor and the postsynaptic tectal cell dendritic arbor using in vivo imaging of DiI labeled neurons [10, 12, 14, 16]. Here, we propose to test the effect of decreasing endogenous tectal cell CaMKII activity on the development of both retinal axon arbor morphology and tectal cell dendritic morphology using the recombinant viruses expressing AIP and AIP_i.

Albino Xenopus are ideally suited for in vivo imaging studies. The animal is transparent so their CNS can be observed in the intact anesthetized animal by placing it on the stage of a laser scanning confocal microscope [7, 9]. Studies on the factors controlling the morphological development of tectal neurons have been limited by the technical difficulty of reproducibly labeling single tectal cells early in their process of differentiation. We have solved this problem by iontophoresing DiI [10, 12, 14] with 1-5nA current in applied in 3-5 pulses of 1-10ms duration. These parameters usually label single cells or a small cluster of cells. The iontophoresis method has opened up the possibility to conduct the experiments on the control of tectal cell development that are proposed here.

Because there are at least 7 types of tectal neurons, defined morphologically, we have chosen to concentrate our efforts on large pear-shaped neurons, which send an axon to the thalamus. Cells that extend an efferent axon typically exhibit the following sequence of morphological development: While still close to the ventricular layer, the cell extends a large growth cone which grows toward the pia, where it turns either rostrally or caudally and heads rapidly toward the target region. Once the axon growth cone has reached the pia and is heading out of the tectum, the dendrites exhibit a burst of growth and rapidly become more elaborate over the next few days (Fig 2).

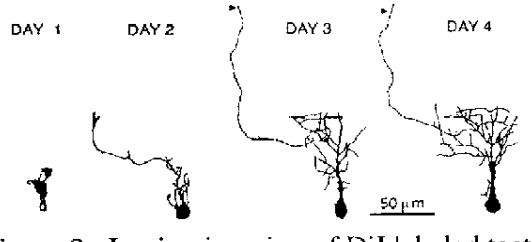

Figure 2. In vivo imaging of DiI labeled tectal neuron reveals timecourse of morphological development. An individual cell near the ventricular layer extends one process to the ventricular layer and another large growth cone oriented toward the pia (Day 1). One day later (Day 2) the axon has reached the pial surface and is oriented toward rostral tectum. Some dendrites have formed. By the next day (Day 3) the axon has reached the thalamus and dendrites become more elaborate. Processes continue to grow in length and number over the next 24 h (Day 4).

We plan to assess the effect of AIP on tectal cell morphological development. In these cases we need a measure of tectal cell maturity that is independent of morphology. Because dendritic branchtip number is correlated with position of the cell body along the RC extent of the tectum [11],. we will use the cell body position along the RC axis as an independent measure of state of maturation of the neuron.

To test the effect of AIP expression on the development of arbor morphology, individual retinotectal axons or individual tectal cells at different positions along the RC axis of stage 44-47 animals will be labeled with DiI. DiI-labeled cells will be repeatedly imaged in vivo using different imaging protocols. To determine the overall effect of AIP expression on the timecourse of the elaboration of the axonal and dendritic arbors cells will be imaged at daily over a period of up to 5 days (see Fig 2). To determine whether AIP expression changes the rates of branch additions or retractions, images will be collected every 2 hours over total observation periods of 8 h, as reported previously [9]. Control experiments will be done to test the effect of the AIP_1 expression on morphological development. Our previous work demonstrates that neurons in animals infected with a virus expressing β-gal are indistinguishable in their morphological development from uninfected control cells. Data will be analyzed as described below.

METHODS

Tectal cells at different positions along the RC axis of the tectum in anesthetized stage 44-47 animals will be labeled with DiI (0.5% in 100% ethanol; Molecular Probes) by iontophoresis using 1-5nA current applied in 3-5 pulses of 1-10ms duration. Retinotectal axons are labeled by iontophoresing dye into the retina. Animal screening and confocal microscopy will be as previously published [16]. Briefly, images of singly labeled axons or tectal neurons in anesthetized animals will be collected in the Z axis with a Noran laser scanning confocal microscope. The confocal microscope permits a high degree of spatial resolution in the X, Y and Z planes of the arbor that is essential for the detailed three-dimensional reconstruction of the arbors. The data analysis in the proposed experiments requires that every individual branch in the arbor be identified at each timepoint. Use of the confocal microscope is essential in order to collect high resolution data on the neuronal morphology.

Morphometric analysis methods are the same as those published [16]. Drawings of the neurons from the initial observation will be compared to those from the succeeding timepoints to detect changes in the arbor structure. For the timecourse experiments, we will quantify the changes in branchtip number, total branch length and branch order, as we have done previously. Each branch is assigned a number for identification and the fates of individual branches will be followed in subsequent observations by recording the maintenance or retraction of each branch. New branches initiated in the subsequent timepoints are also assigned a number and their fate is recorded similarly. The number of branches per arbor at each timepoint will be tabulated. These data are then used to determine the rate of branch initiations and retractions, the relative number of branches which are stable over the observation periodand the rate of growth of branches. Branch lengths will be measured using the NIH Image software on a Macintosh. Changes in morphological parameters in experimental and control neurons will be compared statistically.

2. To test the potential effect of additional activity-regulated genes on the development of neuronal morphology and synaptic function.

I have outlined our present and proposed experiments on CaMKII as a specific example of our efforts to determine the role of activity-regulated proteins on the formation of neural circuits. We have the power of combining several analytic approaches to address this essential question in neuronal development. In addition to examining the role of CaMKII in neuronal development, the gene transfer method with Vaccinia opens up the possibilities of testing the postulated roles of several activity-regulated proteins in neuronal plasticity. I have established several collaborations with Paul Worley at Johns Hopkins University and Elly Nedivi, who is now a Visiting Scientist in my lab, each of whom has isolated several genes and their protein products that are regulated by neuronal activity and during development (see letters of collaboration). We have now generated recombinant Vaccinia viral constructs with several of these activity-regulated genes. Initial experiments with one of the constructs indicates that expression of the activity-regulated protein, named Homer, has a selective effect on the pathfinding of the efferent tectal cell axon. The axon, which in normal cells terminates in thalamus, grows aberrently and fails to develop a terminal arbor in any target area. The dendritic arbor in these infected neurons appears to develop normally. Paul Worley's group has found that Homer associates with a transmembrane receptor, possibly through its PDZ domain. It also associates with a novel microtubule associated protein by the yeast two

hybrid system. They are currently mapping the domains of the protein which are responsible for association with the receptor and with MAP (P. Worley, personnal communication). We will then generate additional viral constructs of truncated forms of Homer, lacking the different functional domains of the protein to test their effect on the phenotype. We propose to test whether expression of the other constructs have an impact on the development of the morphology and synaptic physiology of the circuits underlying visual system processing. These collaborations should result in extremely exciting tests of the postulated roles of these proteins in activity-dependent neural development and plasticity in vivo.

In summary, I have applied several novel technical approaches to the frog retinotectal system which allow us to study the role of activity-regulated proteins on the development of neuronal circuits in vivo. The use of in vivo time-lapse confocal microscopy to observe the patterns of neuronal growth is a unique feature of the albino Xenopus system. We are able to obtain clear high resolution images of both retinal axons and the more deeply situated tectal neurons from early larval stages up through stage 49 animals. Combining our in vivo imaging capacity with our new ability to record retinotectal synaptic activity from neurons at a range of developmental stages provides us with the unusual opportunity to observe directly the effect of changes in synaptic activity patterns on the development of neuronal structure and connectivity.We have already made significant progress toward understanding the complex role of the multifunctional protein kinase, CaMKII, in CNS development and are poised to test the potential roles of other activity-regulated proteins in brain developmental.

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4. LABORATORY SPACE and FACILITIES available to the applicant.
State location if facilities are elsewhere than the applicant's institution.
Indicate any facilities that are required but are not currently available.

My laboratory is comprised of about 1200 square feet. The lab is equipped with a Noran laser scanning confocal microscope controlled from an SGI computer with software packages from Noran. The lab is also equipped with a Macintosh Quadra, which we use for data analysis with the public domain NIH Image software. We have a Hewlett Packard scanner and printer. The lab is also equipped with 2 stereomicroscopes, a light and temperature-regulated incubator, a Kopf vertical pipette puller to make pipettes for DiI iontophoresis and an DiI iontophoresis setup including an old compound microscope, a manipulator and a stimulator. We have an electrophysiology rig for patch clamp recordings from tectal neurons. Shared or borrowed equipment: We pull our patch pipettes on a Sutter puller in a lab in another building. We share a Millipore water system and a -80°C freezer. Cold Spring Harbor Lab has a fee-for-service machine shop. Virus production, maintenance of cell lines is done in another lab using borrowed incubators, shakers, etc. Because of our increased demand for this equipment, we must move this work to my lab. The caged glutamate experiments were done with a borrowed uniblitz shutter and UV light source.

5. BUDGET JUSTIFICATION - Use this space to explain specific needs for items described on budget pages.

Personnel: Funds are requested for 10% of my salary commensurate with the effort devoted to the project. Support is requested for a postdoctoral associate, Isabel Cantallops, who will be joining my lab in Feb '97. She will perform the electrophysiology experiments described in Aims 1 and 2 and as such will be an essential part of the research team. I am requesting support for Song Shi in the 2nd and 3rd years of the grant period. Song is a graduate student at the State University of New York at Stony Brook. He is supported by a Stony Brook training grant until 7/98. Song has learned to perform the in vivo imaging experiments. He will image the effects of activity-dependent proteins on growth of the retinal axons and tectal cells.

Supplies: Amounts and costs for supplies are estimated based on use over the last year.

Travel: The funds will permit the postdoc and graduate student to attend the annual meeting of the Society for Neuroscience.

Other: I am requesting funds for part of the service contracts for the Noran confocal microscope and the SGI computer that runs it. These contracts are essential to maintain the operation of the central piece of equipment in the lab. If the confocal microscope goes down, the lab will be paralyzed and no new data will be collected. Funds are also requested to cover costs for computer services provided by the Cold Spring Harbor Computer facility. All the computers are connected by network. If the network goes down we can't send data from our acquisition system to the analysis station.

Equipment: The uniblitz shutter and incubators equipment are borrowed and must be returned.

6. APPENDIX: Place the appendix materials after the original and each copy of the application form as indicated in the Instructions for New Applications.

a. Biographical Sketches of the professional personnel to be associated with the project.
Each sketch should be NO MORE THAN TWO (2) PAGES. The NIH format is acceptable.
The P.I. should include and indicate by an asterisk the FIVE (5) most significant publications whether or not they relate directly to this application.

b Supporting material (such as letters of collaboration).

c. Copies of not more than FIVE (5) of the applicant's publications or manuscripts that are pertinent to the project.

7. ABSTRACTS of PUBLICATIONS : *Only one set is required.* See Instructions for New Applications.
Submit ONE PHOTOCOPY of the abstract page of each "pertinent publication" included in the appendix (6.c.) above;
For each manuscript, submit a single composite page that includes authors, title, journal, abstract and publication status (for example, "submitted for publication").

P.I. Name Cline, Hollis T.

7. FIRST YEAR'S BUDGET:

A. Salaries. Give % time even if no salary is requested. State names or "to be recruited".

		% time	Amount
Professional Personnel including Principal Investigator			
Hollis T. Cline	P.I.	10%	10,353
Isabel Cantallops	Post Doc	100%	31,740
Songhai Shi	Grad Student	100%	-0-
Technical Support			

A. Salaries Subtotal 42,093

B. Consumable supplies (by major category)

Virus Production	$10,000
Electrophysiology Supplies	5,000
Imaging-related Supplies	5,000

B. Consumables Subtotal 20,000

C. Other Expenses (itemize)

Travel	$1500
SGI Service Contract	$3,000
Computer Services	$2,000

C. Other Expenses Subtotal 6,500

A+B+C Subtotal 68,593

D. INDIRECT COSTS **(15% of A + B + C).** D. Indirect Costs 10,289

E. Permanent Equipment (itemize)

Uniblitz shutter		1,500
Co2 Incub	2@	5,000

E. Permanent Equipment Subtotal 11,500
Indicate here and on Page 1.

F. ..... TOTAL REQUEST 90,382
Indicate here and on Page 1.

8. PROJECTED BUDGET AMOUNTS:

BUDGET PERIOD	Salaries, Supplies and Other expenses	Permanent Equipment *	Indirect Costs	TOTAL
Year 2	92,166	0	13,825	105,990
Year 3 if applicable	95,852	-0*-	14,378	110,230

*You may not use CTR funds to purchase permanent equipment in the terminal grant year.

CURRENTLY ACTIVE GRANTS , CONTRACTS and OTHER SOURCES of FUNDS
List financial support (direct costs, only) from all sources, including own institution.

Title of Project	Sources (give grant numbers)	Total Value of Grant (direct costs)	Current Annual Amount Available to You	Date of Termination of Grant
Control of visual system development	NIH RO1-EY 11261-01	664,178	164,620	11/30/99
Control of neuronal growth and synapto-genesis	NSF IBN-9631756	177,515	59,172	8/31/99

Identify and describe any overlap of this application with the above grants:
There is no overlap with my current grants. The NSF supported experiments on the influence of synaptic activity on growth of retinotectal axons. This work is completed and lays the foundation for part of the present proposal. The NIH supports experiments on the normal pattern of growth of the tectal nuerons and the use of tCaMKII to study the role of CaMKII on the development of neuronal morphology. This work also lays the foundation for the experiments included in the present proposal.

Indicate the total annual funds available to you this year for all research projects under your supervision.	$ 223,792

PENDING OR PLANNED

Title of Project	Sources (give grant numbers)	Total Value of Grant (direct costs)	Avg. Annual Amount Available to You	Total Duration (give inclusive dates)
None				

Identify and describe any overlap of this application with the above project.