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PATTERNED ACTIVITY, SYNAPTIC CONVERGENCE, AND THE NMDA RECEPTOR IN DEVELOPING VISUAL PATHWAYS

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INTRODUCTION

Plasticity, activity-dependent competition, and Hebbian synapes are terms frequently associated with a set of cellular interactions that causally link early neural activity to the final stages of neural circuit differentiation. Similar terms and, in fact, similar interactions are used in discussions of learning and memory. This use of a common vocabulary reflects the hope of neurobiologists that the developmental mechanisms of plasticity will prove similar to those underlying learning and memory in the mature brain. The visual pathway has been the focus of continuous and intense experimental work in developmental plasticity for over three decades, and quite recent work in this area has suggested a molecular mechanism also found in hippocampal long-term potentiation (LTP). Extensive reviews are available in both fields (Movshon & Van Sluyters 1981, Sherman & Spear 1982, Fregnac & Imbert 1984, Shatz & Sretavan 1986, Stryker 1986, Collingridge & Bliss 1987, Teyler & DiSenna 1987, Nicoll 1988, Udin & Fawcett 1988, Brown et al 1990). Consequently, this treatment focuses only on the developing visual pathway and only on those visual system experiments that conceptually link plasticity in the differentiating and mature brain. It presents the arguments and evidence suggesting that temporal correlations in the action potential patterns of young visual synapses determine their relative positions within local regions of neuropil and their convergence onto the same sets of post-synaptic cells. It also

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considers the issue of whether functionally detected developmental changes in synaptic effectiveness are isomorphic with activity-dependent changes detected anatomically. Finally, it reviews recent data on the involvement of the *N*-methyl D-aspartate (NMDA) subclass of excitatory amino acid receptor in use-dependent modifications of the developing visual pathway.

ACTIVITY AND SYNAPTIC CONVERGENCE IN MAMMALS

In the mammalian geniculocortical visual pathway, inputs from the two retinas are segregated in the dorsal lateral geniculate nucleus (dLGN) and in the afferent layers of visual cortex, primarily layer IV. In the nonafferent layers of cortex, synapses from layer IV cells finally converge upon cortical neurons so that left and right retinal pathways are no longer segregated. The segregation of retinally driven inputs as well as their ability to converge on single cortical cells has been shown to be affected by the patterns of activity they convey. As outlined below, this activity dependence was first detected in experiments that perturbed binocular convergence as well as experiments that altered the balance of activity between the two eyes.

Artificially Induced Strabismus: Experimental Deprivation of Binocular Convergence

Animals raised with one eye muscle cut so that their retinas cannot converge on the same point in visual space lose significant numbers of binocular cortical neurons. The changes occur in the apparent absence of sluggish or dying neurons, thus suggesting that the normal convergence of retinally driven inputs rather than cortical cell survival is the parameter that has been affected by the treatment (Hubel & Wiesel 1965). There have been many permutations of experiments in which animals are prevented from seeing the same stimuli through both eyes. All demonstrate the same eye-specific segregation phenomenon and share one principle in common: When visual stimulation does not allow near simultaneous delivery of similar patterns of action potential activity to the same cortical cells through the left and right eye visual pathways, the two pathways fail to maintain the ability to drive those cells. Instead the inputs from the two eyes functionally segregate onto two mutually exclusive sets of cortical neurons (Hirsch & Spinelli 1970, Leventhal & Hirsch 1977, Bruce et al 1981; see Fregnac & Imbert 1984 for review).

Monocular Deprivation Experiments

In monocular deprivation studies one eye of a neonate kitten or monkey is sutured closed for periods ranging from weeks to months. After this period the animal is prepared for physiological recordings, the sutured eye is opened, and the ability of isolated cortical units to respond to either eye is tested. The consistent physiological result is that the previously sutured, deprived eye loses its ability to drive cortical units and all cortical neurons develop pronounced responses to the open eye (Wiesel & Hubel 1963, Hubel et al 1977). The degree of domination is related to the duration of the treatment, but even short exposures during a brief, most sensitive period can produce pronounced changes in eye dominance (Olson & Freeman 1975). If monocular deprivation is begun very early in the postnatal period, this "take-over" occurs in all cortical layers, including layers IV, which normally, in binocular regions of cortex, contains approximately equal numbers of monocular cells driven by either the right or left retina.

The Issue of Structural Versus Functional Changes

It is frequently assumed that activity-dependent developmental changes involve structural "rewiring," whereas changes in the mature brain reflect alterations in the efficacy of existing synapses. In fact there is relatively little evidence to justify generalization for either case. The same issue of assigning an observed change to a structural versus functional cause also arises in the visual development literature. Specifically, structural rewiring in the dLGN and afferent cortical layers is detectable with physiological techniques at the affected level and higher. However, functionally detected changes in the relative efficacy of retinal inputs to cortical neurons do not necessarily imply actual losses or gains in the numbers of synapses to those cells.

In afferent layer IV of visual cortex the functionally detected takeover of a large proportion of neurons by the nondeprived eye has a firm structural correlate in the relative amount of termination space occupied by the geniculate inputs corresponding to the two eyes. Layer IV ocular dominance columns of the nondeprived eye fail to retract at the expense of those from the deprived eye (Hubel et al 1977, Shatz & Stryker 1978, LeVay et al 1980). Furthermore, the dLGN neurons receiving input from the deprived eye show reduced somal size relative to the corresponding cells receiving input from the nondeprived eye. These reductions are found only in binocular regions of the dLGN and they do not occur when animals are binocularly deprived of pattern vision (a procedure that does not unbalance the distribution of terminals in layer IV). Consequently, the somal shrinkage is not due to a loss of activity but probably reflects a loss of ocular dominance termination space in cortex (Guillery & Stelzner 1970, Guillery 1972; see for review Movshon & Van Sluyters 1981, Sherman & Spear 1982).

More recently there have been a number of investigations of deprivation treatment effects on the morphology of single neurons and their geniculate and cortical terminal arbors. The work has generally documented some changes in the structure of single cell arbors consistent with deprivation-induced alterations in function or frequencies of encountering units with particular responses (see Sherman & Spear 1982, Shatz & Sretavan 1986, Sur 1988 for reviews).

Despite this evidence of anatomical change at lower levels in the geniculocortical pathway, however, there is no evidence for actual structural changes in afferent convergence as a result of perturbations in normal activity patterns in nonafferent cortical layers. Thus, some of the physiological observations of developmental plasticity in cortex may reflect actual structural changes in the numbers or positions of synapses while others reflect functional changes in synaptic efficacy. For example rearing a kitten in the dark prolongs the period in which the nondeprived eye can functionally dominate cortical neurons when physiologically assayed. It does not prolong the period during which the ocular dominance columns of the nondeprived eye can show anatomical expansion in layer IV (Mower et al 1985). In addition, when, during a brief period of early life, the initially deprived eye of kittens or monkeys is opened and the open eye sutured shut, there is a rapid recovery of a population of cortical neurons responding to the initially deprived eye (Blakemore & Van Sluyters 1974, Kratz et al 1976, Blakemore et al 1978). The speed of this recovery suggests that some of the effects of monocular deprivation result from increased inhibitory suppression of the initially deprived eye's inputs by the open eye rather than competitive displacement of its terminals from post-synaptic membranes. However, the actual mechanisms involved remain controversial (Duffy et al 1976, Movshon & Van Sluyters 1981).

The Hebbian Synapse Hypothesis

The correlated activity requirement for the developmental maintenance of binocular neurons and the activity-dependent interaction that allows the open eye to take over cortical cells under conditions of monocular deprivation are linked by the theoretical framework provided initially by D. O. Hebb (1949). Hebb's postulate for associative learning (1949) and its modern articulation by Stent (1973) and Changeaux & Danchin (1976) suggests a two-part rule for the use-dependent modification of young labile synapses:

1. Synaptic contacts between synchronously active pre- and post-synaptic neurons are selectively reinforced.

2. Synaptic contacts between asynchronously active pre- and post-synaptic neurons are selectively depressed or eliminated.

The ability of the post-synaptic membrane to temporally summate the synaptic potentials from converging synapses that are synchronously, or near synchronously, active means that inputs with correlated action potential patterns are more likely than asynchronous inputs to have their activity covary with activation of the post-synaptic cell. Hebb's rule, therefore, extended to an array of synapses converging on a single cell, predicts that synchronous inputs are likely to be reinforced whereas asynchronous inputs will be functionally eliminated even though they may carry the same average amount of activity (Stent 1973). In the monocular deprivation paradigm, greater activity in one eye compared to the other provides the opportunity for more frequent correlations of activity among the converging synapses from the active eye, and, consequently, more effective driving of cortical neurons by that eye. The deprived eye not only has a lower probability of driving synaptic events that will sum to activate cortical neurons, but, in addition, its synapses will frequently be silent while the cortical neurons are driven by the open eye. In short, Hebbian ideas explain the results of monocular deprivation as a functional disconnection of the deprived eye from all cortical cells because cortical cell activity is not correlated with synaptic activity from that eye.

In contrast to monocular deprivation, strabismus does not depress either retina's ability to drive cortical neurons, but it does eliminate interocularly correlated synaptic activity when inputs from the two pathways converge on the same cortical cells. Functional segregation of each retina's inputs to separate sets of these post-synaptic cells ensues, according to the Hebb postulate, because that situation allows synapses driven by each eye to maximize their association with synchronously active cells and minimize their association with cells "asynchronously" activated by the other eye.

A key element in the application of Hebbian ideas to normal visual system development as well as monocular domination or binocular segregation within cortex was the demonstration that highly correlated patterns of activity do, in fact, exist *within a retina*. Physiological recordings in goldfish, cats, and rabbits have revealed that action potential patterns of neighboring ganglion cells of the same response type are nearly identical, and this similarity decreases between increasingly distant pairs of neurons. Action potential patterns of neighboring ganglion cells of opposite response type are negatively correlated. Moreover, the correlations remain in the absence of pattern stimulation and even in the spontaneous activity of the cells under conditions of complete dark adaptation (Arnett 1978, Arnett & Spraker 1981, Mastronarde 1983a,b).

The presence of these spatially organized high correlations of activity within a single eye's projection allow a Hebbian explanation for the fact that ocular dominance columns still develop relatively normally under conditions of dark-rearing in cats (LeVay et al 1978, Mower et al 1985, Swindale 1988) or during intrauterine development in monkeys (Rakic 1976, 1977, Hubel et al 1977). High intraretinal correlations in activity and synapses that follow Hebbian rules also provide an explanation for the fact that left and right retinal pathways converge in nonafferent cortical layers and yet segregate in afferent layers. In layer IV, synapses from the left and right eye geniculate laminae constitute virtually all of the inputs to the cortical cells and these synapses begin to sort out before visual experiences and binocular convergence of the eyes are possible. Consequently, intraeye correlations of activity have an opportunity to dominate over the later onset and less frequent correlations of synaptic input between the two eyes. Compared to layer IV, synapses on neurons in nonafferent cortical layers are capable of sorting relatively later in development during periods when the two eyes become capable of good binocular alignment. These neurons have converging inputs from many layer IV cells, resulting in larger visual receptive fields and less tightly correlated intraretinal activity, and they also receive many intracortical and nongeniculate extracortical inputs only indirectly associated with retinal activity (Gilbert 1983). Thus intraretinal correlations in activity are a much smaller proportion of the total number of possible synchronous events on nonlayer IV cortical neurons, whereas events triggered through the retinas of both eyes simultaneously have the better chance of producing simultaneously active synaptic inputs, temporal summation, and effective post-synaptic activation. Several diverse experimental paradigms have been used to manipulate nonretinal activity converging on cortical cells during monocular deprivation. They have all supported the idea that nondeprived eye activity must be correlated with activation of some nonretinal inputs in order to functionally "take-over" cells in nonafferent cortical layers (Freeman & Bonds 1979, Raushecker & Singer 1981, Bienenstock et al 1982, Singer & Raushecker 1982, Singer et al 1982, Fregnac & Imbert 1984).

TTX Blockade in Mammals

A large number of experiments have employed the voltage-dependent Na^+ channel blocker, tetrodotoxin (TTX) to eliminate "spontaneous" activity and demonstrate its importance in utero or before the eye is capable of pattern vision. The anatomical results of these studies are all consistent with Hebb's postulate. Left and right eye inputs do not segregate from each other in the dLGN (Shatz & Stryker 1988) or into cortical layer IV ocular dominance columns (Stryker & Harris 1986). Blocking of all

activity, however, has many effects that could be interpreted as a simple retardation of normal development (Edwards & Grafstein 1984, Kalil et al 1986, Cohan & Kater 1986, Casagrande & Condo 1988), and only two laboratories have actually generated evidence specifically implicating the temporal pattern of synaptic activation as the important missing parameter under TTX blockade.

Recordings in the kitten geniculate after blockade of retinal activity have documented a pronounced disruption of functional segregation in the retinogeniculate pathway that would never have been predicted on the basis of structural data alone (Archer et al 1982, Dubin et al 1986). Neurons within the geniculate laminae of normal kittens generally have well-defined "on-center, off-surround" or "off-center, on-surround" receptive field structure. TTX-treated kittens, recorded from shortly after removal of the retinal blockade, have many geniculate cells that are unusual in that they can be driven by both eyes (Dubin et al 1986). The most significant observation in these experiments, however, is that a large proportion of neurons respond to both "light on" and "light off" throughout their receptive field. The concentric field organization of ganglion cells is not perturbed by TTX treatment (Archer et al 1982). In addition, Dubin et al (1986) were occasionally able to record simultaneously from three cells, a geniculate neuron, an "on-center" ganglion cell that drove it, and an "off-center" ganglion cell that drove it. Thus it appears quite clear from this study that disruption of geniculate neuron functional organization occurs at the level of retinal axon convergence onto geniculate dendrites.

Disruption of a mechanism that stabilizes ganglion cell synapses by virtue of their temporal synchrony and ability to drive geniculate neurons would produce exactly this result. Thus, during normal development, a particular geniculate neuron would by chance receive a majority of its inputs from "on-center" ganglion cells driven by stimuli in the same small region of the visual field. Once this bias is established, synapses from functionally different ganglion cells, responding to cessation of illumination in the same small region, cannot be stabilized on the same neuron: "Off-center" retinal input would never fire in synchrony with the majority of "on-center" inputs to that cell. The "off-center" ganglion cells would, instead, functionally segregate onto closely adjacent geniculate neurons that initially received a slightly larger complement of synapses from the "off-center" ganglion cell type. Elimination of these patterned activity cues through TTX retinal blockade allows both "on-center" and "off-center" ganglion cells to maintain roughly equal ability to drive the same post-synaptic neurons.

In kitten primary visual cortex, TTX retinal treatments in the neonatal period have demonstrated that monocular takeover of cortical cells can

be accomplished by a vision-deprived eye in competition with an eye lacking spontaneous activity (Chapman et al 1986). Binocular treatment of retinas (Stryker & Harris 1986) or direct treatment of cortex with TTX (Reiter et al 1986) will eliminate monocular takeover.

Most significantly, however, fixed temporal patterns of activation in the pathways of the two eyes have been applied to kittens with binocular retinal blockades that would, by themselves, prevent ocular dominance segregation in layer IV. This was accomplished with chronically implanted stimulating electrodes that delivered either simultaneous or out-of-phase volleys of activity to the central visual pathways of both eyes for 1–2 hr per day. The majority of cortical neurons were driven through both eyes in the synchronously stimulated group. Very few neurons were binocular in the asynchronously treated group (Stryker & Strickland 1984, Stryker 1986). Anatomically, in kittens experiencing asynchronous pathway volleys, the TTX effects on blocking segregation were considerably mitigated, whereas synchronously stimulated animals showed no signs of ocular dominance segregation (M. P. Stryker, personal communication).

ACTIVITY AND SYNAPTIC CONVERGENCE IN NONMAMMALIAN VERTEBRATES

Most evidence that temporal parameters in afferent activity patterns have structural effects on visual projections comes from work on the retinotectal systems of goldfish and frogs. These species show retinal ganglion cell response properties that are similar to those of mammals. They exhibit equally refined maps of visual space within their brains, and some tectal neurons receive converging projections from the binocular region of the visual field through both eyes. Nevertheless, the retinal ganglion cells of cold-blooded vertebrates are more resistant than mammalian cells to perturbations of their normal target contacts, and their synapses in tectum are continually broken and remade throughout the larval period and well into adult life (Gaze et al 1979, Reh & Constantine-Paton 1983, Easter & Stuermer 1984). The latter properties permit explorations of dynamic synaptic interactions that are difficult in the internally developing mammals. The three aspects of these cold-blooded vertebrate visual projections that have been used effectively to explore the role played by activity are summarized briefly below.

Synapse Segregation Based On Presynaptic Cell Body Proximity

The first hints of neural activity's role in sculpting the visual pathways of lower vertebrates arose in optic nerve regeneration experiments on goldfish

(Levine & Jacobson 1975, Cronlly-Dillon & Glaizner 1974). In both fish and frogs the retinotectal projection is normally completely crossed. However, when axons from two retinas are normally caused to converge on a single tectal lobe, the inputs from the two eyes do not mix but rather remain segregated into eye-specific "clumps" of retinal terminals.

The similarity between eye-specific clumping in tectum and eye-specific segregation in mammals was first recognized when the tectal terminations of supernumerary amphibian eyes were examined anatomically. In embryonically created three-eyed frogs, two complete visual projections converge on the same tectum from the earliest developmental stages, and anterograde labeling experiments invariably reveal that the continuous retinal projections of both the host and third eye are disrupted into an alternating, periodic pattern highly reminiscent of mammalian ocular dominance stripes (Constantine-Paton & Law 1978). Using the amphibian preparations and embryonic microsurgery, a series of experiments rapidly ruled out right and left eye labels (Law & Constantine-Paton 1981), genotypic differences between the eyes (Ide et al 1983), differences in time of arrival of the two projections (Law & Constantine-Paton 1980), and selective fasciculation within the optic tract (Constantine-Paton et al 1983) as parameters that could provide for recognition and "aggregation" of synapses arising from the same retina. In fact, the only parameter that is invariably correlated with segregation is convergence of terminals from nonneighboring regions of retina within a small region of the target zone (Fawcett & Willshaw 1982). Consequently, given the evidence that retinal neighbors share action potential patterns, it seemed likely that a Hebb-like interaction was causing convergence of each eye's projection into retina-specific zones where nearest presynaptic neighbors have the maximum probability of driving or depolarizing the same tectal neurons. A recent study combining intracellular Lucifer Yellow filling of tectal neurons and simultaneous visualization of eye-specific stripes provided anatomical support for this idea. The majority of tectal neurons restrict either their entire dendritic tree or an entire primary dendritic branch to the terminal zones of only one eye (Katz & Constantine-Paton 1988).

That retinal activity is necessary for eye-specific segregation in fish and frogs has now been confirmed with bilateral TTX-blocking experiments in several different laboratories (Meyer 1982, Boss & Schmidt 1984, Reh & Constantine-Paton 1985). Monocular blockade is not sufficient to eliminate this eye-specific segregation in goldfish (Meyer 1982), although the borders of the terminal "clumps" seem to become more diffuse (Schmidt & Tieman 1985). In addition, in frog larvae with supernumerary eyes it has been possible to rule out the possibility that TTX treatment (i.e. lack of activity) merely delays normal development of the segregated pattern.

In three-eyed tadpoles, because of the normal turnover of retinotectal synapses (Reh & Constantine-Paton 1983), if an activity-dependent mechanism is crucial to the maintenance of segregation, it must remain continually active. In these animals it is possible to observe ocular-dominance stripes in various stages of desegregation as animals are examined after increasingly long durations of TTX retinal blockade (Reh & Constantine-Paton 1985).

Normal Topographic Map Refinement

There is now general agreement that activity-independent differences, in cell surface properties and ability to "read" axon guidance cues, bring visual axons to the vicinity of their topographically appropriate target cells. It is the fine-tuning of these projections through the local ordering of synapses that is established through activity-dependent nearest-neighbor sorting (see Udin & Fawcett 1988 for review).

The prediction from this two-stage mapping hypothesis is that projections lacking retinal activity or lacking correlations in activity that are related to the retinal proximity of ganglion cells should show less refined representations of the retinal surface within the tectal lobe. Conversely, ganglion cells from a larger region of retina should maintain synapses in any given region of the tectal neuropil. Both of these predictions have been borne out by experiments using TTX blockade of retinal activity (Meyer 1983, Schmidt & Edwards 1983) or stroboscopic stimulation of regenerating retinal projections in goldfish (Schmidt & Eisele 1985, Cook & Rankin 1986, Cook 1988).

Binocular Projections of Xenopus *Frogs*

Binocular maps within amphibian tecta arise through the nucleus isthmi (the homologue of the mammalian parabigeminal nucleus), which relays binocular visual field information from the contralateral tectum to the locations representing the same visual field positions in the ipsilateral tectum (Gruberg & Udin 1978). Numerous experiments over the past decade have shown that the development of binocular neurons in these tectal positions depends critically on similar patterns of action potential activity arriving from the contralateral eye and from the ipsilateral eye through the nucleus isthmi. For example, surgical rotation of one retina during tadpole stages so that the location of visual field positions in the contralateral tectum is correspondingly rotated, causes a physiologically detectable shift in the projection of the nucleus isthmi projection to that tectum. The ipsilateral eye projection through the isthmotectal relay therefore reattains registration with the perturbed contralateral eye projection, and inputs carrying information about the same point in the visual field

are still able to converge on a small locus in the tectum (Keating 1974, Udin & Keating 1981). Patterned visual stimulation is crucial to the development of this convergence. Animals maintained in complete darkness during the critical metamorphic period attain diffuse, relatively weak, isthmotectal projections driven by the ipsilateral eye, and the ipsilateral map fails to come into register with the contralateral map (Keating 1975, Keating & Feldman 1975).

Significantly, these physiological observations have striking anatomical correlates. When the nucleus isthmi projection is filled with HRP, the axons carrying ipsilateral eye information can be followed to their termination sites in the tectal lobe. In animals with one eye rotated by 90 degrees, these axons approach the tectum normally and arrive at their normal arborization site. However, they then grow in apparently random fashion until encountering the displaced sites with functionally correlated contralateral eye activity (Udin 1983). They form morphologically normal arbors in these novel positions, leaving little more than a vestigial branch at the tectal position where dense arborization would normally occur (Udin 1985).

CELLULAR MECHANISMS UNDERLYING THE CORRELATED ACTIVITY RULE

Modulation of Synaptic Effectiveness Through Post-Synaptic Cell Excitability

A number of studies have investigated the relationship between action potential activity in single cortical neurons and the efficacy of particular inputs. The results suggest that post-synaptic action potentials produce an increase in the effectiveness of the correlated input (Barany & Feher 1981; see Fregnac & Imbert 1984 for review) and support the idea that near synchrony of pre- and post-synaptic activity is an important factor in modulating cortical synaptic strength. In the visual cortex, pairing of visual stimulation through one eye with post-synaptic cell firing by applied current causes an increase in the effectiveness of the paired eye's input more frequently in kittens than in adult cats. The changes can last from 15 minutes to several hours (Fregnac et al 1988). Similar results have been obtained in experiments in which iontophoresis of excitatory transmitters was paired with activation of one eye's inputs (Greuel et al 1988).

Decreased post-synaptic excitability or reduced sensory signal compared to background "noise" has also been implicated in cat visual cortical plasticity in an extensive literature of studies in which chronic drug treatments of cortex (Kasamatsu et al 1979, Daw et al 1983, 1985, Bear et al 1983, Paradiso et al 1983; see Gordon et al 1987 for review) or lesions of

noncortical regions projecting to cortex (Adrien et al 1985, Daw et al 1984, Singer 1982, Bear & Singer 1986) have eliminated the domination of visual cortical cells by a nondeprived eye. The observations support the notion that a level of post-synaptic activation reflecting both retinal and non-retinal inputs must be reached during sensory stimulation, in order to trigger selective increases in synaptic efficacy (Bienenstock et al 1982, Singer et al 1982, Singer & Raushecker 1982, Bear et al 1987).

One very recent experiment, however, suggests that many of these observations may have to be reexamined for the potentially complicating effects of a competitive disadvantage inflicted upon active inputs that fail to fire post-synaptic cells. Reiter & Stryker (1988) depressed cortical excitability with chronic infusion of the $GABA_A$ receptor agonist muscimol. This treatment suppressed spontaneous activity and inhibited visually elicited responses in kitten cortical neurons. Under these conditions, in which cortical neurons were prevented from firing, the *deprived* (less active) eye came to dominate over the active eye in the monocular deprivation paradigm. This is the first experimental evidence in the visual development literature for a synaptic interaction in which less active inputs appear to have an advantage over more active neighbors (Cooper et al 1979, Levy & Desmond 1985). Synaptic activity seems to be detrimental to an eye's functional dominance of a cortical neuron if that cortical neuron cannot be driven (Reiter & Stryker 1988).

NMDA-Mediated Plasticity in Visual Cortex

Many developmental studies of cortical plasticity have now focused on the properties of the *N*-methyl D-aspartate subclass of excitatory amino receptors as the long sought detectors for correlated synaptic events. This would be equivalent to the documented triggering function of this receptor system in LTP of hippocampal CA1 synapses. The essential idea is that temporal summation of EPSPs results in Ca^{2+} influx through the NMDA receptor channel because the first, non-NMDA channel–mediated responses depolarize the post-synaptic membrane (Wigstrom & Gustafsson 1985). These initial EPSPs relieve the Mg^{2+} block of the NMDA channel so that subsequent excitatory amino acid (EAA)–mediated synaptic events open the channel and let Ca^{2+} into the post-synaptic cell (Mayer et al 1984, Nowak et al 1984).

There is general agreement that the LGN inputs to the visual cortex are predominantly glutaminergic (Hagihara et al 1988). Many of the interlaminar and intracortical projections may also use EAAs (Hicks 1987). However, the particular role of the NMDA receptor in developmental use-dependent plasticity or in visual synaptic transmission is controversial.

EFFECTS OF MANIPULATION OF EAAs The earliest study to demonstrate an effect on plasticity of manipulating EAAs used cortical glutamate infusions in monocularly deprived kittens to demonstrate failure of the nondeprived eye functionally to dominate cortical neurons (Shaw & Cynader 1984). This result was interpreted as demonstrating that any nonspecific imbalances of cortical activity could disrupt plasticity.

The study that actually focused developmental cortical work specifically on the NMDA receptor used chronic infusion of 2-amino-5-phosphonovaleric acid (APV), a specific antagonist of NMDA receptors (Harris et al 1984), into the cortex of neonate, monocularly deprived kittens. The treatment blocks the monocular takeover of cortical neurons by the nondeprived eye, prevents the acquisition or the maintenance of stimulus orientation selectivity, and produces sluggish cells with relatively large receptive fields (Kleinschmidt et al 1987). Singer and his colleagues suggested that the effects arise specifically from blocking the ability of cortical NMDA receptors to detect correlated events rather than a general effect of depression of cortical neuron excitability.

The problem with this interpretation is that there are no definitive experiments tying the level of post-synaptic excitability necessary to induce use-dependent modification of synapses to the threshold for NMDA receptor, or other high threshold Ca^{2+} channel, activation. For example, in the studies such as those of Fregnac et al (1988) and Greuel et al (1988), in which stimulation through one eye is paired with electrical or transmitter stimulation of a binocular cortical cell, it would be extremely interesting to know whether low, NMDA-selective, doses of simultaneously applied APV selectively blocked the increase in the paired eye's effectiveness.

Data from several laboratories suggest that treatments that block cortical NMDA channels also depress neuronal excitability in kitten cortex at least temporarily. Tsumoto and his colleagues (Tsumoto et al 1987, Hagihara et al 1988) tested visually and electrically stimulated cortical responses to application of APV and kynurenate, an antagonist that blocks all EAA receptor types. They found that 70% of visual responses are APV-sensitive in kittens. This dropped to 30% in adult cats. Similar depressions in responsiveness have been observed by Fox et al (1989) and are described in more detail below. Thus, the question has become whether NMDA receptor activation has a unique function as a detector of correlated activity as opposed to having a more general role as one of several transmitter systems that modulate cortical cell excitability. The issue is difficult to address definitively in cortex. In in vivo studies, the absolute concentrations of applied APV at the post-synaptic membrane are never known. At high concentrations, APV loses its selectivity for NMDA receptors and suppresses activation of kainate and quisqualate receptors as well (Collingridge & Bliss 1987). Miller et al (1989) have tried to overcome this

difficulty by infusing APV into cortex with an osmotic pump beginning one day before physiological recording in order to obtain stable concentrations of the drug at fixed distances from the cannulae. They have found a close correlation between sluggishness of cell responses to visual stimulation and selective elevation of responsiveness to applied NMDA but not to similarly applied quisqualate or kainate. They conclude that in normally reared adult cats, NMDA receptor activation is a normal component of the excitatory response of cortical neurons to visual stimulation. Equivalent studies are not yet available for kittens.

This controversy over the function of NMDA receptors in the visual cortex in developmental use-dependent changes will not be easily resolved. The NMDA receptor could still be a unique detector of correlated activity in cortex, even though APV infusions may be acting to decrease cortical excitability. Detection of correlated events via a specific ability to trigger post-synaptic Ca^{2+} influx and participation in visual transmission as an excitatory post-synaptic receptor are not necessarily mutually exclusive functions. In addition, all of the perturbations that depress post-synaptic excitability in cortex and thus block cortical plasticity in the monocular deprivation paradigm would simultaneously decrease the probability of NMDA receptor activation.

SYNAPTIC WEIGHT CHANGES VERSUS ANATOMICAL REDISTRIBUTION OF SYNAPSES Assuming that at least some of the experience dependent plasticity in developing visual cortex is attributable to blocking a specific correlated activity detector function of NMDA receptors, there remains one other major, unresolved, issue in the cortical studies dealing with this issue. Specifically, are the plastic properties attributable to NMDA receptors reflections of structural changes in synaptic convergence, or do they simply reflect long-term changes in synaptic efficacy? Changes in synaptic weight via increased EAA release (Errington et al 1987) or increased EAA receptors (Lynch & Baudry 1984) have been suggested as mechanisms underlying hippocampal LTP. Similarities between the developing cortex and hippocampus are supported by studies in cortical tissue slices that demonstrate an APV sensitive component of a potentiation of cortical synaptic transmission that can be induced with frequent stimulation of cortical white matter (Artola & Singer 1987). Several laboratories have also generated evidence suggesting that visual cortical potentiation may have a sensitive period that corresponds closely to the period of maximal cortical plasticity (Komatsu et al 1981, 1988). Structural changes in the sizes of synaptic contacts or in the number or shape of post-synaptic spines have been, to varying degrees (Coss & Perkel 1985), associated with both cortical (Perkins & Teyler 1988) and hippocampal LTP (Desmond & Levy

1986a,b). Nevertheless, the rapid onset of LTP is generally taken as evidence that most of the functional change results from alterations in the efficacy of existing synapses rather than from the actual loss or repositioning of contacts.

To date only two pieces of evidence even hint that structural rewiring may result from cortical NMDA receptor activation. Rauschecker & Hahn (1987) have detected retrograde inhibition of ocular dominance shifts produced in alert kittens by monocular occlusion. This inhibition was accomplished by intramuscular injections with ketamine-xylazine after each of 15 20-minute exposures to monocular visual experience during the height of the sensitive period for nondeprived eye domination of the cortex. Ketamine, a sigma opiod receptor agonist, is also an activity-dependent blocker of the NMDA channel. Neither saline injections nor xylazine injections alone produced this effect. The authors suggest that an ongoing process that may involve a structural change is implicated by the fact that ketamine treatment is effective after the experience has ceased (Rauschecker & Hahn 1987). If NMDA receptors are involved in a consolidation process necessary for an ocular dominance shift to occur, it is not clear that they are functioning as specific correlated activity detectors, or that the ongoing process involves structural changes.

The final evidence suggesting a structuring role for NMDA channel activation has been generated by physiological studies of cortical neuronal responsiveness to iontophoretically applied D-APV (Tsumoto et al 1987, Fox et al 1989). The reasoning here is that if NMDA receptor function is important as a normal excitatory receptor component of cortical neuron activation, D-APV at low doses should be able to suppress some component of the visual response and the effect should be relatively constant at all ages. However, if absence of NMDA receptor function is a critical factor in limiting plasticity, then *changes* in NMDA receptor sensitivity should correlate with independent measures of changes in plasticity. D-APV will suppress spontaneous activity and visually elicited activity in all visual cortical layers of young kittens. However, the ability of low doses of the drug to block visual activity in layers IV, V, and VI is gradually lost in successively older animals. In adults, visual driving of cells in these layers is insensitive to APV at relatively low doses that are, nevertheless, still successful at significantly suppressing spontaneous activity (Fox et al 1989). The potentially important point is that only in layer IV was the dropoff in APV sensitivity correlated with a sensitive period for plasticity, and this parallel was with the structural segregation of geniculocortical afferents (LeVay et al 1978). Significantly, in layers II and III, APV is able to suppress retinal activation of cortical neurons at all ages (Fox et al 1989).

NMDA-Mediated Plasticity in the Retinotectal System

Evidence that excitatory amino acids are the predominant transmitter in the retinotectal pathway has been obtained, over the past several years, in quantitative autoradiographic and physiological studies of both the goldfish (Langdon & Freeman 1986, 1987, Henley & Oswald 1988) and the frog (Debski et al 1987, Debski & Constantine-Paton 1988, Fox & Fraser 1987, McDonald et al 1989). This information has motivated investigations that capitalize on the ability, in these systems, directly to manipulate, record from, and visualize the cell processes (retinal ganglion cell terminals) believed to be the initiators of plastic synaptic interactions mediated by excitatory amino acids.

APV EXPERIMENTS Chronic application of DL-APV to the doubly innervated tecta of tadpoles or frogs with a supernumerary eye causes complete, anatomically assayed desegregation of the two retinal inputs. This gradual breakdown of ocular dominance stripes is not observed when the biologically inactive isomer L-APV is similarly applied. Furthermore, the effects of the active isomer are fully reversible (Cline et al 1987).

Pharmacological and physiological investigations of EAA transmission in the retinotectal system have been undertaken in normal tadpoles by using an intact, unanesthetized, brain preparation in which all nociceptive inputs have been severed. In this preparation the animals are maintained via vascular perfusion on oxygenated, glucose-supplemented saline solution to which drugs of known concentration can be added and carefully controlled (Debski et al 1987). These studies indicate that the concentrations of APV used in the chronic experiments do not depress action potential activity in retinal ganglion cell axons (Cline et al 1987) or the level of excitability of tectal neurons assessed as the post-synaptic component of the tectal field potential in response to electrical stimulation of the contralateral optic nerve (Debski & Constantine-Paton 1988). In addition, in a completely independent series of experiments, chronic application of APV to the tectum of juvenile *Xenopus*, in concentrations sufficient to block convergence of binocular maps (see below), does not depress retinotectal transmission of visual activity through a tectal relay to the nucleus isthmi (Scherer & Udin 1988). Thus, it appears that in the retinotectal pathway, application of APV at concentrations at which it is selective for the NMDA receptor subtype and at which it has minor, if any effects, on depressing retinal terminal or tectal cell excitability, can effectively block the selective stabilization of synapses from retinal neighbors on tectal dendrites.

In goldfish, chronic treatment of tecta with DL-APV during the final stages of optic nerve regeneration increases the size of multiunit receptive fields recorded in the tectal neuropil (Schmidt 1988). In addition, normal

frog tadpoles chronically treated with APV for six to eight weeks have much larger areas of retina projecting to the same defined point in the tectal lobe as compared to sham-operated or normal tadpoles (Cline & Constantine-Paton 1989). Both of these observations are fully consistent with the idea that the Hebbian synaptic interaction underlying segregation is identical to that which normally increases the fidelity of continuous topographic maps and that the NMDA receptor is similarly critical to both processes.

Finally, Udin and her colleagues have shown that the matching of binocular tectal maps in *Xenopus laevis* with 90 degree rotations of the contralateral eye is completely blocked by chronic treatment of these tecta with DL-APV during the critical period for this plasticity (Scherer & Udin 1988). Thus, NMDA receptors appear to be mediators of binocular convergence in this lower vertebrate preparation as well. This finding is broadly significant. Convergence in the *Xenopus* binocular assay is dependent on the same type of binocular visual experience required for binocular convergence in mammalian cortex, and the system has a limited developmental critical period (Keating 1975). In *Xenopus*, binocular visual experience produces plastic changes by a pronounced structural relocation of synapses (Udin & Keating 1981, Udin 1985).

The most parsimonious explanation for all of the observations in lower vertebrates following APV application is that a functional or selectively activated NMDA channel is a critical trigger in whatever cascade of events ultimately increases the lifetime of visual synapses on the same post-synaptic membrane in response to covariance of pre- and post-synaptic activity. Moreover, in each of these preparations, there is a clear indication, if not direct evidence, that the plastic changes are structural relocations of synaptic contacts and not simply functional alterations in existing synapses.

The idea that a common mechanism underlies developmental and mature plasticity could be strengthened by the demonstration that functional changes in synaptic efficacy similar to hippocampal CA1 LTP accompany structural changes in visual pathways attributable to Hebbian synaptic interactions. Data on this point is only available for the goldfish visual projection. As mentioned above, topographic map refinement in the tectum, during the final stages of goldfish optic nerve regeneration, is blocked by chronic treatment with APV. During the time that this refinement is taking place, visual responses are capable of a potentiation resembling that found in the hippocampus. Schmidt (1987, 1988) has demonstrated that following a short train of low-frequency stimuli, the tectal response to optic nerve stimulation potentiates more quickly and to a greater extent than had been found previously in the mature goldfish optic

nerve projection (Lewis & Teyler 1986). This potentiation can be blocked by APV and is, therefore, presumably, NMDA-mediated (Schmidt 1988).

SINGLE-CELL STRUCTURAL CHANGES UNDERLYING ACTIVITY-DEPENDENT PLASTICITY In our own laboratory we have examined retinal and tectal cell responses to chronic application of NMDA itself. The results suggest a specific relationship between NMDA receptor activation and at least one of the processes that sculpt the morphology and connectivity of single retinal ganglion cell terminal arbors.

Chronic application of nontoxic doses of NMDA to doubly innervated frog tecta produce a pronounced increase in eye-specific segregation (Cline et al 1987, Cline & Constantine-Paton 1987). Stripe boundaries become sharper, and there are fewer fusions and forks in the striped pattern. Thus it appears that the continuous presence of exogenous excitatory ligand for the NMDA receptor improves the ability of the system to discriminate correlated from noncorrelated synaptic events. Although the responses of the stripe pattern to chronic exposure of NMDA or APV seem complementary, closer examination of the morphological and electrophysiological effects of the treatments suggest that the response to NMDA is not the simple opposite of the response of APV. Reconstructions of retinal ganglion cell terminal arbors from NMDA-treated three-eyed animals reveal a dramatic, 50% reduction in the number of terminal branches in the treated arbors compared to the untreated arbors (Cline & Constantine-Paton 1989). Electrophysiological studies in the cannulated tadpole brain preparation were carried out on animals chronically treated for 4 weeks with NMDA in the same concentrations used to produce the anatomical increase in segregation. We found that the response of the retinotectal pathway to applied NMDA is significantly decreased in chronically NMDA-treated tadpoles (E. A. Debski and H. T. Cline, unpublished). We do not yet know whether the decreased sensitivity is due to a down-regulation of receptor number, to a decrease in agonist binding affinity, or to a change in the tectal circuits in which NMDA receptors are involved. Receptor desensitization, however, seems unlikely because NMDA from the implant is washed out of the animal before NMDA sensitivity is assayed. (The receptor would be expected to recover from desensitization during this wash.)

It seems reasonable to suggest that a decreased sensitivity of the receptor system may increase the amount or degree of correlated activity necessary to activate NMDA-gated channels and ultimately stabilize individual retinal ganglion cell synapses. However, it is important to point out that NMDA treatment at any concentration tested does not cause stripe desegregation. Therefore, chronic NMDA treatment does not appear to inactivate the ability of the system to discriminate inputs from the two eyes (Cline & Constantine-Paton 1989).

Stripe boundaries are regions of relatively low correlations in synaptic activity because they are regions of neuropil in which noncorrelated inputs from the two eyes intermix. Quantitative EM analyses of single retinal terminals indicates that most retinotectal synapses are located on the highest order branches of the arbor (Yen & Constantine-Paton 1988). A mechanism in which the survival of these branches is dependent on the formation of some critical number of synapses stabilized via NMDA receptor activation would explain stripe sharpening with decreased NMDA sensitivity. The number of stabilized synapses in boundary regions would be the first to drop below the critical value necessary to sustain a branch. Furthermore, because in the tadpole and young frog the arbors of ganglion cells are constantly withdrawing branches in some regions and initiating new branches in others (Reh & Constantine-Paton 1983), this same reasoning explains the chronic NMDA-induced decreases in branches of individual arbors even within the stripes of one eye. Specifically, with chronic NMDA treatment and decreased NMDA receptor sensitivity, many of the new branches in ganglion cell arbors simply do not establish enough stabilized synapses to survive. The new branches are uniquely vulnerable to decreased NMDA receptor sensitivity because they have the fewest numbers of stabilized synapses to begin with: Initial contact with a post-synaptic process is likely to be by trial and error and only a few of a new branch's synapses can be expected to converge on post-synaptic processes already receiving inputs that are active simultaneously with them. In addition, for each synapse on a distal branch that succeeds in converging along with some nearly synchronized "other" input, the degree of correlation has a high probability of being low.

Over the past ten years it has become clear that the terminal arbors of projection neurons in the developing visual pathways of both cold- and warm-blooded vertebrates are dynamic structures capable of relatively extensive remodeling in response to perturbations of activity. Thus, chick retinal ganglion cells, like those of fish and frogs, appear to make their initial synapses in a region of tectum quite separate from their terminal sites in the mature brain (McLoon 1985). Geniculocortical terminals from the nondeprived eye of young macaque monkeys show some sprouting into the deprived eye's ocular dominance stripes even when monocular occlusion is begun relatively late in development, after segregation is established (LeVay et al 1980). In the kitten geniculocortical pathway, neonatal monocular deprivation or retinal TTX block has been shown to affect selectively and differently the morphology of X and Y ganglion cell arbors in a pattern that is consistent with the type or degree of activity

deprivation to which the system was exposed (Sherman & Spear 1982, Friedlander et al 1982, Sur 1988). Finally, in fetal kittens, as had previously been observed for frog larvae (Reh & Constantine-Paton 1985), TTX blockade results in individual retinal ganglion cells that have greatly expanded central arborizations (Sretavan et al 1988). It is very likely that, in both these studies, the activity blocks suppress post-synaptic as well as presynaptic activity. Is this enlargement simply due to increased growth rates in silent ganglion cells as has been suggested for isolated mollusc neurons in culture (Cohan & Kater 1986)? Is it due to an increased sprouting stimulus from inactive post-synaptic neurons, as has been suggested for the neuromuscular system (Brown et al 1981)? Is it the result of inactivation of a Hebbian selective stabilization system in which no terminal branches are stabilized and none are trimmed because both pre- and post-synaptic processes are silent? Clearly, some of the phenomena described on a system level will soon be addressed at the level of cell biology. However, we know little about morphological changes in post-synaptic neurons and little about structural plasticity in nonprojection neurons. It is also clear that much more information has to be collected on the biochemistry of the changes produced by activity perturbations before the numerous newly raised questions can be answered.

CONCLUSION

It has historically been the case in both the fields of developmental and mature brain plasticity that each new set of observations opens up a new host of questions without necessarily promising that a complete explanation lies immediately around the corner. An association between high sensitivity of NMDA-mediated synaptic transmission and viable "exploratory" contacts of young neurons could be a critical cellular difference between the pronounced structural plasticity of the developing brain and the profoundly less plastic properties of many regions in the mature brain. Only the correlation cited above between the timing of cortical layer IV ocular dominance column plasticity and high NMDA receptor sensitivity of layer IV neurons is currently available to support this hypothesis for the mammalian brain.

From a developmental biologist's point of view, the major unanswered question in all areas of neural plasticity is whether functionally detected changes in synaptic weight represent one end of the same spectrum of interactions that produce structural relocation of synapses at its other extreme. Even in those visual pathways for which data support a specific correlated activity detector function for the NMDA receptor, there is essentially no unambiguous information on the biochemical events subsequent to NMDA receptor activation. Apparently also little agreement has been attained about these events in the more intensively studied hippocampus (Collingridge 1987). Without documentation of biochemical similarity, the argument that brain evolution had adapted exactly the same activity-dependent mechanisms used during development to the mature functions of learning and memory is speculative at best. Nevertheless, for a few, but maybe not all developing visual projections, as for some, but not all hippocampal model systems for learning and memory (Brown 1990), it can now be said that activation of the NMDA subclass of glutamate receptors plays a critical role in modulating the long-term interactions between pre- and post-synaptic neurons.

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NMDA Receptor Antagonists Disrupt the Retinotectal Topographic Map

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Summary

We tested the effect of two NMDA receptor antagonists, APV or MK801 (with NMDA), and the receptor agonist NMDA on the maintenance of retinal topography in frogs. Topography was assayed by measuring the dispersion of retrogradely labeled ganglion cells following a local HRP injection into the tectum. In untreated tadpoles, labeled cells covered about 5% of the retinal area. In APV- or MK801/NMDA-treated tadpoles, labeled ganglion cells covered 17% and 10% of the retinal area, respectively. Neither treatment with L-APV nor with NMDA disrupts the fidelity of the retinotectal projection. Neither APV- nor NMDA-treated ganglion cell terminals differed from untreated terminals with respect to tangential area, branch number, or branch density. These data support a role for the NDMA receptor in visual system development.

Introduction

In visual projections, afferents that register information from adjacent receptive fields terminate in neighboring regions of the central target zone and form a topographic map. The cellular interactions that govern the development of the topographic maps have been extensively studied in the projection from the retina to the optic tectum of amphibians and fish (see Udin and Fawcett, 1988, for review). It is thought that the amphibian retinotectal topographic map forms as a combined result of interactions based on both cell surface and activity-dependent properties in the retinal afferents and tectal neurons. Activity-independent properties, probably cell surface cues, provide a crude topography to the projection, which is then refined by an activity-dependent process such that the neighbor relations of the retinal ganglion cell bodies are maintained in their tectal projections. Neighboring retinal ganglion cells (RGCs) exhibit a high degree of temporally correlated activity compared with non-neighboring RGCs (Arnett, 1978; Arnett and Spraker, 1981; Mastronarde, 1983a, 1983b; Ginsberg et al., 1984). Therefore, a possible means of communicating retinal neighbor relations from the peripheral sensory cell bodies to the terminals in the optic tectum is through their patterns of electrical activity. Specifically, highly correlated patterns of activity in afferent terminals convey a selective survival advantage to coactive terminals that converge on the same postsynaptic neurons (Constantine-Paton et al., 1990; O'Leary et al., 1986).

The NMDA receptor/channel, a type of excitatory amino acid (EAA) receptor, has the unique property of conducting calcium in response to ligand binding only when the postsynaptic membrane is simultaneously depolarized (MacDermott et al., 1986). For this reason, it is a favored candidate for the detection of correlated afferent activity. In other systems, it has been shown that the NMDA receptor is blocked by Mg^{2+} in a voltage-dependent manner (Mayer et al., 1984; Nowak et al., 1984). Initial synaptic events depolarize the postsynaptic membrane by activating colocalized non-NMDA-type EAA receptors (Mayer and Westbrook, 1984; Dale and Grillner, 1985). Sufficient postsynaptic depolarization relieves the voltage-dependent Mg^{2+} block of the NMDA channel so that subsequent synaptic events result in Ca^{2+} influx through the NMDA channel into the postsynaptic cell (reviewed by Mayer and Westbrook, 1987). Numerous studies indicate that the neurotransmitter at the retinotectal synapse is an EAA (Langdon and Freeman, 1986; Cline et al., 1987; McDonald et al., 1989; Debski et al., 1987, 1988, Soc. Neurosci., abstract; Fox and Fraser, 1987, Soc. Neurosci., abstract). It is hypothesized that coordinated activity patterns in overlapping afferent terminals could create the conditions of postsynaptic membrane depolarization and ligand binding necessary for calcium conductance through the NMDA channel. The calcium influx would then trigger events culminating in the stabilization of coactive synapses. Several investigators have suggested a role for the NMDA receptor in the refinement of visual topography, for example, during optic nerve regeneration in goldfish (Schmidt, 1988, Soc. Neurosci., abstract), during segregation of kitten geniculocortical afferents into ocular dominance columns (Kleinschmidt et al., 1987; Tsumoto et al., 1987; Hagihara et al., 1988; Fox et al., 1989), and in the realignment of ipsilateral and contralateral eye maps in Xenopus following experimental rotation of the direct retinotectal projection (Scherrer and Udin, 1988, Soc. Neurosci., abstract).

Our previous work has demonstrated an involvement of the NMDA receptor in the maintenance of eye-specific termination zones or stripes that form in the tecta of frog tadpoles following embryonic implantation of a supernumerary eye primordium (Cline et al., 1987). Treatment with the competitive NMDA receptor antagonist, 2-amino 5-phosphonovaleric acid (APV), causes desegregation of the striped termination zones of the host and supernumerary eye. In this paper we test the hypothesis that the NMDA receptor plays a role in the refinement of the normal retinotectal projection. We examine the effect of chronic treatment with DL-APV, the pharmacologically inactive isomer L-APV, the noncompetitive NMDA receptor antagonist MK801 (Wong et al., 1986), and the receptor agonist NMDA on retinotopy of two-eyed tadpoles using HRP retrograde labeling from the tectum to the retina.

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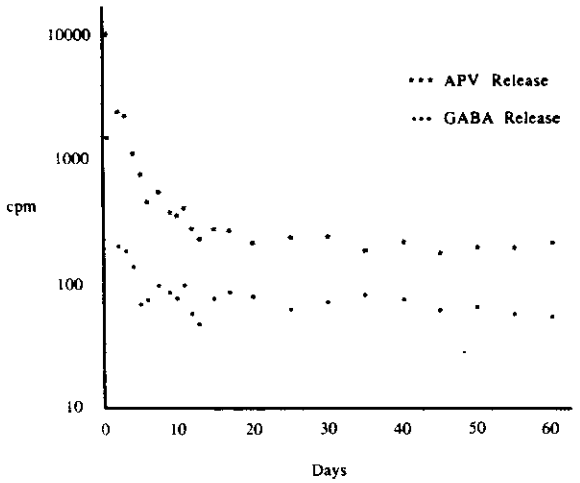

Figure 1. The Rate of Release of [^{3}H]APV and [^{3}H]GABA from Elvax over a Period of 60 Days

After an initial drop, the release remained constant for the 60 days tested.

Results

Elvax Release Characteristics

The small size of the animals used in these experiments (less than 2 grams) required the development of a procedure for prolonged application of drugs to local regions of the brain. For this purpose we adapted the use of the slow release plastic Elvax from earlier studies (Silberstein and Daniel, 1982; Reh and Constantine-Paton, 1985) by cutting the plastic on a cryostat so it would fit within the small cranial cavities. We examined the release of two amino acids from the plastic, by monitoring the release of [^{3}H]GABA or [^{3}H]APV into a small volume of saline at room temperature. Following an initial rapid release of [^{3}H]APV or [^{3}H]GABA from Elvax during the first 5 days, the release was constant over a period of at least 60 days (Figure 1). We estimate that, once the release has reached a plateau, approximately .3% of the original drug concentration in the Elvax is released daily, based on the [^{3}H]APV and [^{3}H]GABA released from the Elvax compared with the amounts added to the Elvax preparation. Exposure of the optic tectum to Elvax containing either [^{3}H]GABA or [^{3}H]APV followed by autoradiographic processing of the brain results in a high density of silver grains within the dorsal tectum under the region covered by the Elvax (H. Cline, unpublished data). Unfortunately, we cannot predict the concentration of drug that actually reaches the retinotectal synapses when Elvax is implanted above the tectal neuropil. These values will depend upon diffusion and transport barriers, as well as inactivation processes, such as glial uptake or enzymatic destruction, which probably differ for each drug.

Retinal Topography

We assessed the refinement of the retinotectal map from the degree of scatter of retinal ganglion cell bodies labeled retrogradely with horseradish peroxidase (HRP) from a controlled injection site in the tectum. With this procedure, a refined map is seen as a low degree of scatter in retrogradely labeled cell bodies, because ganglion cells from a local region of the retina terminate exclusively in a local region of the tectum (Cook and Rankin, 1986). Conversely, a crude map is detected as a high degree of scatter in the labeled cell bodies because ganglion cells from a large portion of the retina are represented in each local region of the tectum. To compare retinal topography between animals requires uniform injection of the retrograde tracer in the tectum. We have accomplished this by using the same injection site in all animals, the same settings on a Picospritzer and the same injection pipette for each stage-matched group of experimental and control animals.

HRP was injected into the rostromedial tectum of tadpoles from stages VII–XVII (Taylor and Kollros, 1946). Each experimental tadpole had a stage-matched control. Usually three to four experimental animals and their controls could be injected with a single pipette, thereby facilitating comparison between animals. Each tectum was examined in flat mount to verify a single injection site. Three tecta displayed either two injection sites or a nonlocalized injection. The corresponding retinae were not used in the study. In the remaining tecta, the area of the HRP-labeled injection site and the total tectal area were measured. The tectal injection sites were uniform and circular with a mean diameter of 111 ± 25 μm. The injection site was about 0. 8 ± .09% of the total tectal area (mean ± SE, range = .3%–1.1%). Following a rostromedial tectal injection, HRP-labeled RGCs were densely packed into a relatively small area of the ventral temporal retina (see top row in Figures 2, 3, 4, 5). The area circumscribing the labeled retinal regions and the total retinal area were measured. The percent of total retinal area occupied by labeled RGCs in all untreated tadpoles was 5.2 ± 0.5% (range = 1.3% – 12%, n = 31). The mean number of HRP-labeled RGCs per retina was 70.5 ± 8.2. We calculated the density of labeled cells to be 13.3 cells/percent retinal area. The percent retinal area with labeled RGCs was not correlated with the stages of the animals or the injection pipette used. Therefore, we pooled all the animals in each experimental group, although they were initially analyzed in groups of stage-matched experimental and control animals, all of which were injected with the same HRP pipette.

APV Treatment

We chronically treated the tectal neuropil of tadpoles with DL-APV for 6–12 weeks to determine whether NMDA receptor activation is required to maintain topography in the developing retinotectal projection. All retinae projecting to APV-treated tecta exhibited a wider distribution of retrogradely labeled RGCs than the retinae from stage-matched controls (Figure 2, Table 1). The percent of retinal area occupied by the retrogradely labeled RGCs was 17.6% ± 3.8% (range = 8%–47%, n = 10 retinae from 6 tadpoles), which was significantly larger than untreated tadpoles ($p < .005$). Retinae from APV-treated animals has a mean of 234 ± 35 HRP-

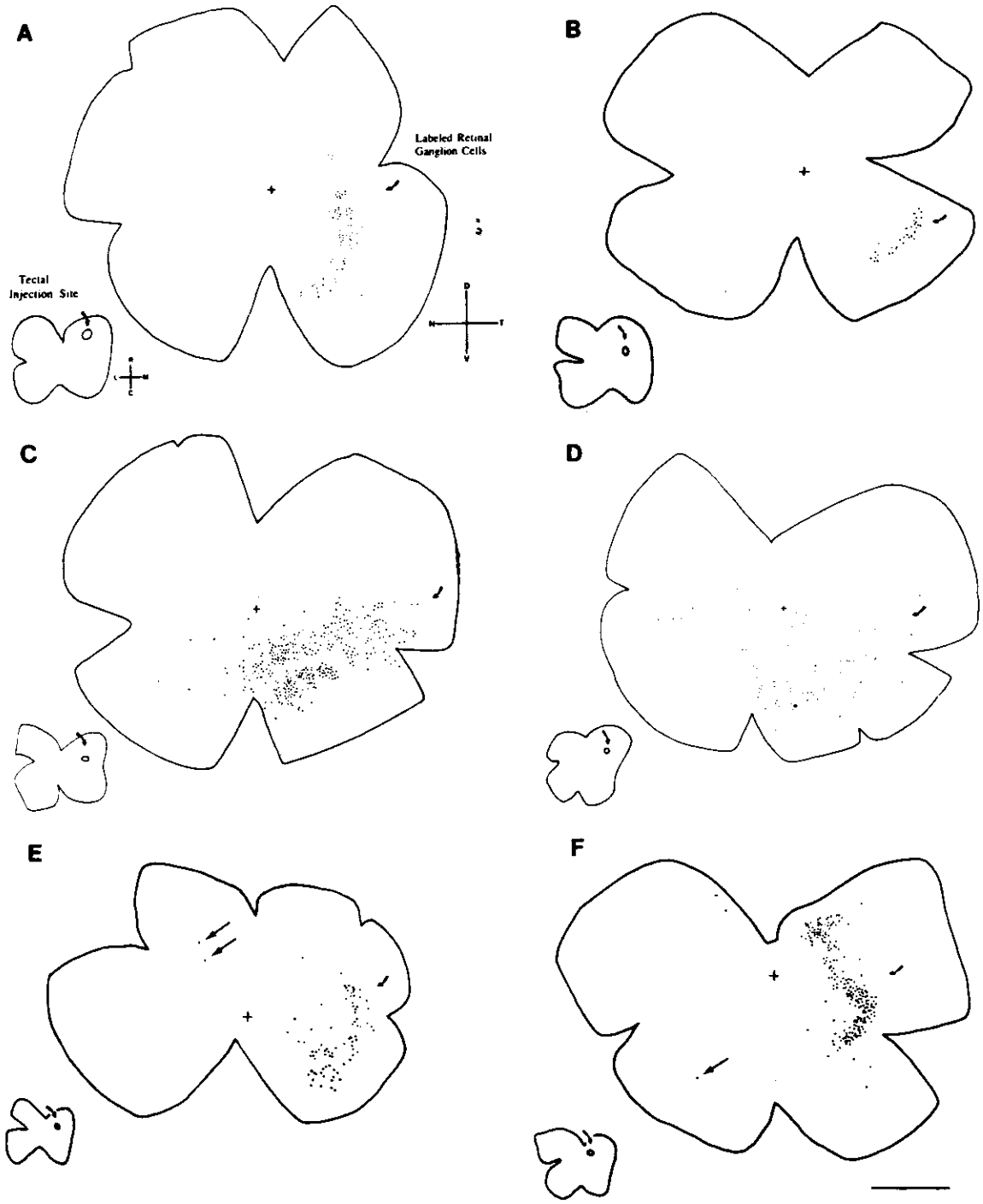

Figure 2. Retinal Topography Is Distorted Following APV Treatment of the Optic Tectum

RGCs were labeled retrogradely with a local HRP injection into the contralateral rostromedial tectal neuropil. Camera lucida drawings of the corresponding retinal and tectal flat mounts show the positions of the HRP-labeled RGCs projecting to the rostromedial tectum and the size and position of the injection site in the tectum (inset). (A) and (B) are two examples of the labeling pattern seen in untreated tadpoles. (C)–(E) are examples of the labeling pattern in APV-treated tadpoles. The animals whose retinae are shown in the left column (A, C, and E) are from stage XIV tadpoles, all of which were injected with the same HRP pipette and processed for HRP histochemistry together. Similarly, the animals whose retinae are shown in the right column (B, D, and F) are from stage XII tadpoles that were injected with the same HRP pipette and processed together. The percent total retinal area occupied by labeled RGCs is 5% in (A), 2.5% in (B), 19.8% in (C), 20.3% in (D), 12.7% in (E), and 14.9% in (F). Straight arrows in (E) and (F) point to ectopic RGCs. There is no consistent difference in the density of labeled ganglion cells between the control and experimental retinae. All retinae and tecta are oriented similarly according to the orientation shown in (A). Drawings of some retinae were flipped along the dorsoventral axis. The scale bar = 0.8 mm for the retinas and 2 mm for the tecta. The labeled RGCs in (F) are in the dorsotemporal retina because the tectal injection site was shifted more toward the lateral margin of the lobe than the other injection sites.

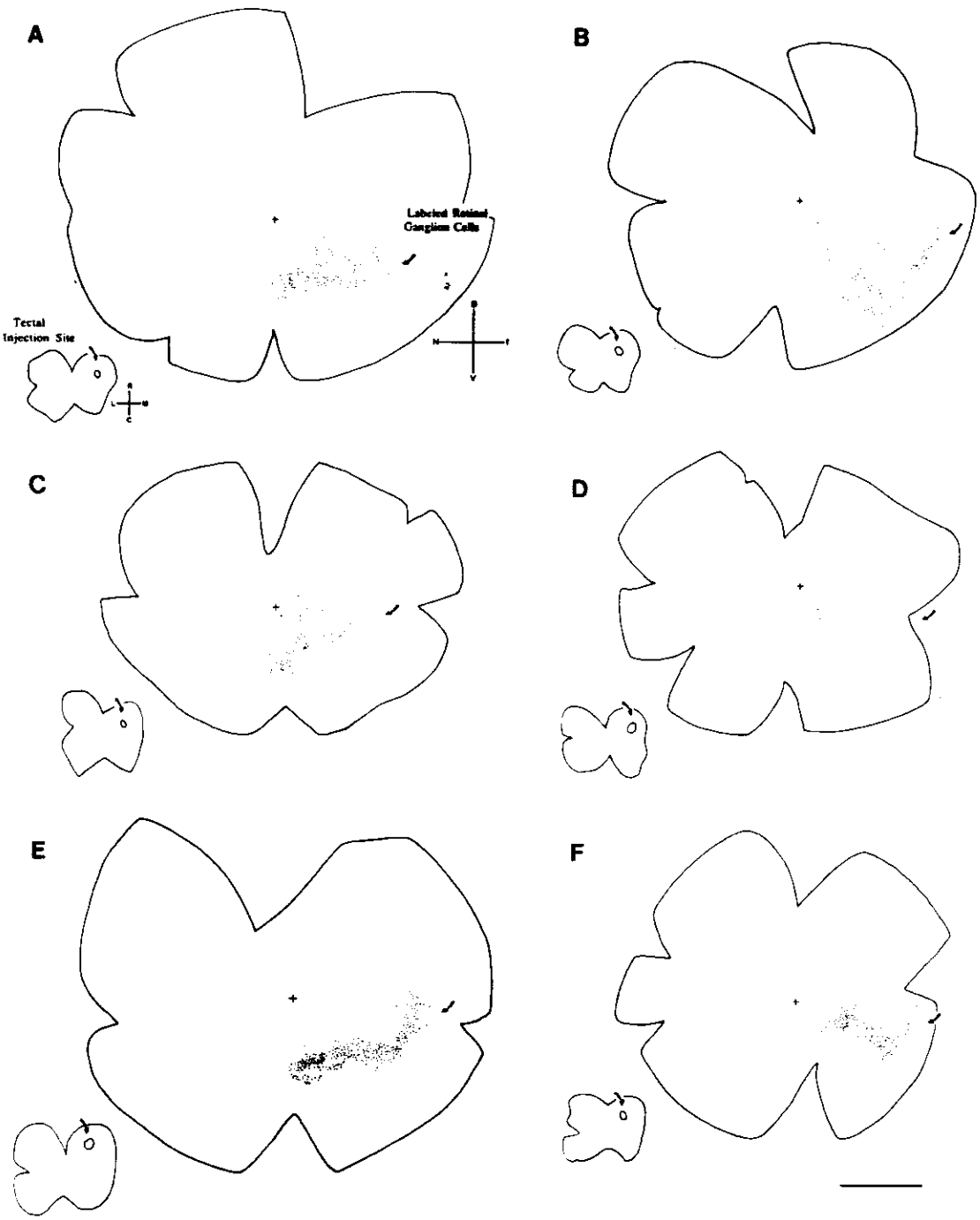

Figure 3. L-APV Treatment of the Optic Tectum Does Not Alter Retinal Topography

(A) and (B) show retinal and tectal flat mounts from untreated control tadpoles and (C)–(E) are from L-APV-treated tadpoles. The left column (A, C, and E) are from stage XIV tadpoles injected with the same HRP pipette and processed together for HRP histochemistry. The right column (B, D, and F) are from stage XII tadpoles that were injected with the same HRP pipette and processed together. The percent total retinal area occupied by labeled RGCs is 5.1% in (A), 6.7% in (B), 5.2% in (C), 7.4% in (D), 4.2% in (E), and 5.6% in (F). Conventions and scales are as in Figure 2.

labeled RGCs, and a mean density of 13.3 HRP-labeled RGCs/percent retinal area. Comparable treatment with the inactive isomer L-APV did not result in any enlargement of the HRP-labeled retinal area (4.7% ± 1.1%, range = 1.2%–7.4%, n = 5 tecta from 3 animals) (Figure 3, Table 1). L-APV treatment did not alter the number of labeled RGCs per retina (61 ± 8.2 RGCs per retina) or the density of labeled cells (13.1 RGCs/percent retinal area).

HRP injections in local tectal regions typically labeled

Table 1. The Percent Retinal Area Occupied by Labeled RGCs in Untreated and Drug-Treated Tadpoles

	Control	DL-APV	MK801	L-APV	NMDA
% Retinal Area Occupied by Labeled RGCs	5.2 ± 0.5 (n = 31)	17.6 ± 3.8[a] (n = 10)	10.4 ± 2.1[a] (n = 8)	4.7 ± 1.1 (n = 5)	6.0 ± 0.8 (n = 12)

[a] $P < .005$

RGCs in well-defined local regions of the retina. Even in the APV-treated animals, where the labeled RGCs are dispersed over a larger area, they are, for the most part, confined to the topographically correct quadrant of the retina. However, in 4 out of 10 retinae examined from the APV-treated animals and in 1 out of 12 retinae examined from NMDA-treated animals, between 1 and 4 HRP-labeled RGCs were located outside the temporal-ventral quadrant of the retina that projects to the dorsomedial tectum (Figure 2). The ectopically positioned HRP-labeled cells are found across the nasotemporal midline of the retina. All the ectopically positioned HRP-labeled RGCs have similar morphological characteristics: they have the largest cell bodies (20–30 μm diameter) and an extensive planar dendritic arbor (250–400 μm) (Figure 7). The axon within the retina is larger caliber than most other retrogradely labeled axons. Figure 7 shows the normal labeling density in a retina projecting to an APV-treated tectum (A) and an isolated HRP-labeled RGC typical of the ectopically positioned RGCs (B). We examined the tecta corresponding to the retinae with the ectopically positioned RGCs to see if the cells were labeled as axons of passage. In the NMDA-treated tectum, the arbor was in fact located in the central tectum, and the trajectory of the axon passed through the injection site, which indicates that the RGC labeled as an axon of passage. However, in the APV-treated tecta, all HRP-labeled arbors were confined to the local rostromedial injection site. No ectopically positioned RGCs were observed in untreated or L-APV treated animals.

MK801 Treatment

We tested the effect of MK801, a noncompetitive use-dependent NMDA channel blocker on the maintenance of the retinotopic map. The drug blocks from within the NMDA channel and can only gain access to the binding site when the channel is open. We had previously noted that MK801 did not cause the desegregation of eye-specific stripes in three-eyed tadpoles. However, the failure of MK801 to desegregate eye-specific stripes might be due to the infrequency of channel opening and the resultant poor access of the drug to the interior of the channel (Huettner and Bean, 1988). Therefore, we treated the tecta of six tadpoles with Elvax prepared with a mixture of MK801, to block the NMDA channel, and NMDA, to increase the frequency of channel opening.

We found that the retinae of tadpoles whose optic tecta were treated chronically with MK801-NMDA for 12 weeks exhibit a wider distribution of retrogradely labeled RGCs than the retinae from stage-matched controls (Figure 4, Table 1). The percent of retinal area occupied by the retrogradely labeled RGCs was 10.4 ± 2.1% (range = 5.4%–24.1%, n = 8 tecta from 5 animals), significantly greater than untreated tadpoles ($p < .005$). The number of HRP-labeled RGCs was 264 ± 30 in retinae from animals treated with MK801 + NMDA. The density of labeled RGCs was 25.4 RGCs/percent retinal area, twice the density of labeled cells from other treatments. However, the density of labeled RGCs from the control animals injected with the same pipette was 26.8 ± 3.1, also twice the density seen in the remainder of the controls.

NMDA Treatment

We have reported that exposure of the tecta of surgically produced three-eyed tadpoles to the receptor agonist NMDA results in eye-specific stripes with sharper borders (Cline et al., 1987). This observation suggested that NMDA treatment might enhance the point-to-point specificity of the retinotopic projection. Exposure of the tectal lobes to NMDA for periods of 6 to 10 weeks did not alter the percent retinal area occupied by labeled RGCs (6.0% ± 0.8%, range = 1.6%–9.8%, n = 12 tecta from 6 animals; Figure 5, Table 1). NMDA treatment did not alter the number or the density of HRP-labeled RGCs, compared with controls. The number of labeled cells was 76± 9.1 RGCs per retina and the density was 12.8 RGCs/percent retinal area. Exposure of the tectum to ten times greater concentration of NMDA did not alter retinal topography (data not shown).

RGC Arbor Morphology

The distortion of the retinal projection seen in tecta treated with NMDA receptor antagonists could result from the enlargement of individual RGC terminal arbors or from less precise targeting of normal sized arbors. To distinguish these possibilities, we reconstructed individual HRP-labeled RGC terminal arbors in the rostromedial tectum of untreated, APV-treated, and NMDA-treated tadpoles of similar stages (Figure 6). Terminal arbors from untreated tadpoles are elongated in the rostrocaudal direction and most major branches of arbors from the rostromedial tectum are directed caudally. The branches are uniform in diameter between branch points, and decrease in diameter after branching. The branches do not have local thickenings along the length, as seen in arbors from adult frogs (Constantine-Paton et al., 1983; Stirling and Merrill, 1987). In the main part of the arbor, branches often end in forks and elaborate growth cones. Several short, blunt-ended branches leave the primary axon proximal to the major terminal arbor. These are probably remnants of an arborization from a

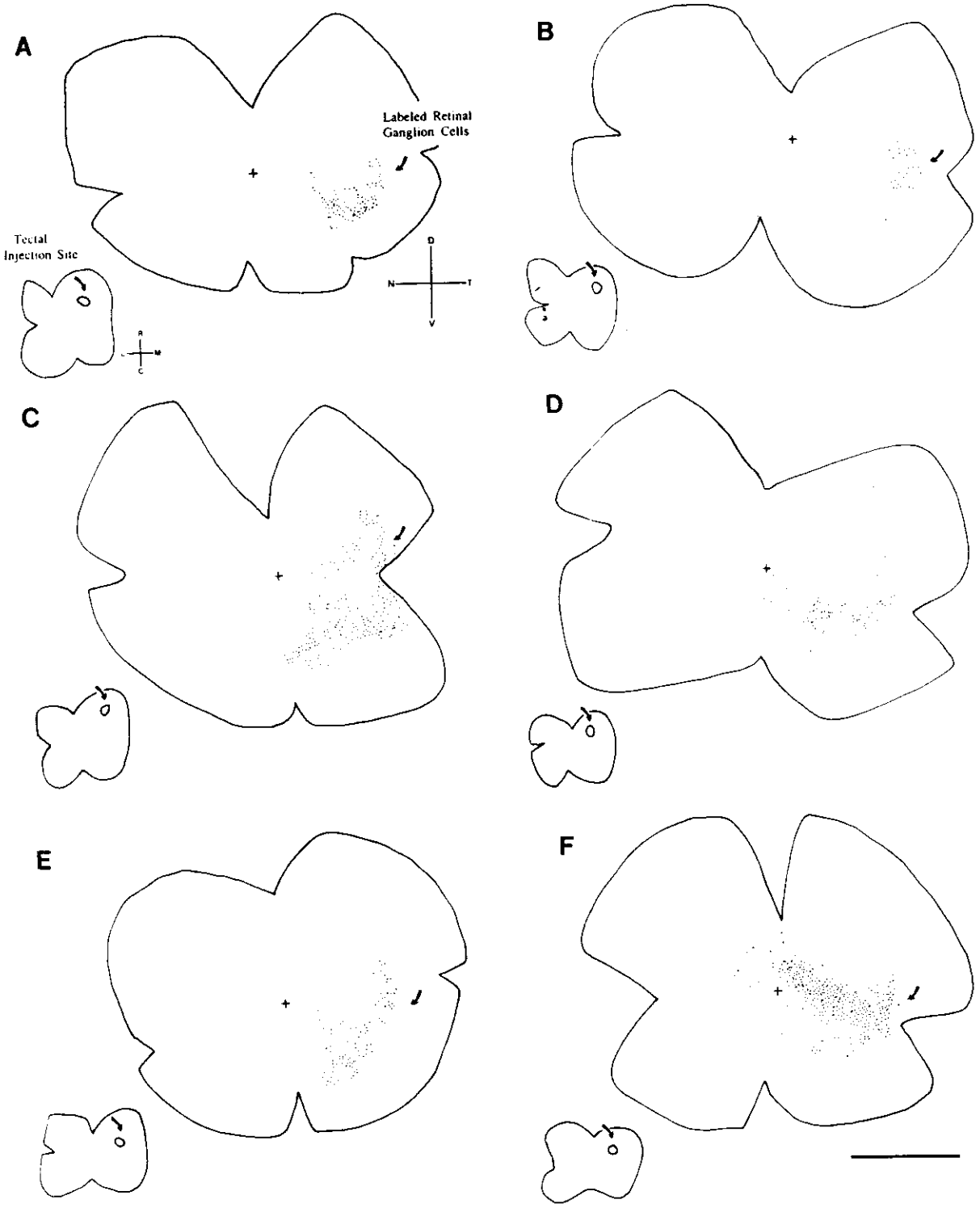

Figure 4. MK801 Treatment of the Optic Tectum Disrupts Retinal Topography

(A) and (B) show retinal and tectal flat mounts from untreated control tadpoles and (C)–(E) are from tadpoles treated with a combination of MK801+ NMDA. The left column (A, C, and E) are from stage XVII tadpoles and the right column (B, D, and F) are from stage VII tadpoles. All the MK801-treated tadpoles were injected with the same HRP pipette and processed together. The percent total retinal area occupied by labeled RGCs is 7.0% in (A), 3.8% in (B), 18.4% in (C), 14.3% in (D), 8.8% in (E), and 16.5% in (F). Conventions and scales are as in Figure 2.

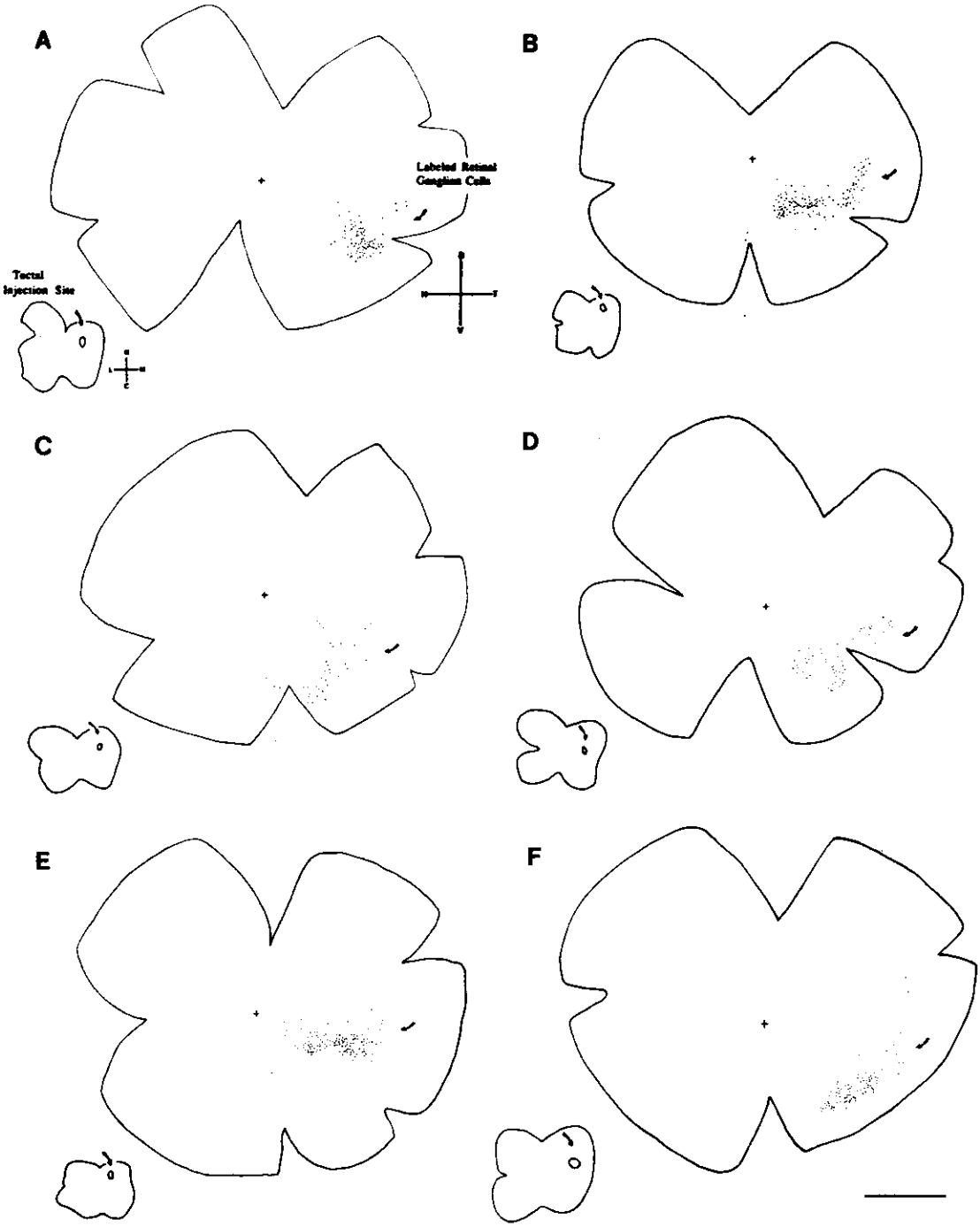

Figure 5. NMDA Treatment of the Optic Tectum Does Not Alter Retinal Topography

(A) and (B) show retinal and tectal flat mounts from untreated control tadpoles and (C)–(E) are from NMDA-treated tadpoles. The left column (A, C, and E) are from stage XII tadpoles injected with the same HRP pipette and processed together for HRP histochemistry. The right column (B, D, and F) are from stage XIV tadpoles that were injected with the same HRP pipette and processed together. The percent total retinal area occupied by labeled RGCs is 5.5% in (A), 6.1% in (B), 7.0% in (C), 6.3% in (D), 4.5% in (E), and 7.1% in (F). Conventions and scales are as in Figure 2.

former, more rostrally situated, termination site which are retracting during the growth process (Reh and Constantine-Paton, 1984). We determined the length, width, tangential area, and number of branch endings per arbor (Table 2). Tadpole RGC terminal arbors cover approximately 27×10^3 μm^2 in tangential area (27.6×10^3 $\mu m^2 \pm 7.3 \times 10^3$ μm^2, n = 7, range = 10.1×10^3 μm^2 – 51×10^3 μm^2). These values are uniformly larger than values reported in earlier studies because previous fixing and clearing regimes caused more severe shrink-

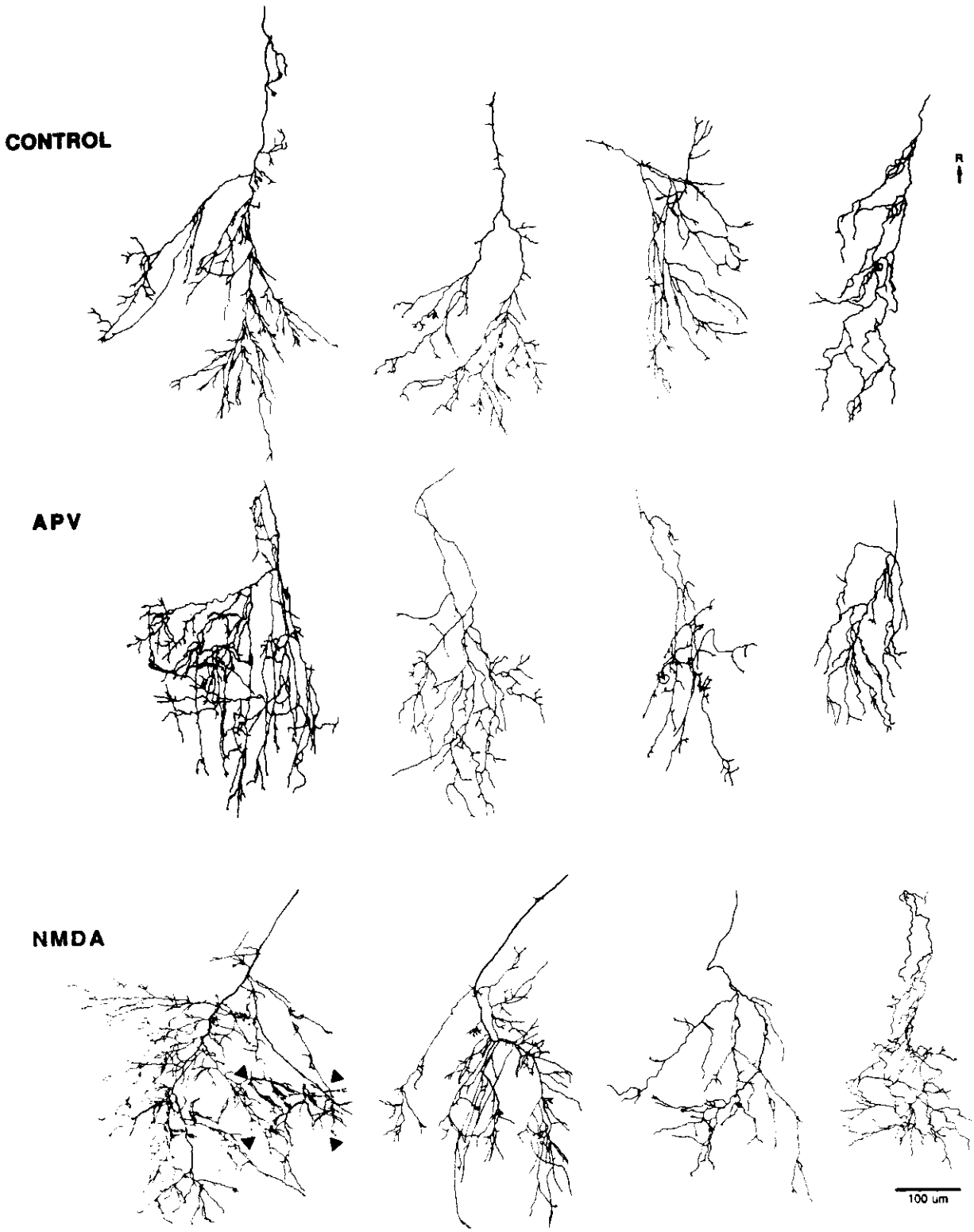

Figure 6. APV and NMDA Treatment Do Not Alter RGC Terminal Arbor Morphology

Camera lucida drawings of RGC terminal arbors from untreated (top row), APV-treated (middle row), and NMDA-treated (bottom row) tecta of two-eyed tadpoles. Arbors in each column are matched approximately for tangential area with the largest arbors on the left. Arrowheads on the lefthand NMDA-treated arbors mark areas in which terminal branches grow in close apposition. Scale bar = 100 μm.

Table 2. Descriptive Parameters of Arbor Morphology in Untreated and Drug-Treated Terminal Arbors

Treatment	Length (μm)	Width (μm)	Area (10^3 μm^2)	Branch #	Branch #/Area (n/10^3 μm^2)
Untreated (n = 5)	340 ± 44	170 ± 40	27.6 ± 7.3	89 ± 20	3.3 ± 0.6
DL-APV (n = 7)	438 ± 50[a]	162 ± 28	27.4 ± 7.8	95 ± 26	3.9 ± 0.6
NMDA (n = 8)	400 ± 43	273 ± 36[a]	32.0 ± 8.0	122 ± 32	4.1 ± 0.6

[a] $P < .001$

age of tissue. The mean rostrocaudal length was 340 ± 44 μm (range = 200–450 μm), and the mean width was 170 ± 40 μm (range = 90–230 μm). The arbors have 89 ± 20 branch endings per arbor (range = 45–146). The branch density, or branch endings per unit area, is 3.3 ± 0.6 branches/10^3 μm^2 (range = 1.8–4.5 branches/10^3 μm^2) (Table 2).

RGC terminal arbors from APV-treated tecta did not differ from arbors from untreated tecta with respect to their tangential area, their width, the number of branch endings per arbor, or branch density (Table 2). However, APV-treated arbors are significantly longer than untreated arbors (438 ± 50 μm for APV-treated arbors vs. 340 ± 44 μm for untreated arbors, $p < .001$). NMDA-treated arbors did not differ from untreated arbors with respect to their tangential area, length, branch number, or branch density. However the NMDA-treated arbors are significantly wider than either untreated or APV-treated arbors (273 ± 36 μm for NMDA treated arbors, compared with 170 ± 40 μm for untreated arbors, $p < .001$). In addition, we observed a unique branching pattern in the NMDA-treated arbors. Often the finer branches grow parallel to one another for considerable distances, to the extent that the two branches appear superimposed. In the NMDA arbor to the left in Figure 6, several examples of the terminal branchlets growing in close association can be seen within the area marked by arrowheads.

Toxicity Controls

We did not detect any toxic effects of drug treatment in any of our experimental groups. Chronic exposure of tecta to DL-APV, L-APV, or NMDA did not alter the density of retinal ganglion cell bodies from that observed in control groups (Table 3). Tectal cell density in layer 6, the cellular layer containing the majority of retinorecipient tectal neurons, also showed no change between drug-treated and control animals (Table 3).

Discussion

Effects of Drug Treatments on Topography

The current experiments, like those conducted earlier on the developing retinotectal projection, rely on the constant mobility of the RGC terminal arbor in the rap-

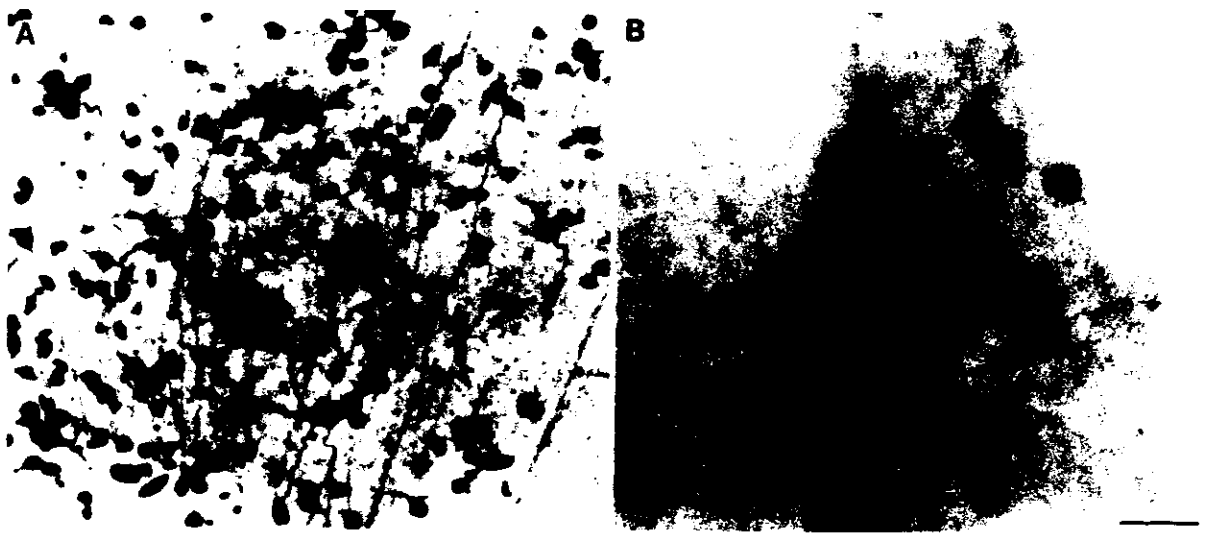

Figure 7. Ectopic RGCs Label in Retinae Projecting to APV-Treated Tecta

(A) Typical density of HRP-labeled cell bodies in the ventrotemporal retina of an APV-treated animal. Many RGC types are labeled and cell bodies are close together.

(B) An "ectopically labeled" RGC in the dorsonasal quadrant of the same retina as in (A). The RGC is well isolated from other labeled cell bodies. The RGC has a distinctive morphology, characterized by the large cell body and the extensive planar dendritic arbor. This morphological subtype is also seen in the field in (A), although the dendritic arbor is obscured by other labeled cell bodies. The blurred dark spots are bits of pigment epithelium. Scale bar = 50 μm for (A) and (B).

Table 3. Retinal Ganglion Cell and Tectal Cell Density in Untreated and Drug-Treated Tadpoles

	Untreated	APV	L-APV	NMDA
RGC/100 μm	16.1 ± .3	16.9 ± .5	15.9 ± .4	15.0 ± .8
Tectal cells per 6250 μm^3	16.5 ± 1.6	17.8 ± .7	–	18.4 ± .4

idly growing tadpole. Entire RGC terminal arbors shift along the rostrocaudal axis of the tectum during larval development, when the tectum is adding cells primarily at the caudomedial border and the retina is adding cells equally along its perimeter (Gaze et al., 1979; Reh and Constantine-Paton, 1984). Despite the mismatched growth patterns of the retina and tectum, retinal topography is maintained at every stage of development (Gaze et al., 1979). It is thought that the retinotectal synaptic connections are shortlived and that the arbors shift in a topographically ordered fashion. The shifting of the terminals would be accomplished by the addition of new branches in the growing, caudal region of arbors and the retraction of older branches from the rostral region of the arbor. The point-to-point topography would be maintained by the coordinated migration of neighboring RGCs. In this context, the present study tests the ability of the growing retinotectal system to maintain coordinated migration of neighboring RGC terminals in the presence of an antagonist or agonist of the NMDA receptor.

Our data are consistent with the following interpretation, which links NMDA receptor/channel activation with the cellular mechanism for synapse stabilization. We are assuming that the NMDA receptor in the frog tectal neuropil shares the properties described for this receptor/channel in other systems (Mayer and Westbrook, 1987). We suggest that selective activation of the NMDA receptor by coactive retinal afferents triggers the stabilization of those coactive synapses. By virtue of the release of neurotransmitter and the spatiotemporal summation in the postsynaptic tectal neuron, the synapses driven by coactive neighboring RGCs are thought to activate the NMDA receptor, which would lead to the selective stabilization or increased lifetime of those coactive synapses. Exclusion of afferents with dissimilar activity patterns from a postsynaptic membrane could occur through an active process (Stent, 1973) or through an ongoing rapid turnover of those synapses that are not stabilized (Changeaux and Danchin, 1976). Our observations suggest that, in the presence of APV or MK801, constant release of the NMDA receptor antagonist from the Elvax produces a maintained disruption of normal NMDA receptor function. The continuous presence of NMDA receptor antagonists essentially inactivates the mechanisms that recognize coactive afferents and initiate their selective stabilization when they converge on a common postsynaptic neuron. In the absence of selective synapse stabilization based on correlated activity, the retina and tectum continue to grow, the RGC terminals continue to migrate, but RGC synapse lifetime is unrelated to retinal activity patterns. New contacts survive irrespective of whether they are localized among coactive contacts from retinal neighbors.

In untreated tadpoles, a local HRP injection into the rostromedial tectum labels a cluster of RGCs in the temporoventral retina. Often the cluster of HRP-labeled RGCs is crescent-shaped and includes a small portion of an age-related annulus of RGCs (Constantine-Paton et al., 1983; Hitchcock and Easter, 1987), whose axons were labeled as they passed through the injection site. Therefore a local HRP injection typically labels the RGCs whose terminals arborize at that site and a small group of age-related axons projecting from the more nasal retina to a slightly more caudal site in the tectum. The rostromedial tectum was chosen for the injection site because it was possible to verify that the Elvax was present at the time of the injection and because few axons of passage traverse the rostromedial tectum to reach other tectal sites.

The percent tectal area covered by the tectal injection site was consistently smaller than the percent retinal area labeled from that site. The tectal area measured was the densely labeled region where the injection pipette penetrated the tectal neuropil. It is thought that the ganglion cell body labels with HRP when any portion of its arbor is damaged by the injection pipette and therefore takes up HRP from the broken branches. Typically the entire arbor and the axon back to the cell body are well labeled with HRP. The larger retinal area labeled, relative to the area of the tectal injection site, might be due to the labeling of cell bodies whose arbors only extended a few branches into the injection site. Consequently, the cell body would label, but the entire arbor would not overlap with the measured injection site.

The cresent-shaped labeling pattern is seen in retinae from untreated, sham-operated, NMDA-treated, and L-APV-treated animals, which indicates that the surgical manipulations do not perturb the the axonal termination patterns. Furthermore, the retrogradely labeled axons in the tecta of animals treated with either APV or MK801 follow the same pathway from the injection site to the medial optic tract as seen in tecta from animals with normal topography. Therefore, the disrupted topography cannot be accounted for by a perturbed trajectory of the RGC axons.

Following treatment with NMDA receptor antagonists, RGC terminal arbors that normally (and originally) projected to distinct tectal sites now converge on the same region of neuropil. As might be predicted from the disrupted topography, the absolute number of HRP-labeled RGCs is significantly greater in APV- and MK801-

treated animals, compared with controls. An increase in the number of labeled RGCs may result from an increased efficiency of labeling of the same number of terminals in the injection site, or an increase in the number of arbors, a portion of which overlaps with the injection site. An increased efficiency of labeling would increase the density of labeled cells, because number of labeled cells would increase, but the percent retinal area labeled would not. An increase in the number of arbors with branches penetrating the injection site would be seen as an increase in the number of labeled RGCs, but not necessarily as an increase in the density of labeled cells. The increased number of labeled RGCs we observe in animals treated with NMDA receptor antagonists probably reflects an increase in the number of arbors that aberrently branch into the injection site. We suggest that the increased convergence results from the inability of the tectal neurons to coordinate the direction of terminal migration, based on activity patterns in the afferent terminals when the NMDA receptor/channel is blocked.

The density of labeled RGCs in retinae from APV-treated treated animals is the same as the density of labeled RGCs in control retinae, although the density of labeled RGCs from MK801-treated animals is about twice that density. However, the control animals injected with the same pipette as the MK801-treated animals also had twice the density of labeled cells compared with the remainder of the controls. Therefore, it is likely that the injections made in the MK801 series were more effective at damaging terminals than the other injections. This might be expected if the broken pipette tip had more jagged edges than other tips. It is important to note, however, that although the controls from the MK801 series had more labeled RGCs, they were confined to a limited retinal area, comparable to other controls. This indicates that the efficiency of labeling was not a determinant in the percent retinal area labeled and that the increase in retinal area labeled in the animals treated with MK801 was not an artifact of the increased labeling efficiency.

Two alternative results may have been expected from the animals exposed to NMDA. Based on our observation that NMDA treatment of the tecta of three-eyed tadpoles sharpens the borders of the eye-specific stripes, we anticipated that NMDA treatment of the two-eyed tadpoles might increase the refinement of the retinal projection. However, we did not observe a decrease in the percent retinal area with labeled RGCs. It is possible that we could not detect such a difference with our anatomical assay. A second possible result was that the NMDA receptor would desensitize with chronic exposure to the receptor agonist, resulting in a loss of retinal topography, comparable to that seen with NMDA receptor antagonists. Although electrophysiological studies demonstrate a decreased sensitivity to NMDA following chronic exposure to NMDA in Elvax (Debski et al., 1989, Soc. Neurosci., abstract), the maintenance of both eye-specific stripes and retinal topography despite chronic exposure to NMDA indicates that the receptor system is not desensitized to the extent that retinal terminals can no longer sort out based on patterns of correlated activity. It is possible that the desensitization is counterbalanced by the constant presence of the NMDA.

Although NMDA-treatment does not change retinal topography, we are confident that the NMDA is diffusing out of the Elvax and exerting an effect on the tectal cells for several reasons. NMDA-treated RGC terminal arbors exhibit a slightly different morphology than arbors from either untreated or sham-operated (i.e., implanted with fast green-Elvax). In addition, our experiments with three-eyed tadpoles exposed to either MK801 alone or MK801 + NMDA indicate that NMDA is required for MK801 to cause stripe desegregation, presumably by increasing the frequency of NMDA channel openings. Finally, recent electrophysiological experiments performed on tadpoles treated with NMDA in the same manner as the animals used for these experiments provide convincing evidence that the drug is reaching the tectum in a biologically active form (Debski et al., 1989, Soc. Neurosci., abstract). The observation that higher concentrations of NMDA do not alter retinal topography is consistent with the observation that eye-specific stripes are present in three-eyed tadpoles even at toxic concentrations of NMDA (Cline and Constantine-Paton, submitted).

Detailed morphological analysis of drug-treated and untreated RGC terminal arbors indicate that APV-induced disruption of retinotectal topography is not due to an enlargement of individual RGC terminal arbors. These data are consistent with our earlier observation that chronic treatment of doubly innervated tadpole tecta with APV produced desegregation without enlarging the RGC terminal arbors (Cline et al., 1987). It is also unlikely that the 30% increase in length of APV-treated arbors can account for the decrement in retinotopic precision. The increase in HRP-labeled RGC distribution is too large to be accounted for by the increase in arbor length alone. Furthermore, increasing rostrocaudal arbor length would only increase labeling in the retina along one axis, rather than the dramatic concentric expansion observed. Thus, the pronounced distortion of the highly refined retinal topography in tecta treated with APV is not due to the disruption of the mechanism(s) that controls arbor size.

It is important to point out that even though NMDA receptor antagonists impair the coordinated migration of terminals arising from neighboring RGCs, none of the drugs tested appeared to alter the overall caudally directed migration of RGC terminals. The drugs neither increased nor decreased the area of the tectum innervated, determined by comparing the caudal extent of HRP labeling in treated and untreated tectal lobes (H. Cline, unpublished data). These data support previous suggestions that the mechanisms underlying the growth shifting toward uninnervated tecta involves different mechanisms than those involved in the activity-dependent sorting (Constantine-Paton and Reh, 1985; Meyer and Wolcott, 1988). However, APV-treated termi-

nals are elongated along the rostrocaudal tectal axis. We have suggested that NMDA receptor antagonists prevent the selective stabilization of coactive synapses and thereby decrease the lifetime of terminal branches with correlated activity patterns (Cline and Constantine-Paton, submitted). The elongation of the arbors along the axis of shift may reflect a decreased stabilization of terminal branches that could allow more rapid sprouting of terminal into less innervated caudal tectal regions. Our work on the striped tecta of three-eyed frogs suggests that chronic NMDA treatment increases the stabilization of RGC terminal branches in regions where afferent activity is well correlated (Cline et al., 1987; Cline and Constantine-Paton, submitted). In the shifting retinotectal projection of two-eyed tadpoles, the highest degree of correlated activity would occur between RGC terminals that are neighbors along the axis perpendicular to displacement. Thus increased synapse stabilization in regions of arbors with well-correlated activity could explain the mediolateral expansion of NMDA-treated arbors.

Ectopically Positioned HRP-Labeled Retinal Ganglion Cells

A surprising observation in this study was the occurrence of ectopically positioned HRP-labeled ganglion cells following APV treatment. The single ectopically labeled RGC seen in NMDA-treated animals probably labeled because its axon trajectory passed through the injection site. However, disruption of axons of passage does not explain the ectopically labeled RGCs in the nasodorsal and nasoventral retina of APV-treated animals because the axons of the RGCs from nasodorsal retina project to the caudolateral tectum though the lateral optic tract, far from the rostromedial HRP injection site. Similarly, axons from the nasoventral RGCs follow the medial optic tract to the caudomedial tectal quadrant before exiting the tract to grow into the tectum. Each tectum was carefully examined for axons passing through the injection site and for spillover into the medial optic tract.

The ectopically labeled RGCs seen in APV-treated animals appear to fall into a single morphological class, characterized by their large cell body, their extensive, planar dendritic tree, and their relatively thick axon within the retina. These features correspond well to the Type IV cells whose tectal arbor often includes a secondary termination site in a non-neighboring region of the tectum (Stirling and Merrill, 1987). It is possible that labeling through the secondary termination site is increased by APV-treatment, perhaps by allowing an increased degree of arborization of this arbor in a region of poorly correlated activity. APV-treatment may deter or prevent pruning of incorrectly projecting branches. We did not see any terminal arbors with secondary termination sites. This may have been due to the low number of reconstructed terminals.

Several studies have suggested that the mechanisms underlying segregation of eye-specific afferent terminals in frogs, fish, and cats are the same as those which determine topographic order in afferent sensory projections (Constantine-Paton et al., 1990; Fraser, 1985; Schmidt, 1985). Our previous work demonstrated that the NMDA receptor is critically involved in the mechanism that maintains eye-specific segregation. Here, we have shown that treatment of the tectum of normal tadpoles with NMDA recept or antagonists results in a loss of the point-to-point retinal topography seen in untreated tadpoles. These results strengthen the parallels between the mechanisms underlying eye-specific terminal segregation and those underlying the formation and maintenance of retinal topography in a developing sensory system.

Experimental Procedures

Preparation of Elvax

Elvax plastic polymer (Dupont, Inc) was prepared as described in detail elsewhere (Silberstein and Daniel, 1982). Briefly, the plastic was solubilized in methylene chloride (100 mg Elvax/1 ml solvent). Concentrated solutions of drugs, prepared in either distilled water or saline, were added to the plastic solution along with fast green (.01% final concentration). The Elvax was mixed with a vortex, frozen rapidly in a dry ice/acetone bath, and stored for 2 days to 2 months at −20°C. After 1–2 days under gentle vacuum, the Elvax was cut into small pieces, embedded in OCT embedding solution, and cut into 30 μm slabs on a cryostat for surgical implantation.

Calibration of Release from Elvax

[^{3}H]APV-Elvax was prepared with [^{3}H]DL-APV (Tocris Neuramin; 20–40 Ci/mmol) diluted in 10^{-3} M DL-APV. [^{3}H]GABA-Elvax was prepared with [^{3}H]GABA (NEN; 56 Ci/mmol) diluted in 10^{-4} M GABA. Slices of Elvax were cut on the cryostat, weighed, and soaked in 100 μl saline solution. Each day the total volume (100 μl) of the soaking solution was replaced with fresh saline and the soaking solution was counted on a Beckman LS5000 Scintillation Counter. Samples were taken in triplicate. The radioactivity released was normalized to the weight of the Elvax slab and plotted as cpm released/mg Elvax/day over a period of 60 days.

Surgery

Elvax implants: The optic tectum of tadpoles, anesthetized by submersion in 0.05% MS222 (amino benzoic acid; Sigma), was exposed. The dura was opened along the midline, and the pia was either lifted away from the tectal lobe in a sheet or peeled away to expose the bare tectum. Elvax pieces (about 1 × 1 × .03 mm) were laid over the entire tectal surface and held in place by the pial and/or arachnoid membranes. The brain covering and skin were replaced and the wound was sealed with Histoacryl glue (Tri-Hawk, Montreal). Animals recovered from surgery in oxygenated dilute Instant Ocean supplemented with Penn/Strep (100 U/l; Sigma).

Retrograde HRP Labeling

Tadpoles were anesthetized by submersion in 0.05% MS222 and the optic tectum was exposed. Prior to the HRP injection, contact of the Elvax with the dorsal tectum was verified. The Elvax was lifted away from the tectum and HRP was injected with a General Valve, Inc., Picospritzer into the rostromedial tectum underneath where the Elvax had been. Untreated or sham-operated tadpoles of the same stages as the experimental animals were injected with the same HRP pipette using the same injection parameters.

A preliminary series of experiments was performed to determine the factors involved in obtaining a reproducible injection of HRP into a local region of the tectum. We tested several concentrations of HRP and WGA-HRP, as well as tip diameter of the injection pipettes, depth of the pipette tip within the tectum, and the pressure applied to the pipette. Injection pipettes were pulled on a horizontal Narashige puller, and the tips were broken to a tip diameter of approximately 50 μm. Uniform size of the tectal injection site and labeling in the retina were obtained using a 20% HRP solution (Sigma, Type VI or Boehringer Mannheim, Grade 1) in a 50 mM Tris, NaCl solution injected into the superficial tectal layer. After 5–6 days, the animals were sacrificed and the eyes and brains

were reacted in whole mount with DAB as described previously (Reh and Constantine-Paton, 1984). The retinal epithelium was dissected free from the retina, taking care to place a radial cut at the ventral, dorsal, nasal, and temporal poles to aid in subsequent alignment. Tecta were dissected free from the brain and major cuts were made at the rostral and caudal poles of the lobe. The retinae and tecta were flattened between cover slips, and fixed in 2% paraformaldehyde and 2% glutaradehyde in 0.1 M phosphate buffer for 3–4 days at 4°C. They were subsequently rinsed in 0.1 M phosphate buffer, dehydrated through graded alcohols, cleared in xylene, and mounted in Permount. The positions of labeled RGCs were verified at 400× magnification and drawn with a camera lucida at 112× magnification. The axons of labeled RGCs converge on the optic nerve head as a small fan of fibers originating from cells in the labeled region of the retina. Examination of the optic nerve head easily revealed any labeled axons of RGCs outside the densely labeled region.

HRP Labeling of RGC Terminals

To label RGC terminal arbors, tungsten needles tipped with HRP crystals were introduced into the ventrotemporal retina. After 2 days survival, the brains were processed for HRP histochemistry in wholemount as described previously. The tecta were dissected free from the brain, flattened between cover slips, and fixed for 3–4 days in 2% paraformaldehyde and 2% glutaradehyde. After rinsing in 0.1 M phosphate buffer, the tissue was dehydrated through graded alcohols, cleared in xylene, and mounted in Permount. Individual HRP-labeled retinal ganglion cell terminal arbors were drawn with a camera lucida using a 63× oil immersion lens. Arbors were selected for reconstruction from the rostromedial quadrant of the tectum where it was possible to verify that the Elvax had remained in contact with the tectum throughout the exposure period. To control for variation in arbor morphology with the stage of the animal, we have drawn arbors from stage-matched untreated control animals.

Arbor Morphology Analysis

Tangential area was determined using a Terak computer equipped with a bitpad. The perimeter of the arbor was traced so that the cursor outlined the highest order branches of the arbor rather than the overall envelope of the arbor. The area of each arbor was measured three times and the average taken as the most accurate estimate of tangential area covered. The area measurements are not particularly sensitive to the shape or branching patterns of arbors. For example, an extremely sparce arbor could extend across a large portion of a tectum, yet have the same area mesurement as a densely branched arbor having half the linear dimensions. Consequently, we determined three other measures of arbor morphology: length, width, and branch number per unit area. Length of an arbor along the rostrocaudal axis was measured as the length of a straight line extending from the first branch off the major axon to the caudalmost tip. Arbor width was taken as the widest part of the arbor perpendicular to the straight line established as the rostrocaudal length. Branch density of the arbors were calculated by counting the number of branch endings in the arbor and dividing this value by arbor area measured as described above.

Tectal cell density in layer 6 was determined in toluidine blue or hematoxylin stained histological sections by counting the number of cell bodies in a 625 μm^2 × 10 μm volume under 400× magnification (Constantine-Paton and Ferrari-Eastman, 1987). Retinal ganglion cell density was determined in hematoxylin stained sections by counting the number of retinal ganglion cell bodies in four alternating 10 μm sections through the optic nerve head. The length of the RGC layer was measured with a Terak computer and the cell number/unit length was determined.

Statistical analyses were performed using a two-tailed Student's t test (Snedecor and Cochran, 1967) and the Bonferroni correction.

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The Differential Influence of Protein Kinase Inhibitors on Retinal Arbor Morphology and Eye-Specific Stripes in the Frog Retinotectal System

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Summary

We investigated retinal axon morphology and eye-specific afferent termination zones in the optic tectum of three-eyed tadpoles that were chronically treated with protein kinase inhibitors. The kinase inhibitors sphingosine, H-7, and phorbol ester, which down-regulates protein kinase C with chronic exposure, were applied to the tecta in a slow release plastic, Elvax. In vivo protein phosphorylation assays in drug-treated tadpoles indicated that the treatments decreased ^{32}P incorporation into some protein bands by as much as 60%. Although the drugs did not cause a desegregation of the eye-specific stripes, treated retinal axon arbors covered about half the area covered by untreated arbors or arbors treated with inactive analogs of the drugs. We conclude that eye-specific segregation can be maintained under conditions that markedly alter retinal ganglion cell axon arbor size and that significantly perturb protein phosphorylation. Furthermore, we conclude that the protein kinase(s) that we blocked with these treatments is involved in the growth of axon arbors.

Introduction

Recent interest has been spurred by the findings that both the plasticity of developing connections in the visual system and long-term potentiation in the hippocampus, a cellular model of learning and memory, can be blocked by NMDA receptor antagonists (Collingridge et al., 1983; Harris et al., 1984; Cline et al., 1987; Kleinschmidt et al., 1987; Scherer and Udin, 1989; Cline and Constantine-Paton, 1989; Schmidt, 1990). These findings suggest that common mechanisms may underlie synaptic plasticity in developing and mature nervous systems. Roles for protein kinases in many forms of synaptic plasticity have been suggested (reviewed by Nairn et al., 1985; Routtenberg, 1986; Schwartz and Greenberg, 1987). In the hippocampus, tetanic stimulation of glutamatergic afferents activates postsynaptic glutamate-sensitive NMDA receptors (Collingridge et al., 1983). The resulting calcium influx through the NMDA channels (MacDermott et al., 1986) is thought to stimulate calcium-sensitive protein kinases such as protein kinase C (PKC) and/or calcium calmodulin-dependent protein kinase type II (CaMKII) and thereby to increase synaptic efficacy. Similarly, in the retinotectal system, it is thought that NMDA receptor activation by coactive retinal inputs initiates a series of events culminating in the stabilization of the coactive retinal ganglion cell (RGC) synapses and the refinement of point-to-point order in the projection (Cline et al., 1987; Cline and Constantine-Paton, 1989). One possible scenario is that a calcium transient through either pre- or postsynaptic NMDA channels (Cline and Constantine-Paton, 1990) modulates a calcium-sensitive protein kinase(s), which in turn increases or prolongs the stability of the coactive synapses. Therefore, it would be the kinase activity that is ultimately responsible for synapse stabilization.

PKC is a particularly attractive candidate for involvement in the formation of retinotectal topographic maps. This process involves both continual axon sprouting and stabilization of coactive synapses in growing axons (Reh and Constantine-Paton, 1984; Easter and Stuermer, 1984; Sakagushi and Murphey, 1985; O'Rourke and Fraser, 1989, Soc. Neurosci., abstract; Nakamura and O'Leary, 1989). PKC is one of the major protein kinases in growth cones (Katz et al., 1985; Van Hooff et al., 1988). In addition, several growth-associated proteins (reviewed by Skene, 1989) are PKC substrates (Patel and Kligman, 1987; Hyman and Pfenninger, 1987; Nelson et al., 1989), and their phosphorylation, in addition to their synthesis, is developmentally regulated (Jacobson et al., 1986; Neve et al., 1987). Spatial and temporal variations in the presence of growth-associated proteins, which correlate with the regeneration of topographic projections, have been used to implicate PKC in axon outgrowth and developmental synaptic plasticity (Benowitz and Lewis, 1983; Benowitz and Schmidt, 1987; McGuire et al., 1988; Skene, 1989; Nelson et al., 1989).

CaMKII is also thought to have a role in the developing nervous system. CaMKII activity is present in growth cones of cultured neurons (Scholz et al., 1988), in preparations of isolated growth cones (Katz et al., 1985), and in preparations of isolated nerve terminals (Katz et al., 1985; Wang et al., 1988). CaMKII constitutes one of the major developmentally regulated proteins in postsynaptic densities (Kennedy et al., 1983; Goldenring et al., 1984; Kelly et al., 1984; Ouimet et al., 1984). However, in contrast to PKC activity, CaMKII activity has been more closely correlated with periods of synaptogenesis (Newman-Gage and Graybiel, 1988; Sahyoun et al., 1985) and synapse maturation (Weinberger and Rostas, 1988), rather than axon growth.

In the developing retinotectal system in which synaptogenesis, synapse withdrawal, and axon growth are concurrent, activation of either PKC or CaMKII could be part of the cellular mechanisms that lead to the increased stability of local synapses subsequent to N-methyl-D-aspartate (NMDA) receptor activation

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(Cline and Constantine-Paton, 1989, 1990; Constantine-Paton et al., 1990).

To test this hypothesis, we chronically treated the optic tecta of Rana pipiens tadpoles with drugs that alter protein kinase activity: sphingosine (SPH), 1-[5-isoquinolinesulfonyl]-2-methyl piperazine (H-7), and phorbol esters. Although acute treatment with phorbol esters stimulates PKC (Nishizuka, 1984), chronic treatments of neuronal cultures down-regulate the enzyme (Matthies et al., 1987). SPH blocks PKC and CaMKII activity by competing with their activators, diacylglycerol and calmodulin, respectively, at the regulatory binding site (Hunnan et al., 1986; Jefferson and Schulman, 1988). H-7 competes with ATP at the catalytic domain of several kinases, including PKC and CaMKII (Hidaka et al., 1984).

The animals receiving these chronic treatments had been implanted as embryos with a supernumerary eye primordium. Consequently, the normal retina shares the target space, the optic tectum, with the projections from the extra retina. Labeling the supernumerary optic nerve of three-eyed animals with horseradish peroxidase (HRP) reveals a retinal projection pattern of interdigitating, rostrocaudally oriented stripes of RGC axon arbors, which can be viewed on the surface of the tectum without sectioning the brain (Constantine-Paton and Law, 1978). We have previously shown that the presence or absence of the striped projection pattern after chronic drug treatments is a useful assay for the integrity of the cellular mechanisms involved in both refinement of retinal topography and ocular dominance segregation (Reh and Constantine-Paton, 1985; Cline et al., 1987; Cline and Constantine-Paton, 1989).

We reasoned that if protein kinase activity were required for the development and maintenance of the segregated retinal projections, then treatment with agents that decrease kinase activity should result in the desegregation of eye-specific stripes in three-eyed tadpoles.

We found that treatments with phorbol esters, SPH, or H-7 decrease protein phosphorylation in the optic tectum, but they do not desegregate eye-specific stripes despite prolonged (up to 8 weeks) treatments. Nevertheless, the drugs do alter RGC arbor morphologies. We discuss these results in terms of the lifetimes of synapses in the highly mobile RGC arbors and 2-amino-5-phosphonovaleric acid-sensitive, protein kinase-independent, transient potentiation of synaptic transmission seen in the hippocampal slice preparation (Collingridge et al., 1983; Kauer et al., 1988; Malinow et al., 1988, 1989; Malenka et al., 1989).

Results

We treated the optic tecta of 9 three-eyed tadpoles with phorbol 12,13 dibutyrate (PDB) at concentrations of 10^{-3}, 10^{-4}, and 10^{-5} M in Elvax (n = 3 for each concentration). Six additional tadpoles were treated with the inactive phorbol ester, 4α-phorbol 12,13 didecanoate (4αPDD) at concentrations of 10^{-4} and 10^{-5} M in Elvax, (n = 3 for each concentration). In addition, groups of 3 tadpoles each were treated with SPH at 10^{-3} or 10^{-4} M in Elvax, with the inactive analog N-acetyl sphingosine (NAS) at 10^{-3} and 10^{-4} M in Elvax, or with H-7 and 10^{-2} or 10^{-3} M in Elvax. All treatments were started at T&K stage X-XI (Taylor and Kollros, 1946). After 4–8 weeks, when the tadpoles were stage XII–XV, the supernumerary retinal projection was labeled with HRP to test whether the drug treatments interfered with the maintenance of eye-specific stripes. Drugs were delivered to the tectal lobes by implanting thin, drug-infiltrated pieces of the slow-release plastic Elvax over the optic tectum. Previous studies have shown that the release of small molecular weight compounds from Elvax is constant for at least 60 days and that approximately 0.3% of the drug in the Elvax is released daily (Cline and Constantine-Paton, 1989). The supernumerary retinae from all of the animals tested projected a striped pattern of termination zones to the optic tectum. Examples of the striped projection pattern in the optic tectum from untreated and PDB-treated tadpoles are shown in Figures 1A and 1B, respectively; examples of the projection pattern in tadpoles treated with SPH and H-7 are shown in Figures 1C and 1D, respectively.

In contrast to the expected stripe desegregation, the stripe borders in some of the PDB-, SPH-, and H-7-treated tecta appeared sharper than those in the untreated, 4αPDD-, or NAS-treated tecta. We have previously observed such a "stripe-sharpening" effect following NMDA treatments (Cline and Constantine-Paton, 1990). To quantify the stripe sharpening, we counted the HRP-labeled axons that left a stripe and traversed at least 50% of the interstripe zone for similar border lengths in treated and untreated tadpoles. Untreated tadpoles had a mean of 12.5 ± 0.7 axons crossing into the interstripe zone per 550 μm of stripe border. PDB treatment reduced the numbers of crossing axons to 6.1 ± 0.8 processes per 550 μm, whereas SPH and H-7 caused a more drastic decrease to 4.1 ± 0.6 and 4.0 ± 0.3 processes per 550 μm, respectively (Table 1). Tecta treated with NAS or 4αPDD had similar numbers of axons crossing into the interstripe zone as the untreated tecta.

Retinal Axon Arbor Morphology

Protein kinase activity has been reported to be involved in the regulation of process outgrowth in cultured neurons. We have examined the retinal afferent axon arbors to determine whether manipulation of protein kinase activity in vivo produced effects comparable to those seen in vitro.

For these experiments, groups of 5 animals each were treated with PDB or SPH at 10^{-4} M and groups of 3 animals each were treated with the inactive analogs 4αPDD or NAS, also at 10^{-4} M. All tadpoles were stage X-XI at the start of the treatments. After 4–6 weeks, when the tadpoles were between stages XII

Figure 1. Inhibitors of Protein Kinase Activity Do Not Cause Eye-Specific Stripe Desegregation

The supernumerary retinal projections, visualized by HRP labeling the optic nerve, show a striped pattern of RGC arbors within the tecta treated with saline (A), PDB (10^{-4} M in Elvax) (B), SPH (10^{-4} M in Elvax) (C), or H-7 (10^{-3} M in Elvax) (D). The photographs of the flattened tecta were all taken and processed identically. Bar, 100 μm.

and XV, a few RGCs in the ventrotemporal or dorsotemporal quadrants of the supernumerary retina were labeled with HRP-tipped tungsten needles. The HRP-labeled RCG axon arbors from the drug-treated tadpoles were drawn with a camera lucida and compared with arbors drawn from comparable tectal regions of sham-operated, stage-matched control tadpoles. Arbors drawn from the dorsomedial tectum of PDB- and SPH-treated tadpoles, which had been overlaid with Elvax, had aberrant morphologies compared with controls (Figure 2, the six arbors on the right), whereas arbors drawn from the lateral tectum, which was not in direct contact with the Elvax, fell into the normal range of arbor morphologies (Figure 2, the two arbors on the left). The treated arbors from the dorsomedial tectum were smaller than untreated arbors drawn from dorsomedial tectum, which have been described previously (Cline and Constantine-Paton, 1990). The mean area of the dorsomedial PDB-treated arbors (n = 10 arbors drawn from 5 animals) was only 9.9 ± 1.7 × 10^3 μm², significantly less than the 31.3 ± 4.3 × 10^3 μm² or 29.3 ± 2.2 × 10^3 μm² covered by untreated and 4αPDD-treated arbors, respectively (Table 2). The PDB-treated arbors were significantly shorter (350 ± 20 μm

Table 1. Number of HRP-Labeled Axons Crossing from a Stripe Border through 50% of the Interstripe Zone

	Untreated	PDB	4αPDD	SPH	NAS	H-7
Axons crossing per 500 μm	12.5 ± 0.7	6.1 ± 0.8	12.8 ± 0.9	4.1 ± 0.6[a]	12.0 ± 0.9	4.0 ± 0.3[a]

HRP-labeled axons were counted at 160× magnification at 9 stripe borders in 3 tecta for each drug treatment and at 12 stripe borders in 4 tecta for the untreated tadpoles.

[a] $p < 0.001$.

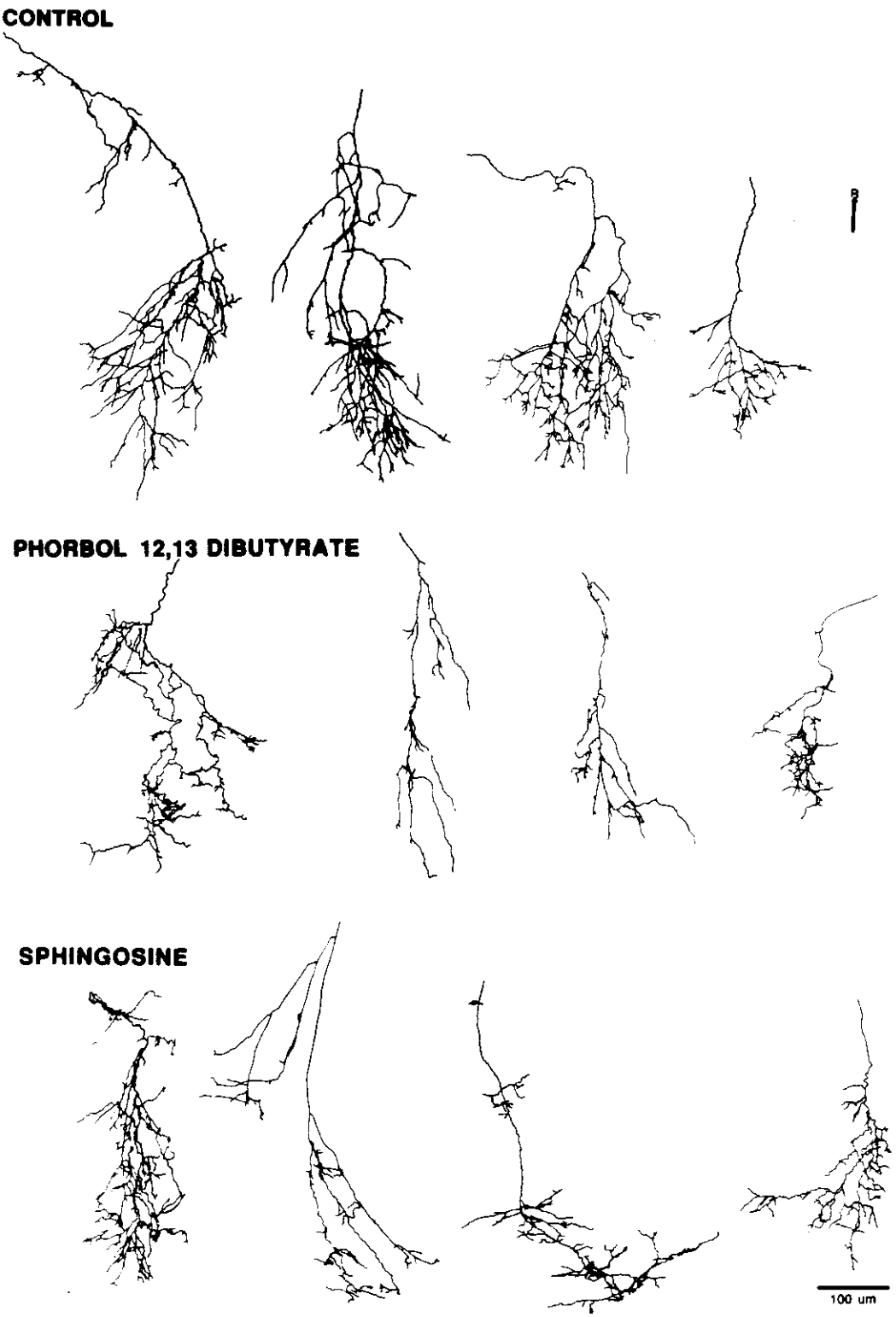

Figure 2. PBD-Treated and SPH-Treated RGC Arbor Morphologies Differ from Controls

Camera lucida drawings of RGC arbors from control (top row), PBD-treated (middle row), and SPH-treated (bottom row) tecta. All control arbors are drawn from the dorsomedial tectum. The arbors on the left in both the middle and bottom rows were drawn from the lateral tectum, and the remaining arbors were drawn from the dorsomedial tectum, where it was possible to verify that the Elvax had remained in contact with the tectum throughout the exposure period.

Table 2. Features of Untreated and Drug-Treated RGC Arbors

Treatment	Length (μm)	Width (μm)	Area (10^3 μm^2)	Number of Branch	Branches/Area (n/10^3 μm^2)
Untreated (n = 12)	540 ± 43	230 ± 28	31.3 ± 4.3	169 ± 24	5.6 ± 0.6
PDB (n = 10)	350 ± 20[a]	130 ± 21[a]	9.9 ± 1.7[a]	73 ± 8[a]	6.2 ± 0.9
4αPDD (n = 5)	529 ± 33	242 ± 37	29.3 ± 2.2	151 ± 36	5.1 ± 0.9
SPH (n = 10)	438 ± 39[a]	201 ± 16	16.4 ± 1.4[b]	121 ± 14[b]	6.1 ± 0.6
NAS (n = 5)	510 ± 45	210 ± 36	29.0 ± 3.1	161 ± 10	5.5 ± 0.5

[a] $P < 0.001$.
[b] $P < 0.01$.

compared with 540 ± 43 μm for untreated arbors) and narrower (130 ± 21 μm compared with 230 ± 28 μm for untreated arbors) than untreated or 4αPDD-treated arbors. The branchtip number (72.8 ± 8.3 branchtips per arbor) was also significantly less than the untreated or 4αPDD-treated arbors. SPH treatment (n = 10 arbors drawn from 4 animals) also reduced arbor area to about 50% of the control value (to 16.4 ± 1.4 × 10^3 μm^2) and reduced branchtip number by about 30%, to 121.3 ± 14.6 branches per arbor). SPH-treated arbors were significantly shorter than control arbors (438 ± 39 μm compared with 540 ± 43 μm for untreated arbors). Although these results suggest that the treatments may have slowed neurite outgrowth, both PDB- and SPH-treated axon branches often ended in growth cones, indicating that the HRP labels the entire extent of the arbor and that the treated arbors are capable of growth.

Protein Phosphorylation

We assayed protein phosphorylation in vivo in tadpole tecta treated with Elvax containing PDB, 4αPDD, SPH, NAS, H-7, and Elvax alone. We found that ^{32}P incorporation into some proteins, but not others, was decreased by treatments with PDB, SPH, and H-7 relative to the incorporation seen in animals treated with either 4αPDD, NAS, or Elvax alone (Figures 3 and 4). Individual animals incorporated different amounts of ^{32}P, according to the radioactivity recovered in the TCA soluble fraction. To compare the densitometric readings of ^{32}P incorporation into protein bands from different animals, we normalized the readings of ^{32}P incorporation in individual bands against the incorporation in a protein band (band 1) whose level of ^{32}P incorporation was insensitive to drug treatments and correlated with the radioactivity in the trichloroacetic acid (TCA)-soluble fraction. One protein band (band 3 in Figure 3), with approximate molecular mass of 43 kd, showed a consistent decrease in ^{32}P incorporation, ranging from 24.6% of control in PDB-treated animals to 67% of control in SPH-treated animals; H-7-treated animals had an intermediate value of 62.5% (Figure 4). A second band (band 4 in Figure 3), with approximate molecular mass of 85–90 kd, also exhibited a decreased ^{32}P incorporation ranging from 56% of control in PDB-treated animals to 69% of control in H-7-treated animals (Figure 4). In contrast, band 2 (about 30 kd) of Figure 3 is an example of a band whose ^{32}P incorporation did not vary with any of the treatments. Since many proteins comigrated in these gels, the values for each band may represent the decreased phosphorylation of several unresolved proteins.

We believe that these values are an underestimate

Figure 3. Coomassie Blue–Stained Gel and Fluorograph from PDB- and 4αPDD-Treated Tadpoles

Each lane represents one animal. Only one of the 4αPDD-treated controls is shown on the left of each panel. Standard molecular weight markers (S) are 23, 43, and 68 kd. Bands analyzed by densitometry are labeled 1 through 4 and are marked with small arrows.

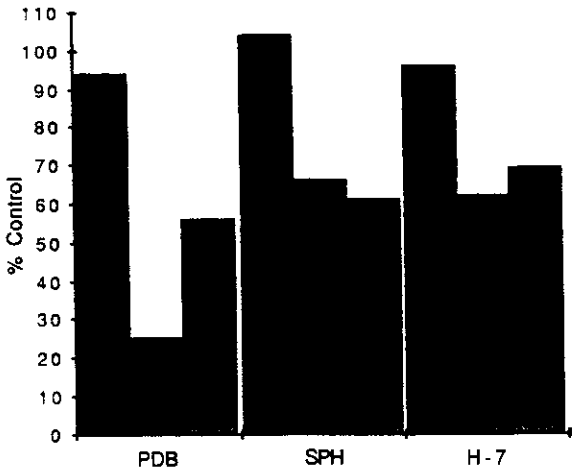

Figure 4. PDB, SPH, and H-7 Treatments Decrease ^{32}P Incorporation in Some Protein Bands

The densitometric readings from bands 2, 3, and 4 were normalized against band 1, which covaried with the radioactivity in the TCA precipitate and did not vary with drug treatments. Band 2 (solid bars) did not vary with drug treatments. Band 3 (dark gray stippling) and band 4 (light gray stippling) both showed a decrease in ^{32}P incorporation with each treatment. The values for the changes in ^{32}P incorporation for bands 3 and 4 are the same whether they are normalized against band 1 or against band 2. Each experimental group was compared with its own control group, such that the ^{32}P incorporation into proteins from PDB-treated animals was compared with that from 4αPDD-treated animals, SPH-treated animals were compared with NAS-treated animals, and H-7-treated animals were compared with sham-operated controls.

of the degree of protein kinase inhibition in the intact animals because the Elvax containing the drugs was removed from the surface of the tectum during the incubation period with the ^{32}P. Therefore any inhibition or down regulation of the enzymes as a result of drug treatment might begin to be reversed during the in vivo assay.

Discussion

We investigated the role of protein kinase activity in the maintenance of eye-specific stripes and the regulation of retinal axon growth in the retinotectal system of the three-eyed tadpole. We tested the hypothesis that NMDA receptor activation, which we have shown to be required for the selective stabilization of coactive retinal inputs, triggers an increase in protein kinase activity, which in turn would be required by the subsequent cellular events underlying synapse stabilization. If this hypothesis were correct, we would have expected to see a desegregation of the eye-specific stripes in three-eyed tadpoles treated with protein kinase inhibitors. However, eye-specific stripes remained intact despite treatments that result in significant inhibition of in vivo protein phosphorylation. Indeed, these treatments accentuate the segregation pattern by sharpening stripe borders. Our data do not support the hypothesis that NMDA receptor activation exerts its effect on synapse stabilization by triggering an increase in protein kinase activity. However, because our treatments did not block all protein phosphorylation, the possibility that phosphorproteins unaffected by our treatments participate in the selective stabilization of coactive synapses remains open.

In addition, we report that the protein kinase blockers alter RGC arbor morphology by decreasing arbor size, a type of change not observed following any previous drug treatments. These data suggest that elevated kinase activity in retinal axons does appear to be necessary for normal arbor growth.

Chronic in vivo drug treatments have both advantages and disadvantages. The primary advantage is that they provide a means of influencing ongoing developmental events over an extended period within an otherwise normal environment. One disadvantage is that it is difficult to determine the site of action of the drugs. For example, the influence of the protein kinase inhibitors on RGC arbor morphology could reflect a direct action on the retinal axons or an indirect effect exerted through changes in protein phosphorylation at another site. A second disadvantage of chronic in vivo treatments is that it is difficult to control for the possibility that the mechanisms which regulate protein phosphorylation may have compensated for the effects of the kinase inhibitors, for instance, by a decrease in protein dephosphorylation. A detailed analysis of the proteins whose phosphorylation patterns have been altered by our treatments would be needed to address these issues.

Nevertheless, the fact that the drug treatments decrease protein phosphorylation while also altering the morphology of retinal axons suggests a causal relationship between the two processes. Although we do not know which kinases are blocked with our treatments, SPH, H-7, and PDB all block or downregulate PKC. Therefore, it is likely that the treatments have the most consistent influence on PKC activity.

We have previously reported that treatment of three-eyed tadpoles with the NMDA receptor agonist NMDA both sharpens stripe borders and alters RGC axon arbor morphology (Cline and Constantine-Paton, 1990). Based on our previous studies on the role of the NMDA receptor in the segregation of eye-specific stripes and the modification of RGC axon arbor morphology, we proposed that activation of the NMDA receptor increases the lifetimes of coactive retinotectal synapses and the supporting axon branchtips and that NMDA receptor activation or the resulting synapse stabilization cause a local inhibition of branch initiation in regions of the presynaptic arbor that converge with coactive neighbors (Cline and Constantine-Paton, 1990). We suggested that chronic NMDA treatment sharpens stripes and decreases branchtip number because the lowered NMDA receptor sensitivity of treated tecta (Debski et al., 1989, Soc. Neurosci., abstract) reduces synapse lifetimes and branch survival at stripe borders, where activity in converging

afferents is poorly correlated. At the same time, the continuous presence of agonist enhances the efficacy of NMDA receptor activation and synapse stabilization within the stripe, where inputs are well correlated, and this in turn inhibits local sprouting.

However, the effects of phorbol esters or SPH treatments on arbor morphology differ significantly from the effects of NMDA treatment. Chronic NMDA treatment does not affect arbor area, although the number of branchtips supported by each arbor is decreased. This results in a 50% reduction in the branch density, or number of branchtips per unit area. As outlined above, we suggest that the decreased axon arbor branch density reflects changes in synapse stabilization and an associated regulation of axon branch sprouting. In contrast, the treatments used in this study decrease both the total arbor area and the branchtip number so that the branch density within the smaller arbor is not significantly different from that of untreated controls. Therefore, exposure to protein kinase inhibitors results in RGC arbors that are smaller than normal, but otherwise have normal branch density. We think that the stripe sharpening effect of the protein kinase inhibitors is only superficially similar to that seen with NMDA treatment. Specifically, we suggest that the kinase blockers decrease the number of axons crossing into the interstripe zone because they decrease the total branch number without selectively altering synapse stabilization or branch density.

Our data on arbor morphology are consistent with a well-documented role for protein kinases in neurite outgrowth (Ishii, 1978; Hsu et al., 1984) and support earlier reports that PKC, in particular, is involved in the regulation of axon branching (Hall et al., 1988; Hsu et al., 1988, Soc. Neurosci., abstract; Bixby, 1989). In concurrence with Bixby (1989), our observation that treatments with protein kinase blockers decrease arbor size suggests that protein kinase activity in growing axons is elevated and that reducing the kinase activity decreases the arbor extent. This could be accomplished by either decreasing branch initiation or increasing branch retraction. A kinase-linked decrease in branch initiation would be consistent with the idea that the NMDA receptor–mediated down regulation of RGC axon branch initiation near stabilized synapses may occur through a highly localized decrease in protein kinase activity that is triggered by the active receptor.

It is important to point out that treatments with protein kinase inhibitors do not arrest the caudally directed growth of the retinal afferents. In two-eyed tadpoles, in which one tectal lobe was treated with kinase blocker and the other tectal lobe was treated with the Elvax alone, the caudal extent of the retinal innervation, assayed by labeling the projection with HRP, was not retarded in the drug-treated tectal lobe compared with the control lobe (H. Cline, unpublished data). This, along with the frequent occurrence of growth cones in treated arbors, indicates that the stripe sharpening effect of the drugs is not simply because all growth of the arbors has been arrested. It also indicates that the effect of the drugs is not simply due to the retraction of previously existing branches, since this would be observed as a decrease in the caudal extent of the tectal innervation in the drug-treated tectum.

The ability of NMDA receptor antagonists to block synaptic plasticity in the visual system and in the hippocampus has led to the suggestion that the same cellular processes underlie the modification of synaptic connections in developing and mature nervous systems. Consistent with this idea, Schmidt (1990) and Artola and Singer (1987) have described activity-dependent potentiation of synaptic transmission in regions of the visual pathway that also display NMDA receptor–dependent synaptic rearrangements during regeneration or development. In addition, axon sprouting (Sutula et al., 1988) and modifications of synaptic terminal morphologies (Desmond and Levy, 1986a, 1986b) seen in the potentiated hippocampus may be similar to structural changes occurring during development.

A further similarity is suggested by our data and some recent work in the hippocampal slice preparation. The hippocampal slice exhibits a transient decaying potentiation of synaptic transmission that lasts for 30–60 min (Kauer et al., 1988; Malinow et al., 1988, 1989; Malenka et al., 1989). Notably, both the slowly decaying potentiation and the eye-specific segregation are sensitive to 2-amino-5-phosphonovaleric acid and insensitive to either SPH or H-7. Focal NMDA application to the hippocampus mimics the transient potentiation (Collingridge et al., 1983), and the NMDA-induced potentiation is also insensitive to SPH treatment (Kauer et al., 1988). Perhaps eye-specific segregation in the retinotectal system is dependent on an NMDA receptor–mediated, protein kinase–independent mechanism that increases synapse lifetime for a limited period of time. Therefore, the degree of coactivity of that synapse with the neighboring synapses would be continually assessed by the magnitude of the NMDA receptor–mediated calcium influx. If the coactivity is sufficient to elevate intracellular calcium levels above a threshold value, then the cellular machinery would be activated to prolong the lifetime of the synapse for a limited period of time. Additional coactive events would further prolong synapse lifetime. If the degree of coactivity of the synapses is too low to allow spatiotemporal summation of the excitatory postsynaptic potentials, and thereby relieve the voltage-dependent magnesium block of the NMDA channel, then intracellular calcium levels would not rise, the life of the synapse would not be prolonged, and the synapse would retract.

The attractive feature of this transient signal hypothesis is the degree of mobility that a short-term stabilization would offer the retinal axon branches. Individual RGC arbors migrate over the surface of the tectum during development (Reh and Constantine-Paton, 1984) and make retinotectal synapses at differ-

ent tectal locations as they migrate. Recent experiments on individually labeled retinal axons followed over a period of several days have demonstrated that during a 24 hr period a large number of branches in an arbor are retracted and replaced by new branches (O'Rourke and Fraser, 1989, Soc. Neurosci., abstract). These data provide an upper limit to the lifetimes of the synapses on those branches. A long-lasting synapse stabilization, comparable to the time course of long-term potentiation, which has been recorded for up to 3 weeks (Bliss and Lomo, 1973), would not permit the degree of branch movement known to occur within the tectum. The mobility of synapse-bearing branchtips is more consistent with synapse lifetimes in the range of hours: a time course which closely parallels that of the slowly decaying potentiation in the hippocampal slice. In short, stabilization of the retinotectal synapses through an NMDA receptor-mediated, kinase-independent mechanism, would satisfy the requirement for the selective maintenance of coactive synapses within a highly dynamic developing system. Although a kinase-independent mechanism for synapse stabilization has not been described in the visual system, arachidonic acid and its metabolites (Belardetti et al., 1989; Piomelli et al., 1989), which have been shown to play a role in long-term potentiation (Williams and Bliss, 1988; Williams et al., 1989), may also be involved in developmental plasticity.

In conclusion, we examined retinal axon termination patterns and arbor morphologies following chronic treatments with protein kinase inhibitors to determine whether protein kinases are involved in the processes of axon growth and maintenance of eye-specific termination zones in the optic tectum of three-eyed frogs. Chronic treatments with agents that we show decrease protein phosphorylation sharpen the borders of eye-specific stripes rather than cause stripe desegregation as expected. In addition, the treatments alter the growth of individual RGC axon arbors in a unique way. We conclude that kinase activity, most likely PKC activity, is involved in the regulation of axon growth, as has been suggested previously from in vitro studies. We have previously shown that the maintenance of segregated afferents requires NMDA receptor activation in the frog visual system. The current data indicate that selective stabilization of coactive synapses, assayed by stripe maintenance, is not dependent on normal kinase activity. The results are more consistent with an NMDA receptor-mediated mechanism that is independent of kinase activity and that confers an increased lifetime on the order of hours to the highly labile retinotectal synapses.

Experimental Procedures

Preparation of Elvax

Elvax plastic polymer (Dupont, Inc.) was prepared as described in detail elsewhere (Silberstein and Daniel, 1982). Briefly, the plastic was solubilized in methylene chloride (100 mg of Elvax per 1 ml of solvent). Concentrated solutions of drugs, prepared in either distilled water or ethanol, were added to the plastic solution along with fast green (0.01%, final concentration). The Elvax was mixed with a vortex, frozen rapidly in a dry ice/acetone bath, and stored for 2 days −20°C. The Elvax was put under gentle vacuum for 1–2 days, embedded in OCT embedding solution, and cut into 30 μm slabs on a cryostat for surgical implantation. Elvax was prepared containing the following drugs: sphingosine (SPH), the inactive analog, N-acetyl sphingosine (NAS), phorbol 12,13 dibutyrate (PDB), the inactive 4α-phorbol 12,13 didecanoate (4αPDD), and 1-[5-isoquinolinesulfonyl]-2-methyl piperazine (H-7). All drugs except NAS were obtained from Sigma. NAS was a gift from R. Malinow. The drug concentrations listed in the text are those in the Elvax preparation. The estimated concentration of drug released from the plastic is approximately 2 orders of magnitude lower than that in the plastic (Cline and Constantine-Paton, 1989).

Surgery and Elvax Implants

Tadpoles were anesthetized by submersion in 0.05% 3-aminobenzoate (MS222; Sigma) prior to all surgery. The optic tectum was exposed by deflecting the skin and cartilaginous skull from the brain caudal to the eyes. The dura was opened along the midline, and the arachnoid was either lifted away from the tectal lobe in a sheet or pealed away to expose the bare tectum. Elvax pieces were laid over the tectal surface and held in place by the dural membrane. The brain covering and skin were replaced, and the wound was sealed with Histoacryl glue (Tri-Hawk, Montreal). Animals recovered from surgery in oxygenated dilute Instant Ocean supplemented with penn/strep (100 U/liter; Sigma).

HRP Labeling

To label the entire supernumerary projection with HRP, the supernumerary optic nerve was cut immediately behind the eye and a small piece of gelfoam soaked with saturated HRP solution (Sigma, Type VI, or Boehringer Mannheim, Grade 1) was placed at the cut end of the nerve. After 2 days, the animals were sacrificed by submersion in 0.1% MS222, and the brains were reacted in whole-mount with diaminobenzidine as described previously (Reh and Constantine-Paton, 1984). The tectal lobes were dissected free from the brain, flat-mounted between coverslips, and fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer for 3–4 days at 4°C. Then they were rinsed in 0.1 M phosphate buffer, dehydrated, cleared in xylene, and mounted in Permount.

To label a smaller population of RGC axons for arbor reconstruction, tungsten needles tipped with HRP crystals were introduced into the supernumerary retina. The brains were processed as described above. Individual HRP-labeled RGC arbors were drawn with a camera lucida using a 63× oil immersion lens. To control for variation in arbor morphology with the stage of the animal, we have drawn arbors from stage-matched, untreated or sham-operated control animals. All arbors were drawn from the central two laminae of the tectal neuropil, layer 9. Previous studies have shown that arbors drawn from these central laminae of tadpoles between stages X and XVII are morphologically similar and do not yet display the extreme variations in arbor type typical of arbors from adult frogs (Cline and Constantine-Paton, 1989, 1990; Cline, unpublished data). In particular, significant increases in arbor areas are not seen in untreated arbors from tadpoles of stages X–XVII. Therefore, we grouped the arbors from the tadpoles without specifying stages. Arbor area was determined using a Terak computer as described previously (Cline and Constantine-Paton, 1990) or using the SigmaScan program by Jandel Scientific. Arbor length was taken from the first branch off the main axon to the furthest tip. Arbor width was measured at the widest point perpendicular to the long axis of the arbor. Statistical analyses were performed using a two-tailed Student's t-test (Snedecor and Cochran, 1967).

Protein Phosphorylation Assay

Normal tadpoles (T&K stages X–XII; Taylor and Kollros, 1946) were treated with PDB, 4αPDD, SPH, NAS, all at 10^{-4} M, and with H-7 at 10^{-3} M in Elvax for 4 weeks as described above. The tadpoles were reanesthetized in 0.1% MS222 and wrapped in tissue

soaked in MS222. The skin, cartilaginous skull, and dura were folded back from the tectal lobes. The position of the Elvax over the dorsal tectum was verified, and the plastic was removed. A piece of Whatman #1 filter paper (2 × 3 mm^2) soaked with ^{32}P (0.1 mCi in physiological saline containing 100 mM NaCl, 2 mM KCl, 2.5 mM $CaCl_2$, 3 mM $MgCl_2$, 2.5 mM HEPES, and 5 mM glucose) was positioned over the tectal lobes. After a 1 hr 15 min incubation, blood flow in the brain was verified, and the brain was removed and rinsed in a large volume of saline. The dorsal tectal lobes were dissected free from the brain and frozen in a dry ice/isopropanol bath until all samples were collected. The tissue was prepared for electrophoresis as described previously (Jacobson et al., 1986). Briefly, the tecta were homogenized in 0.5 ml of homogenization buffer (10 mM Tris [pH 7.5], 5 mM dithiothreitol, 5 mM EDTA) and centrifuged at 100,000 × g for 30 min to separate the soluble and particulate fractions. TCA (100%) was added to the supernatant to a final concentration of 10%, and the precipitate was separated by centrifugation at 8000 × g for 15 min. An aliquot of the trichloroacetic acid supernatant was taken to determine the radioactivity in each sample by liquid scintillation counting. The pellet was solubilized in 1% SDS, and duplicate aliquots were taken for protein determination (Bradford, 1976). Tris buffer (10 mM [pH 7.5]) was added to the remainder of the sample followed by incubation at 95°C for 2 min. Samples were run on 12% polyacrylamide gels and stained with Coomassie (brilliant) blue. The gels were dried and exposed to Kodak XAR X-ray film at −70°C for 1–5 days to obtain comparable grain densities in the different lanes.

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in dendrites[2]. Both spine-bearing and aspinous dendrites have ribosomes. In spine-bearing dendrites they are usually located at the base of the spines, but may also be present in the spine head and thus close to the postsynaptic density of the synapse. In a more recent study, Steward and Reeves[6] analysed the ribosomes in dendritic spines of neurones in the dentate gyrus and hippocampus by serial section reconstruction and stereo-imaging of thick sections in the electron microscope. They found that about half of the polyribosomes at the base of the spine were associated with membrane cisterns. However, in most cases the ribosomes were not directly attached to the membrane as they are in rough endoplasmic reticulum. Nonetheless, the association may have functional implications and Steward and Reeves suggested that plasma membrane proteins may be synthesized on these ribosomes and inserted into the synapse. Since all transmembrane proteins are believed to be glycosylated, this idea begs the question of where such proteins would acquire their sugars. Many dendritic spines in the cerebral cortex have a spine apparatus. This enigmatic organelle, first described by George Gray[7] nearly 30 years ago, has still not been ascribed a function. It is conceivable that the spine apparatus is involved in the post-translational modification of membrane proteins though other functions for it have been proposed, for instance Ca^{2+} sequestration[8]. Consistent with the former idea is Steward and Reeves' observation that the polyribosomes in the spine base were more likely to be associated with a membrane cistern when a spine apparatus was present than when it was not.

Is it necessary to have ribosomes at all post-synaptic sites in the neurone? If so, what about axo-axonic synapses? Most of these are on the axon hillock, and therefore near to the ribosomes in the cell body. Synapses on axonal presynaptic terminals are in fact relatively rare[9].

These observations on ribosomes and RNA transport in dendrites lead to a variety of interesting questions, of which the most pressing is: what proteins are being synthesized? Are they the proteins that form part of the postsynaptic membrane such as neurotransmitter receptors and ion channels or are they, for example, cytoskeletal proteins of the postsynaptic density? Whatever proteins are being synthesized in the dendrite, it is clear that the local presence of the machinery for protein synthesis may allow a more precise and rapid control of the production and turnover of proteins at the synapse. Such control may be essential in the changes resulting from experience and its storage in memory.

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viewpoint

What, if anything, is a neurotrophic factor?

Y-A. Barde

The phenomenon of naturally occurring neuronal death and the protein nerve growth factor (NGF) are often referred to when new, putative neurotrophic factors make their débuts in the literature. Although these factors are shown to support the survival of embryonic neurons in vitro, it is doubtful if many of them are true neurotrophic factors in the sense that, like NGF, they participate directly in the regulation of naturally occurring neuronal death in vivo.

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It is a well-established feature of the vertebrate nervous system that many (apparently redundant) neurons are eliminated during the course of normal development. This happens soon after the axons of these neurons reach their targets, and there is very good evidence that the target plays a major role in regulating the number of neurons it needs to be adequately innervated[1]. One of the reasons why nerve growth factor (NGF) is a protein of considerable interest is that it fits perfectly into this biological framework. First, NGF has the spectacular property of keeping alive vertebrate neurons that would simply not exist in its absence, as demonstrated *in vivo* by the administration of NGF-antibodies[2,3]. Second, it is known to be present in its biologically active form[4] and synthesized in the target tissues of the very neurons that need it for survival[5,6]. Third, the amounts of NGF in the target are very small[4], and there is convincing evidence that these amounts are limiting: the administration of exogenous NGF to developing embryos prevents neuronal death in ganglia whose neurons are known to depend on NGF for survival[7]. One characteristic of NGF is its neuronal specificity, in that it acts on sympathetic and most neural crest-derived sensory neurons in the PNS, and on some cholinergic neurons in the CNS. Since naturally occurring neuronal death also affects many neurons that are not dependent on NGF, it is probable that other proteins with properties analogous to those of NGF exist. However, although many recent publications describe putative neurotrophic factors that at first sight seem to fulfil such a role (see examples below), further consideration of their characteristics makes it unlikely that they regulate neuronal survival during development. One of these features is quantitative in nature: only very small amounts of such factors

would be expected to be present if neuronal death is to be explained by their availability in limiting quantities. In fact, in this field, there is a long and regrettable tradition of neglecting quantitative considerations, and this, too, started with NGF. The providential discovery of very large amounts of NGF in the adult male mouse submandibular gland, however convenient from a practical point of view, was (and probably still is) conceptually misleading. There have been many erroneous reports in the past suggesting NGF to be present more or less everywhere, at any time, and in large quantities[8]. A critical, but appropriate statement like '[the] lack of information about the source of endogenous NGF and the site of interaction with sensitive neurones argues for continued caution in accepting the idea that NGF normally acts after peripheral uptake and retrograde transport' was at the time (in the late 1970s) extremely rare[9]. Indeed, the first report of accurate NGF determinations was published as late as 1983, and showed it to be present in the target organs of the sympathetic nervous system[4]. The seminal point of this demonstration was that even in the most densely innervated organs, NGF was present only in minute quantities – as little as a few nanograms per gram tissue.

The problem of tissue culture

In-vitro assays can hardly be avoided in the process of identifying and characterizing new neurotrophic factors. Typically, parts of the embryonic PNS or CNS are dissociated and the cells placed in culture. The key feature of these cultures is that the neurons present usually die quite rapidly, and the rationale behind such assays is that one cause of death is separation of the neurons from their targets and consequently any target-derived trophic factor. Thus, anything added to the medium that promotes neuronal survival can be used as an operational test of a putative neurotrophic factor. The problem with this working definition of neurotrophism is that many nutritive components of a normal tissue culture medium, including oxygen, or conversely agents destroying peroxides and other toxic substances, can be defined as neurotrophic factors if they are essential to the survival of the cultured neurons. Thus, pyruvate[10] and catalase[11] have been shown to support the survival of neurons in culture. To my mind however, a clear distinction should be made between these effects which, while of importance to anybody trying to grow neurons in culture, are unlikely to reflect a physiological role in the regulation of naturally occurring cell death.

A commentary on some proteins recently purified and proposed as putative neurotrophic factors is outlined below.

Neuroleukin

Neuroleukin is a 56 kDa protein that was purified from the adult male mouse submandibular glands[12]. Initially identified from the fact that antibodies to it were able to inhibit sprouting of motor axon terminals (induced at the mouse neuromuscular junction by injections of botulinum toxin)[13], it has subsequently been reported to support the survival of cultured, NGF-independent spinal sensory neurons, and of unidentified spinal cord neurons[12]. Additionally, it has been shown that neuroleukin is produced by T lymphocytes and can stimulate immunoglobulin production by B lymphocytes[14]. A study of the distribution of neuroleukin and its mRNA in various mouse tissues revealed that it is very abundant. Assuming a yield of 100% after purification, there are over 4 $\mu g\ g^{-1}$ wet weight in the salivary glands, and often much more in many other adult mouse tissues, including skeletal muscle. It is important to note that unlike NGF, the large amount of neuroleukin to be found in the submandibular glands is not an exception. The wide range of regulatory functions proposed for neuroleukin, coupled with its broad and abundant distribution are difficult to reconcile with a possible role as a neurotrophic factor preventing neuronal death during development. If it has such a role, then one might expect it either to be present in dramatically smaller amounts during development or, alternatively, to be compartmentalized so that only limiting amounts are available to its responsive cells. The mechanisms involved in compartmentalization are unclear, and unlikely to involve the classical secretory pathways.

Fibroblast growth factor (FGF)

FGF is a potent 16 kDa mitogen that comprises two distinct but related molecules, one acidic and one basic. Both forms have been shown to be mitogenic for a variety of cells in culture, in particular fibroblasts, myoblasts and endothelial cells (reviewed in Ref. 15). A number of *in-vivo* roles have been suggested for FGF, including wound healing (FGF stimulates the proliferation of most cell types involved in this process), limb regeneration in amphibia, lens regeneration, and mesodermal induction in the *Xenopus* blastula[16,17]. In addition, some recent reports have shown FGF to support the survival of several types of embryonic neurons in culture[18–20]. However, it is still not entirely clear whether the effects of FGF on most neurons are direct or indirect, with the possible exception of purified embryonic chick ciliary neurons[20]; the reason for this reservation is that in most primary cultures of the nervous system, it is very difficult to eliminate all non-neuronal cells, so that the observed effects of FGF might be mediated by the numerous other cell types known to be affected by FGF. There is no question that FGF stimulates fiber outgrowth from PC12 cells[21,22], but these cells cannot be considered as post-mitotic, embryonic neurons, and fiber outgrowth is not synonymous with neuronal survival. In addition, implicit in the target-derived model is the notion that the putative neurotrophic protein should be released from the target, but it appears that FGF is not a secretory protein[23]. Though FGF could reach the extracellular space upon disintegration of the cells that synthesize it, this possibility, while plausible for a wound-related factor, is difficult to envisage for a target-derived neurotrophic factor regulating neuronal

survival during development. Indeed, the extreme consequence of such a mechanism would be the survival of those neurons whose target cells have disappeared! Finally, FGF is not a rare protein: up to 0.5 $\mu g\ g^{-1}$ wet weight of basic FGF can be isolated from the pituitary[24]. Indeed, FGF has been described as 'the most abundant growth factor found in mammalian tissue'[24], and its temporal and spatial distribution is very broad: basic FGF in particular can be found in most tissues, starting with the oocyte, where large amounts of FGF mRNA have been found, it being as easy to detect as fibronectin mRNA in the *Xenopus* blastula[17].

Ciliary neurotrophic factor (CNTF)

CNTF is a 20 kDa acidic protein that supports the survival of most chick embryonic neurons from the PNS *in vitro*[25]. This action is seen not only at very low protein concentrations, but also at very low cell density. Furthermore, there is no indication that CNTF is a mitogen for non-neuronal cells, so that a direct action of CNTF on neurons is very likely. Nevertheless, it is not clear that CNTF plays a role in the regulation of naturally occurring neuronal death *in vivo*, its concentration in the target tissues of chick ciliary neurons being very high. The purification factor necessary to obtain a pure preparation from these tissues at embryonic day 15 is only about 400-fold[25]. It is worth noting that to purify NGF from 'normal' target tissues (which, understandably, has never been done), a purification factor of a few millionfold would be necessary[4]. From the adult male mouse submandibular gland (generally accepted as being irrelevant to the developing nervous system), the figure calculated for β-NGF is only slightly lower than for CNTF, about 100-fold[26]. Interestingly, CNTF is present in ciliary neuron target tissues early in development (embryonic day 8)[25], though in lower but still inexplicably high amounts before the period of neuronal death actually starts in the ciliary ganglion. CNTF is also present in large amounts in peripheral nerves[27]. Since neuronal cell death does take place, and is even dramatically increased after administration of NGF-antibodies in sympathetic and sensory ganglia (whose nerves presumably also contain large amounts of CNTF), it is difficult to understand how CNTF would operate in the context of naturally occurring neuronal death. As mentioned above, mechanisms allowing release, but nevertheless restricting the availability of CNTF would have to be postulated. In any event, the *in-vitro* effects of CNTF are so clear and dramatic on peripheral neurons, whose axons can regrow after lesion, that it is a very intriguing and interesting molecule. Clearly, more work remains to be done before it is possible to assign to CNTF its biological role *in vivo*.

Brain-derived neurotrophic factor (BDNF)

BDNF is a 12 kDa basic protein purified from the brain on the basis of its ability to support the *in-vitro* survival of embryonic sensory neurons[28]. Of all the putative neurotrophic factors purified so far, BDNF is by far the rarest protein. Based on its specific activity *in vitro*, a purification factor of over 1 000 000-fold was needed to obtain a pure sample[28,29]. Taking into account an overall yield of about 20%, the purification procedure described indicates that there is about 5 $ng\ g^{-1}$ wet weight of BDNF in adult pig brain[29]. The hypothesis that the amounts of BDNF might be limiting during normal development *in vivo* has been tested by showing that microgram amounts injected into quail embryos decrease naturally occurring neuronal death in sensory ganglia[29]. Although the data obtained until now are consistent with the idea that BDNF is, like NGF, a neurotrophic factor present in limiting amounts, much more needs to be done to prove that BDNF is the endogenous protein that regulates neuronal survival during normal development. In particular, it is unclear where BDNF is synthesized, as there is no direct demonstration that it is made specifically in the targets of neurons needing it for survival. Finally, it remains to be shown that BDNF is a secretory protein. The availability of a cDNA probe and recombinant BDNF should greatly help to resolve some of these questions.

Conclusion

While extremely useful in characterizing new proteins, *in-vitro* tests of neuronal survival have limited predictive value: for the reasons discussed, the ability to rescue neurons *in vitro* is, alone, no longer sufficient to imply that a new molecule will rescue neurons during normal development. Thus, in my opinion, the term 'neurotrophic factor', when used in connection with the physiological phenomenon of naturally occurring neuronal death, should be used to refer to agents likely to affect the development or maintenance of neurons *in vivo* on the basis of additional criteria. In particular, considerations such as tissue distribution, mechanisms of release and, above all, quantity, are necessary. It remains to be seen what physiological roles many new agents can play *in vivo*, having been characterized on the basis of their *in-vitro* activity. Elucidation of their roles is an exciting task for the future.

Note:

After submission of this manuscript, two studies were published demonstrating that the protein neuroleukin discussed here is identical with the ubiquitous, cytoplasmic enzyme glucose-6-phosphate isomerase[30,31].

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techniques

Confocal microscopy: applications in neurobiology

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New methods of confocal microscopy permit the observation at high resolution of structures deep within neural tissue. The resulting images are often striking and informative. Used in conjunction with fluorescent voltage-sensitive or ion-sensitive dyes, confocal microscopy may make possible a wide range of investigations of brain function and plasticity that were difficult or impossible with previous techniques.

Neuroscientists dream of being able to watch the brain work and change. The difficulty in *seeing* these things is one reason why many important questions about CNS function and plasticity remain unanswered. This difficulty is diminishing with the introduction of new optical methods, many involving fluorescence or reflectance microscopy. Intracellular or membrane-bound fluorescent dyes, for example, have been used to follow the growth and long-term changes of nerve terminals[1–3], and retrogradely transported fluorescent markers have been used to identify living neurones with particular projections for subsequent electrophysiological or structural study[4]. Voltage- and Ca^{2+}-sensitive indicator dyes have been used to observe patterns of electrical activity[5–10] without the constraints that microelectrodes impose on the size and number of structures that can be monitored. Such voltage-sensitive dyes have already helped to reveal aspects of the functional organization of vertebrate forebrain structures[11–13].

Unfortunately, fluorescence and reflectance images are often severely degraded by scattered, emitted or reflected light from tissue structures outside the plane of focus. The usefulness of the new optical methods has been further limited by the poor depth discrimination of ordinary light microscopy. These limitations have been only partly overcome by video image processing[14–16] and deconvolution[17,18] techniques.

Principle of confocal microscopy

An alternative solution to this problem is offered by confocal microscopy, which achieves dramatic increases in resolution by physical means. The principle of confocal microscopy was described by Minsky as early as 1957[19] and first used successfully by Egger and Petran in 1967[20] to view unstained neural tissue. The technique uses light from a point source, combining focal illumination of a single point in the specimen with imaging of the illuminated specimen point on a detector pinhole. Light from out-of-focus elements can thereby be virtually eliminated (Fig. 1A). There is also a theoretical 1.4-fold improvement in resolution in the plane of focus compared with normal microscopy.

A confocal optical system can be realized in a number of ways. For example, a pinhole in the incident light path can be made to correspond with a second pinhole in the image plane; a complete image of a specimen at the highly restricted focal depth can then be built up either by scanning the specimen under the beam or, as in Egger and Petran's design[20], by coordinated (tandem) scanning of the pinholes across the specimen and image planes.

Such tandem scanning confocal microscopes can produce extremely high resolution images, scanning fast enough for the full image to be visible[21]. But because the apertures exclude such a high proportion of light, only extremely bright light sources and highly reflective or fluorescent specimens can be used. This difficulty was overcome by the use of laser illumination with a mechanically scanned specimen, as pioneered by Brakenhoff and colleagues[22]. Mechanical scanning of the specimen, however, imposes limits on the mass and stability of the preparation and on the speed of the scan. These limitations have in turn been largely overcome through the development in Sweden[23] and the UK[24] of beam-steering methods to scan the illuminating spot within the fixed microscope optics.

A practical confocal microscope

The instrument developed at the Medical