The Development of Functional Inputs to a Neural Circuit: Synaptic Strength Before and After the Activity-Dependent Maturation of the Retinogeniculate System

by

Carsten Dietrich Hohnke

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Abstract

The activity-dependent development of appropriate connections in the central nervous system relies on mechanisms that are also used in the modification of the efficacy of synaptic transmission. Consequently, it has been proposed that long-term changes in the efficacy of synapses mediate the stabilization and withdraw of axon branches. This thesis comprises four sets of experiments designed to explore this hypothesis. That is, does synaptic efficacy, by some measure, change during a period when neural circuitry is being shaped by neural activity? The experiments were conducted using whole-cell patch-clamp recordings of excitatory postsynaptic currents (EPSCs) to probe synaptic efficacy throughout a period of activity-dependent axonal reorganization, ON/OFF sublamination, in the ferret lateral geniculate nucleus of the thalamus (LGN). Because, in the context of information transfer, synaptic efficacy should be considered relative to the voltage deflection required to initiate an action potential in the postsynaptic cell, I first extensively explored the development of the electrophysiological properties of relay cells in the LGN. While some of these properties change significantly during ON/OFF sublamination, the difference between action-potential threshold and resting membrane potential is fixed. Thus, the contribution to information transfer at a single synapse can be compared by examining synaptic currents before and after this period. This was accomplished in the next set of experiments by recording spontaneous EPSCs (sEPSCs) at resting membrane potentials as a measure of charge transfer at a single synapse. These conductances have been shown to increase in many types of long-term enhancement of synaptic efficacy. However, I show that the properties of sEPSCs recorded at the beginning of ON/OFF sublamination were not different from those recorded after its completion. The third set of experiments, then, investigated the development of charge transfer that is active at depolarized membrane potentials as well as the development of the ratio of the two types of conductances. These two measures likewise are held constant during this period of axon reorganization. The last set of experiments takes a slightly different view of synaptic efficacy. Namely, because it is the retinogeniculate axon *as a whole,* that is undergoing reorganization, I asked whether the contribution to membrane depolarization of a single retinogeniculate axon, rather than one of its synapses, changes during ON/OFF sublamination. As an extension of that exploration, I investigated whether the number of retinogeniculate axons converging on a particular relay cell in the LGN is modified during this

period in order to determine whether there is, in fact, a functional reorganization of input taking place. The number of retinogeniculate fibers innervating a cell in the LGN decreases during ON/OFF sublamination while the strength of the remaining fibers increases. As a whole, the results from the experiments in this thesis provide evidence for the functional reorganization of retinogeniculate input in parallel with a remarkable stability and normalization in synaptic transmission during a period of intense anatomical and physiological, activity-dependent change.

Thesis Supervisor: Mriganka Sur
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Introduction

We are shaped, to an extent, by our environment. In early life the environment takes on a particularly important role. A wealth of experimental data show that the acquisition of language, appropriate social behavior, and other cognitive abilities are exquisitely dependent on external stimuli during critical or sensitive periods (or "experience-expectant" periods [Greenough et al., 1987]). For example, while the *ability* to develop language may be largely guaranteed by "hard-wired" mechanisms, the specific phonetic make-up of a child's language and the rate at which it is learned depend heavily on the feedback the child receives from the environment (for review see Gleitman and Newport, 1995). Similarly, the classic experiments of Harlow demonstrate that inadequate social interaction in early life impairs a rhesus monkey's ability to respond appropriately to normal social cues (Gleitman, 1995).

Even more striking and well documented is the effect of the environment on normal sensory development. Children with visual deficits (e.g., strabismus or amblyopia) who are not treated in early life recover with much greater difficulty (if at all) than those who are. Similarly, newborn kittens and primates deprived of normal visual experience subsequently show deficits on a wide range of visual behaviors (Movshon, 1976; Carlson, 1990). These effects are not limited to mammals. Certain species of birds must receive appropriate auditory signals during early development if they are to learn species-specific songs (for reviews see Mooney 1995; Bottjer et al., 1997; Ball and Hulse, 1998). Work in the visual system suggests that deficits in visual behavior are

almost certainly due to the inappropriate wiring or patterns of connectivity between neurons that also results from visual deprivation (Hubel and Wiesel, 1970; LeVay et al., 1980; Sur et al., 1982; Antonini and Stryker, 1996).

Consequently, a fundamental question of developmental neurobiology is: How do the more than 100 billion neurons in the human brain establish the *correct* connections amongst themselves and the motor output (i.e., muscles)? Classic experiments have shown that neural activity is crucial for the establishment of the appropriate neural circuitry in many systems. More recent experiments have uncovered important components of the mechanisms involved in this activity-dependent development. Interestingly, these components are also involved in the modification of synaptic efficacy[1]. In this thesis I explore the connection between those two phenomena. That is, I measure the development of synaptic efficacy during a time when neural circuitry is being shaped by neural activity.

Outline of the Dissertation

Chapter one is a brief review of the body of research on which the work presented here draws, namely, activity-dependent development of neural connectivity and the possible

[1] I use the term "efficacy" (and sometimes "strength") throughout this thesis and wish to clarify exactly what I mean by it here. The power of a synapse to produce an effect stems from multiple sources. These include: the probability that a vesicle of transmitter is released from the presynaptic terminal in response to an action potential, the number of molecules of transmitter contained within a vesicle, the degree to which transmitter is neutralized by reuptake and/or enzymatic agents in the synaptic cleft, the affinity of a postsynaptic receptor for a ligand, and the current flux through the receptor-mediated channel. The experiments presented here do not rigorously assay the probability of transmitter release. Consequently, the term "efficacy," when referring to the results, is overly broad, but the alternatives are unwieldy.

role therein for activity-dependent modifications of synaptic efficacy. As described above, appropriate stimulation by the environment is crucial for the normal development of animals. Chapter One continues by recalling work characterizing early, activity-*in*dependent axon development and contrasting that with investigations of the activity-dependent refinement of the initial neural circuitry. In particular, I review the results of experiments that detail the effects of stimulus deprivation on the anatomical and physiological properties of the nervous system. Those experiments were followed by attempts to characterize the specific molecular components of activity-dependent development including postsynaptic receptors, diffusible, retrograde messengers, and growth factors. In the following section I go on to describe the regulation of the time periods during which activity-dependent mechanisms are most critical. Thereafter, the review explores the similarities and possible relationship between activity-dependent development and the modification of synaptic efficacy. Finally I introduce the mammalian visual system as a model for examining activity-dependent development and describe the system that I've used in the experiments presented in this thesis, the ferret LGN. I explain why the ferret LGN is the right system to use and summarize what is known about its development during the period when neural activity is shaping its neural circuitry.

Chapter Two describes the general methods that were used in all the experiments presented in this thesis. It begins with a description of the experimental setup: slice preparation, whole-cell patch-clamp recording from relay cells in the LGN, and pharmacological and electrical manipulations of synaptic inputs. Next I discuss how the

data were acquired in general, and the unavoidable corruption of the measured signals by the access resistance of the recording pipette. The last section explains the automated signal detection routines that I coded and used, and an explanation of the types of statistical analyses that I performed.

Chapter Three through Chapter Six present the data collected in four sets of experiments. The first set of experiments presents a large body of data that I've gathered on the development of the electrophysiological properties of relay cells in the LGN. These data are important for determining the relative effect of synaptic input on information transfer. In the discussion I compare the results to what is known about the development of relay cell anatomy. The next set of experiments investigated the development of single inputs onto relay cells of the LGN that activate conductances at resting membrane potentials. These experiments were designed as a first-pass at answering the question of whether, on average, the strengths of synapses onto a neuron are increased after the axons converging on that neuron have stabilized. The third set of experiments investigated conductances that are active at depolarized membrane potentials. These experiments recognized that, at times when the postsynaptic neuron is depolarized, an additional type of neurotransmitter receptor contributes to synaptic efficacy. They also allowed me to examine the development of the ratio of resting and voltage dependent synaptic currents. The last set of experiments explored the development of the strength of retinogeniculate axons and the convergence of retinal axons onto relay cells in the LGN. That is, what is the development of the contribution to information transfer of a single fiber, how many different retinal axons contact a

particular relay cell, and how does this number change over the course of the activity-dependent reorganization of retinal axons in general? Or, just how much *functional* reorganization of the neural circuitry is going on?

Because, for the most part, chapters three through six were written as stand-alone articles (indeed, chapter four has been published, more below), there is some repetition of content between them. Overwhelmingly the repetition is found in the introduction sections of the chapters (the method sections have been collapsed as much as possible into *Chapter Two: General Methods*). Hopefully (and I believe this to be true) the redundancy will be found to be more useful than burdensome in that it is the main themes of the "big picture" which are repeated.

Chapter seven summarizes the findings from the data chapters and presents overall conclusions.

Publications

Chapter Four has been published (Journal of Neuroscience, 1999, 19:236-247). The literature review relies heavily on two reviews that I wrote together with Mriganka Sur as do many of the themes presented in the introduction. One of the reviews is published (Mental Retardation and Developmental Disabilities Research Reviews, 1999, 5:51-59) and the other is in press. The former is referred to throughout the thesis as "Hohnke and Sur, 1999b". Parts of those reviews benefited enormously from discussions with and comments from Sherri Hitz.

Chapter One: Literature Review

Activity-Independent vs. Activity-Dependent Development

At some point during the term in most introductory psychology courses a discussion of the "nature vs. nurture" question arises (or innate vs. acquired, modularity vs. domain-generality, Chomsky vs. Piaget, etc.). The question is to what extent are an organism's traits and abilities inherited vs. learned during its lifetime? Keeping in mind that the separation of developmental influences into genetic and environmental is probably a false dichotomy (see Barkow et al., 1992 for an interesting discussion), it is useful to restate the question in neurophysiological terms: To what extent is the wiring of a nervous system activity-independent vs. activity-dependent? Research on the initial growth, target recognition, and final connectivity of axons suggests that there are, broadly speaking, two phases of axon development. Initially, axons from sensory neurons grow from the periphery into their targets in the central nervous system. Upon arriving at their destination, axons probe large areas of tissue, but finally are restricted to a smaller area within which they make the "right" connections. The first of these phases, target recognition, does not require neural activity while the latter, appropriate point-to-point connectivity, does.

Target Recognition is Activity-Independent

Experiments in systems in which the growth of individual axons could be manipulated and observed, showed that "pioneer" neurons lay down paths that subsequent axons

discriminate amongst and follow to appropriate targets. Growth of these axons along the correct paths and into the correct targets is not affected by the absence of activity; indeed, the initial phase of axon growth largely proceeds prior to the formation of synapses and the onset of neural activity (e.g., Blagburn et al., 1996). How then do the pioneer neurons find their way? The answer seems to be that the path of the earliest axons is determined by myriad attractive and repulsive cues in the extracellular matrix. It is likely that neurons rely on several of the available cues in order to select the correct pathway (for reviews see Tessier-Lavigne and Goodman, 1996; Kolodkin, 1996).

Once a retinal axon, for example, reaches its target structure, it must still determine an appropriate place to contact specific neurons within that structure. Early work on axon guidance in the optic tectum by Roger Sperry and his colleagues in the 1950s led to the chemoaffinity hypothesis. According to this view, axons from the retina grow to the correct location in the tectum by following specific chemical labels (Sperry,1963). Thus, when the optic nerve is crushed, retinal axons regenerate and grow into the tectum with remarkable specificity, recreating an ordered map of visual space. Sperry's view led to the search for chemical "labels", many of which have been discovered to be cell surface molecules on the projecting and target neurons. However, when only a subset of retinal axons is permitted to regrow into the tectum, they are not restricted to those areas that they would occupy had all the retinal axons been allowed to regenerate. Rather, this subset of axons grows to occupy the entirety of the available tissue, suggesting that chemical labels are only one (early and coarse) mechanism by which connectivity proceeds (for review see Goodman and Shatz, 1993).

Precise Addressing is Activity-Dependent

When axons reach their targets they must finally make connections with the neurons that reside there. Is it possible that the molecular guides that led the axons this far can finish the job? Sperry proposed exactly that. In particular Sperry suggested that specific surface molecules on incoming fibers "recognize" matching molecules on target neurons. Given a sufficient diversity of these types of molecules, precise point-to-point connectivity could develop. Yet, there are over a 100 billion neurons in a human brain each of which usually make hundreds (if not thousands) of contacts (Kandel et al., 1991). The volume of "data" that would be required to "program" appropriate connectivity is prohibitively large. That is, genetic coding of specific connectivity is a prohibitively large task.

Indeed, axons and dendrites do not initially grow into the appropriate wiring scheme. In the visual system, axons from both retinae grow into areas of the LGN that later in development are occupied by axons from only one or the other retina (Rakic, 1977; Linden et al., 1981; Shatz, 1983). Likewise, axons from the LGN carrying inputs from the two eyes are initially intermingled in layer IV of the visual cortex (LeVay et al., 1978; 1980), but subsequently reorganize such that the input from one eye is segregated from the other (Hubel and Wiesel 1963; Hubel et al., 1977).

If not molecular cues, then what informs the formation of the correct connectivity between neurons? Neural activity plays an important role during this stage of development. While in some cases the appropriate patterning of inputs does not require neural activity, in many cases it does (for reviews see Goodman and Shatz, 1993; Cramer

and Sur, 1995). For example, in the ferret, retinogeniculate axons segregate in an activity-dependent manner during the first four postnatal weeks to form laminae and sublaminae (Linden et al., 1981; Stryker and Zahs, 1983; Hahm et al., 1991; more on the ferret LGN below).

Activity is also crucial to the development of the appropriate wiring in structures involved in later stages of visual processing. The segregation of left and right eye inputs in the visual cortex is disrupted after neonatal lid suture (Hubel and Wiesel, 1970; Hubel et al., 1977; Sherman and Spear, 1982) and intraocular injections of tetrodotoxin[1] (TTX; Stryker and Harris, 1986). That is, normally, individual axon arbors projecting from the LGN to the visual cortex develop distinct clusters of terminations consistent with the segregation of the inputs from the two eyes. However, when retinal activity is blocked by TTX, most of the axon arbors fail to develop terminal clusters (Antonini and Stryker, 1993).

Interestingly, many of the activity-dependent events in the visual system occur prior to any environmentally driven visual experience by the animal. The formation of eye-specific laminae and ON/OFF sublaminae in the ferret LGN, ocular dominance columns in monkeys, and orientation tuning properties of visual cortical neurons all occur prior to eye-opening (and in monkeys, *in utero*). The stereotyped neural activity required during this early period of development may derive from endogenously generated patterns of activity. Retinal ganglion cells fire bursts of activity during prenatal life

(Galli and Maffei, 1988). In the ferret, these bursts form spontaneous waves of neural activity that sweep across the retina during the time that retinogeniculate axons are reorganizing (Wong et al., 1993). Axons from the two eyes that overlap in the LGN rely on these waves of activity to compete for space (Penn et al., 1998). Similar kinds of correlated activity during early development have also been described in the cerebral cortex (Katz and Shatz, 1996).

After precise connectivity has been achieved, it must, in some systems, be maintained. Experimentally induced eye-specific stripes in the frog optic tectum desegregate if neural activity is blocked after their formation (Reh and Constantine-Paton, 1985; Cline and Constantine-Paton, 1989). Similarly, while maps of orientation and ocular dominance in the kitten visual cortex develop in the absence of neural activity, they deteriorate if activity is not present after three weeks of age (Crair et al., 1998).

Activity May Play an Instructive Role in the Development of Connections

It seems clear that activity is necessary for the development of precise connectivity in the visual system. However, it does not automatically follow that activity is *sufficient*. The results that I have reviewed show only that neural activity is permissive, not that it is instructive. That is, activity is required for axon growth and connectivity to move forward, but may not necessarily play a role in determining appropriate connections. The recognition of correct contacts may be mediated by a mechanism that does not rely on

[1] TTX is a potent toxin found in certain species of puffer fish that prevents action potentials by blocking voltage-gated sodium channels.

activity.

Yet, *patterned* activity is crucial. When neural activity in the retina is eliminated, simultaneously stimulating both of the optic nerves with electrodes does not restore the pattern of ocular dominance columns. Only when the optic nerves are stimulated asynchronously does the development of the ocular dominance columns proceed normally (Stryker and Strickland, 1984). Similarly, when all retinal ganglion cells of the retinae are induced to fire together by stroboscopic illumination, the retinotopic maps formed by retinal axons in the optic tectum do not fine-tune their connections (Schmidt and Eisele, 1985; Becker and Cook, 1990; Brickley et al., 1998). In these cases retinal axons experience a normal amount of activity, but it is not patterned appropriately. These experiments, then, suggest that patterned activity is instructive as well as permissive.

The term "instructive" is used here only to imply that particular patterns of activity (and not simply activity itself) are required for the normal development of the visual system. It is not meant to imply a particular view vis-a-vis the ongoing debate between contructivism and selectionism (e.g., Jerne 1967; Changeux and Danchin 1976; Edelman 1987; Rakic et al., 1986; Purves et al., 1996; Quartz and Sejnowski, 1997). That the environment "instructs" the developing brain is the central tenet of contructivism which contrasts with the selectionist claim that the environment merely 'selects' appropriate neural circuits from a preexisting repertoire. Much of the activity-dependent literature reviewed here is often taken as evidence for selectionism (and the data presented in Chapter Six is also consistent with that view), though there is equally compelling evidence pointing to the elaboration of correctly positioned axons and

dendrites. As with many such debates, the final resolution is likely to be a subtle and complex mixture of the two; perhaps along the lines suggested by Greenough and colleagues (e.g., Black and Greenough, 1986). Indeed, both elaboration in correct target areas (i.e., instructed growth) and pruning of incorrect connectivity (i.e., selection) are known to occur.

Mechanisms of Activity-Dependent Development

We know that blocking neural activity disrupts the normal development of precise connectivity, but why? What are the subsequent links in the chain that translate neural activity into morphological changes (i.e., changes in the structure of the nervous system)? It appears that N-methyl-D-aspartate (NMDA) receptors, nitric oxide (NO), and neurotrophins are involved. These molecules are also involved in most types of long-term potentiation (LTP) of synaptic transmission, a mechanism proposed for altering synaptic strength in the adult hippocampus and cerebral cortex. Thus, activity-dependent development and LTP appear to be closely related phenomena.

NMDA Receptors and Nitric Oxide

NMDA receptors are one of two types of neurotransmitter receptors that respond to the amino acid glutamate, a major excitatory neurotransmitter. Other receptors do not respond to NMDA, but rather to α-amino-3 hydroxy-5 methyl-4 isoxazole proprionic acid (AMPA) and are grouped together as the AMPA receptors. The role of the NMDA receptor in plasticity is intriguing because it acts as a type of coincidence detector. That is, both glutamate from the presynaptic terminal and depolarization of the postsynaptic

terminal are required simultaneously for calcium flux through the associated ion channel.

The existence of coincidence detection at synapses was implied by Hebb (1949) who proposed that a synapse is strengthened in proportion to the product of the activity of its pre- and postsynaptic sides. In the early 1970s Bliss and Lomo (1973) discovered that synaptic strengthening could, indeed, be induced in the nervous system. They showed that brief, high frequency stimulation of pathways in the hippocampus, a region critical for learning and memory, produced an increase in synaptic strength subsequently termed LTP. A decade later it was discovered that LTP in the CA1 region of the hippocampus could not be induced if the coincidence detecting NMDA receptors were blocked, thus providing evidence of "Hebbian" synapses in the brain. Subsequent experiments have examined whether NMDA receptors are also involved in activity-dependent development.

For example, the development of the relay cells of the LGN is upset in the ferret when NMDA receptors are blocked (more below). Interestingly, postsynaptic NMDA receptors also play a role in the development of their presynaptic inputs. Early evidence that NMDA receptors might be involved in activity-dependent plasticity of retinal axons came from experiments in which an extra eye was implanted into a frog embryo. The retinal projections from the third eye grow into the optic tectum and must share that space with the projections from one of the normal retinae. Suprisingly, the projections from the two retinae do not intermingle, but rather segregate into rostrocaudally oriented zones that are reminiscent of the ocular dominance columns found in the visual cortex of higher vertebrates (Constantine-Paton and Law, 1978). Chronically blocking the NMDA

receptors in the optic tectum results in the gradual desegregation of these eye-specific zones (Cline et al., 1987; Cline and Constantine-Paton, 1989) and blockade in the LGN disrupts ON/OFF sublamination (Hahm et al., 1991; Figure 1). In the frog, treatment with NMDA reduces the number of retinal axon branches (Yen et al., 1995). These experiments suggest that NMDA receptor-mediated activity may serve to modulate the size, complexity and location of retinal axons.

NMDA receptors are also involved in the development of the physiological properties of neurons in the visual cortex, presumably as a consequence of their role in anatomical plasticity. In the mature visual cortex, neurons are responsive to stimulation from either eye. However, when one eye is deprived of visual stimuli during early development, neurons become responsive only to the non-deprived eye. When NMDA receptors are chronically blocked during monocular deprivation, more neurons than normal continue to be responsive to both eyes (Bear et al., 1990). Not only are NMDA receptors involved in this activity-dependent decoupling of inappropriate inputs, they are also involved in the strengthening of appropriate ones. When kittens are reared in the dark, visual cortical neurons remain unselective for orientation. When, subsequently, one eye receives normal visual experience, neurons develop normal, strong orientation selectivity. Chronic blockade of NMDA receptors during the late period of visual experience, however, blocks that development of orientation selectivity (Bear et al., 1990).

In some cases, however, NMDA receptors do not seem to be involved in plasticity. The segregation of retinogeniculate axons into eye-specific laminae does not

depend on NMDA receptor activation (Smetters et al., 1994), nor do certain types of LTP (more below). Additionally, there is an important caveat to the interpretation of NMDA-blockade experiments. NMDA receptors are involved in the normal transmission of patterned visual information and inhibiting their activation may simply result in a generalized attenuation of postsynaptic responsiveness to stimuli. That is, it may not be the special coincidence detection properties of NMDA receptors that are involved in developmental plasticity, but rather their contribution to normal synaptic transmission (Fox and Daw, 1993). However, recent experiments that reduced the expression of a particular *subunit* of the NMDA receptor have shown that the ocular dominance shift resulting from monocular deprivation continues to be disrupted, but with minimal effects on the general responsiveness to and selectivity of visual stimuli (Roberts et al., 1998).

If blocking NMDA receptors (which are found on postsynaptic membranes) results in the reorganization of presynaptic retinal axons, then there must be a signal that travels back from the post- to the presynaptic terminal. NO is an attractive candidate for the required retrograde messenger: its production requires the presence of calcium which enters through NMDA receptors, and it can diffuse out of the postsynaptic terminal and signal the presynaptic terminal. There is some evidence that NO plays exactly that role in LTP (more below). Additionally, NO synthase (NOS), the precursor of NO, is developmentally regulated in the ferret LGN (more below). Similarly, NOS expression peaks in the chick tectum coincidentally with the retraction of an aberrant retinal projection (Williams et al., 1994), and the inhibition of NOS during this period prevents that retraction (Wu et al., 1994).

Neurotrophins

Neurotrophins mediate growth and survival in a number of systems and may be the mechanism whereby activity is translated into structural changes. For example, the levels of mRNA that encode a receptor for a particular neurotrophin vary with visual experience in the rat visual cortex (Castren et al., 1992). Additionally, neurotrophins are involved in the formation of ocular dominance columns in the cat visual cortex. The expression of truncated trkB, a neurotrophin receptor, is well correlated with the critical periods in visual development (Allendoerfer et. al., 1994) and blocking the neurotrophins that act on trkB prevents ocular dominance column formation (Cabelli et al., 1995). Similarly, neurotrophin expression in the frog retina coincides with the patterning of retinal axons in the tectum (Cohen-Cory and Fraser, 1994). More specifically, neurotrophins have been shown to modulate both axonal and dendritic growth in the visual system. When a particular neurotrophin is injected into the optic tectum of live tadpoles, the branching and complexity of optic axon terminal arbors is rapidly increased (Cohen-Cory and Fraser, 1995). Localized delivery of neurotrophins to the visual cortex during monocular deprivation rescues geniculocortical axons of the deprived eye from the atrophy that normally results (Riddle et al., 1995). Neurotrophins also modulate the growth of dendrites in the developing visual cortex (McAllister et al., 1996). Lastly, neurotrophins can induce increases in long-term synaptic efficacy that may lead to the stabilization of appropriate inputs (Kang and Schuman, 1995).

Long-Term Potentiation

The type of activity-dependent development described above has components that seem to be shared with LTP (Shatz, 1990; Goodman and Shatz, 1993; Cramer and Sur, 1995). For example, like activity-dependent development, many types of LTP require patterned electrical activity, NMDA receptor activation and NO production (for review see Bliss and Collingridge, 1993). Consequently, numerous researchers have suggested that LTP underlies the stabilization of synapses in developing sensory structures (Constantine-Paton, et al., 1990; Kandel and O'Dell, 1992; Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996).

LTP can be induced in visual structures as well as in the hippocampus where it has been most actively studied. In the rat visual cortex, the ability to induce LTP is tightly correlated with the critical period for developing binocular connections. When the critical period is delayed by rearing rats in the dark, the period during which LTP can be easily induced is also shifted (Kirkwood et al., 1995). LTP of inhibitory synapses is also more easily induced in young visual cortex than in old (Komatsu, 1994). Additionally, in the ferret LGN, NMDA-dependent long-term enhancement of synaptic efficacy can be induced during the time that retinogeniculate axons are reorganizing (Mooney et al., 1993). However, in the mouse visual cortex, the development of ocular dominance columns can proceed normally despite genetically induced defects in several forms of LTP (Hensch et al., 1998).

That LTP can be induced in particular regions of the nervous system and that the ease of induction is correlated with periods of exceptional plasticity does not imply that LTP actually occurs *in vivo* during development. More direct demonstrations that LTP

is, in fact, occurring during development comes from investigations of so-called "silent synapses." Silent synapses are synapses at which no functional AMPA receptors are present (Liao et al., 1995; Isaac et al., 1995). Shortly after birth a significant proportion of silent synapses are found in the rat and mouse thalamocortical system and rat visual cortex, but the proportion declines during early development (Isaac et al., 1997; Rumpel et al., 1998; Golshani and Jones, 1999). The early, silent synapses are converted to functional synapses using LTP induction protocols, require NMDA receptor activation to do so, and the ease of LTP induction declines in parallel with the reduction in the proportion of silent synapses (Isaac et al., 1997; Rumpel et al., 1998). Morphological evidence suggests that AMPA receptors are entirely absent from silent synapses. That is, it is not the case that they are present, but non-functional or "turned-off," rather, at least in a significant proportion of hippocampal synapses, AMPA receptors do not appear until later in development although NMDA receptors are present early on (Petralia et al., 1999; Liao et al., 1999).

Research on the Mammalian Visual System

Research on the role of neural activity in the development of the nervous system has taken place in many systems. For example, the development of vocalization in songbirds has proven to be a valuable model for examining the neural mechanisms underlying behavior (for reviews see Mooney 1995; Bottjer et al., 1997; Ball and Hulse, 1998). Studies in the peripheral nervous system were the first to suggest that neural activity prunes the number of connections made onto a target (Purves and Lichtman, 1985). In

the auditory, somatosensory, and olfactory areas of the central nervous system, many experiments have demonstrated the crucial role of neural activity in early development (e.g., Sanes and Constantine-Paton, 1983; Rees et al., 1985; Baker et al., 1993; Sanes and Takacs, 1993; Pasic et al., 1994; Killackey et al., 1995; Hyson and Rubel, 1995; Zirpel and Rubel, 1996; Philpot et al., 1997a; Philpot et al., 1997b; Sigg et al., 1997). The majority of the research on the activity-dependent development of neuronal connectivity, however, has taken place in the mammalian visual system. Visual experience is easy to manipulate—incoming light to one or both eyes can be precisely controlled, contact lenses or goggles can provide calculated distortions, images can be presented on computer screens, and so on. In many mammals, the visual system, being the primary sensory system, occupies the largest portion of sensory processing tissue in the brain. Both subcortically and in the cortex, dramatic anatomical patterns of connectivity (Figure 2), such as ocular dominance columns in layer IV of the cortex and eye-specific laminae and sublaminae in the thalamus, provide convenient assays of manipulations of visual experience. The precise "tuning" of neurons in the visual system to features in the environment (e.g., the orientation and direction of contours or the binocular disparity of an object) also provides a useful physiological measure of appropriate connectivity. These anatomical and physiological properties of the mammalian visual system are very similar from species to species.

Lastly, vision can be used to probe general mechanisms of development. For example, in animals in which retinal projections are induced to provide input to the auditory cortex, neurons in that area develop properties characteristic of neurons in visual

cortex (Sur et al., 1988; Roe et al., 1990; Roe et al., 1992). Additionally, many areas of the developing neocortex have the ability to take on the characteristics of other, functionally different areas when transplanted at an early age (for review see O'Leary et al., 1995). Consequently, whatever developmental mechanisms are at play in the visual system are likely to be involved in other sensory systems.

The Ferret Lateral Geniculate Nucleus as a Model System

The ferret LGN, in particular, is an excellent system in which to explore the relationship between activity-dependent development and changes in synaptic efficacy. The activity-dependence of ON/OFF sublamination is well-characterized and requires many of the components also required for the modification of synaptic efficacy. Additionally, it has been demonstrated that retinogeniculate synaptic efficacy can be modulated and developmental measurement of synaptic efficacy is aided by the characteristics of the neural circuitry of the LGN.

As noted briefly above, retinogeniculate axons in the ferret segregate in an activity-dependent manner during early postnatal life. More specifically, they segregate to form eye-specific laminae during the first two postnatal weeks (Linden et al., 1981) and subsequently, within each of the eye-specific laminae, inputs from ON-center and OFF-center retinal ganglion cells segregate to form sublaminae by then of the fourth postnatal week (Stryker and Zahs, 1983; Hahm et al., 1991). Segregation of retinal inputs into eye-specific laminae is modulated by retinal activity (Shatz and Stryker, 1988; Penn et al., 1998) and the segregation into ON/OFF sublaminae is disrupted entirely by intraocular injections of TTX (Cramer and Sur, 1997).

Development of the LGN during ON/OFF segregation is also disrupted by the blockade of NMDA receptors or NO (Hahm et al., 1991; Cramer et al., 1996; Figure 1). For example, blocking NMDA receptors during the third postnatal week upsets ON/OFF sublamination. Retinogeniculate axon arbors terminate in inappropriate areas of the LGN and/or have arbors that are too large (Hahm et al., 1991; Figure 1). Finally, NOS is developmentally regulated in the ferret LGN. Between one and five weeks after birth, NADPH-diaphorase (which is colocalized with NOS) is expressed in LGN cells, but not before or after. The peak of expression at four weeks is coincident with the segregation of retinogeniculate axons into ON/OFF sublaminae (Cramer et al., 1995). Indeed, inhibiting NOS during the third and fourth postnatal weeks significantly reduces normal sublamination (Cramer et al, 1996; Figure 1).

Not only is normal neural activity critical for appropriate retinogeniculate axon development, it is also involved in the development of its major postsynaptic target, the relay cells of the LGN. Normally, relay cell dendrites increase in complexity in the weeks after birth, adding branches and small appendages along the branches (Sutton and Brunso-Bechtold, 1991). The addition of branches and appendages ordinarily pauses during the third postnatal week. However, when d-APV, an NMDA receptor antagonist, is infused into the thalamus during this time, relay cells show an increase in branching and appendage addition (Rocha and Sur, 1995). Intracranial infusions of TTX in fetal cats cause an increase in the density of dendritic spines in the LGN (Dalva et al., 1994). Similarly, relay cells show an increase in dendritic branching and in the number of dendritic spines when a type of postsynaptic receptor is blocked (more below; Rocha and

Sur, 1995). Later in development, after eye-opening, the normal elimination of transient dendritic spines is delayed following early eye enucleation (Sutton and Brunso-Bechtold, 1993).

In addition to being a good model of activity-dependent development, the LGN is a useful system for studying the development of synaptic efficacy. First, there are only two major classes of neurons in the LGN, relay cells and interneurons, allowing for sampling from a relatively homogeneous population of cell types (Sherman and Koch, 1990). The relay cells can be distinguished from interneurons by both anatomical and physiological characterisitcs (Pape and McCormick, 1995). Second, because the optic tract forms an easily identifiable bundle along the lateral edge of the LGN, access to the input fibers in question, the retinogeniculate fibers, is unencumbered. Third, Mooney et al. (1993) have shown that the efficacy of retinogeniculate synapses can be modified in response to high-frequency stimulation. Fourth, the largely feed-forward connectivity of retinogeniculate inputs eliminates any contamination (i.e., disynaptic responses) from lateral inputs, and more generally, an enormous amount of information is known about the circuitry of the LGN (for reviews see Sherman and Koch, 1990; Sherman and Guillery, 1996).

Summary

Both activity-dependent and activity-independent mechanisms are at play during the early development of the visual system. Activity-independent mechanisms are involved in coarse target recognition, but activity is required for establishing precise connectivity.

One major question is whether, during the refinement of connections, activity is instructive or permissive. In the adult, neural activity involved in learning and memory must, at some level, be instructive (e.g., the neural activity associated with learning a new concept from reading this chapter must instruct synaptic change rather than simply permit unguided synaptic change to occur) and experiments suggest that the quality as well as the quantity of visual input is important. One model that emerges from these considerations is that neural activity is exploited by the nervous system at different stages of development for progressively more precise manipulations. At the earliest stages of development axons find their targets without the aid of activity. Subsequently, patterned spontaneous activity is generated by the nervous system itself to guide precise connectivity within the target. Later, stimulus driven activity is required to dynamically maintain the complex synaptic relationships that mediate routine, but sophisticated, perceptual and cognitive processing (retinotopy in the three-eyed frog and the orientation maps in the visual cortex require ongoing activity after relatively precise connections have already been laid down). Lastly, after sufficient wiring has been established, activity instructs specific changes in synaptic connectivity that result in learning and memory.

Activity-dependent development employs mechanisms that are similar to those used for long-term increases in synaptic strength. NMDA receptors, nitric oxide and neurotrophins play critical roles in both phenomena. However, there are more than just a few cases of both types of events that do not rely on these three components. It is likely that many mechanisms exist for the crucial purpose of mediating plasticity in the nervous

system.

The ferret retinogeniculate system is a good model for investigating the link between activity-dependent development and changes in synaptic efficacy. Retinogeniculate axon segregation into laminae and sublaminae requires neural activity and, more specifically, NMDA receptor activation and NO production. Additionally, long-term changes in synaptic efficacy can be induced in the LGN during the period that segregation is occurring. Throughout this period the electrophysiological and anatomical properties of the relay cells in the LGN are undergoing numerous changes that are relevant for considering the functional impact of their inputs.

Figure Legends

Figure 1. The segregation of ON- and OFF-center retinal axons within each of the eye-specific layers in the ferret LGN depends on neural activity during the third and fourth postnatal weeks. (A) At birth, denoted by postnatal day 0 (P0), retinal axons from both eyes have invaded the LGN and arborize throughout its extent. (B) At the beginning of the second postnatal week (P7), axon arbors from the left and right eyes have largely segregated from one another forming an ipsilateral eye lamina (A1) and a contralateral eye lamina (A). (C) Within the A1 and A laminae, axons from ON- and OFF-center retinal ganglion cells are intermingled during the first two to three postnatal weeks. (D) Finally, by the fourth postnatal week, ON- and OFF-center axons have segregated from one another to form sublaminae (denoted by dashed lines) in both of the eye-specific laminae. (E) If NMDA receptors in the LGN are blocked during the period that ON/OFF sublaminae segregate, or if the production of NO is blocked, normal sublamination is disrupted and axons can be too large or not restricted to their appropriate layer. Sublamination is also prevented if retinal activity is blocked by intraocular TTX injections, presumably because of similar abnormalities in axon arbor size and placement.

Figure 2. The primary visual pathway and examples of the activity-dependent development of brain circuits. (A) Retinal ganglion cells mediating perception of a visual hemifield project their axons to the contralateral LGN. Relay cells of the LGN innervate layer IV of the visual cortex. (B) Early in development axons from the ipsilateral and contralateral eyes are intermingled in the LGN. Later, axons from the two eyes segregate

to from eye-specific laminae and, shortly thereafter, sublaminae that receive inputs from either ON- or OFF-center retinal ganglion cells. (C) Similarly, axons from both LGN laminae initially overlap at their target, layer IV of the visual cortex, and are subsequently refined such that they occupy distinct ocular dominance columns. (D) Maps of the orientation preference of neurons (depicted as shades of gray) in visual cortex show relatively little clustering of neurons with like orientation preferences in early development. As the circuitry matures, maps reveal extensive clustering of similar orientation preferences in a pinwheel-like organization. (E) Developing pyramidal neurons in the superficial layers of visual cortex extend their axons horizontally in a relatively crude manner showing little preference for any particular area within their reach. As development proceeds, the axonal arborization is elaborated in areas of similar orientation preferences and branches are removed from dissimilar areas.

Figure 1

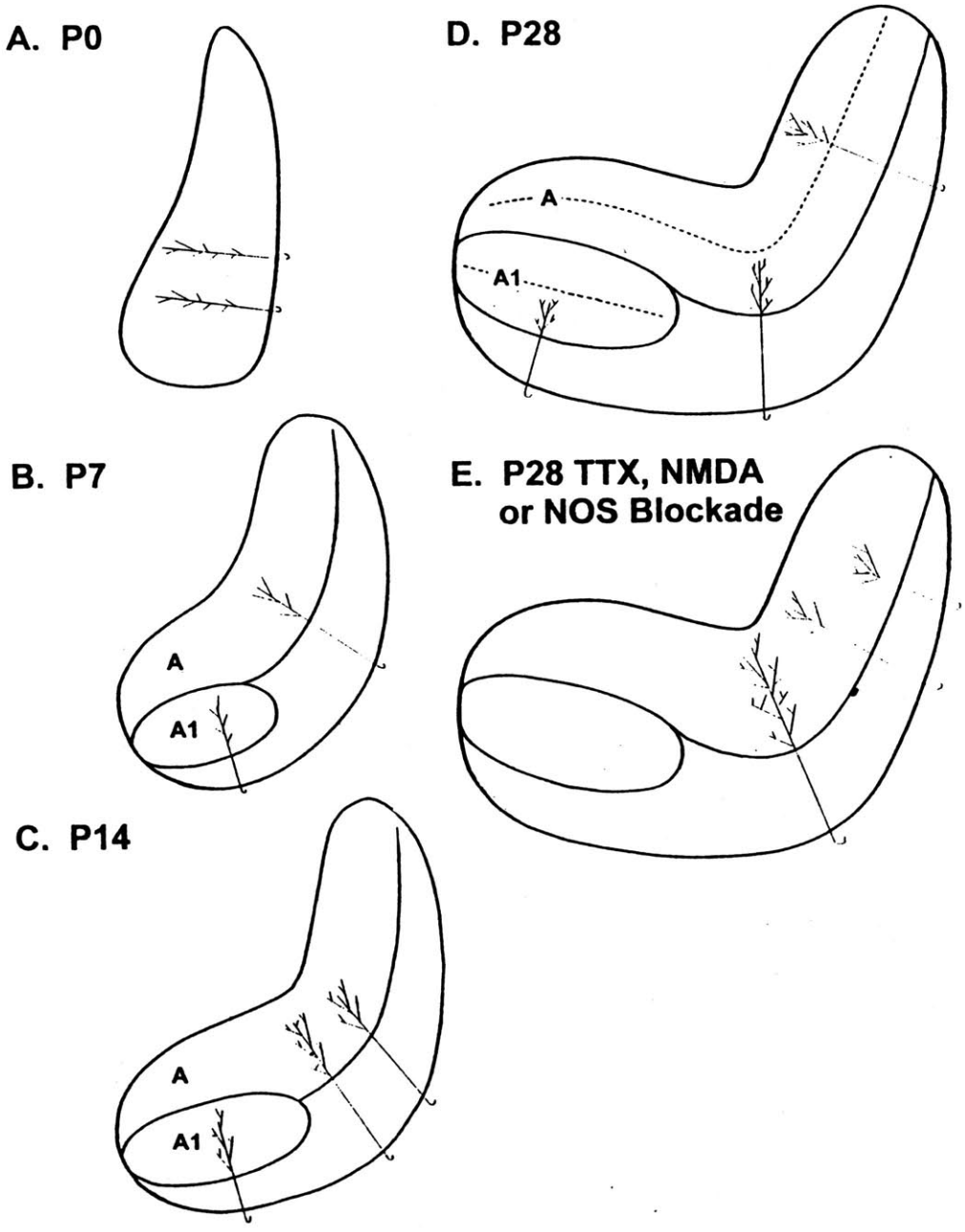
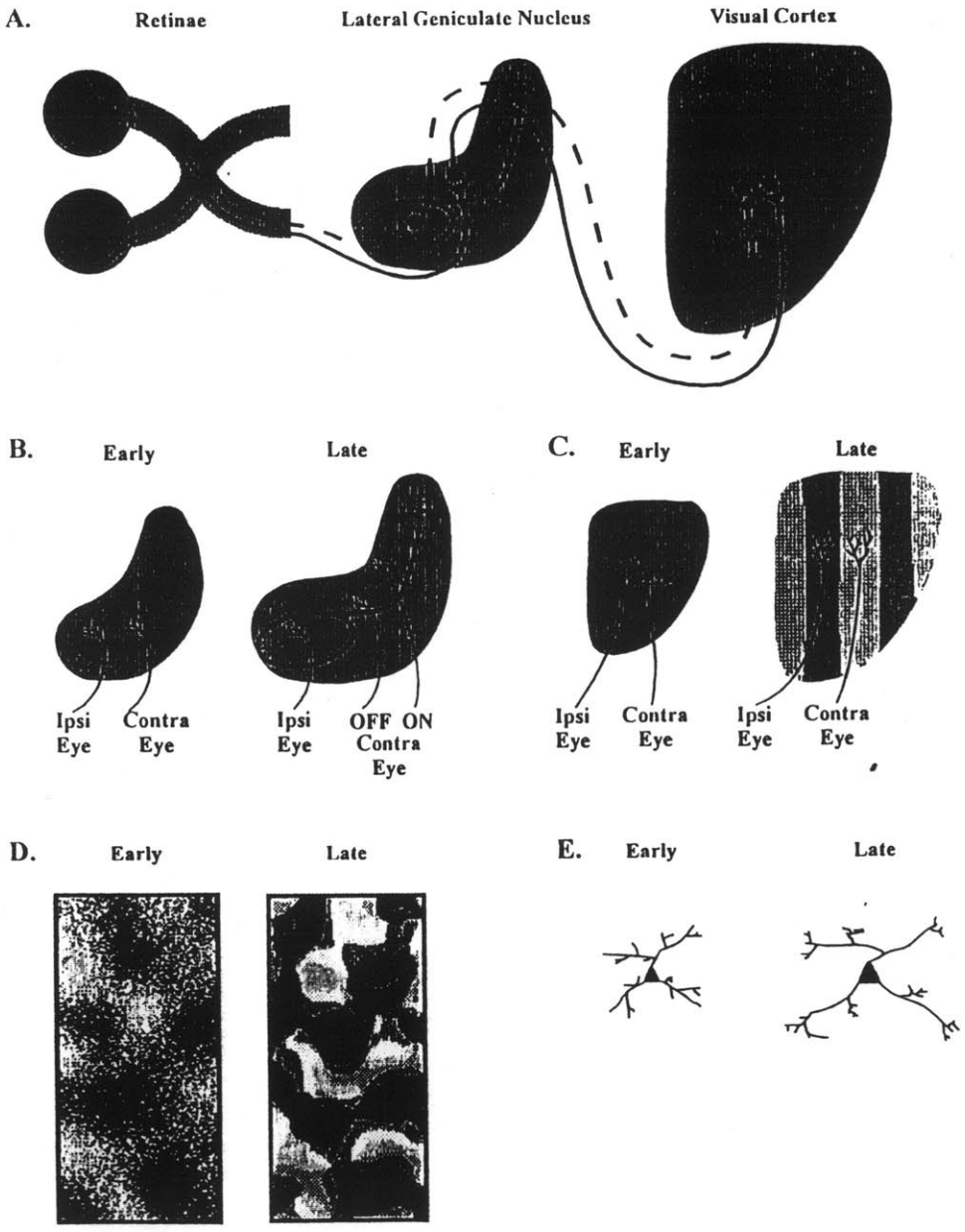


Figure 2


A.
Retinae
Lateral Geniculate Nucleus
Visual Cortex
B.
Early
Late
Ipsi Eye
Contra Eye
Ipsi Eye
OFF
ON
Contra Eye
C.
Early
Late
Ipsi Eye
Contra Eye
Ipsi Eye
Contra Eye
D.
Early
Late
E.
Early
Late

Chapter Two: General Methods

Experimental Setup

Whole-cell patch-clamp recordings (Blanton et al., 1989) were made during current- and voltage-clamp of LGN relay cells in thalamic slices (400 μM thick) from young (postnatal day 12 [P12] to P31) ferrets (Figure 1). The animals were deeply anesthetized with sodium pentobarbital (35 mg/kg, i.p.) and decapitated. A block of tissue including the thalamus was rapidly removed and placed in a cold solution (4°C) containing (in mM): NaCl (126), KCl (3), $MgSO_4$ (2), $NaHCO_3$ (25), $NaHPO_4$ (1), $CaCl_2$ (2.5), and dextrose (10), saturated with 95% O_2, 5%CO_2 (pH 7.4). In some experiments NaCl was replaced with equiosmolar sucrose (252 mM; Aghajanian and Rasmussen, 1989), and kynurenic acid (0.5 mM) was added to minimize excitotoxicity during slicing. The cortex was dissected away and the remaining thalamus was sliced in the horizontal or coronal plane with a Vibratome (Ted Pella, Model 1000).

Slices were maintained at room temperature and continuously superfused with the NaCl based solution described above. Before "blind" recording, a slice was transferred to an interface-type chamber and given 45 minutes to equilibrate to 32°C. In later experiments slices were transferred to a submersion chamber and recordings were performed at room temperature under visual control with DIC-enhanced optics.

In both cases, the boundaries of the LGN and the position of the recording electrode were visible and recordings were made from relay cells in the A or A1 lamina.

I identified, and limited recordings to, relay cells based on a number of criteria. Class 3 cells (e.g., Guillery, 1966), presumptive interneurons (McCormick and Pape, 1988), generally have somata smaller than relay cells. In experiments using DIC-enhanced optics, it was possible to target recordings to cells with medium and large somata. During blind recordings, the smaller size of Class 3 cells, their oval shape[1] (Sutton and Brunso-Bechtold, 1991), and their low proportion of total LGN cells (Sherman and Koch, 1990; Sutton and Brunso-Bechtold, 1991), minimized the probability of successfully patching onto such a cell. These facts were confirmed during the experiments using DIC-enhanced optics. More importantly however, are the electrophysiological differences between interneurons and relay cells. Most (White and Sur, 1992) relay cells, but not interneurons, have low-threshold calcium currents that are activated after hyperpolarization (McCormick and Pape, 1988; Pape and McCormick, 1995). The vast majority of the cells that I recorded from had hyperpolarization deinactivated low-threshold calcium currents (Figure 1A). Addtionally, interneurons have significantly narrower action potential widths and higher frequency action potential generation than relay cells (McCormick and Pape, 1988; Pape and McCormick, 1995). Histograms of these parameters for the cells that I recorded from do not suggest more than one population of cell type (Figure 2A,B). Finally, the smaller soma sizes of interneurons should translate into smaller membrane capacitance. However, as with action potential width and frequency, membrane capacitance is not obviously distributed into two

[1] It is more difficult to form a patch recording onto small, oval-shaped somata. These cells tend to "roll"

populations (Figure 2C), though it is more broadly distributed and may represent multiple, overlapping distributions; perhaps due to the different somata sizes of interneurons and relay cells, but more likely due to the different somata sizes of X and Y type relay cells.

Patch pipettes were pulled from borosilicate glass (World Precision Instruments) on a horizontal pipette puller (Sutter Instruments) to tip resistances of 3-8 MΩ. The pipettes were filled with (in mM): potassium gluconate (125), KCl (10), HEPES (10), sodium EGTA (1), $CaCl_2$ (0.1), $MgCl_2$ (2), Na-ATP (2), Na-GTP (0.2), or cesium gluconate (120), HEPES (10), sodium EGTA (1), $CaCl_2$ (0.1), $MgCl_2$ (2), Na-ATP (2), Na-GTP (0.1); the pH was adjusted to 7.3.

Retinal afferents were stimulated by delivering constant current through a bipolar stimulating electrode positioned in the optic tract at the lateral edge of the slice. Cortical afferents were stimulated in the perigeniculate nucleus just medial to the LGN. The particular stimulation protocols used are detailed in the method sections of each of the data chapters.

Likewise, the particular use of pharmacological agents is described in the method sections of each of the data chapters. In summary, the agents used were: lidocaine N-ethyl bromide quaternary salt (QX-314, Research Biochemicals International [RBI]) for the intracellular blockade, and tetrodotoxin (TTX, Sigma) for the extracellular blockade, of sodium channels; bicuculline methiodide (BMI, Sigma), 6-cyano-7-nitroquinoxaline-

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out from under the recording electrode.

2,3-dione (CNQX disodium, RBI), D-2-amino-5-phosphonopentanoic acid (D-AP5, RBI), and α-methyl-4-carboxyphenylglycine (MCPG, RBI) for the extracellular blockade of $GABA_A$, AMPA, NMDA, and metabotropic glutamate receptors, respectively. BMI (50 μM) was used in all recordings in Chapters Five and Six. The data in Chapter Three is not affected by synaptic currents so recordings both with and without BMI present are pooled. The effect of the presence or absence of BMI is examined explicitly in Chapter Four.

Data Acquisition

Recordings were obtained with an Axopatch-200 amplifier (Axon Instruments). Data were acquired off-line with the pClamp data acquisition software (Axon Instruments), digitized using a Neurocorder encoding unit (Neurodata), and stored on video tape and computer disk for off-line analysis. Recordings were low-pass filtered at 1 to 5 kHz and sampled at 10 kHz.

Cells that had resting membrane potentials more hyperpolarized than -40 mV upon patch rupture and generated overshooting action potentials were considered for analysis. For examination of resting potential properties, all cells were voltage-clamped at -60 mV.

Cell membrane capacitance was charged using the independent circuitry in the Axopatch-200 designed for that purpose. Briefly, values for access resistance and membrane capacitance can be accurately determined through canceling whole-cell capacity transients by shifting current load to separate circuits. However, it should be

noted that the maximum value for membrane capacitance that can be achieved in this way (due to the limitations of the amplifier) is 100 pF. Consequently, analyses of membrane capacitance did not include cells with membrane capacitance greater than 100 pF. Input resistance was calculated by measuring the steady-state current required to clamp a 5 mV square wave (at line frequency).

Access resistance was monitored throughout the experiment, but not compensated for. Relay cell soma size in the LGN is quite small during the period of interest (10-15 μm; Sutton and Brunso-Bechtold, 1991; Rocha and Sur, 1995) and the filtering effects of access resistance (R_a) are related to soma size (via C_m; i.e., corner frequency = $\frac{1}{2\pi R_a C_m}$). An understanding of the effect of the access resistances of recordings on the parameters measured is critical because it can often be significant and, more importantly for these experiments, age-related.

The Effects of Access Resistance

Access resistance is the electrical resistance, in series with the cell membrane, encountered between the recording electrode and the inside of the cell. Any non-zero access resistance will have three effects on the accuracy of voltage-clamp measurements. First, there is an increase in the response time of the cell membrane potential to a voltage step that is proportional to the access resistance. Second, there is a deviation of the membrane potential from the command potential equal to the command current multiplied by the access resistance. Third, access resistance in series with membrane capacitance acts as a low-pass filter with a cut-off that is inversely proportional to the

product of the two.

The first two effects are not serious issues for the data presented in this thesis. First, none of the questions posed in the following chapters is concerned with the dynamics associated with voltage steps. Second, given the very small currents measured in these experiments, the deviation from the command potential is not likely to be greater than 3 mV.

The third effect is a much more serious one. In typical recordings the filtering effects of access resistance can lead to a bandwidth of around 450 Hz. As a consequence, as access resistance increases, the high-frequency rise times, whose components can be up to 1 kHz, will become longer and the widths and decay times will increase. In multicompartmental models of reconstructed cortical, pyramidal cells, for example, rise times from proximal events recorded in the presence of a 30 MΩ access resistance are reduced by up to 75% when compared to the theoretical 0 MΩ recording (e.g., Smetters, 1995). Many of the parameters in these experiments are, in fact, correlated with the access resistance of the recordings used to measure them including both cellular current kinetics and amplitudes. For example, under voltage-clamp the rise times of sEPSCs become significantly longer as access resistance increases ($r_s = 0.54$, $n = 32$, $p < 0.05$; Figure 3A) while their amplitudes become significantly smaller ($r_s = -0.42$, $n = 32$, $p < 0.05$; Figure 3B). The filtering effect of access resistance is even more pronounced during current-clamp measurements of action potential amplitude ($r_s = -0.62$, $n = 152$, $p < 0.05$; Figure 3C), an extremely "high-frequency" parameter.

However, while minimizing access resistance is always a goal throughout a set of

experiments, I am concerned mostly with the trend of a parameter with age and not the precise value of that parameter *per se*. That is, as long as the access resistance-mediated deviation from the true value is relatively low and constant across the development of ON/OFF sublaminae, then the true trend of a parameter will not be skewed. Unfortunately, access resistance itself is correlated with age ($r_s = -0.28$, $n = 311$, $p < 0.05$; Figure 3D). While it is not clear why this is the case, there are number of possible causes. First, there may have been an unintentional bias toward slightly smaller electrode tip sizes used to record from slightly smaller cell bodies at the beginning of ON/OFF sublamination. Second, younger cell membranes may reseal more efficiently after membrane patch rupture. Last, the configuration in which the electrode contacts the cell body may be less amenable to low access resistance recordings at P14 than at P28 (e.g., due to slight difference in cell body shape, size, orientation, etc.).

Whatever the cause may be, a developmental decrease in access resistance can lead to artifactual developmental changes of parameters that are sensitive to it. Consequently, I have limited analysis of miniature currents to recordings with maximum access resistances in a range that does not correlate with the parameter under question—between 20 and 35 MΩ.

Data Analysis

Detection of Miniature Events

Data were analyzed using Matlab (MathWorks) routines that I coded. A moving average of the current detected fluctuations greater than a threshold. If the fluctuation remained

over threshold for a given amount of time, the event was tagged as a possible miniature event and detection resumed after a given amount of time intended to allow the current to return to baseline. Events were then reviewed by visual inspection to eliminate any noise-induced detection.

Different combinations of detection parameters were optimal for event detection depending on the baseline noise of a particular recording. Therefore, all detection parameters were user-defined for each run of the program. However, for the sake of consistency, I determined generally reliable values for the width of the moving average window and for the time-below-threshold and used these values throughout the data analysis. To compensate for differences in baseline noise, only the detection threshold was tuned. The length of time (the width of the moving window) over which the current was averaged was set at 10 ms, time below threshold at 1 ms, and threshold at 1.5 to 2.5 times the standard deviation of the noise.

A significant concern is whether one can detect the smallest of the miniature events, that is, whether the signal is sufficiently large relative to the noise. There are two ways in which events might fall under the "detection window." The first is that significant signals are reaching the recording electrode, but the detection threshold chosen by the user (me) is greater than the amplitude of the smallest events. This is not the case. First, the threshold is set relatively close to the noise—only 1.5 to 2.5 standard deviations away. Second, while reducing the threshold even further results in the detection of an increased number of events, visual inspection shows these to be overwhelmingly noise-induced. The second possibility is that the smallest events have

fallen below the detection window due to cable filtering prior to reaching the recording electrode. This, too, does not appear to be the case. Increasing the driving force of synaptic currents be hyperpolarizing the cell from –60 mV (i.e., resting potential) results in the detection of larger amplitude events, but not more frequent events suggesting that there is not a significant subpopulation of events "flying below the radar."

The events were fit using a least-squares algorithm with the sum of three (one rising and two decaying) exponentials (Soltesz and Mody, 1995):

$$y = Ae^{\frac{-x}{a}} + B\,e^{\frac{-x}{b}} - (A + B)\,e^{\frac{-x}{c}}.$$

The area of the event was calculated by integrating the fitted curve between the beginning and end of the event. Rise times were calculated from 20% to 80%, widths at 50%, and decay times at e^{-1}% of peak amplitude.

Evoked EPSCs amplitudes were measured as the peak amplitude in a 10-100 ms window following the stimulus artifact subtracted from a baseline defined by a 5-10 ms window prior to the stimulus artifact. Rise times and widths were measured as described above for those events whose onset occurred with sufficient latency[2].

Statistics

Recordings from P7 to P24 animals, termed the "younger" group, were compared to recordings from animals ranging in age from the end of PW4 to the beginning of PW5, the "older" group. Cells from the older group can, for the most part, be considered adult.

[2] That is, if the latency is too short, 20% of peak amplitude can be buried in the stimulus artifact while peak

Relay cell morphology reflects adult characteristics by the end of PW4 (Sutton and Brunso-Bechtold, 1991; Rocha and Sur, in preparation), although spine density, especially on proximal segments, is higher than in the adult (Sutton and Brunso-Bechtold, 1991). Both the resting membrane potentials and input resistances of LGN relay cells have reached adult values by P33, if not earlier (compare White and Sur, 1992 and Esguerra et al., 1992). The major afferent input to the LGN shows adult patterns by the second half of PW4. Retinogeniculate afferents have segregated into eye-specific laminae by P14 (Linden, 1981). Sublaminae can first be seen by P21 (Hahm et al., 1991), and are sharply segregated by P26 (Cramer et al., 1996). Finally, corticogeniculate afferents in the ferret have achieved adult patterning by P20 (Johnson and Casagrande, 1993; Clasca et al., 1995).

Five types of non-parametric statistical analyses were performed as none of the data appeared to be normally distributed, and, given the highly skewed nature of the distributions, median values were used as the preferred measure of central tendency. Values are presented as (medians, mean ± standard deviations) throughout. Spearman's Rank Correlation was used to test for significant correlations. Median parameter values between groups were compared with the Mann-Whitney U test, or, if the number of groups was greater than two (e.g., when testing for interactions between intracellular solution and age), with the Kruskal-Wallis non-parametric ANOVA. Parameter distributions from different conditions within the same recording were compared with the

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amplitude itself is unambiguous.

Kolmogorov-Smirnov test.

Finally, I sought to test for differences in the variance of parameters in the younger and the older groups. An F-Test can be used to compare the variances of two groups whose sample measures are normally distributed. However, as noted above none of the parameter means or medians appeared to be normally distributed, but rather are significantly skewed toward larger values. In this case, the test is not reliable (Markowski and Markowski, 1990 as cited in Sokal RR and Rohlf FJ, 1995, *Biometry*, W. H. Freeman and Company, p. 397; Zar JH, 1974, *Biostatistical Analysis*, Prentice-Hall, p. 103). The Scheffe-Box test, which uses the log of the variance of subsamples, is less sensitive (but not insensitive) to departures from normality, but single degree-of-freedom comparisons (i.e., number of groups is less than 3 which is the case in this study) are unreliable (*Biometry*, p. 401). In cases where the medians of the groups are not significantly different, one may be tempted to user a non-parametric test for continuous data (which is the case in these studies) such as the Kolmogorov-Smirnov (K-S) to test for differences in the variance of two groups. The reasoning is that since we know that any significance in the K-S D statistic must be due to differences in the variances of the two groups if the medians are known to be equal. Unfortunately, the K-S test is most sensitive to changes in the median value of populations and is not reliable for finding differences at the tails, and hence the variances, of the distributions (Press WH, Teukolsky SA, Vetterling WT, and Flannery BP, 1992, *Numerical Recipes in C*, Cambridge University Press, p. 626). Nonetheless, one can express values as standard deviations from the mean and then apply the K-S test (Hsia AY, Malenka RC, and Nicoll

RA, 1998, J. Neurophys. 79:2013). While this method does not directly address whether the variance of the two groups are significantly different, it does allow for a comparison of the shapes of the pooled young and the pooled old distributions (Figure 4). A better solution in these cases is to test directly for differences in variances between the two groups using the Siegel-Tukey test (Siegel S, Castellan NJ Jr, 1988, *Nonparametric Statistics for the Behavioral Sciences*).

Non-parametric tests trade some diagnostic power for having the advantage of not assuming a particular distribution. A concern that arises in these experiments is that, given many parameters show considerable variability, the non-parametric tests may be "missing" some significant developmental events that are, perhaps, occurring. There are a number of answers that one can provide to suggest that the risk has been minimized here. The first is to simply say, "Tough." That is, most parameter distributions analyzed here are highly skewed and so don't meet the assumption of normality upon which the more frequent t-test and ANOVA rely[3]. However, a more reassuring answer is that the loss of power is not that significant. The Mann-Whitney, for example, is one of the most powerful of non-parametric tests and 95% as powerful as the t-test when the latter is applicable (Zar, 1999, p. 149). The Spearman rank-correlation is 91%, and the Kruskal-Wallis 95%, as effective as their parametric analogues (Zar, 1999, pp. 196, 395).

[3] Some will feel a need here to point out that these tests are quite robust even with departures from normality. This is true for groups with equal *n*s and variances, but much less so when this is not the case (see, for example, Zar, 1999, pp. 127, 188). The former is not true in the experiments described in this thesis, and the latter may very well also not be true (although in some cases I have shown that the variances are not significantly different using the Siegel-Tukey test). At any rate, given the additional points in favor of the non-parametric tests, assuming a normal distribution when it's clear that that is not the case seems

In cases where populations of miniature events are detected, one may chose to identify multiple peaks in the histograms. This might allow one to make conclusions about the quantal nature of synaptic transmission or, in general, identify subpopulations of synapses. The requirements for quantal analysis have been discussed in the literature and I feel that many thousands of events are required to unambiguously detect multiple peaks (for example, see Thomson AM, 1992, TINS 15:167-168). Additionally, even if multiple peaks could be distinguished, one would not be able to determine unambiguously (without further experiments) the source of the separate peaks (e.g., differences in signal filtering that arise from differences in input location, differences in quantal content, etc.). Not being able to reliably detect peaks in the histogram does not preclude detection of a shift in the median value that would be associated with a developmental change from one "peak" to another (Figure 4).

Interpretation of Minimal Stimulation

Chapters Five and Six include results from the minimal stimulation of LGN afferents. The goal of minimal stimulation is to consistently stimulate one and only one of the multiple fibers that innervate the cell under investigation. There are number of issues of interpretation surrounding this technique (Allen and Stevens, 1994). How can one be sure that only one fiber is being stimulated (and not multiple fibers with near-identical relative thresholds for activation)? How can one be sure that any failure of synaptic transmission is due to a probabilistic failure of transmitter release and not due to a failure

unwise.

in activating an input fiber? How temperature-dependent is the technique? Are experiments performed at room temperature (20°C) reflective of what occurs at physiological temperatures (37°C)? The first two issues are difficult to determine experimentally, but strong circumstantial arguments can be made. The last question can be answered explicitly.

Theoretically, one could determine whether one and only one fiber was being stimulated consistently. Taking the retinogeniculate system, for example, one could stimulate the optic tract while recording simultaneously from every retinal ganglion cell. Detecting antidromic stimulation in only one cell would confirm the simulation of a single fiber. Likewise, detecting antidromic stimulation on every trial would allow for the interpretation of a failure of transmission as a synaptic failure (or as a branch-point failure, more on this below; but in any case one would be assured of consistent fiber activation). Of course, recording simultaneously from all (or even many) of the neurons that give rise to a particular projection is certainly prohibitively difficult and may be impossible given current technology. Alternatively, one could determine a) the probability of synaptic vesicle release given the arrival of an action potential, b) the size of a unitary (single synapse) response, and c) the number of synapses made by the typical afferent onto a target cell. If the variability of these parameters were not too great, than strong conclusions could be drawn as to whether only a single fiber was being activated and whether it was being activated consistently. However, the variability of the parameters is rather large, but, perhaps more importantly, the determination of these parameters, too, is surrounded by non-trivial issues of interpretation (Edwards, 1991;

Korn and Faber, 1991; Thomson, 1992; Hessler et al., 1993; Rosenmund et al., 1993; Stevens, 1993; Bekkers, 1994; Murthy et al., 1997; Liu et al., 1999).

Nonetheless, one can determine that the probability is quite high that only one fiber is being stimulated given minimal stimulation. While the threshold for action potential generation in an axon is variable, the variability is quite small. The coefficient of variation (CV) is only about 3.0% in the hippocampus (Allen and Stevens, 1994). Thus, as long as the minimal response is stable over a 5.0% or greater range in stimulus intensity, then stimulation failures should not be an issue. Additionally, the small CV implies that the likelihood that there is significant overlap of thresholds among axons that are different distances away from the stimulating electrode is quite small. It is possible, of course, that some small number of axons in the three-dimensional slice are equidistant from the stimulating electrode. Even if two of these axons were stimulated simultaneously the probability that they both innervate the cell being recorded from is vanishingly small—at least in the retinogeniculate system. In the cat, there are approximately 100,000 retinogeniculate axons only one or a very few of which innervate a particular cell in the LGN (Sherman and Koch, 1998).

Findings on the temperature-dependence of minimal stimulation are mixed. Allen and Stevens (1994) report no correlation between the failure rate of minimal stimulation and slice temperature in the hippocampal slice. However, Hardingham and Larkman (1998) show that in the visual cortex the reliability of minimal stimulation is significantly enhanced at physiological temperatures. Hardingham and Larkman (1998) suggest that difference in their findings is due to the fact that Allen and Stevens (1994) were

interpreting activation failures as failures of synaptic transmission. While possible, it is unlikely that activation failures contributed significantly to their findings. Allen and Stevens (1994) showed that axon threshold fluctuations occur over a narrow range and, consequently, minimal stimulation that is stable over approximately a 5% range of stimulus intensities is occurring outside that range. Hardingham and Larkman (1998) consider it unlikely that the differences in the two studies are due to the different brain areas investigated. However, the effects of temperature are different in different species (Gabriel et al., 1998) and are quite sensitive to the extracellular milieu within the same system (Barret et al., 1978).

In any case the debate surrounding the effects of temperature is not relevant to the interpretations used in Chapters Five and Six. In those chapters I'm not concerned with the reliability of transmission *per se*. In Chapter Five I compare the difference in reliability at positive and negative holding potentials. There is no evidence (and, indeed, no good reason) to suggest that cooler than physiological temperatures would differently affect reliability at –60 mV and +40 mV. In Chapter Six I gradually increase stimulation intensities in order to identify the recruitment of additional afferents. Here, too, any temperature-dependent processes would presumably act similarly at all stimulation intensities.

Figure Legends

Figure 1. Examples of whole-cell patch-clamp recordings during current- and voltage-clamp in the ferret LGN. (A) Hyperpolarizing and depolarizing voltage steps in current-clamp allow for the activation of low-threshold calcium currents and the measurement of action potential onset, threshold, amplitude, width and frequency. (B) The high signal to noise ratio of whole-cell patch-clamp recordings enable the detection of spontaneous and (C) evoked miniature synaptic currents.

Figure 2. Histograms of indicative parameters do not suggest more than one population of cell type. (A) Action potential width is tightly, and relatively normally, distributed around 1.2 ms with no obvious secondary peaks. (B) As with most measured values, action potential frequency is somewhat skewed toward larger values. The skew is light, however, and does not suggest a distinct subpopulation. (C) Soma size, as assayed by membrane capacitance is broadly distributed. The breadth of the distribution may reflect the two populations of relay cells that are known to have significantly different soma sizes: X cells and Y cells.

Figure 3. Cellular current kinetics and amplitudes are correlated with access resistance which, in turn, is correlated with age. (A) The rise times of sEPSCs become significantly longer, and (B) their amplitudes significantly smaller, with increasing access resistance. (C) The filtering effect of access resistance is even more pronounced during current-clamp measurements of action potential amplitude. (D) While extremely variable, access resistance is strongly correlated with age.

Figure 4. Robust peaks in the histograms are detected by the standarized cumulative probability distributions. Histograms for the (meaningless) inter-event intervals of (A) young and (B) old evoked, miniature EPSCs. The peak in the histograms of emEPSCs is an artifact of the detection software, but is robust and (C) shown clearly as a "kink" in the associated cumulative probability distribution. The cumulative probability distributions, then, can be used to detect substantially different distributions which may, in some cases, be due to multiple peaks.

Figure 1

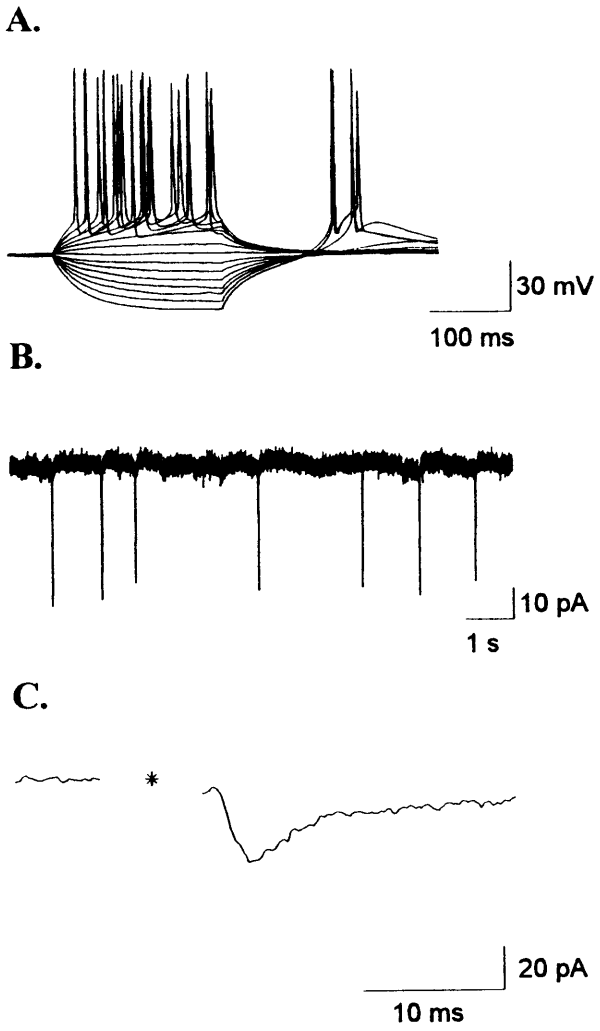


Figure 2

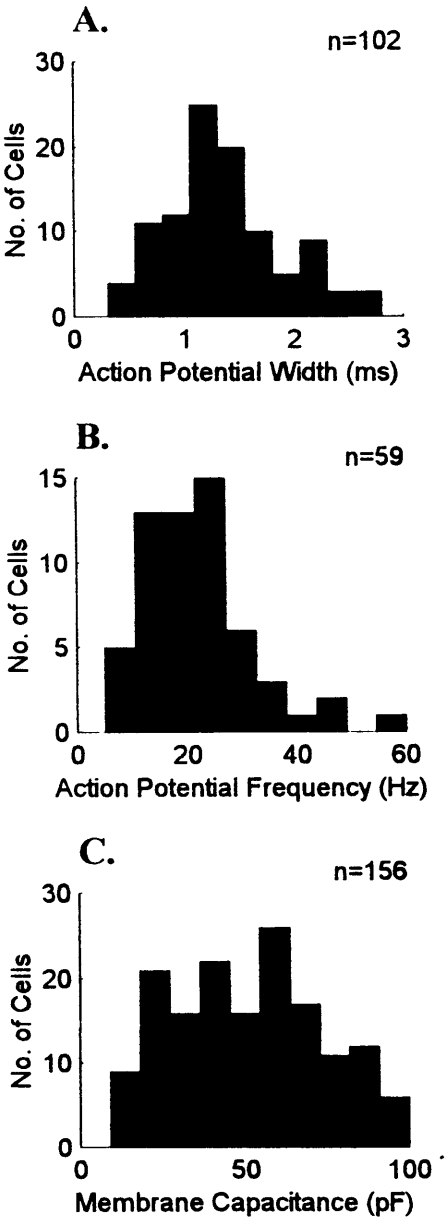

A.
n=102
30
20
10
0
No. of Cells
0
1
2
3
Action Potential Width (ms)
B.
n=59
15
10
5
0
No. of Cells
0
20
40
60
Action Potential Frequency (Hz)
C.
n=156
30
20
10
0
No. of Cells
0
50
100
Membrane Capacitance (pF)

Figure 3

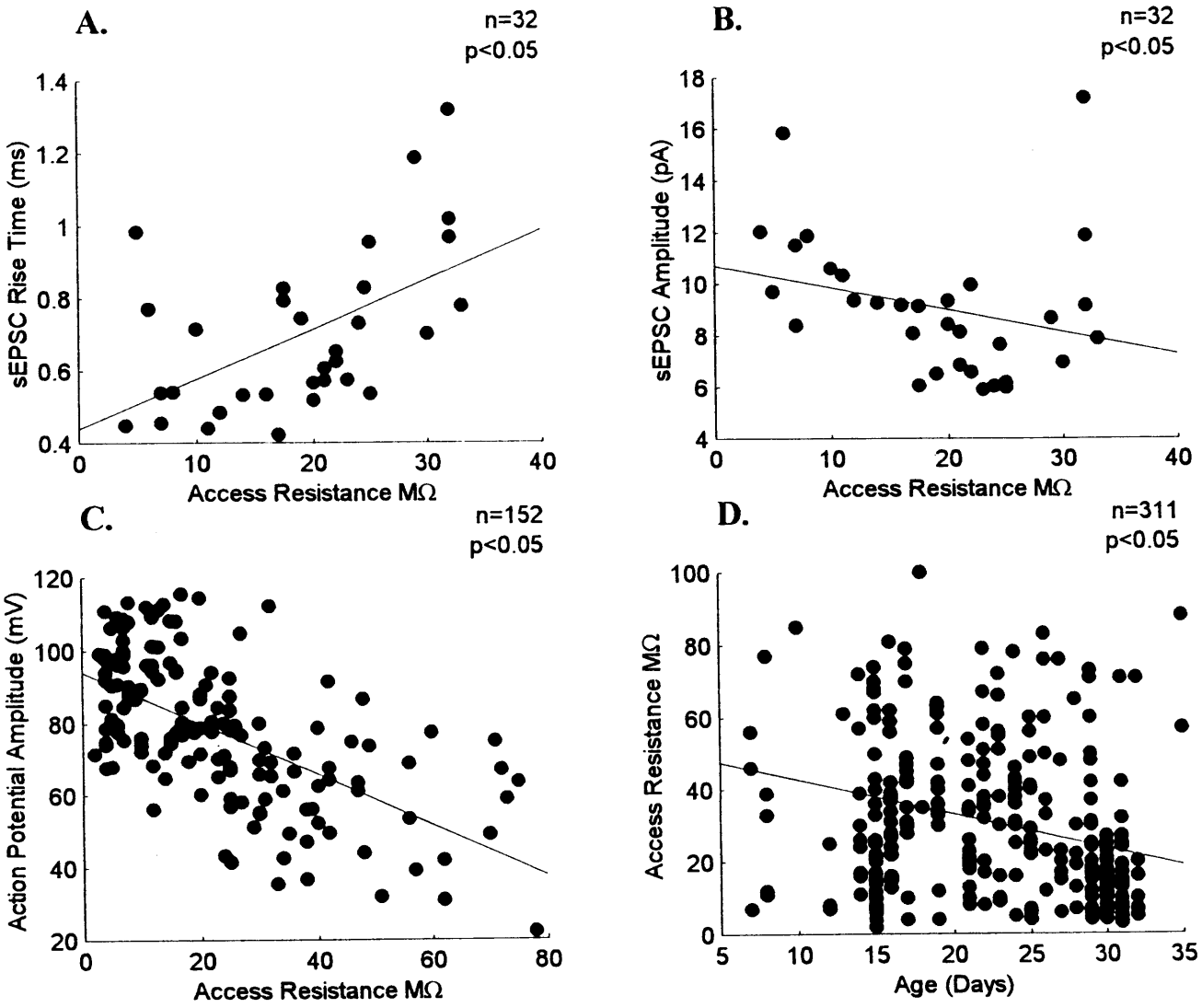
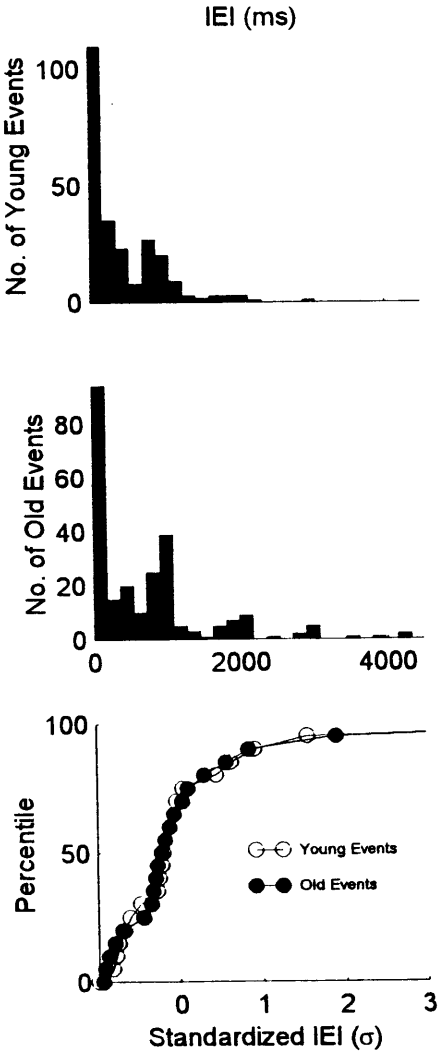

A.
n=32
p<0.05
sEPSC Rise Time (ms)
Access Resistance MΩ
B.
n=32
p<0.05
sEPSC Amplitude (pA)
Access Resistance MΩ
C.
n=152
p<0.05
Action Potential Amplitude (mV)
Access Resistance MΩ
D.
n=311
p<0.05
Access Resistance MΩ
Age (Days)

Figure 4


IEI (ms)
No. of Young Events
100
50
0
No. of Old Events
80
60
40
20
0
0
2000
4000
Percentile
100
50
0
Young Events
Old Events
0
1
2
3
Standardized IEI (σ)

Chapter Three: The Development of Relay Cell Membrane Properties during the Development of ON/OFF Sublamination in the Ferret Lateral Geniculate Nucleus

Abstract

In the ferret, ganglion cell axons from each of the retinae project to each of the lateral geniculate nuclei (LGNs) of the thalamus. The retinogeniculate axons from each eye initially overlap, but subsequently segregate into laminae that only receive inputs from either the left or the right eye. Shortly thereafter, retinogeniculate axons segregate further to form sublaminae that consist only of axons from either ON-center or OFF-center retinal ganglion cells; both events require neural activity. It has been hypothesized that this and other activity-dependent development of neural connectivity relies on long-term changes in synaptic efficacy. It is of interest, then, to carefully explore the development of the membrane properties of target cells in order to determine the contribution of any change in synaptic efficacy to information transfer (i.e., membrane depolarization). I explored the development of membrane properties of relay cells in the LGN during ON/OFF sublamination. Action potential threshold and frequency are stable during this period. While resting membrane potential becomes slightly, but significantly more hyperpolarized at the end of ON/OFF sublamination, the difference between it and action potential threshold was not significantly changed. While action potential and low-threshold calcium current amplitudes increase, and input resistance decreases, during this period, most membrane properties have reached mature values. The stability of relay cell membrane properties during ON/OFF sublamination suggests that changes in synaptic efficacy during this period of axon reorganization would result in a proportional change in the contribution of single inputs to information transfer.

Introduction

The mammalian visual system is characterized by extremely precise and patterned connectivity (for review, see Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Hohnke and Sur, 1999b). For example, during the first four postnatal weeks in ferrets, retinogeniculate axons segregate first into eye-specific laminae (Linden et al.,

1981) and, subsequently within each of the eye-specific laminae, into sublaminae that receive input from either ON-center or OFF-center retinal ganglion cells (Stryker and Zahs, 1983; Hahm et al., 1991). The development of the retinogeniculate circuitry is dependent on neural activity during this period (Shatz and Stryker, 1988; Cook et al., 1996; Cramer and Sur, 1997; Penn et al., 1998). More specifically, the formation of the ON/OFF sublaminae requires NMDA receptor activation (Hahm et al., 1991) and nitric oxide (NO) production (Cramer et al., 1996).

Neural activity, NMDA receptor activation and NO production are also involved in many types of long-term potentiation (LTP) of synapses (for review see Bliss and Collingridge, 1993). Consequently, it has been noted that LTP in the CNS may mediate the rearrangement of neural circuitry that occurs in many systems in early development (Constantine-Paton, et al., 1990; Kandel and O'Dell, 1992; Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Constantine-Paton and Cline, 1998). Indeed, at the neuromuscular junction changes in connectivity are preceded by experience-driven changes in synaptic efficacy (Colman et al., 1997).

In addition to identifying "appropriate" synaptic connections during development, LTP would act to modulate information transfer between cells. This modulation can occur as a uniform increase in postsynaptic responses (Selig et al., 1999) or as changes in the frequency dependence of postsynaptic responses (Markram et al., 1998). In any case, in the context of information transfer, synaptic efficacy should be considered relative to the voltage deflection required to initiate an action potential in the postsynaptic cell. That is, other things being equal, potentiated synapses are presumably

more capable of driving the target cell. However, it is not clear whether other things are, in fact, equal. The development of relay cell membrane properties during ON/OFF sublamination has not been examined in detail.

Cell membrane properties determine the relative contribution that a synaptic input makes to the generation of an action potential and thus, to information transfer. The most straightforward example of this is a developmental change in the action potential threshold. More relevant, however, is the difference between action potential threshold and resting membrane potential. Also important in determining the amount of current flux required to generate an action potential are the cell membrane's capacitance and resistance. All of these properties are known to change to various degrees during early development in numerous CNS areas and species (Ramoa and McCormick, 1994a and references therein; Vincent and Tell, 1997; Pirchio et al., 1997; Guido et al., 1998)

Here I investigate the development of the membrane properties of LGN relay cells during the period when retinal axons are segregating into ON/OFF sublaminae. While some membrane properties continue to mature throughout ON/OFF sublamination, the difference between resting membrane potential and action potential threshold was not significantly changed. These findings provide a context for understanding the impact of developmental changes in synaptic efficacy during activity-dependent development.

Methods

The data included in this chapter were acquired over a range of experimental sets. Both the potassium and cesium-based solutions were used. There were no group differences in

the membrane properties recorded with the two solutions[1]. In some experiments 50 μM BMI was added to the bath, but, given that the data presented in this chapter is independent of synaptic currents, its presence or absence would not affect the results.

Action potential thresholds were determined at the point at which the derivative of the voltage exceeded a threshold. Action potential width was calculated at 50% amplitude and action potential frequency was calculated by dividing the number of action potentials by the time of depolarization rather than by calculating the first inter-spike interval.

Results

I recorded from 318 relay cells in the A or A1 laminae of the LGN. Of these, ~60% (187) met criteria[2] for inclusion in the data analysis I present in this section. These cells were evenly distributed in age throughout, and just subsequent to, ON/OFF sublamination. Cells at all ages responded to a series of current injections with low-threshold and high-threshold action potentials (Figure 1). A small number of cells were younger than postnatal day 14 (P14). I present the data from those cells for completeness, but limit discussion to postnatal week three (PW3) through PW5.

The resting membrane potential of relay cells in the ferret LGN becomes

[1] Of course the cesium-based solution impeded re-polarization following an action potential. Consequently, those recordings were excluded from analyses of action-potential frequency. Also see Chapter Four (i.e., Hohnke and Sur, 1999a) which presents a quantitative discussion of the equivalence of sEPSCs recorded with different intracellular solutions.

[2] See Chapter Two.

significantly more hyperpolarized after the completion of ON/OFF sublamination. While quite variable, resting membrane potentials are significantly correlated with age (r_s = -0.22, n = 187, $p < 0.05$; Figure 2A) during this period. Values are stable between PW2 through PW4 (e.g., -50, -51 ± 9 mV, n = 40 for PW4), but hyperpolarize significantly after the completion of ON/OFF sublamination (-57, -57 ± 10, n = 92, $Q_{PW4\ vs.\ PW5}$ = -3.07, $p < 0.05$; Figure 2B).

One possible cause of the depolarized resting membrane potentials in younger cells is that, given smaller soma sizes at these ages, the seal between the recording pipette and the cell membrane is looser. Consequently, the resting membrane potential will depolarize as ions escape into the extracellular matrix. This would result in the resting membrane potential being negatively correlated with τ, where τ is the membrane time constant, i.e., the input resistance multiplied by the membrane capacitance[3]. However, resting membrane potential does not correlate with τ in these recordings (r_s = 0.2, $p > 0.05$; Figure 2C), confirming that differences in recording configurations do not account for the hyperpolarization observed after ON/OFF sublamination.

I also investigated the development of τ during ON/OFF sublamination. τ is correlated with age (rs = -0.36, $p < 0.05$, n = 59; Figure 3A) falling from a median value of 0.014 (0.015 ± 0.009, n = 22) at PW3 to 0.008 (0.0132 ± 0.020, n = 25) at PW5

[3] See Chapter Two for a description of how input resistance and membrane capacitance were determined. In effect, the measured parameter, τ, more accurately reflects the *cell* time constant rather than the membrane time constant. That is, the activation of voltage-gated channels during the measurement was not accounted for. However, it is precisely the development of the behavior of the whole cell that is of interest in these studies so the measurement serves the intended purpose. I've chosen not to modify the term

(Figure 3B). The decline in τ was due almost entirely to a significant decrease in cell input resistance. Input resistance was highly variable, but strongly correlated with age (r_s = -0.54, $p < 0.05$, n = 68; Figure 3C), decreasing 53% between PW3 (305, 425 ± 335 MΩ, n = 22) and PW5 (143, 212 ± 292 MΩ, n = 33, $Q_{PW3\ vs\ PW5}$ = -3.81, $p < 0.05$; Figure 3D). In contrast, relay cell membrane capacitance has reached adult values near the beginning of ON/OFF sublamination and does not correlate with age during this period (r_s = 0.06, n = 156, $p > 0.05$). Membrane capacitance during PW3 was 52 fC (50 ± 22 fC, n = 48) and it held steady through PW5 (54, 53 ± 22, n = 77, $Q_{PW3\ vs\ PW5}$ = 0.78, $p > 0.05$).

The depolarization required to bring relay cell membrane potentials from rest to action potential threshold (V_d) is stable throughout ON/OFF sublamination. The difference between the two values does not correlate with age during this period (r_s = -0.14, n = 123, $p > 0.05$; Figure 4A). The required depolarization at PW3 is 13 mV (14 ± 7 mV, n = 35), which is not significantly different from that required at PW5 (18, 16 ± 11 mV, n = 67, $Q_{PW3\ vs\ PW5}$ = -1.66, $p > 0.05$; Figure 4B). The slight, but non-significant increase in V_d observed during PW4 is a result of a significant "dip" in the action potential threshold that occurs at the same time. The membrane potential at which action potentials are triggered does not correlate with age during retinogeniculate axon reorganization (r_s = -1.67, n = 123, $p > 0.05$), but does depolarize significantly during PW4 (-33, -30 ± 13 mV, n = 17) and subsequently rebounds during PW5 (-41, -40 ± 9

("membrane time constant"), but rather note here that the meaning I apply to it is somewhat different from

mV, n = 67, $Q_{PW4\ vs\ PW5}$ = -3.22, $p < 0.05$) to PW3 values (-41, -38 ± 8 mV, n = 35, $Q_{PW3\ vs\ PW5}$ = -1.54, $p > 0.05$; Figure 4C,D).

Action potential amplitude increases during ON/OFF sublamination while action potential width rebounds to initial values after a decline. Action potential amplitudes are quite variable at all ages, but are significantly correlated with age throughout PW2 to PW5 (r_s = 0.26, n = 123, $p < 0.05$; Figure 5A). The increase in action potential amplitude comes at the completion of ON/OFF sublamination (91, 88 ± 18 mV, n = 67) after remaining stable throughout that period (PW3: 78, 76 ± 15 mV, n = 35, $Q_{PW3\ vs\ PW5}$ = 3.24, $p < 0.05$; PW4: 71, 73 ± 16 mV, n = 17, $Q_{PW3\ vs\ PW4}$ = -0.77, $p > 0.05$; Figure 5B). Action potential width, like action potential threshold, does not change monotonically with age during ON/OFF sublamination (r_s = -0.05, n = 102, $p > 0.05$; Figure 5C). There is, however, a narrowing of action potential width during PW4 (0.9, 1.0 ± 0.4 ms, n = 14) relative to PW3 (1.6, 1.6 ± 0.7 ms, n = 30, $Q_{PW3\ vs\ PW4}$ = -3.17, $p < 0.05$) which subsequently rebounds during PW5 (1.3, 1.4 ± 0.5 ms, n = 57, $Q_{PW3\ vs\ PW5}$ = -1.66, $p > 0.05$; Figure 5D).

Both high-threshold and low-threshold action potential frequencies increase in response to increasing current injection intensities. While the increase in action potential frequency is similar for both younger (r_s = 0.50, n = 163, $p < 0.05$) and older cells (r_s = 0.32, n = 316, $p < 0.05$; Figure 6A1), older cells consistently generate more low-threshold action potentials (r_s = 0.61, n = 359, $p < 0.05$) than younger cells (r_s = 0.54, n = 239, p <

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what is ususal.

0.05) with any given current injection (Figure 6B1). As was to be expected, high-threshold, but not low-threshold, action-potential onset time was inversely proportional to the magnitude of current injection. The high-threshold action potential onset times of younger cells and older cells are correlated with increasing current injections (r_s = -0.32, n = 124, r_s = -0.35, n = 218, respectively, both $p < 0.05$), but do not differ from each other ($p > 0.05$, Kolmogorov-Smirnov test; Figure 7A,B). In contrast, low-threshold action-potential onset times for neither younger cells (r_s = 0.1, n = 85, $p > 0.05$) nor older cells (r_s = 0.01, n = 184, $p > 0.05$) are correlated with the intensity of the current injection (Figure 7C,D).

Discussion

While the development of electrophysiological properties of relay cells in the ferret LGN have been examined previously (White and Sur, 1992; Ramoa and McCormick, 1994a), I have contributed a much more detailed account of the period surrounding ON/OFF sublamination. This section will focus on comparison with the pervious studies of the developing ferret LGN. For comparison of the development of electrophysiological properties of LGN relay cells with cells in other areas of the nervous system, see the discussion in Ramoa and McCormick (1994a).

Previous studies in ferret LGN do not report a developmental decrease in resting membrane potential at the beginning of PW5 (White and Sur, 1992; Ramoa and McCormick, 1994a). These differences are likely due to the limited number of recordings in those studies from cells during PW5 (n ≅ 7 in White and Sur [1992] and n ≅

8 in Ramoa and McCormick [1994] compared to n = 92 in the present study) and the notable variability of the values recorded. On the whole, however, the resting membrane potentials observed in all studies were similar to each other[4] and to adult values (i.e., -62 mV, Esguerra et al., 1992).

The variability and values of, and trend in, input resistances are consistent with previous reports (White and Sur, 1992; Ramoa and McCormick, 1994a). Values are difficult to relate to those in the adult because that study (Esguerra et al., 1992) used sharp electrodes causing a several-fold decrease in apparent input resistance. Cell membrane capacitance was not reported in any previous study of ferret LGN relay cells. The membrane time constant, τ, however, was, and because τ is the product of cell membrane capacitance and input resistance, which was also reported, membrane capacitance can be inferred. White and Sur (1992) report no change in τ and a slight decrease in input resistance between P10 and P33 suggesting little or no increase in membrane capacitance. Likewise, the data presented by Ramoa and McCormick (1994a) suggest no change in τ after P10 and little or no decrease in input resistance suggesting little or no increase in membrane capacitance. Those findings are consistent with the non-significant increase in membrane capacitance that I report here.

Action potential threshold is reported to decrease in the first two postnatal weeks

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[4] Although slightly more depolarized in the current study (-57 mV) compared to White and Sur (1992; -62 mV) and Ramoa and McCormick (1994a; ≅-62 mV). It is likely that this is partly due to the difference in inclusion criteria between the studies (-50 mV in White and Sur [1992] and –40 mV here), and partly to the slightly higher potassium content of the intracellular solution used here compared with that used in Ramoa and McCormick (1994a). White and Sur (1992) used sharp electrodes as opposed to the whole-cell

and appears to hold steady between PW3 and PW5 (Ramoa and McCormick, 1994a). This finding is generally consistent with what I report, but does not include the slight, but significant "dip" in action potential threshold I observe during PW4. Again, the difference is likely due to the five-fold increase in the number of recordings I base my observations on, the variability in the parameter, and the quantitative analysis of values during ON/OFF sublamination; Ramoa and McCormick (1994a) present only qualitative results. Both studies are in agreement, however, that the difference between resting potential and action potential threshold before and after ON/OFF sublamination is the same.

All studies (i.e., White and Sur, 1992; Ramoa and McCormick, 1994a), including the current one, report that action potential amplitude increases with age. However, Ramoa and McCormick (1994a) report peak action potential amplitudes of ~70 mV, whereas I regularly measured peak amplitudes between 100 and 120 mV. White and Sur (1992) report a range of 36 to 98 mV. Data from Ramoa and McCormick (1994a) suggest little if any change in action potential width between PW3 and PW5, generally consistent with my finding. Here again, however, as with action potential threshold, I report a significant decrease and then rebound in action potential width at PW4 and PW5, respectively. White and Sur (1992) report a significant, negative correlation between age and action potential width which ranges from 6 to 1 ms. I measure action potential widths at no greater than 3 ms and as narrow as 0.3 ms. These difference are likely due

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electrodes used here and in Ramoa and McCormick (1994a) and so the intracellular solution, not diffusing

to the filtering effects of the sharp electrodes used by White and Sur (1992).

Numerous anatomical developments occur in ferret LGN relay cells throughout the formation of the ON/OFF sublaminae (Sutton and Brunso-Bechtold, 1991; Rocha and Sur, in preparation). These include changes in soma size, dendritic length and area, the number of dendritic spines and branch points, and the orientation of the dendritic arbor.

Somatic area and dendritic area, length, branch points and spine density increase markedly between birth and the end of PW1 (Sutton and Brunso-Bechtold, 1991; Rocha and Sur, in preparation). While the number of dendritic intersections generally increases during the first four postnatal weeks (Sutton and Brunso-Bechtold, 1991; Rocha and Sur, in preparation), there is a "dip" in this value during PW4 (Rocha and Sur, in preparation).

Relay cells can begin to be classified by P14 and have reached adult morphology by the beginning of PW5. The two classes of relay cells consist of those with large somata and large dendritic arbors and those with medium sized somata and smaller arbors (Sutton and Brunso-Bechtold, 1991; Rocha and Sur, in preparation). No developmental study of the electrophysiological properties of relays cells has distinguished between these two cell types. This may, in part, account for the variability in properties that I and others (White and Sur, 1992; Ramoa and McCormick, 1994a) observe. However, the relative, anatomical homogeneity of these cells until the end of ON/OFF sublamination may reflect a similar electrophysiological similarity between, what in the adult are, two separate classes of cells.

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as readily into the cell body, was less of a factor.

Interestingly, the detailed study of Rocha and Sur (in preparation) reports time-courses of anatomical change that are similar to ones that I observe during the development of electrophysiological properties. While, in most cases, the relationship between those anatomical and electrophysiological properties is unclear, it is nonetheless worthwhile to compare their maturation. Somatic area and dendritic area and length hold stable during PW3 and PW4 and subsequently increase at the beginning of PW5. I observe the same time-course for resting membrane potential and action potential amplitude. Similarly, the decrease seen in dendritic branch points reported by Rocha and Sur (in preparation) during PW4 and the subsequent rebound in PW5, mirrors the changes that I observe in action potential threshold and width.

Despite the important changes in both the physiological and anatomical properties of relay cells that occur during ON/OFF sublamination, the gap between their resting potential and their action potential threshold is maintained within a narrow window. As a consequence, the contribution of a constant synaptic input during this period has a constant relationship to information transfer.

Figure Legends

Figure 1. Examples of membrane properties in relay cells prior to and subsequent to ON/OFF sublamination. A series of current steps in a P14 (A) and a P30 (B) cell reveals the membrane properties of the cells. The same current injection protocol was used in both cells. Note the larger voltage deflections in the younger cell compared to the older cell which reflect the higher input resistances of younger cells. Sufficient depolarizing current injection elicits a train of action potentials both prior to (A, middle trace) and subsequent to (B, middle trace) ON/OFF sublamination. Action potentials in younger cells have smaller amplitudes than those generated in older cells. Hyperpolarizing current injections elicit low-threshold action potentials at all ages examined. However, fewer are generated prior to ON/OFF sublamination (A, bottom trace), than after (B, bottom trace). Arrows indicate low-threshold calcium currents.

Figure 2. The resting membrane potential of relay cells in the ferret LGN becomes significantly more hyperpolarized after the completion of ON/OFF sublamination. (A) A scatter plot of resting potential against age shows a significant, negative correlation embedded in a great deal of variability. The ceiling effect is due to the fact that only cells with resting potentials of –40 mV or lower were included in the analysis. (B) A bar graph of the mean and standard deviation of the resting potentials grouped by postnatal week shows that, while remaining stable during ON/OFF sublamination, the resting potential becomes more hyperpolarized after its completion (* = $p < 0.05$ relative to the preceding group). (C) Resting membrane potential is not negatively correlated with τ.

Consequently, differences in the membrane seals achieved with younger vs. older cells do not account for the age-related hyperpolarization of the resting membrane potential.

Figure 3. Measurement of passive membrane properties during ON/OFF sublamination. (A) The membrane time constant, τ, decreases with age. (B) The decrease occurs primarily during PW3 and PW4 and (C) the decrease in τ is principally due to a parallel decrease in the input resistance during this period. (D) Relay cells have input resistances with adult values by the fourth postnatal week (* = $p < 0.05$ relative to the preceding group).

Figure 4. The difference between action potential threshold and resting membrane potential (V_d) is held constant over ON/OFF sublamination. (A) V_d is not correlated with age. (B) Although there is an increase in V_d prior to PW3, it remains constant thereafter. (C) The slight, but non-significant increase in V_d during PW4 is the result of a significant "dip" in the action potential threshold that occurs at the same time. (D) Examples of action potentials elicited at the beginning of PW3, PW4, and PW5. Action potential threshold depolarizes between PW3 and PW4, but returns to initial values by PW5 (* = $p < 0.05$ relative to the preceding group).

Figure 5. Action potential amplitude increases during ON/OFF sublamination while action potential width rebounds to initial values after a decline. (A) While quite variable, action potential amplitude is significantly, positively correlated with age throughout PW2 through PW5. (B) The increase in action potential amplitude comes at the completion of ON/OFF sublamination after remaining stable throughout that period. (C) Action

potential width does not change monotonically with age during ON/OFF sublamination. (D) There is, however, a narrowing of action potential width during PW4 which subsequently rebounds to initial values (* = $p < 0.05$ relative to preceding group).

Figure 6. Action potential frequency, both high-threshold and low-threshold, increases in response to increasing current injection intensities. (A1) Younger cells (filled circles, bottom best-fit line, $p < 0.05$) and older cells (unfilled circles, top best-fit line, $p < 0.05$) generate action potentials in proportion to the magnitude of current injections (normalized current is the current injected divided by the magnitude of the first current step). (A2) Example traces of increasing (bottom to top) current injections from a P14 and (A3) a P30 cell. (B1) Similarly, both younger and older cells generate an increased number of low-threshold action-potentials with increased current injection. However, older cells consistently generate more with any given current injection. (B2) Example traces of increasing (top to bottom) current injections from a P14 and (B3) a P30 cell.

Figure 7. High-threshold, but not low-threshold, action-potential onset time is inversely proportional to the magnitude of current injection. (A) The high-threshold action potential onset times of younger cells (filled circles, top best-fit line, $p < 0.05$) and older cells (unfilled circles, bottom best-fit line) are correlated with increasing current injections, but do not differ from each other. Indeed, the cumulative distributions of onset times overlap (A, inset; $p > 0.05$). (B) Onset times of high-threshold action potentials for both younger (filled bars) and older (unfilled bars) cells are significantly reduced with current injections larger than 200 pA. (C) In contrast, low-threshold action-potential onset times are not correlated with the intensity of the current injection.

Younger and older cells behave similarly (C, inset; $p > 0.05$). (D) At no block of current intensities do the onset times of low-threshold action potentials of younger and older cells differ (* = $p < 0.05$ relative to first group).

Figure 1

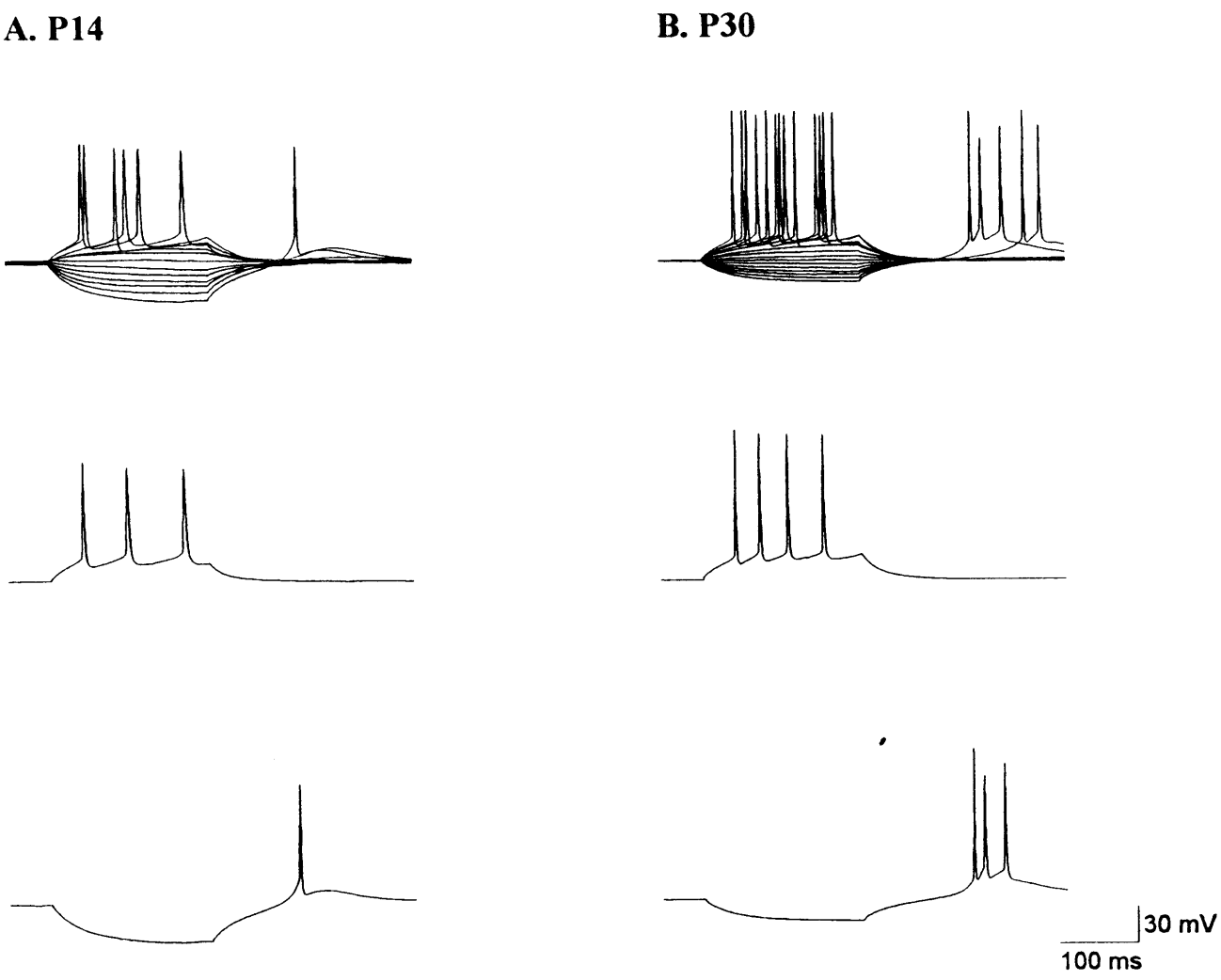


Figure 2

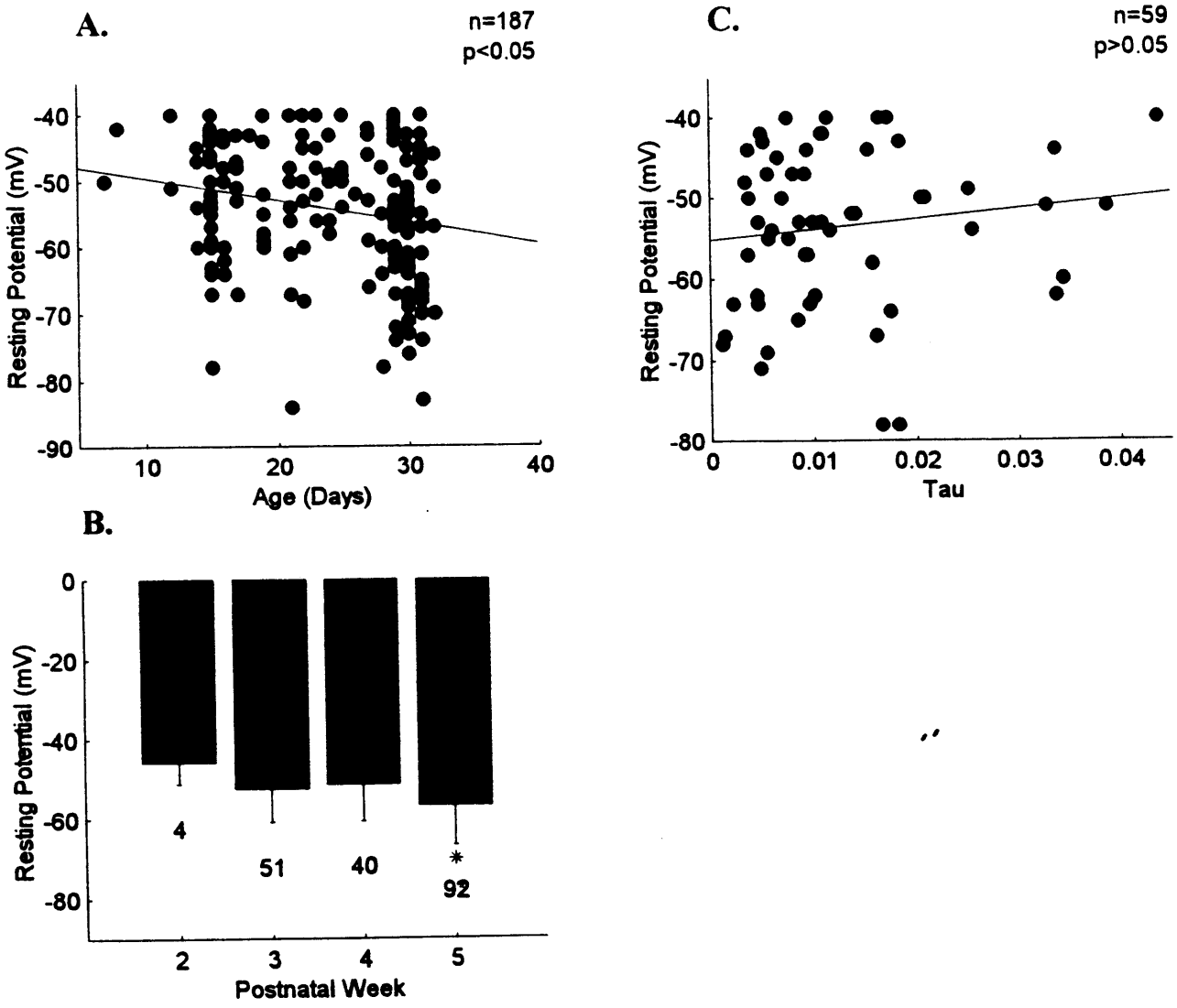

A.
n=187
p<0.05
Resting Potential (mV)
-40
-50
-60
-70
-80
-90
10
20
30
40
Age (Days)
C.
n=59
p>0.05
Resting Potential (mV)
-40
-50
-60
-70
-80
0
0.01
0.02
0.03
0.04
Tau
B.
Resting Potential (mV)
0
-20
-40
-60
-80
4
51
40
92
*
2
3
4
5
Postnatal Week

Figure 3

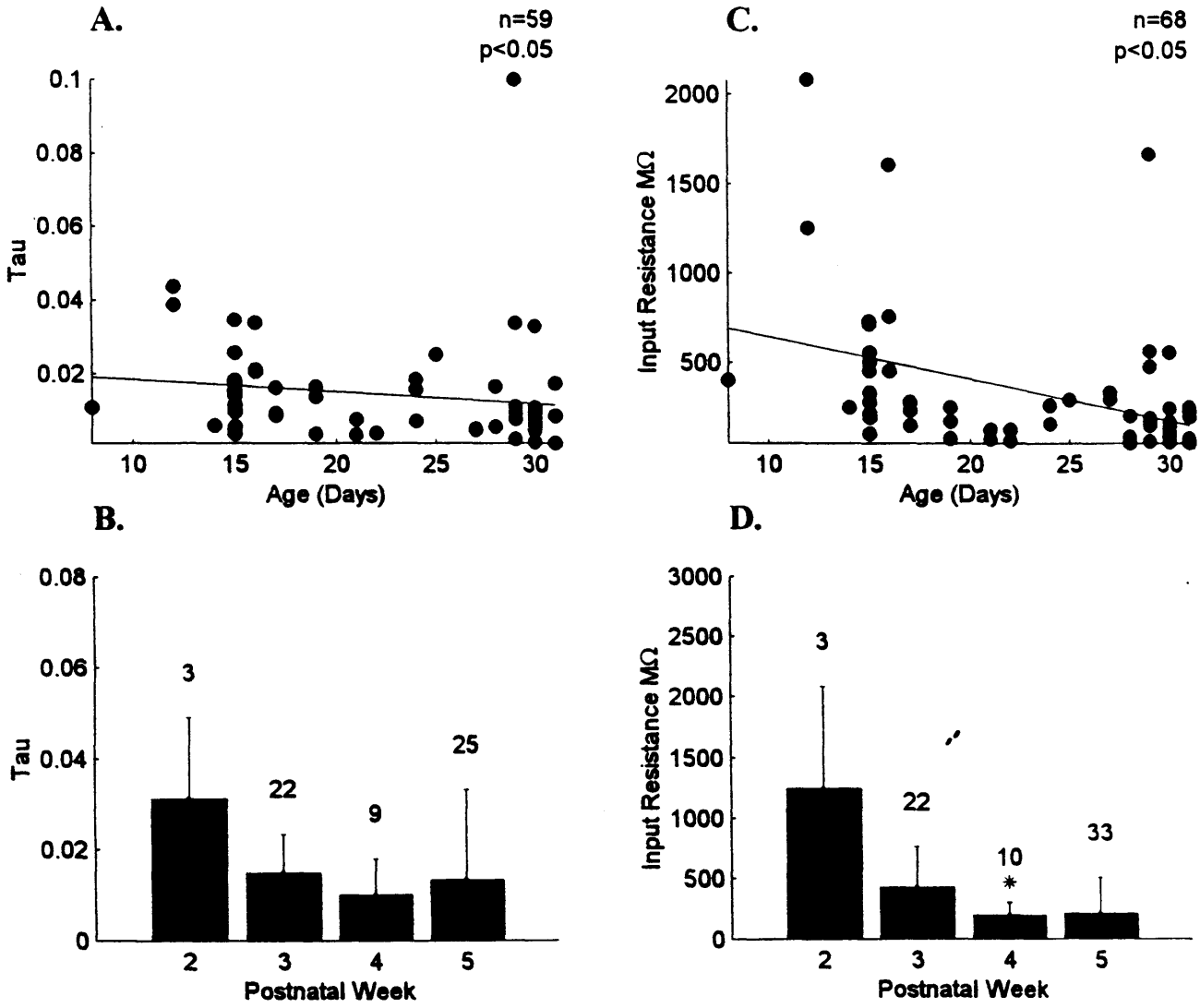

A.
n=59
p<0.05
Tau
Age (Days)
B.
Tau
3
22
9
25
Postnatal Week
C.
n=68
p<0.05
Input Resistance MΩ
Age (Days)
D.
Input Resistance MΩ
3
22
10
33
Postnatal Week

Figure 4

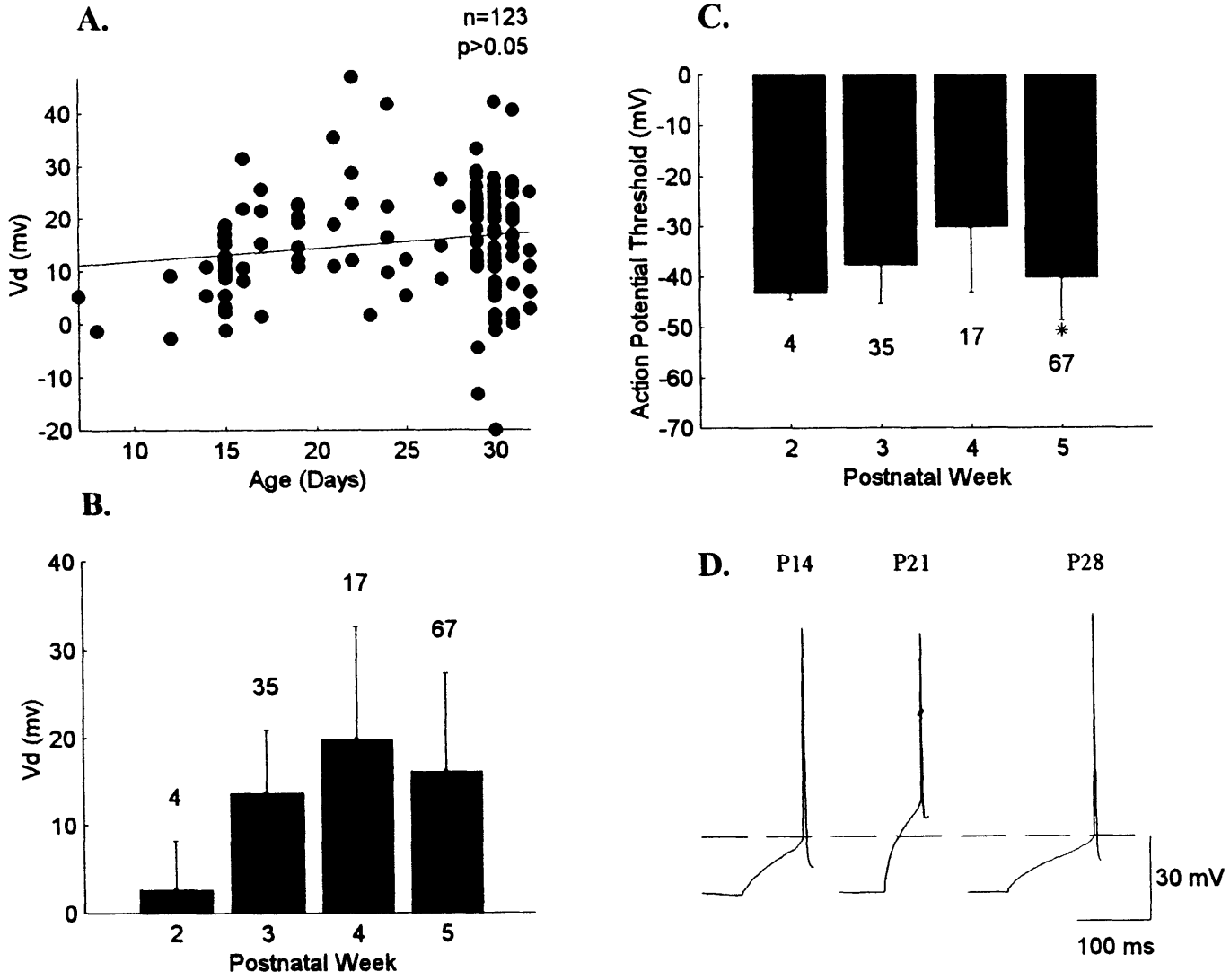

A.
n=123
p>0.05
Vd (mv)
Age (Days)
B.
Vd (mv)
Postnatal Week
4
35
17
67
C.
Action Potential Threshold (mV)
Postnatal Week
D.
P14
P21
P28
30 mV
100 ms

Figure 5

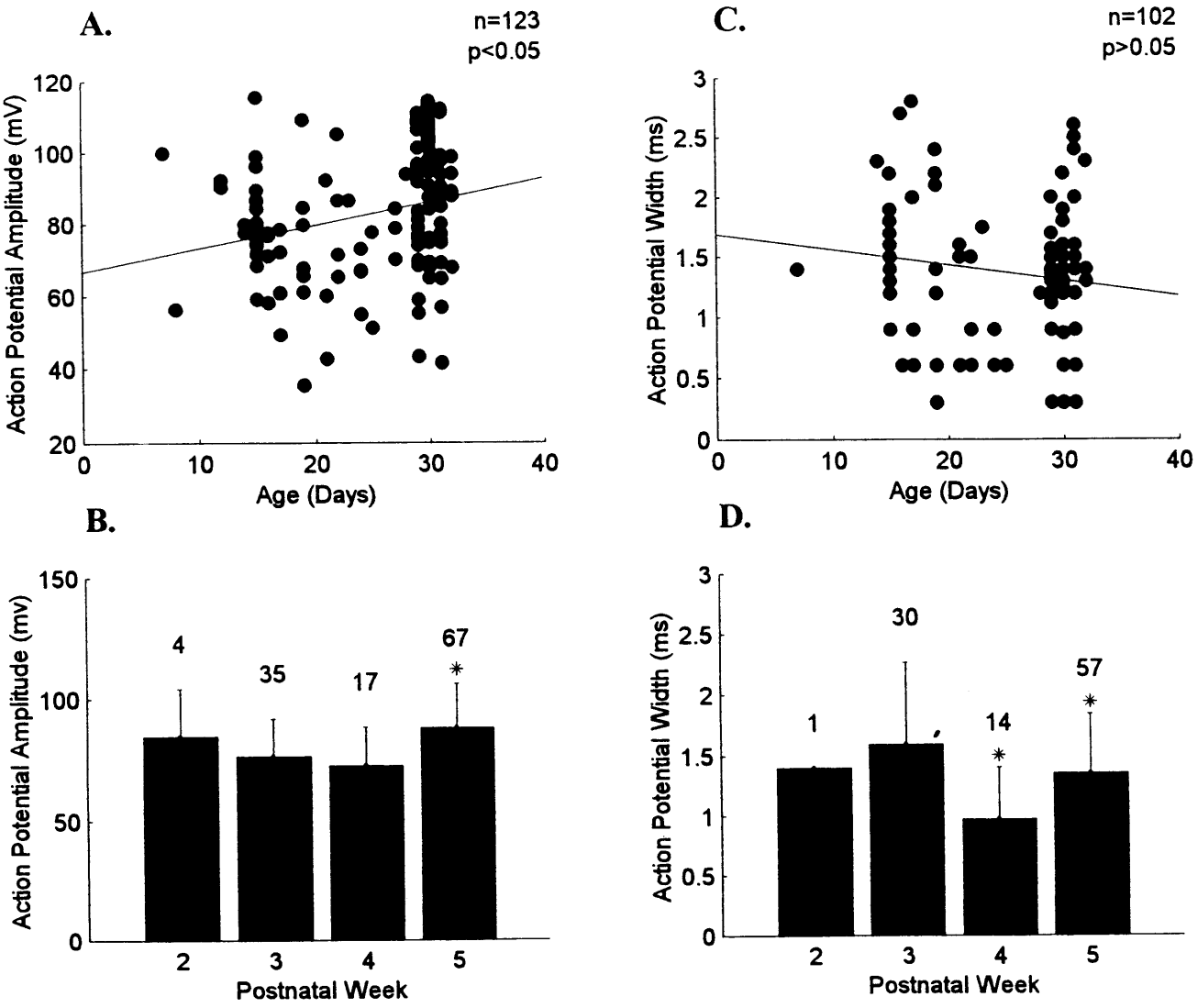

A.
n=123
p<0.05
Action Potential Amplitude (mV)
Age (Days)
C.
n=102
p>0.05
Action Potential Width (ms)
Age (Days)
B.
Action Potential Amplitude (mv)
4
35
17
67
Postnatal Week
D.
Action Potential Width (ms)
1
30
14
57
Postnatal Week

Figure 6

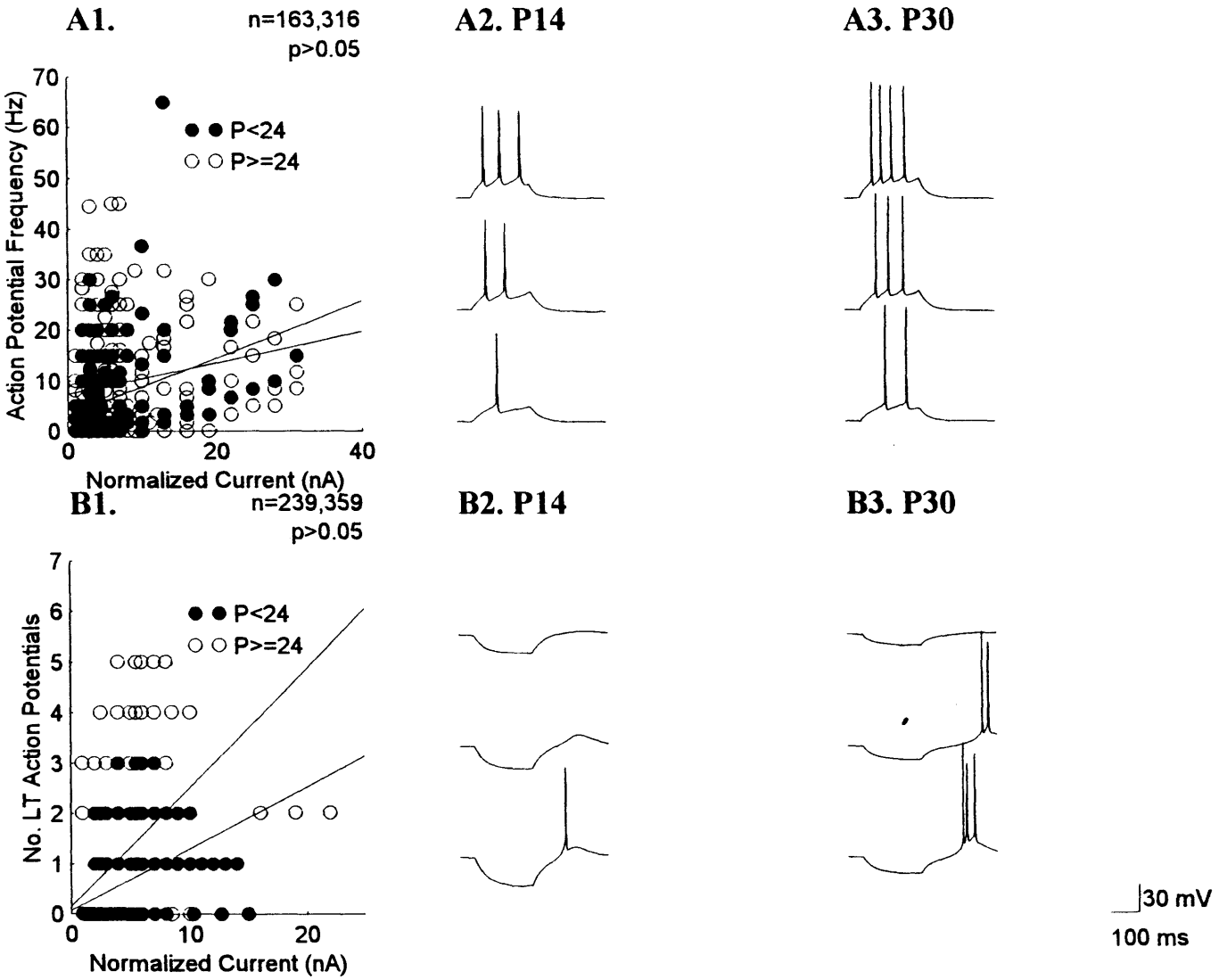
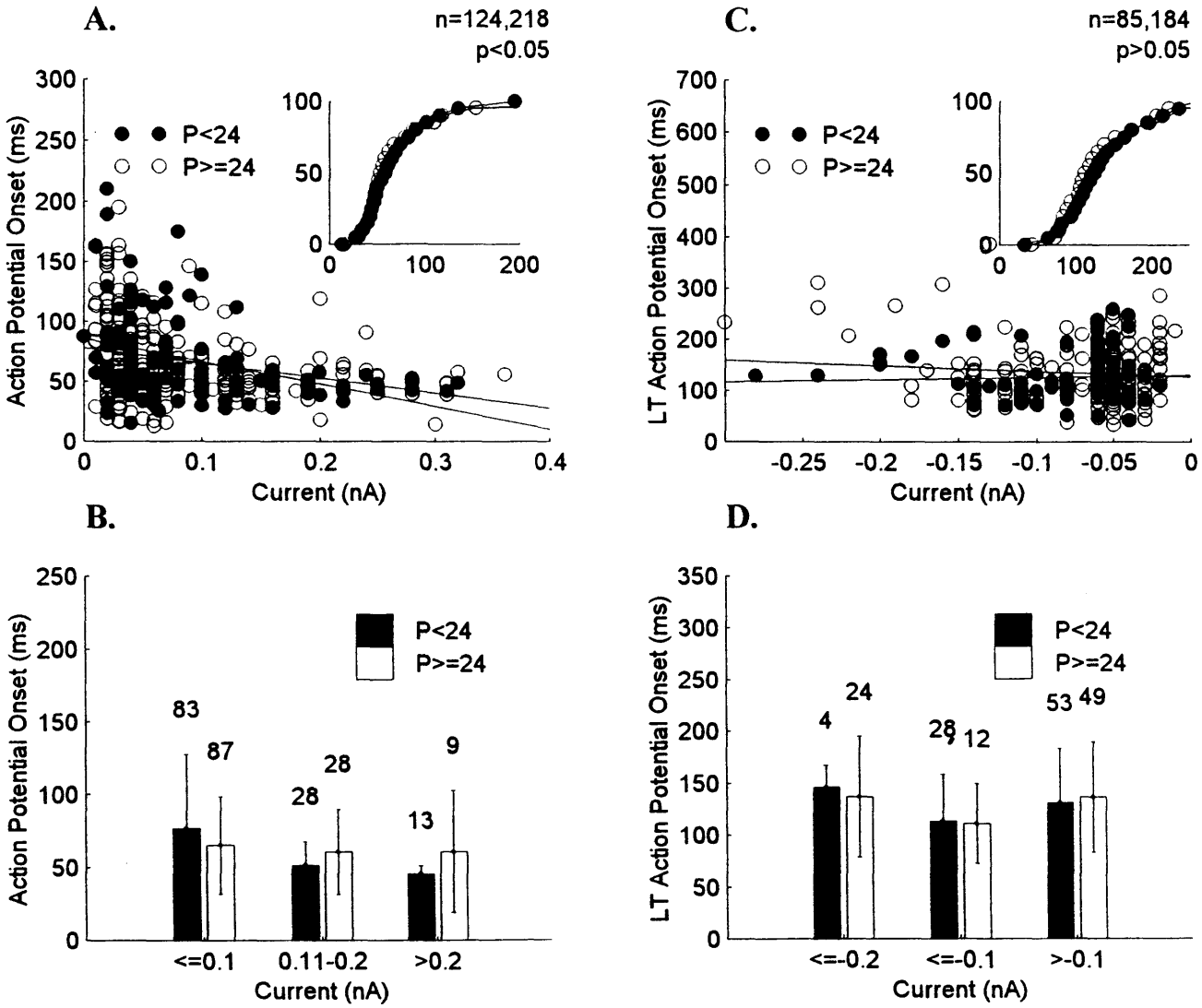

A1.
n=163,316
p>0.05
Action Potential Frequency (Hz)
P<24
P>=24
Normalized Current (nA)
A2. P14
A3. P30
B1.
n=239,359
p>0.05
No. LT Action Potentials
P<24
P>=24
Normalized Current (nA)
B2. P14
B3. P30
30 mV
100 ms

Figure 7


A.
n=124,218
p<0.05
P<24
P>=24
Action Potential Onset (ms)
Current (nA)
C.
n=85,184
p>0.05
LT Action Potential Onset (ms)
Current (nA)
B.
Action Potential Onset (ms)
P<24
P>=24
83
87
28
28
13
9
<=0.1
0.11-0.2
>0.2
Current (nA)
D.
LT Action Potential Onset (ms)
P<24
P>=24
4
24
28
12
53
49
<=-0.2
<=-0.1
>-0.1
Current (nA)

Chapter Four: Stable Properties of Spontaneous EPSCs and Miniature Retinal EPSCs during the Development of ON/OFF Sublamination in the Ferret Lateral Geniculate Nucleus

Abstract

Retinal projections to the lateral geniculate nucleus (LGN) in ferrets progressively segregate into eye-specific laminae and subsequently into sublaminae that receive inputs from ON-center and OFF-center afferents. In order to study the development of synaptic efficacy during a period of activity-dependent growth and reorganization in the CNS, I recorded spontaneous excitatory postsynaptic currents (sEPSCs) from cells of the LGN during ON/OFF sublamination. We also examined retinal inputs specifically by stimulating the optic tract in the presence of strontium and recording evoked miniature EPSCs (emEPSCs). The rise times, areas, half-widths and decay times of sEPSCs and emEPSCs, and inter-event intervals of sEPSCs, recorded at the beginning of ON/OFF sublamination were not different from those recorded after its completion. Typically EPSC areas were small (10-20 fC), but varied greatly both within and between neurons. The frequency of sEPSCs was also quite variable, ranging from 0.2 to 5 Hz. sEPSCs were equivalent to miniature EPSCs recorded in the presence of tetrodotoxin and both sEPSCs and emEPSCs were CNQX sensitive. No difference was observed between sEPSCs recorded at room temperature and those recorded at 34°C, and strontium could be substituted for calcium with no effect on sEPSC shape. These data argue for a remarkable stability in the components of at least AMPA-mediated synaptic transmission during a period of major synaptic rearrangement in the LGN.

Introduction

The development of precise connectivity in the mammalian visual system depends on normal neural activity (for reviews see Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996). For example, the segregation of left and right eye inputs in the visual cortex is disrupted after neonatal lid suture (Sherman and Spear, 1982) or intraocular injections of tetrodotoxin (TTX; Stryker and Harris, 1986). Activity is also

crucial to the development of the appropriate wiring in structures involved in earlier stages of visual processing. In the ferret, retinogeniculate axons from the two eyes segregate during development to form eye-specific layers (Linden et al., 1981). Subsequently, within each of the eye-specific layers, inputs from ON-center and OFF-center retinal ganglion cells segregate to form sublaminae (Stryker and Zahs, 1983; Hahm et al., 1991). Segregation of retinal inputs into eye-specific laminae is modulated by activity (Shatz and Stryker, 1988; Cook et al., 1996; Penn et al., 1998), and the segregation into ON/OFF sublaminae is disrupted entirely by intraocular TTX injections (Cramer and Sur, 1997), inactivation of NMDA receptors (Hahm et al., 1991), or nitric oxide blockade (Cramer et al., 1996).

Not only is normal neural activity critical for appropriate retinogeniculate axon development, it is also involved in the development of its major postsynaptic target, the relay cells of the lateral geniculate nucleus (LGN). When d-APV, an NMDA receptor antagonist, is infused into the thalamus during the third postnatal week, LGN relay cells show an increase in dendritic branching and in the number of dendritic spines (Rocha and Sur, 1995). Likewise, intracranial infusions of TTX in fetal cats cause an increase in the density of dendritic spines (Dalva et al., 1994). Later in development, after eye-opening, the normal elimination of transient dendritic spines is delayed following early eye enucleation (Sutton and Brunso-Bechtold, 1993).

Activity-dependent development of connections in the visual system has components that seem to be shared with long-term potentiation, or LTP, of synapses in the CA1 region of the hippocampus (Shatz, 1990; Goodman and Shatz, 1993; Cramer and

Sur, 1995). For example, both phenomena require patterned electrical activity, but may also require NMDA receptor activation, nitric oxide production (Bliss and Collingridge, 1993) and neurotrophin signaling (Cabelli et al., 1995; Kang and Schuman, 1995). Consequently, it has been suggested that LTP and its counterpart, long-term depression (LTD), underlie the stabilization and withdrawal of synapses in developing sensory structures (Constantine-Paton, et al., 1990; Kandel and O'Dell, 1992; Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Constantine-Paton and Cline, 1998). A similar mechanism may operate at the neuromuscular junction where changes in connectivity are preceded by experience-driven changes in synaptic efficacy (Colman et al., 1997). Yet, exactly how changes in the wiring between two neurons are reflected by changes in synaptic transmission between them is not well understood.

There is, however, a growing body of evidence that suggests that NMDA receptor-mediated activity plays an important role in initiating the stabilization of appropriate inputs during development (for reviews see Hofer et al., 1994; Cramer and Sur, 1995; Constantine-Paton and Cline, 1998). NMDA receptors undergo developmental changes in their subunit composition in several regions of the nervous system (for review see Scheetz and Constantine-Paton, 1994) that coincide with decreases in NMDA-mediated currents at the end of periods of axonal reorganization (Shi et al., 1997; Ramoa and Prusky, 1997). The longer duration of NMDA receptor-mediated responses at immature synapses may facilitate the induction of a form of LTP expressed as an increase in the effectiveness of AMPA responses (Constantine-Paton and Cline, 1998).

Chapter Four: Stable Properties of Spontaneous and Miniature EPSCs

As a first step toward examining changes in AMPA-mediated synaptic transmission during a period of activity-dependent, axonal reorganization in the CNS, I have recorded spontaneous excitatory postsynaptic currents (sEPSCs) and optic tract-evoked miniature EPSCs (emEPSCs) in brain slices from ferrets of varying ages during the period of ON/OFF sublamination in the LGN; at the retinogeniculate synapse sEPSCs have been shown to be quantal and equivalent to mEPSCs (Paulsen and Heggelund, 1994). In the LGN dramatic changes in connectivity are thought to be occurring during ON/OFF sublamination. Retinal axons are refined in an activity-dependent manner during the third and fourth postnatal weeks (Cramer and Sur, 1996, 1997). Anatomical data from the cat suggest that the number of retinal synapses on LGN cells increases by about 25% during the first eight weeks postnatally (Kalil and Scott, 1979; Mason 1982a), while the number of cortical inputs increases eight-fold during the first ten weeks (Weber and Kalil, 1987). In addition to changes in NMDA receptor-mediated current and a possible increase in the efficacy of AMPA synapses, it is possible that developmental changes in the subunit composition of AMPA receptors occur during ON/OFF sublamination as they do in other regions of the nervous system (Durand and Zukin, 1993; Rossner et al., 1993; Jakowec et al, 1995). Thus, there are a number of reasons to expect changes in sEPSCs and emEPSCs over this period.

Surprisingly, I find no change in the properties of AMPA-mediated spontaneous and miniature retinal EPSCs over the period of ON/OFF sublamination. These data have interesting implications for activity-dependent development of retinogeniculate synapses. A preliminary report has been published in abstract form (Hohnke and Sur, 1996, 1998).

Methods

This set of experiments included a test of the effect of various recording conditions on sEPSCs. Both the potassium and cesium-based solutions were used. In some experiments 50 μM BMI and/or 1 μM TTX was added to the bath and the effects of their presence investigated. 10 μM CNQX was used to block AMPA receptors. In a subset of experiments extracellular calcium was replaced with strontium (1-3 mM).

Electrical stimuli, averaging 5 MA for 0.02 ms, were delivered at 0.5 Hz and emEPSCs were detected during the 1-second period following the synchronous response. Stimulus strength was set at a value slightly greater than the value that produced the minimal response.

In Figures 2B and 8B, values are expressed as standard deviations from the mean in order to allow the pooling of events across cells (Hsia et al., 1998). In order to represent the younger and older groups in the pool equally, 50 randomly selected events were chosen from an equal number of the youngest cells and oldest cells for which there were at least 50 events.

Results

Spontaneous EPSCs

Spontaneous EPSCs were analyzed in 32 cells from animals between P12 and P31. sEPSCs were observed in approximately 66% of the cells, and were recorded from animals at all ages throughout the period of sublamination. We observed no correlation

between the age at which recordings were made and the observation of sEPSCs. We recorded 2,222 sEPSCs from younger animals and 739 sEPSCs from older animals. Typical distributions of areas and the averaged sEPSC for one younger and one older cell are shown in Figure 1. The histograms show skewed distributions that were observed at all ages for all sEPSC parameters measured, and are similar to those seen in other regions of the brain, including the hippocampus (McBain and Dingledine, 1992, 1993; Jonas et al., 1993) and cerebral cortex (Hestrin 1992, 1993; Stern et al., 1992). Pooled data from 10 of the youngest and 10 of the oldest cells show sEPSC area and inter-event interval (IEI) histograms that are similar (Figure 2A). Indeed, the shapes of the distributions were the same for the two groups ($p > 0.05$, Kolmogorov-Smirnov test; Figure 2B) as were the distributions for rise, width and decay times ($p > 0.05$).

There was a significant correlation between access resistance and age ($r_s = -0.41$, $p < 0.05$), decreasing in the older animals (14, 13 ± 7 MΩ; n = 13 [from 10 animals]) as compared to the younger animals (23, 23 ± 8 MΩ; n = 19 [from 13 animals]). Consequently, sEPSC parameters that are sensitive to access resistances would show artifactual developmental changes (Hohnke and Sur, 1996). However, the two parameters that I report on here are relatively insensitive to changes in access resistance. Neither the areas of the sEPSCs ($r_s = 0.01$, $p > 0.05$) nor their inter-event intervals (IEIs; $r_s = -0.17$, $p > 0.05$) were correlated with the access resistances. In contrast, peak amplitudes were significantly correlated with access resistance ($r_s = -0.42$, $p < 0.05$) and remained so when analysis was limited to recordings with access resistances less than or equal to 20 MΩ ($r_s = -0.71$, $p < 0.05$). Whole-cell capacitance was also significantly

correlated with age ($r_s = 0.48$, $p < 0.05$) increasing from a median value of 57 pF (51 ± 20 pF) in the younger animals to 77 pF (76 ± 22 pF) in the older animals. The increase is likely due to the developmental increase in soma size during this period (Sutton and Brunso-Bechtold, 1991; Rocha and Sur, 1995).

There was no correlation between age and resting potential ($r_s = 0.09$, $p > 0.05$; Figure 3A). Median resting membrane potential was –50 mV (-51 ± 8 mV, n = 19) in younger animals and –53 mV (-54 ± 12 mV, n = 13) in older animals[1]. Input resistances, however, were significantly correlated with age ($r_s = -0.53$, $p < 0.05$). Input resistances were quite variable in the younger animals (217, 384 ± 493 MΩ) and decreased by 33% in the older animals (147, 166 ± 109 MΩ; Figure 3B). The variability in input resistances has been noted in other reports of adult and developing LGN cells (Ramoa and McCormick, 1994a; White and Sur, 1992; Bloomfield et al., 1987) and is likely due to a large variability in the specific membrane resistance of specific cell types (Bloomfield et al., 1987). Part of the decrease in input resistance was due to the large input resistances of the two youngest neurons (P12); nonetheless excluding these two neurons from the analysis did not change the significance of the correlation ($r_s = -0.43$, $p < 0.05$).

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[1] This finding conflicts with the significant hyperpolarization reported in the previous chapter. The rather large variability in this parameter will, on occasion, result in a sample of the population that doesn't show the same degree of change. In both sets of experiments the trend was toward hyperpolarization with age. In the larger study of the previous chapter (n = 187) V_m significantly decreased from –50 to –57 mV. In this smaller study (n = 32) V_m decreased from –50 to –53. These results are not wildly inconsistent with one another. The statistical significance in the first case and it's absence in the second case illustrate the need for cautious conclusions when dealing with these highly variable parameters. Given the ubiquity of reports of a developmental hyperpolarization of membrane potential throughout the nervous system, I feel reassured in making the conclusion in Chapter Three (significant hyperpolarization). No arguments in this chapter rely on the stated stability of the resting membrane potentials in this subpopulation, so the fact that

There was no significant change in the charge transfer of sEPSCs during the period of ON/OFF sublamination (Figure 3C). Specifically, median area in the younger animals was 15 fC (21 ± 19 fC) and median area in the older animals was 15 fC (18 ± 9 fC, $p > 0.05$). Neither was the frequency of sEPSCs significantly changed after reorganization of the retinal axons (Figure 3D). The median IEI in the younger animals (529, 647 ± 439 ms) was slightly shorter than in the older animals (705, 1,059 ± 1,096 ms, $p > 0.05$). Limiting the analysis to those recordings with a 20 MΩ or less access resistance ($n = 17$) demonstrated that there was no correlation between access resistance and rise times ($r_s = 0.07$, $p > 0.05$), half-widths ($r_s = 0.12$, $p > 0.05$), and decay times ($rs = 0.13$, $p > 0.05$). In this subpopulation of recordings no correlation was observed between age and rise times ($r_s = 0.27$, $p > 0.05$), half-widths ($r_s = 0.45$, $p > 0.05$), or decay times ($r_s = 0.45$, $p > 0.05$). Both areas and IEIs remained uncorrelated with access resistance and age. Thus, the younger and older groups had equivalent sEPSCs (Table 1).

The sEPSCs I report on in this study were recorded in a number of different conditions that had little effect on their areas or IEIs. In order to fully characterize sEPSCs in the ferret LGN, I was interested in recording sEPSCs in the presence of an alternate divalent cation. We tested whether replacing calcium with strontium, the most effective substitute for calcium with regard to transmitter release (Miledi 1966; Goda and Stevens, 1994; Oliet et al., 1996; Abdul-Ghani et al., 1996; Morishita et al., 1997; Choi et al., 1997), would affect the size or frequency of sEPSCs. My experiments show that

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the trend in V_m may in fact reflect a significant hyperpolarization is not worrisome.

strontium is equivalent to calcium in supporting sEPSCs in the developing LGN; both the sEPSC areas and IEIs were unchanged in the presence of strontium (Figure 4). Recordings in 0 mM calcium and 1-3 mM strontium revealed sEPSCs with areas (16, 20 ± 12 fC) and IEIs (655, 825 ± 396 ms; n = 6) that were not different from those recorded in normal calcium (15, 20 ± 17 fC, $p > 0.05$; 607, 812 ± 859 ms, $p > 0.05$; n = 26). No developmental change in the efficacy of strontium was observed. In strontium solutions, the areas (13.5,13.5 ± 2.7 fC) and IEIs (583, 583 ± 126) in the younger group (n = 2) were not significantly different from the areas (18.1, 23.4 ± 13.4 fC; $Q = 1.23$, $p > 0.05$, Kruskal-Wallis non-parametric ANOVA) and IEIs (932, 946 ± 444 ms; $Q = 0.86$, $p > 0.05$) in the older group (n = 4; Table 2).

The vast majority of sEPSCs that I recorded were CNQX-sensitive, quantal events: CNQX (10 μM) blocked all sEPSCs (n = 3; Figure 5A) and sEPSCs under TTX blockade were similar to normal sEPSCs (Figure 5B). The distributions of the IEIs in control (759, 668 ± 249 ms) and TTX (483, 612 ± 358 ms) solutions were not significantly different (in all cases $p > 0.05$, Kolmogorov-Smirnov test, n = 4; Figure 5C). Likewise, there was no significant change in sEPSC areas in TTX (13, 13 ± 5 fC) as compared to control (13, 13 ± 2 fC) solutions (in one case $p = 0.02$, in three cases $p > 0.05$, Kolmogorov-Smirnov test; Figure 5D). By clamping the cells at -60 mV, close to the chloride reversal potential, I expected to not detect any spontaneous inhibitory postsynaptic currents. Indeed, no significant differences in areas or IEIs were observed between control (n = 7) and BMI solutions (n = 21) when recording from animals of similar ages (Table 2).

Additionally, eight recordings with cesium gluconate in the recording electrode produced sEPSCs similar to those recorded with potassium gluconate ($n = 24$). A slight, but significant difference was observed between sEPSC areas recorded from younger animals with cesium gluconate and sEPSC areas recorded from older animals with potassium gluconate ($Q = 2.75$, $0.02 < p < 0.05$). However, no significant differences in areas or IEIs were observed between the intracellular solutions when recording from animals of similar ages. There were also no significant differences in areas or IEIs between sEPSCs recorded at room temperature and those recorded at 34°C ($p > 0.05$; Table 2.).

Finally, I examined the coefficient of variation (CV) of the areas and IEIs of the sEPSCs to determine whether changes in the variability of these parameters occurred during ON/OFF sublamination. There was no age-dependent change in the variability of sEPSCs within a recording for any of the parameters measured. The median CV (of the individual CVs) for area was 0.58 in the younger animals and 0.57 in the older animals. The median CV for IEI was 1.1 in both the younger and older animals. There was also no change in the variability of sEPSCs between recordings. The variance of the median values in the younger group was not different from the variance in the older group for all the parameters measured ($p > 0.5$, Siegel-Tukey test).

Evoked[2] Miniature EPSCs

In order to examine the retinal synapses specifically, I evoked asynchronous transmitter release by stimulating the optic tract after replacing extracellular calcium with strontium (Miledi, 1966; Goda and Stevens, 1994; Oliet et al., 1996; Abdul-Ghani et al., 1996; Morishita et al., 1997; Choi et al., 1997). Replacing extracellular calcium with strontium caused a marked decrease in the normal, synchronous release of transmitter, and caused an after-discharge of miniature EPSCs lasting up to one second, but occurring primarily in the first 500 ms (Figure 6).

Evoked miniature EPSCs were analyzed in 11 additional cells from animals between P15 and P31. We recorded 762 emEPSCs from younger animals and 529 emEPSCs from older animals. Typical distributions of areas and the averaged emEPSC for one younger and one older cell are shown in Figure 7. As with sEPSCs, histograms of emEPSC parameters showed skewed distributions at all ages. More specifically, pooled data from the youngest (n = 5) and the oldest cells (n = 5) show emEPSC area and IEI histograms with similar shapes (Figure 8A). The cumulative histograms were the same for the two groups ($p > 0.05$, Kolmogorov-Smirnov test; Figure 8B) as were the distributions for rise, width and decay times ($p > 0.05$).

The access resistances of the recordings in this set of experiments were less than

[2] The term "miniature" has classically been used in neurophysiological studies of single inputs to describe sEPSCs occurring in the presence of TTX. Consequently, it has been suggested to me that it is misleading to embed that word in the phrase that I use to describe the events that I characterize in this chapter. When TTX is not present, the practice has been to simply use the term "spontaneous." Clearly, "evoked spontaneous" EPSCs is oxymoronic. Additionally, evidence will be provided that evoked miniature EPSCs

20 MΩ. The areas (r_s = 0.22, $p > 0.05$), rise times (r_s = 0.08, $p > 0.05$), and decay times (r_s = 0.05, $p > 0.05$) of the emEPSCs were not correlated with access resistance. The peak amplitudes of the emEPSCs, however, tended to correlate with the access resistance (r_s = -0.54, $p = 0.06$) as they did for the recordings of sEPSCs.

The input resistances of the recordings in this set of experiments revealed the same developmental decrease that was observed during the first set of experiments. Input resistances in the younger animals was 455 MΩ (424 ± 116 MΩ) and input resistances in the older animals was 116 MΩ (191 ± 193 M; r_s = -0.58, $p < 0.05$).

The properties of emEPSCs recorded from younger animals (n = 5 [from 3 animals]) prior to ON/OFF sublamination were not different from those made from older animals (n = 6 [from 4 animals]) after sublamination was complete. Median area in the younger animals was 16 fC (14 ± 4 fC) and median area in the older animals was 22 fC (23 ± 11 fC, $p > 0.05$; Table 3). Rise times, widths and decay times were also unchanged after the completion of sublamination ($p > 0.05$ for all comparisons; Table 3). No correlation was observed between age and emEPSC rise times (r_s = 0.31, $p > 0.05$), areas (r_s = 0.29, $p > 0.05$), half-widths (r_s = 0.36, $p > 0.05$), or decay times (r_s = 0.41, $p > 0.05$).

In general, emEPSCs and sEPSCs were quite similar. emEPSC areas in the younger animals (16, 14 ± 4 fC) were not different from sEPSCs in the younger animals (15, 21 ± 19 fC, $p > 0.05$), nor were emEPSCs in the older animals (23, 23 ± 11 fC) different from sEPSCs in the older animals (15, 18 ± 9 fC, $p > 0.05$). Rise times, widths

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(emEPSCs) are not significantly different in size from mEPSCs, as has been demonstrated in other studies

and decay times of emEPSCs were not different from those calculated for sEPSCs (compare Tables 1 and 3). More specifically, the areas and decay times of emEPSCs and sEPSCs from the same recordings were not different from one another (n = 2; in both cases $p > 0.05$, Kolmogorov-Smirnov test). In one cell, the rise times of the emEPSCs (0.41, 0.44 ± 0.15 ms) were slightly shorter than those of the sEPSCs (0.48, 0.54 ± 0.19 ms, $p < 0.05$, Kolmogorov-Smirnov test) from the same recording. emEPSCs, like sEPSCs, were AMPA-mediated at resting membrane potentials and could be blocked completely with CNQX (10 μM, n = 6; Figure 6). Lastly, the within-cell CV showed no age-dependent change for any of the parameters measured, nor was there a change in the variability of sEPSCs between recordings ($p > 0.05$, Siegel-Tukey test).

Discussion

We have demonstrated that sEPSCs are present in ferret LGN cells as early as P12 and occur throughout the period of ON/OFF sublamination. The results presented here suggest that the basic physiological properties of these unitary inputs onto LGN cells are present at the beginning of ON/OFF sublamination and do not change significantly, although at least the retinal inputs are undergoing dramatic, activity-dependent anatomical reorganization and development. Because the population of sEPSCs recorded from a neuron may be generated from a heterogeneous set of synapse types (e.g., retinal and cortical synapses), it is possible that changes occurring among one of those types are

as well (Oliet et al., 1996).

masked in my data. We examined retinal synapses specifically, and show that unitary, AMPA-mediated, *retinal* EPSCs remain unchanged during ON/OFF sublamination and are not different from the population of sEPSCs. Indeed, the similarity of sEPSCs and emEPSCs suggests that sEPSCs might derive largely if not solely from retinal synapses.

We analyzed these data for group differences ("younger" vs. "older" group) as well as for correlation with age. Sublaminae can first be seen by P21 (Hahm et al., 1991), and are sharply segregated by P26 (Cramer et al., 1996). There were very few cells between P21 and P24 (see Figure 3); their properties were not different from the "younger" group of cells and they were included in this group for analysis. Furthermore, the correlation analyses showed no trend in any of the EPSC parameters with age.

Three caveats to these data should be noted. First, I recorded from a heterogeneous population of cells in the A layers of the LGN that include X cells, Y cells and interneurons. It is unlikely that I recorded from many interneurons: recordings from cells with small somas (which are characteristic of interneurons [Friedlander et al., 1981]) were avoided and all cells included in the analysis displayed easily evoked low-threshold calcium spikes, a characteristic of LGN relay cells (McCormick and Pape, 1988). However, while I observed no systematic variations in these data that might correlate with different LGN cell types, such a relationship cannot be excluded. Second, I recorded EPSCs only at resting membrane potentials. Hence, changes at synapses mediated by conductances not active at rest (NMDA receptors, for example) cannot be excluded. Third, to the extent that changes in presynaptic transmitter release are not well assayed by the frequency of sEPSCs (more below), they would not have been detected by

these experiments.

Another issue deserves comment. It is possible that immature inputs may have such low sEPSC amplitudes and/or frequencies that they can not be reliably detected. However, the detection thresholds I used allowed me to detect sEPSCs with extremely small amplitudes and areas (e.g., I detected events with amplitudes that were less than two times the standard deviation of the baseline noise). Additionally, I observed no increase in sEPSC frequency which would be expected if undetectably low, immature sEPSC frequencies matured to the frequencies I observed during ON/OFF sublamination.

Comparison with Other Systems

The lack of developmental changes in sEPSCs that I observe in the LGN are similar to those reported for unitary EPSCs in the rat neocortex (Burghard and Hablitz, 1993). That is, the characteristics of sEPSCs are present early in development and do not change significantly during the third and fourth postnatal weeks. The development of sEPSCs, miniature EPSCs (mEPSCs), and minimal evoked excitatory postsynaptic potentials (meEPSPs) have also been examined in other systems. The amplitude of sEPSCs and mEPSCs does not change in early development in the rat superior colliculus and visual cortex (Hestrin, 1992; Carmignoto and Vicini, 1992; Shi et al., 1997). A developmental increase in meEPSP amplitudes between the third and fifth postnatal weeks has been reported in the rat hippocampus to be due to an increase in quantal content as opposed to quantal size (Dumas and Foster, 1995). In fact, as in this study, mEPSCs do not increase in size during hippocampal development (Hsia et al., 1998). In the rat superior colliculus, NMDA-mediated mEPSC and sEPSC decay times decrease during the second

and third postnatal weeks (Hestrin, 1992; Shi et al., 1997); NMDA-mediated decay times also decrease with age in visual cortex (Carmignoto and Vicini, 1992). The differences observed in the development of mEPSCs in various mammalian brain regions may be due to intrinsic differences in those regions or differences in the recording conditions. For example, the experiments in visual cortex and superior colliculus were concerned with isolating the NMDA components of the mEPSCs and recorded events in the presence of zero magnesium and/or at extremely depolarized potentials. In addition, Hestrin (1992) reports a qualitative, but not a quantitative, decline in mEPSC decay time in the superior colliculus. Shi and Constantine-Paton (1997) report a decrease in sEPSC frequency following retinocollicular map refinement in the rat that they suggest may be due to the onset of $GABA_A$-mediated inhibition. $GABA_A$ and $GABA_B$-mediated inhibition first appear in the developing ferret LGN at P15 and between P21 and P30, respectively (Ramoa and McCormick, 1994b), but I observe no significant decrease in the frequency of sEPSCs at those times. In contrast, mEPSC frequency is reported to increase dramatically during hippocampal development (Hsia et al., 1998). In any case, a change in unitary EPSC frequency would be difficult to interpret in developmental slice physiology studies. Changes in frequency could be due to the slightly different planes of section (Staley and Mody, 1991) and/or changes in the number of preserved contacts in a slice resulting from the growth and movement of a structure during development. Lastly, Blanton and Kriegstein (1991) report increases in frequency, rise and decay times of mEPSCs during embryonic development of the turtle cortex. These changes contrast with what is observed in the rat and ferret and may be due to species differences or a

difference in the developmental stage between an embryonic turtle cortex and postnatal cortical and subcortical structures in mammals.

Retinogeniculate Transmission during Sublaminar Segregation

We show that strontium can substitute for calcium in mediating sEPSCs at retinogeniculate synapses. No change in sEPSC size or frequency is observed when calcium is replaced with equal concentrations of strontium. Strontium has been shown to be the most efficient substitute for calcium at the neuromuscular junction (Miledi, 1966; Dodge et al., 1969; Meiri and Rahamimoff, 1971; Bain and Quastel, 1992). Fast synchronous release is inhibited when calcium is replaced with strontium, but the slower, asynchronous release is facilitated. Transmitter release is quantal in strontium and spontaneous release continues. Similar findings have been reported more recently in the CNS (Goda and Stevens, 1994; Oliet et al., 1996; Abdul-Ghani et al., 1996; Morishita et al., 1997; Choi et al., 1997). In hippocampal slices, as in this study, the size and frequency of sEPSCs is unchanged in the presence of strontium (Oliet et al., 1996). In addition to showing that strontium can be substituted for calcium, my findings show that the efficacy of that substitution is not altered during a period of activity-dependent plasticity in early development.

We observe stable resting membrane potentials (RMPs) and a decrease in input resistances of LGN cells during sublamination. Two previous reports of the physiological development of ferret LGN cells, one using intracellular recordings (White and Sur, 1992) and the other using whole-cell patch-clamp (Ramoa and McCormick, 1994a), likewise report a developmental decrease in input resistance and stable RMPs

during the third and fourth postnatal weeks. A developmental decrease in input resistance has also been reported in mouse thalamus (Warren and Jones, 1997), rat thalamus (Perz and Carlen, 1996), rat superior olivary complex (Kandler and Friauf, 1995), rat hippocampus (Spigelman et al, 1992), and rat neocortex (McCormick and Prince, 1987; Annis et al., 1993). In the LGN, it is possible that the observed developmental decrease in input resistance reflects a bias toward recording from Y-cells towards the end of sublamination. Y-cells have larger somata and lower input resistances than X-cells in the adult cat (Crunelli et al., 1987), and response properties that develop later than those of X-cells (Daniels et al., 1978). It is also possible that the decrease in input resistance reflects the developmental increase in soma size of both X and Y cells (Rocha and Sur, 1998).

The developmental changes in receptor subunit composition and the putative role for LTP during development suggest a change in spontaneous and evoked miniature EPSCs during ON/OFF sublamination, at least of synapses mediated by AMPA receptors on LGN cells. What might account for the stability of synaptic efficacy during a period of dramatic reorganization of afferents and pattern formation?

It is possible that homeostatic mechanisms maintain a constant synaptic efficacy during the period of ON/OFF sublamination. Such homeostasis may be of two types. First, it has been shown that quantal amplitudes in cultured neocortical pyramidal neurons are globally modulated by activity (Turrigiano et al., 1998), so that blockade of activity results in a scaling up of sEPSC amplitudes. Similarly, quantal amplitudes in cultured hippocampal neurons are negatively correlated with the number of synaptic

contacts on these cells (Liu and Tsien, 1995). On this view, if the number of synaptic contacts (particularly retinal synapses) on LGN cells increases during sublamination, the amplitude (or areas) of sEPSCs and emEPSCs should decrease. The fact that sEPSC and emEPSC areas remain similar through the period of sublamination may be indicative of no major change in the overall number of at least retinal synapses during this period. Indeed, the available data on postnatal cats (Kalil and Scott, 1979) is difficult to relate to postnatal ferrets, and can be interpreted as reflecting only a modest increase in retinogeniculate synapses over a time period similar to the P14-28 period in ferrets.

Second, synaptic rearrangement during ON/OFF sublamination implies that some synapses—presumably incorrect ones—are lost or retracted from cells while new synapses are added. One interpretation of the data presented here is that the new synapses have properties very similar to the ones they replace (Figure 9). It is possible that the postsynaptic cell provides such stability, with synapses targeted to specific dendritic sites regulated to have specific properties. An alternative possibility is that retinogeniculate synapses are correctly targeted in location and number by P14 and sublamination consists of the separation of ON-center and OFF-center axon-cell pairs to the appropriate sublayer by P28 (Figure 9).

These results add complexity to the hypothesis that developmental changes in connectivity are mirrored by changes in synaptic efficacy (Constantine-Paton et al., 1990; Kandel and O'Dell, 1992; Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Constantine-Paton and Cline, 1998). While I did not follow individual synapses over time, the population data do not indicate that a subset of synapses is

strengthened while another is weakened and retracted over the time period of sublamination. Rather, the results presented here suggest that total synaptic input to a neuron is "normalized" toward some acceptable level and that if LTP is involved in the stabilization of appropriate synapses during development, a subsequent mechanism counteracts its effects. For example, an LTP-like mechanism by which changes in synaptic efficacy decays over a period of hours (Cline, 1991), but which initially mediates synapse stabilization, may exist and would not be detected by these experiments.

Figure Legends

Figure 1. sEPSC areas in the LGN are stable during ON/OFF sublamination. Histograms of sEPSC areas and example current traces from (A) a P19 and (B) a P30 neuron. Histograms for all properties measured were skewed toward larger values and showed considerable variability. The lower traces for each neuron are averages of 50 consecutive sEPSCs.

Figure 2. The shape of sEPSC area and IEI distributions is stable over ON/OFF sublamination. (A) Histograms of pooled data from the youngest cells (n = 10) and the oldest cells (n = 10) are similar for both sEPSC area and IEIs. (B) Cumulative histograms of the standardized areas and IEIs show that the shapes of the distributions did not change with age ($p > 0.05$, Kolmogorov-Smirnov test).

Figure 3. Development of electrophysiological and sEPSC properties of LGN cells during ON/OFF sublamination. (A) Resting membrane potentials of cells was unchanged

while (B) input resistances decreased significantly. The large input resistances recorded at P12 did not alter the significance of the developmental decrease (see text for details). Neither (C) sEPSC areas nor (D) IEIs changed during this developmental period.

Figure 4. Strontium supports spontaneous synaptic transmission in the developing LGN. Sample histograms of sEPSC areas and IEIs for a P19 neuron recorded in (A) calcium are similar to those for a P15 neuron recorded in (B) strontium. Traces are averages of 25 sEPSCs from each neuron. Strontium was as effective in mediating sEPSCs as calcium in both the younger and older groups.

Figure 5. sEPSCs at resting membrane potential are AMPA-mediated, quantal events. A. CNQX blocked all sEPSCs. B. Five individual sEPSCs and their average traces are shown for control and TTX conditions. Cumulative amplitudes of sEPSC (C) IEIs and (D) areas show no difference between sEPSCs recorded in control and TTX solutions. The sEPSCs from four experiments are combined in each condition for illustrative purposes. Statistical analyses were performed for each experiment individually (see text for details).

Figure 6. Replacing extracellular calcium with strontium allows for the analysis of unitary, *retinal* EPSCs. A. Stimulation of the optic tract in the presence of normal calcium results in a large EPSC. B. Replacing calcium with strontium causes a marked decrease in the synchronous response to stimulation and generates a discharge of asynchronous transmitter release. C. In the absence of stimulation, the frequency of sEPSCs is extremely low suggesting that there is little contamination of the emEPSCs by

sEPSCs of unknown origin. D. CNQX blocked all emEPSCs as well as what remains of the synchronous response in strontium. E. Responses to stimulation recovered after removal of CNQX from the bath.

Figure 7. emEPSC areas in the LGN are stable during ON/OFF sublamination. Histograms of emEPSC areas and example current traces from (A) a P15 and (B) a P31 neuron. The lower traces for each neuron are averages of 50 consecutive emEPSCs.

Figure 8. The shape of emEPSC area distributions is stable over ON/OFF sublamination. (A) Histograms of pooled data from the youngest cells (n = 5) and the oldest cells (n = 5) are similar for sEPSC area. (B) Cumulative histograms of the standardized areas show that the shapes of the distributions did not change with age ($p > 0.05$, Kolmogorov-Smirnov test).

Figure 9. Efficacy of synapses on LGN cells appears to be "normalized" such that it remains stable throughout changes in neural activity and connectivity. A. Prior to ON/OFF sublamination retinal axons arborize in "inappropriate" areas of the LGN and may or may not make significant synaptic contacts outside of the areas which they will later be restricted to. B. After sublamination is complete, relay cells in the LGN receive inputs exclusively from either ON- or OFF-center retinal axons. C. A schematic of two possible explanations for the stability of synaptic efficacy during the activity-dependent reorganization of retinal axons in the LGN. If synaptic efficacy in the LGN is inversely proportional to the number of synaptic contacts as it is in the hippocampus (see text for details), then either inappropriate inputs (open circles) have similar synaptic efficacies

and are replaced by an equal number of appropriate inputs (closed circles), or only appropriate inputs are in place prior to sublamination and their number does not increase.

Table 1

Table 1. Properties of sEPSCs in the LGN during and after ON/OFF Sublamination

	N	Rise (ms)	Width (ms)	Decay (ms)	Area (fC)	IEI (ms)
Younger Group	6	0.54, 0.57±0.14	2.2, 2.5±0.1	2.1, 2.4±0.1	14.5, 15.1±2.8	576, 536±160
Older Group	11	0.54, 0.63±0.18	2.7, 2.8±0.1	2.7, 2.8±0.1	15, 19±9.4	744, 1,181±1,153

Access resistance ≤ 20 MΩ. Parameters calculated as described in Methods.

Table 2

Table 2. Size and Frequency of sEPSCs in the LGN under Different Recording Conditions

	Younger					Older				
		Area (fC)		Inter-Event Interval (ms)			Area (fC)		Inter-Event Interval (ms)	
	N	Median	Mean±Std	Median	Mean±Std	N	Median	Mean±Std	Median	Mean±Std
All	19	15.0	21.3±19.2	529	647±439	13	15.1	18.1±8.7	705	1,060±1,096
Sr^{2+} ACSF	2	13.5	13.5±2.7	583	583±126	4	18.1	23.4±13.4	932	946±444
BMI	13	13.5	14.7±6.1	540	657±414	8	16.5	18.8±10.4	655	1,117±1,405
Cs Gluconate	5	10.6	11.0±2.4	540	536±176	3	13.6	13.7±3.3	744	595±355
34°C	7	15.3	28.6±28.2	500	692±590	7	15.1	20.8±13.1	672	829±515

Sr^{2+} ACSF=0 Ca^{2+}/1-3 mM Sr^{2+} in ACSF. BMI=50 μM bicuculline methiodide. For all recording conditions, $p>0.05$ when comparing recordings from younger and older animals. Access resistance ≤ 33 MΩ.

Table 3

Table 3. Properties of Evoked Miniature Retinal EPSCs in the LGN during and after ON/OFF Sublamination

	N	Rise (ms)	Width (ms)	Decay (ms)	Area (fC)
Younger Group	5	0.37, 0.41±0.08	2.09, 2.17±0.25	1.78, 1.75±0.13	15, 14±4
Older Group	6	0.56, 0.54±0.17	3.83, 3.89±1.77	3.03, 2.97±1.13	22, 23±11

Figure 1

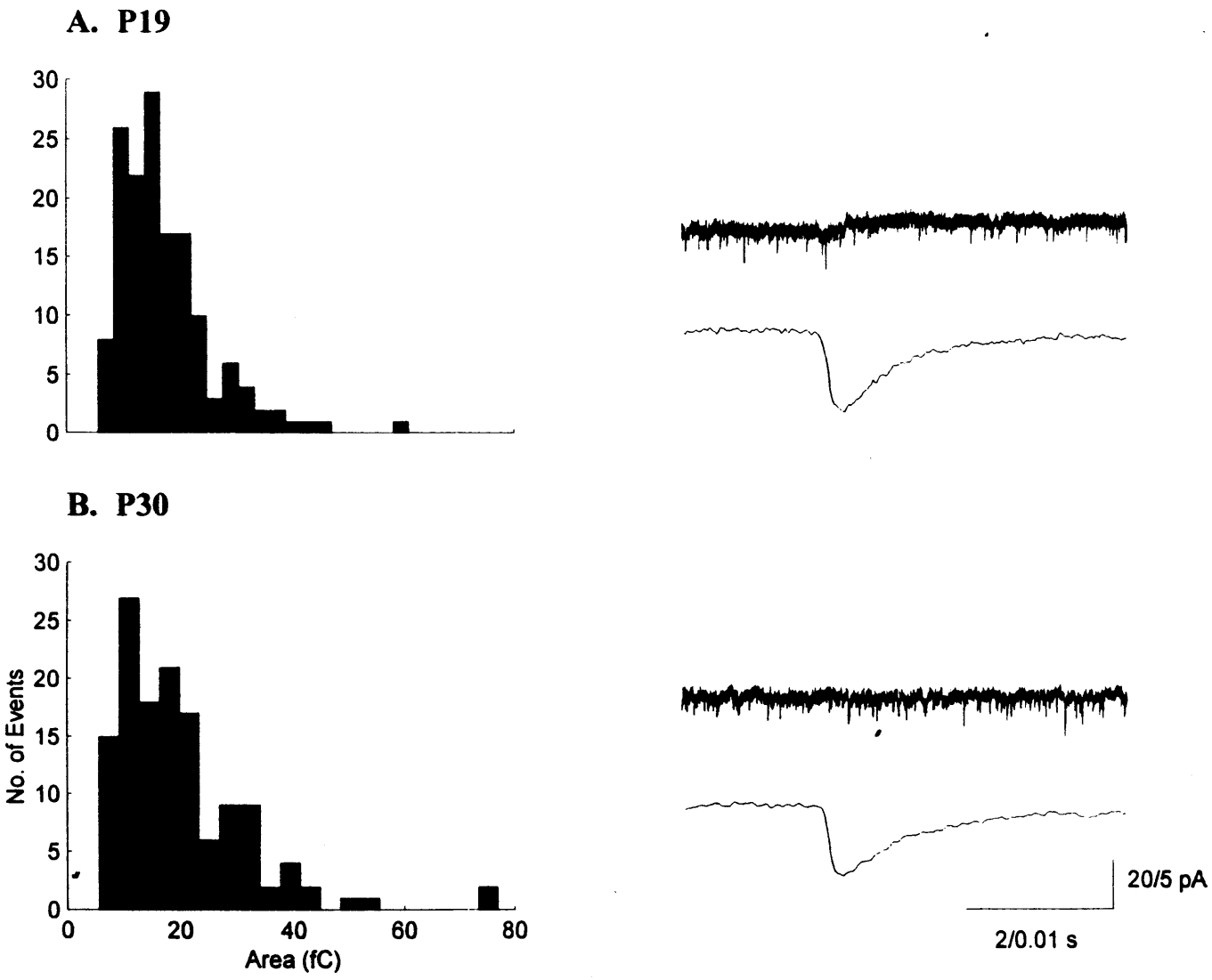

A. P19
30
25
20
15
10
5
0
B. P30
No. of Events
0
20
40
60
80
Area (fC)
20/5 pA
2/0.01 s

Figure 2

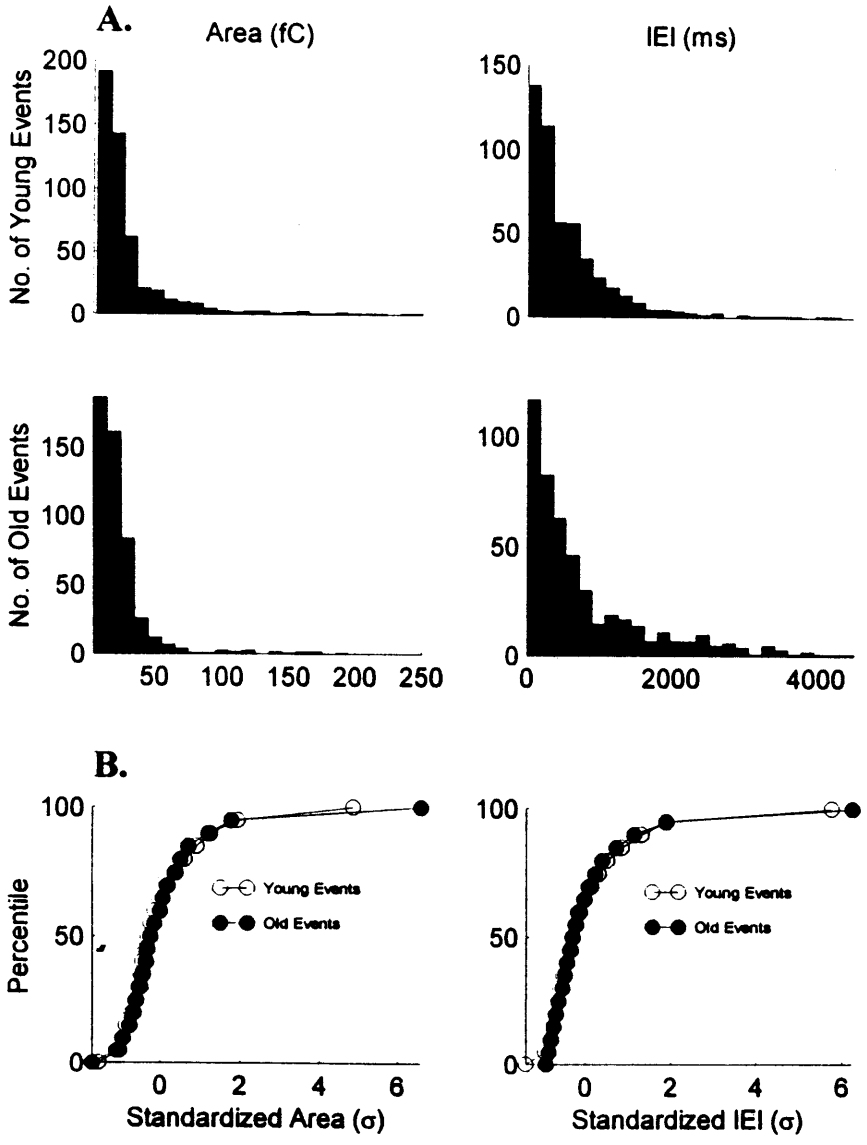

A.
Area (fC)
IEI (ms)
No. of Young Events
No. of Old Events
200
150
100
50
0
150
100
50
0
100
50
0
50 100 150 200 250
0 2000 4000
B.
Percentile
100
50
0
Young Events
Old Events
0 2 4 6
Standardized Area (σ)
Standardized IEI (σ)

Figure 3

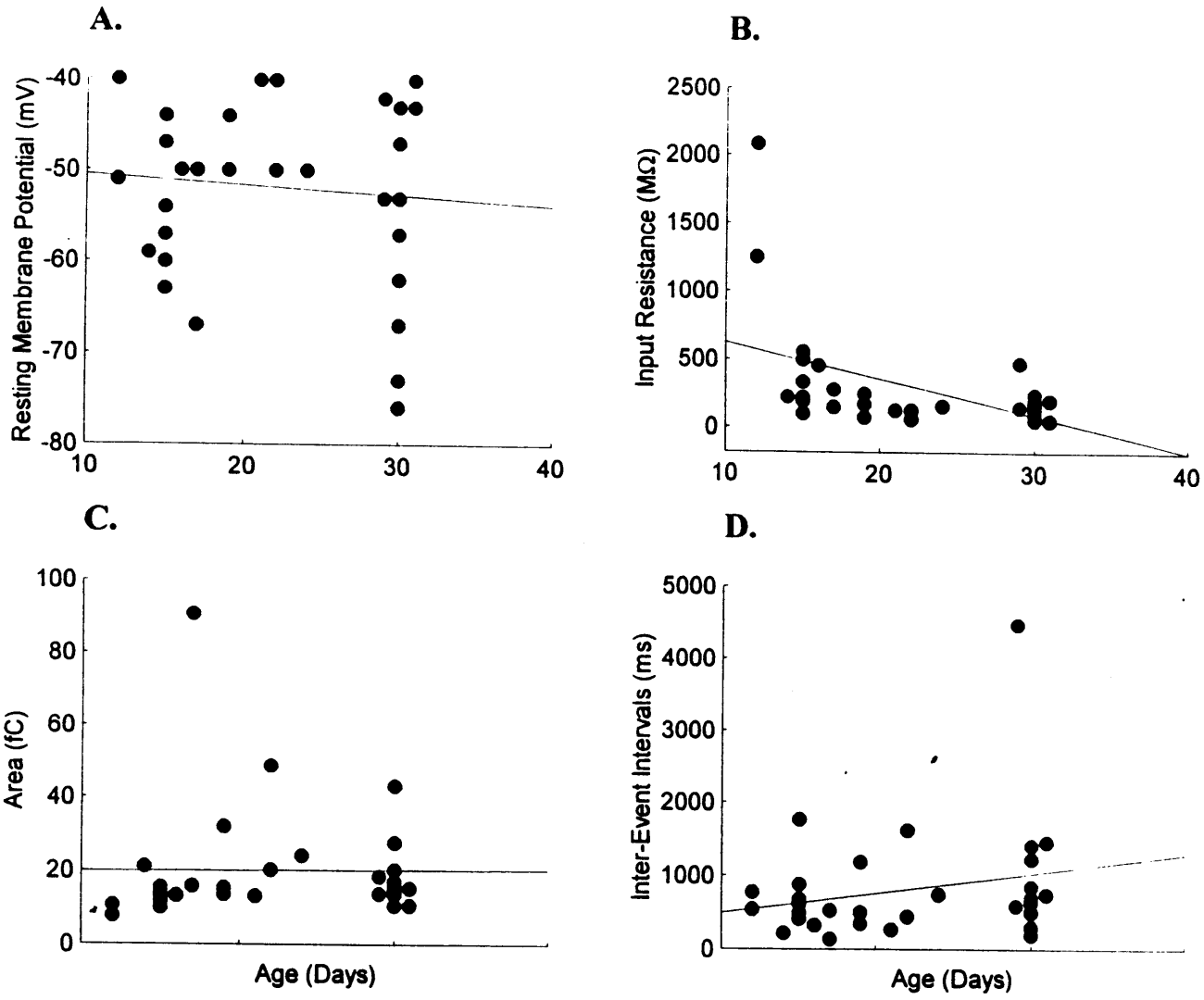

A.
Resting Membrane Potential (mV)
-40
-50
-60
-70
-80
10
20
30
40
B.
Input Resistance (MΩ)
2500
2000
1500
1000
500
0
10
20
30
40
C.
Area (fC)
100
80
60
40
20
0
Age (Days)
D.
Inter-Event Intervals (ms)
5000
4000
3000
2000
1000
0
Age (Days)

Figure 4

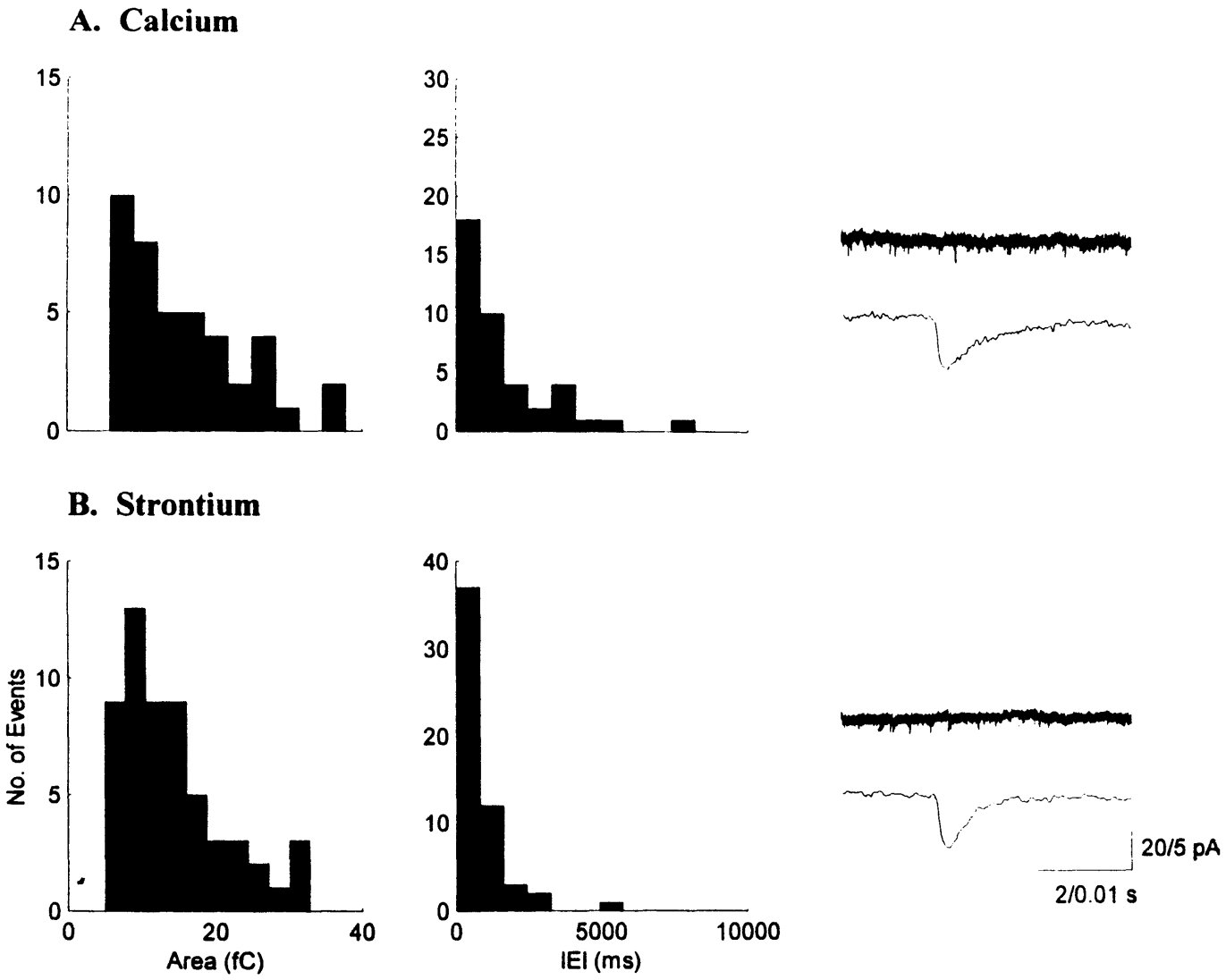

A. Calcium
15
10
5
0
30
25
20
15
10
5
0
B. Strontium
No. of Events
15
10
5
0
0
20
40
Area (fC)
40
30
20
10
0
0
5000
10000
IEI (ms)
20/5 pA
2/0.01 s

Figure 5

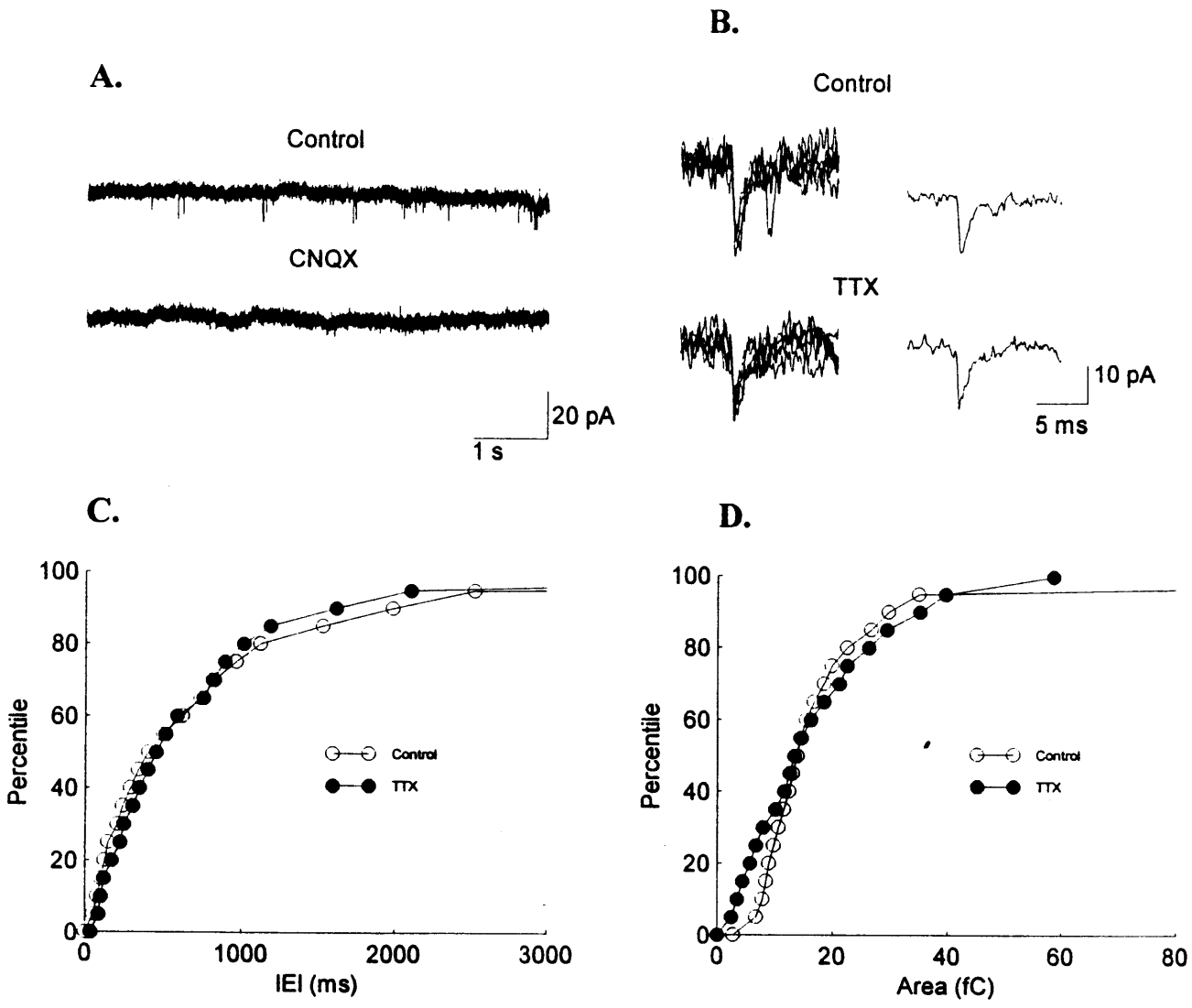

A.
Control
CNQX
20 pA
1 s
B.
Control
TTX
10 pA
5 ms
C.
Percentile
IEI (ms)
Control
TTX
D.
Percentile
Area (fC)
Control
TTX

Figure 6

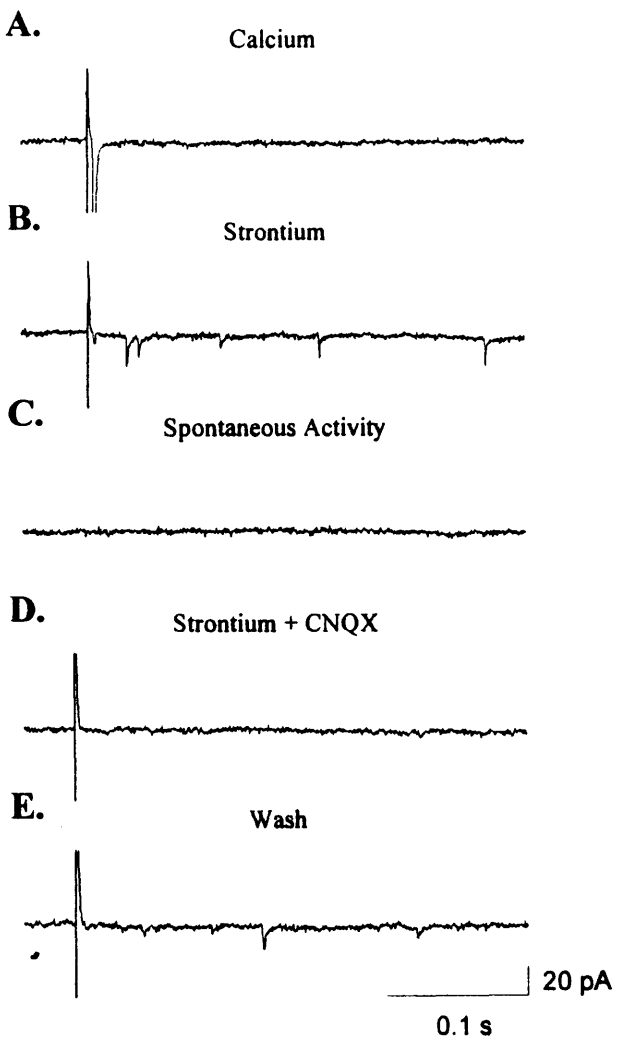

A.
Calcium
B.
Strontium
C.
Spontaneous Activity
D.
Strontium + CNQX
E.
Wash
20 pA
0.1 s

Figure 7

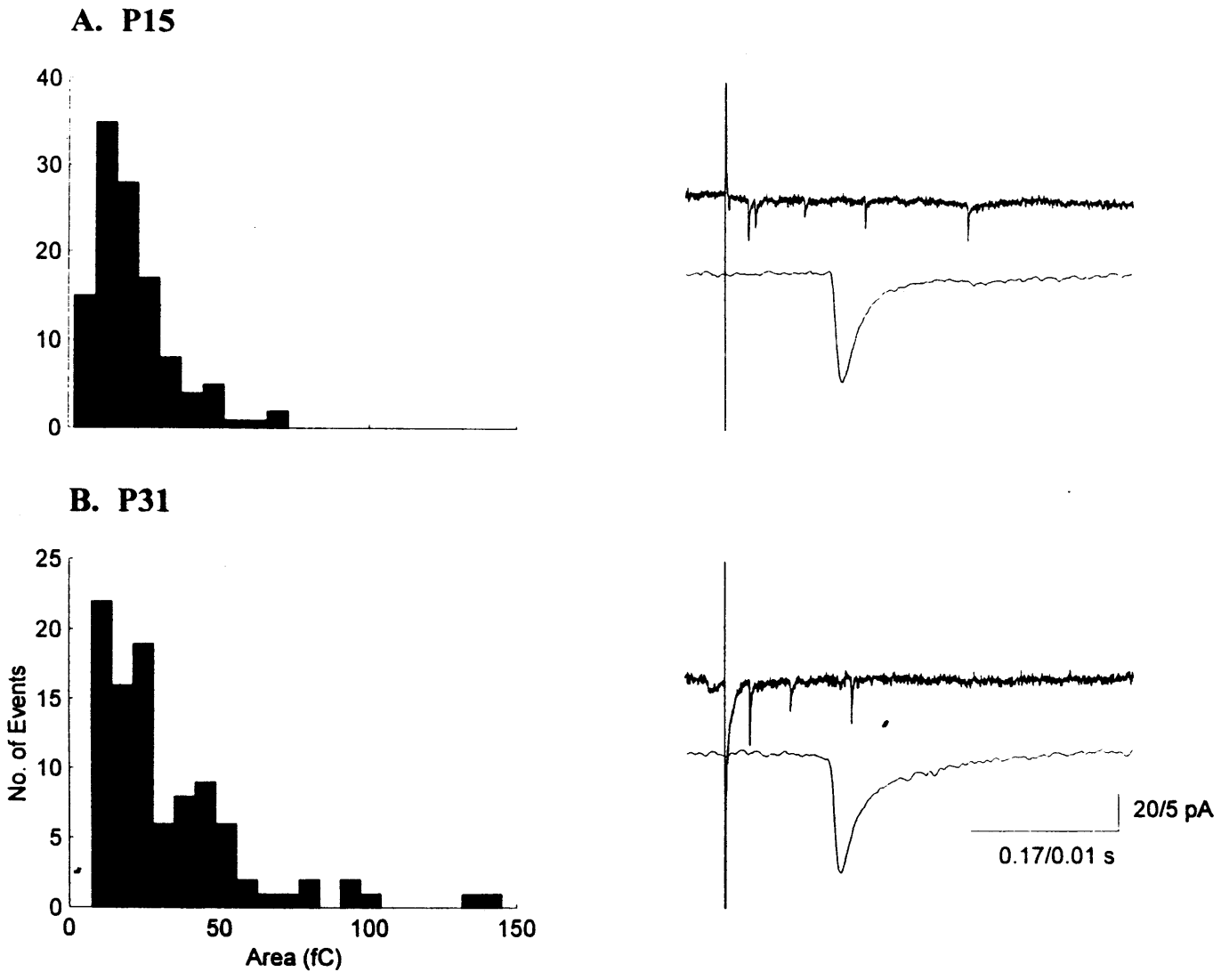

A. P15
40
30
20
10
0
B. P31
25
20
15
10
5
0
No. of Events
0
50
100
150
Area (fC)
20/5 pA
0.17/0.01 s

Figure 8

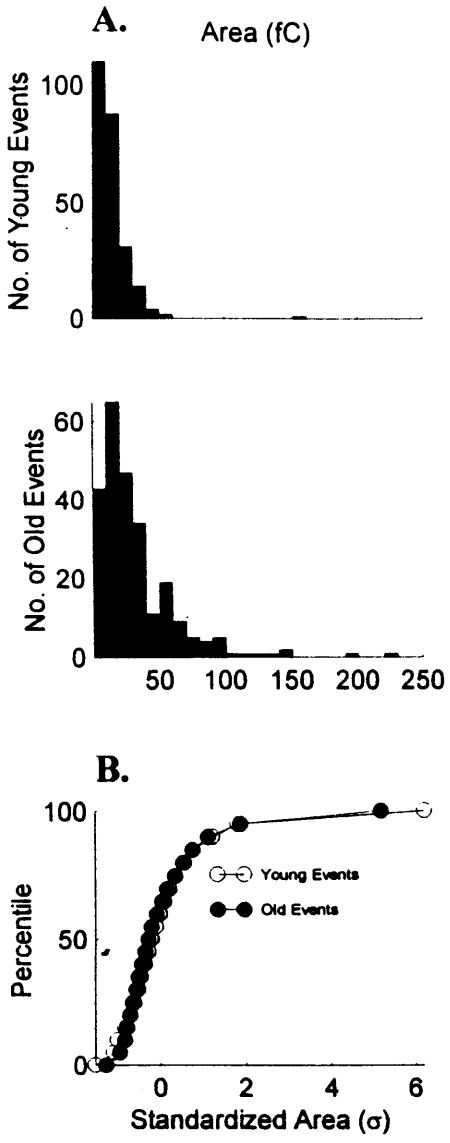
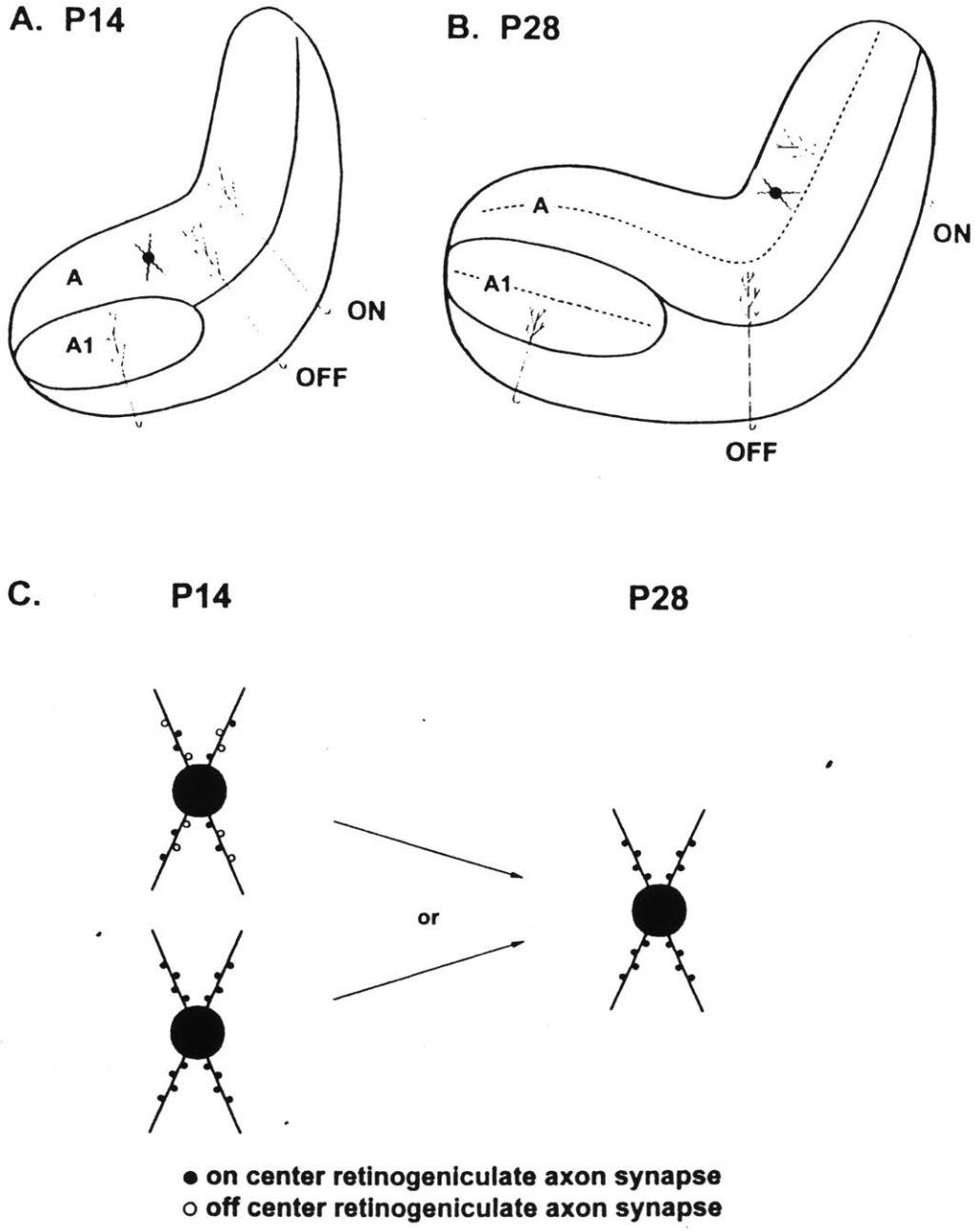

A.
Area (fC)
No. of Young Events
100
50
0
No. of Old Events
60
40
20
0
50
100
150
200
250
B.
Percentile
100
50
0
Young Events
Old Events
0
2
4
6
Standardized Area (σ)

Figure 9


A. P14
A
A1
ON
OFF
B. P28
A
A1
ON
OFF
C.
P14
P28
or
● on center retinogeniculate axon synapse
○ off center retinogeniculate axon synapse

Chapter Five: The Development of the AMPA/NMDA Ratio of EPSCs during ON/OFF Sublamination in the Ferret Lateral Geniculate Nucleus

Abstract

In the ferret, retinal projections to the lateral geniculate nucleus (LGN) from each eye initially overlap, but subsequently segregate into laminae that only receive inputs from either the left or the right eye. Shortly thereafter, retinogeniculate axons from ON-center and OFF-center retinal ganglion cells also segregate to form distinct sublaminae. The segregation in to ON/OFF sublaminae is activity-dependent and thought to rely on signals mediated by changes in synaptic efficacy. Yet, the development of synaptic efficacy during this period is not well understood. In the previous chapter I demonstrated that AMPA-mediated synaptic efficacy is stable throughout this period of axon reorganization. Here I extend those results by reporting on both the development of NMDA-mediated synaptic efficacy and the development of the ratio of synaptic current mediated by AMPA receptors to that mediated by NMDA receptors (A/N ratio). The properties of spontaneous NMDA receptor-mediated excitatory postsynaptic currents (sN-EPSCs[1]) are also stable throughout ON/OFF sublamination. Median charge transfer is large and highly variable at all ages. Likewise, the frequency of sN-EPSCs is quite variable. The A/N ratio has reached mature values prior to ON/OFF sublamination. Amplitudes of sN-EPSCs in control and AMPA receptor-blockade conditions are similarly affected at all ages. Additionally, the amplitude of optic-tract evoked EPSCs before and after this period are equally sensitive to CNQX. Finally, I observed a population of so-called "silent synapses" at which there are no functional AMPA-receptors. Consistent with the other assays of the A/N ratio, the presence of silent synapses is held constant during axon reorganization; however, their proportion of total synaptic input is quite small. These results add to the view of retinogeniculate synapses as surprisingly stable or normalized during a period in which retinogeniculate axons and their target cells are undergoing dramatic anatomical and electrophysiological change.

[1] "EPSC", a widely used acronym, continues to be modified as modern experiments proceed to reduce the EPSC to its component parts. Thus, "mEPSC" and "sEPSC" for miniature and spontaneous EPSCs, respectively. I use this most recent augmentation in an attempt to remain consistent with the literature (cf., Stocca and Vicini, 1999).

Introduction

In many sensory systems, the development of normal neural circuitry depends critically on the presence of normally patterned neural activity. In the mammalian visual system, for example, both thalamic and cortical circuits are refined in an activity-dependent manner during early development (for review, see Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Hohnke and Sur, 1999b). However, it is not well understood how neural activity provides instruction for changes in neural morphology (i.e., circuitry). Given the similarities between the mechanisms involved in activity-dependent development and those involved in changes to synaptic efficacy, it has been proposed that the latter phenomenon mediates the former (Constantine-Paton, et al., 1990; Kandel and O'Dell, 1992; Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Constantine-Paton and Cline, 1998).

However, the maintenance of changes in synaptic efficacy can take many forms (Collingridge and Bliss, 1993; Kullmann and Siegelbaum, 1995; Malinow, 1998). For example, increased synaptic efficacy may be maintained by an increase in current flux through particular receptor-mediated ion channels, by the additional recruitment of these channels, and/or by an increase in the probability or amount of neurotransmitter released from the presynaptic terminal. An examination of the role of the modulation of synaptic efficacy in activity-dependent development, then, involves the characterization of the development of all of these properties.

The ferret LGN is a useful system for characterizing these properties. In the ferret, retinal projections from each eye initially overlap in the LGN, but subsequently

segregate into laminae that only receive inputs from only one or the other eye. Subsequently, retinogeniculate axons from ON-center and OFF-center retinal ganglion cells also segregate to form ON/OFF sublaminae. This segregation is activity-dependent and has components that are similar to those required for the modification of synaptic efficacy (Cramer and Sur, 1995; Hohnke and Sur 1999b). In the previous chapter I demonstrated that AMPA-mediated synaptic efficacy is stable throughout this period of axon reorganization. Here I extend those results by reporting on the development of NMDA-mediated synaptic efficacy.

I also characterize the development of the ratio of synaptic current mediated by AMPA receptors to that mediated by NMDA receptors (A/N ratio). This investigation was prompted by recent reports that changes in synaptic efficacy may be mediated by changes in the presence of "silent synapses." Developmental changes in the prevalence of silent synapses, then, might reflect a role for changes in synaptic efficacy in activity-dependent development. Silent synapses are synapses at which no functional AMPA receptors are present (Liao et al., 1995; Isaac et al., 1995). Shortly after birth a significant proportion of silent synapses are found in the rat and mouse thalamocortical system and rat visual cortex, but the proportion declines during early development (Isaac et al., 1997; Rumpel et al., 1998; Golshani and Jones, 1999). The early, silent synapses are converted to functional synapses using protocols that also induce long-term changes in synaptic efficacy (Liao et al., 1995; Issac et al., 1995; Isaac et al., 1997; Rumpel et al., 1998); the impact of these protocols declines in parallel with the reduction in the proportion of silent synapses (Isaac et al., 1997). The conversion also requires NMDA

receptor activation (Rumpel et al., 1998).

The results presented in this chapter demonstrate the presence of a small number of silent synapses during ON/OFF sublamination, but no change in their proportion. Likewise, the A/N ratio by other measures is held constant during this period of important axonal growth and reorganization. These results add to the view of retinogeniculate synapses as surprisingly stable or normalized during a period in which retinogeniculate axons and their target cells are undergoing dramatic anatomical and electrophysiological change.

Methods

In this set of experiments cells were clamped at the resting membrane potential (-60 mV) and, in order to record sN-EPSCs, at +40 mV. During all experiments, recording pipettes contained the cesium-based solution with 5 mM QX-314, and the bath contained 50 μM BMI. 10 μM CNQX and 100 μM D-AP5 were used to block AMPA and NMDA receptors, respectively.

Afferents were stimulated as described in Chapter Two. Electrical stimuli, averaging 1 nC, were delivered at 0.2 Hz. For minimal stimulation, appropriate stimulus intensities were based on those used in previous investigations of minimal or single-fiber stimulation (Raastad et al., 1992; Stevens and Wang, 1995)[2]. Briefly, a stimulus intensity was sought that a) resulted in both EPSCs and failures of synaptic transmission, b)

[2] See Chapter Two for a discussion of the issues surrounding the interpretation of minimal stimulation.

produced constant EPSC latencies, and c) did not produce a significantly different response than those intensities just less than or great to it (that is, the minimal response was stable over a range of stimulus intensities). These intensities were typically just greater than the maximum at which no synaptic transmission occurred. For non-minimal stimulation, stimulus strength was set at the value just greater than that which produced no transmission failures.

The A/N ratio for sN-EPSC amplitudes was calculated as $\frac{\text{Control}}{\text{CNQX}} - 1$, i.e.,

$$\frac{\text{AMPA + NMDA}}{\text{NMDA}} = \frac{\text{AMPA}}{\text{NMDA}} + \frac{\text{NMDA}}{\text{NMDA}} = \frac{\text{AMPA}}{\text{NMDA}} + 1.$$

Results

I measured the A/N ratio throughout ON/OFF sublamination by comparing evoked and spontaneous EPSCs (sEPSCs) in two conditions: one in which only AMPA receptors were available and one in which both AMPA and NMDA receptors were available (Figure 1A). Both sEPSCs and evoked EPSCs were recorded in control solutions at a holding potential of –60 mV (AMPA receptor only condition), at +40 mV (AMPA + NMDA receptor condition), and at +40 mV in the presence of CNQX (NMDA receptor only condition; Figure 1B).

The Development of Spontaneous NMDA Receptor-Mediated EPSCs

In some measures (e.g., the difference in amplitude between AMPA + NMDA sEPSCs and spontaneous NMDA receptor-mediated EPSCs [sN-EPSCs]), developmental changes in the magnitude and/or kinetics of the current passed by the activation of a particular

receptor type would be reflected as a change in the A/N ratio. In the previous chapter I demonstrated that no such change occurs during ON/OFF sublamination for AMPA receptor-mediated sEPSCs. Here I extend those results by examining the development of sN-EPSCs during this period.

I recorded sN-EPSCs in 16 cells from animals between P14 and P31. Typical distributions of areas and the averaged sN-EPSCs from a cell prior to and subsequent to ON/OFF sublamination are shown in Figure 2. Distributions at all ages and for all sEPSC parameters measured were skewed towards larger values.

The properties of sN-EPSCs are stable throughout ON/OFF sublamination. The charge transfer of sN-EPSCs was not correlated with age (r_s = -0.24, $p > 0.05$). More specifically, median area in the younger cells (179 sN-EPSCs from 9 cells and 5 animals) was 366 fC (426 ± 248 fC) and median area in the older cells (118 sN-EPSCs from 7 cells and 4 animals) was 373 fC (477 ± 308 fC, $p > 0.05$; Figure 3A). Similarly, sN-EPSC amplitude in younger cells (6.4, 7.4 ± 2.5 pA) was unchanged in older cells (7.8, 7.8 ± 2.4 pA, $p > 0.05$). Neither was the frequency of sN-EPSCs correlated with age during retinogeniculate axon reorganization (r_s = -0.23, $p > 0.05$). The median inter-event interval (IEI) in the younger animals (3,578, 6,210 ± 5,141 ms) was not significantly different from that in older animals (2,016, 5,146 ± 5,441 ms, $p > 0.05$; Figure 3B).

The mature kinetics of sN-EPSCs have been established prior to ON/OFF sublamination. Rise times (r_s = 0.16), half-widths (r_s = -0.18), and decay times (r_s = -0.16) were fixed throughout this period and did not correlate with age ($p > 0.05$ for all

measures). Median rise times in younger cells (4.0, 5.1 ± 2.8 ms) were not significantly different from those in older cells (4.6, 4.7 ± 1.6 ms, $p > 0.05$; Figure 3C). Likewise, half-widths in the younger cells (114, 116 ± 42 ms) were unchanged in older cells (97, 98 ± 23 ms, $p > 0.05$; Figure 3D). Finally, the long decay times characteristic of NMDA receptor-mediated currents were similar in the younger (124, 147 ± 58 ms) and older (118, 123 ± 34 ms, $p > 0.05$) cells.

As is true of sEPSC properties in the CNS in general, and of AMPA receptor-mediated sEPSCs in the ferret LGN in particular (see Chapter Four), the properties of sN-EPSCs in the LGN at any age during ON/OFF sublamination is highly variable. For example, the coefficient of variation (CV) of sEPSC areas in younger cells was 0.7 (1.0 in older cells) and the CV of the IEI was 1.1 in younger cells (0.9 in older cells). The CV for all parameters measured was 45% or greater and consistent between younger and older cells ($p > 0.05$ for all measures).

The Development of the A/N Ratio

For one measure of the A/N ratio, I recorded sEPSCs in control and CNQX solutions at positive holding potentials and compared their amplitudes. Overall CNQX had a relatively small effect on sEPSCs recorded at a holding potential of +40 mV, well above the reversal potential for both AMPA and NMDA receptor-mediated currents. NMDA receptors, then, appeared to dominate synaptic currents at all ages during this period.

The properties of sN-EPSCs showed the expected effects in the presence of CNQX, but these effects were not age-dependent. Overall, the ratio of sN-EPSC amplitudes in control to CNQX conditions was significantly different from unity (1.1, 1.2

± 0.3, $p < 0.05$). In younger cells, sEPSC amplitudes in control solution (7.1, 9.5 ± 4.2 pA, n = 9) were larger than those recorded with CNQX in the bath (6.4, 7.4 ± 2.5 pA, n = 7). In older cells, sEPSC amplitudes in control solution (7.5, 7.8 ± 2.6 pA) were affected slightly less by the addition of CNQX (7.8, 7.8 ± 2.4 pA; Figure 4A). However, there was no significant difference in the effect of CNQX, and therefore in the A/N ratio, between the younger and older cells ($p > 0.05$). Similarly, the ratio of normalized sN-EPSC rise times in control to CNQX conditions was significantly different from unity (0.9, 0.9 ± 0.3, $p < 0.05$). Here too, the difference between sN-EPSC rise times in younger cells in control solution (3.8, 3.8 ± 0.8 ms) and in the presence of CNQX (4.0, 5.1 ± 2.8 ms, $p > 0.05$) was similar to the difference observed at older ages (control: 4.2, 4.3 ± 1.0 ms, CNQX: 4.6, 4.7 ± 1.6 ms, $p > 0.05$).

I also compared the frequency of sEPSCs at hyperpolarized (-60 mV) and depolarized (+40 mV) holding potentials to probe for NMDA receptor-only synapses. That is, if a significant population of silent synapses are present on LGN relay cells, the frequency of sEPSCs would be expected to be higher at the depolarized potentials at which NMDA receptors are active. However, in neither younger nor older cells was sEPSC frequency markedly increased at +40 mV. Frequency was typically low in both conditions at all ages. At a holding potential of –60 mV, the sEPSC frequencies in younger cells (0.2, 0.3 ± 0.2 Hz) and older cells (0.2, 0.2 ± 0.1 Hz) were no different than at +40 mV either before ON/OFF sublamination (0.2, 0.2 ± 0.1 Hz, $p > 0.05$) or after its completion (0.2, 0.2 ± 0.1 Hz, $p > 0.05$; Figure 5A,B). The ratio of sEPSC frequencies observed at +40 mV to those observed at –60 mV in younger cells (0.5, 1.0 ± 1.4) was

stable, not changing in older cells (1.1, 1.8 ± 0.6, $p > 0.05$).

Relay cells in the LGN receive input from numerous sources other than retinogeniculate input (Sherman and Koch, 1986, 1998). While it has been demonstrated that evoked, miniature, retinogeniculate EPSCs are indistinguishable from sEPSCs in this system (Chapter Four), it is not clear whether the sEPSCs recorded here are corticogeniculate or retinogeniculate in origin. In order to more specifically assay the A/N ratio of *retinogeniculate* EPSCs, I compared minimally evoked, putatively single-fiber, EPSCs at resting potentials and at +40 mV. I also compared EPSCs evoked at depolarized potentials and in control and CNQX solutions.

Minimal stimulation was established at +40 mV and repeated at resting potential, or, conversely, established at resting potential and repeated at +40 mV. The success of synaptic transmission in response to minimal stimulation is significantly greater at a holding potential of +40 mV than at resting potential (Figure 5C,D). Specifically, in younger cells ($n = 5$) the success of transmission at +40 mV was 0.6 (0.6 ± 0.3) compared to 0.5 at -60 mV (0.3 ± 0.3, $p < 0.05$). Similarly, older cells ($n = 9$) displayed greater success of transmission at a depolarized holding potential (0.8, 0.6 ± 0.3) than at resting potential (0.4, 0.4 ± 0.3, $p = 0.06$). However, there was no age-dependent change in the decrease in minimal stimulation failures. The difference between success rates in younger cells (0.2, 0.3 ± 0.2) was the same as in older cells (0.1, 0.2 ± 0.4, $p > 0.05$).

Optic tract stimulation in control and CNQX conditions also suggests that no developmental change occurs in the A/N ratio during ON/OFF sublamination. That is, the presence of CNQX had the same relative effect on evoked EPSCs in younger ($n = 3$)

and older cells (n = 2). In younger cells, the A/N of EPSC amplitude (1.1, 0.8 ± 0.7) was no different from that in older cells (2.5, 2.5 ± 3.0, $p > 0.05$).

Discussion

The results presented here demonstrate the stability of sN-EPSCs and the proportion of silent synapses during ON/OFF sublamination. The median charge transfer and frequency of sN-EPSCs is highly variable at all ages, but their median values are held constant throughout this period of activity-dependent axon reorganization. Likewise, silent synapses are observed at all ages during this period, but with no significant, age-dependent change in their proportion. I also show that the A/N ratio, as assayed by multiple manipulations, does not change dramatically during ON/OFF sublamination.

In the LGN, ON/OFF sublaminae can first be seen by P21 (Hahm et al., 1991), and are sharply segregated by P26 (Cramer et al., 1996). In order to compare the properties of EPSCs in younger and older cells, I compared recordings from cells between P14 and P24 to recordings from cells between P25 and P31. However, there were few cells between P17 and P27 (see Figure 3); their properties were not different from the "younger" group of cells and they were included in this group for analysis. While the distinction between younger and older cells is somewhat arbitrary (i.e., for my purposes somewhere between P21 and P26), the correlation analyses would show (but did not) any consistent change in EPSC properties.

I also note that I recorded from a heterogeneous population of cells in the A layers of the LGN, that is both X cells and Y cells. I saw no obvious clustering of EPSC

properties that might suggest a difference between the two cell types, but I cannot exclude the possibility that EPSCs recorded from X and Y cells have overlapping, but significantly different properties. If this were the case, relatively small changes in the properties of one cell type may be obscured by the properties of the other. However, there is no evidence to suggest that X and Y cells have different glutamate receptor profiles (Sherman and Koch, 1998).

A final issue deserves comment. The holding potential used to record sN-EPSCs (+40 mV) results in a somewhat decreased driving force through the excitatory ligand-gated ion channels (for which the reversal potential is approximately 0 mV) compared to resting potential (-60 mV). Consequently, it is possible that at +40 mV some sEPSCs went undetected below the threshold. However, three factors suggest that this was not a significant effect. First, my detection thresholds allowed me to detect sEPSCs with extremely small amplitudes (i.e., less than two times the standard deviation of the baseline noise). Though, it should be noted that the baseline noise at +40 mV was approximately twice that at –60 mV which may lead to an under-representation of small sEPSCs. Second, by visual inspection, very few (almost no) sEPSCs had amplitudes below the detection threshold (but above the noise). Last, while holding potentials more depolarized than +40 mV resulted in unacceptable baseline noise due to both the large driving forces and the activation of voltage-gated channels, it was nonetheless possible to visually identify the occurrence of an sEPSC. There was no increase in the frequency of sEPSCs at +50 mV.

Development of Spontaneous NMDA Receptor-Mediated EPSCs

The development of spontaneous and miniature N-EPSCs have been investigated in other systems. Consistent with the observations presented in this chapter, sN-EPSC properties in the rat neocortex do not change over early development (P3-14; Burgard and Hablitz, 1993). Perhaps more analogously, sN-EPSC and mN-EPSC amplitudes and rise times show no age-dependent changes in the rat superior colliculus during the time when retinocollicular axons are refining (Hestrin, 1992; Shi et al., 1997). However, in that system sN-EPSC and mN-EPSC decay times decrease during map refinement, while the results presented here show no such change in the ferret. This difference is likely due to the difference in timing of a developmental shift from NR2B to NR2A NMDA receptor subunits. This study encompasses the entirety of ON/OFF sublamination, but the NR2B to NR2A shift in the LGN does not occur until well thereafter (Ramoa and Prusky, 1997), in parallel with the decrease in the decay times of evoked N-EPSCs (eN-EPSCs; Ramoa and McCormick, 1994b). In contrast, the NR2B to NR2A shift in the rat superior colliculus, and a parallel decrease in the decay times of eN-EPSCs occur within the time period examined in those studies (Shi et al., 1997). The role of the subunit shift vis-a-vis periods of plasticity is not completely clear. While it has been proposed that the subunit shift occurs during, or near the end, of a period of axon reorganization (Carmignoto and Vicini, 1992; Flint et al., 1997), it may in fact usher in the *onset* of a period of plasticity (Roberts and Ramoa, 1999).

In any case, the subunit shift is thought to be involved in modulating plasticity, not reflecting it. That is, in this study I was interested in measuring changes in those

parameters associated with NMDA receptor-mediated maintenance of long-term changes in synaptic efficacy; for example, the amplitudes of N-EPSCs (Bashir et al., 1991; Berretta et al., 1991; Xie et al., 1992; Asztely et al., 1992). The results in this context reinforce discussions from previous investigations reporting a robust stability of sEPSCs at resting potentials during development (Burgard and Hablitz, 1993; Chapter Four). While noting the possibility that developmental changes may occur in sN-EPSCs during ON/OFF sublamination (here shown not to occur), I suggested that the magnitude of sEPSCs may be normalized over development. Indeed, recent evidence suggests that absolute synaptic efficacy could remain constant while relative synaptic efficacy is modulated by activity-dependent changes in the intrinsic excitability of neurons (Desai et al., 1999; Stemmler and Koch, 1999) or in the connectivity of cell pairs (Hsia et al., 1998).

The Development of the A/N Ratio

Numerous studies have examined the development of either AMPA or NMDA receptor-mediated mEPSCs or sEPSCs (Carmignoto and Vicini, 1992; Hestrin, 1992; Burgard and Hablitz, 1993; Shi et al., 1997; Hsia et al., 1998; Bellingham et al., 1998; Chapter Four). However, the ratio of the AMPA and NMDA receptor components of miniature EPSCs or sEPSCs in a system undergoing activity-dependent development has not been extensively studied.

There is evidence in some systems that, like the results reported here, the A/N ratio of sEPSC amplitudes is stable during early development. In the rat optic tectum, sEPSCs have both AMPA and NMDA receptor components throughout the period of

map refinement and their respective peak amplitudes do not change during this time, indicating a constant A/N ratio (Shi et al., 1997). Likewise, sEPSCs in the rat neocortex consist of both AMPA and NMDA receptor-mediated currents and their ratio is constant throughout early development (Burgard and Hablitz, 1993).

However, there is some evidence that the relative NMDA receptor component of sEPSCS and mEPSCs decreases with age. In recordings from immature, caudal neurons in *Xenopus* optic tectum, mEPSCs are present at depolarized holding potentials, but not at hyperpolarized potentials. Recordings from progressively more mature, rostral neurons reveal faster rise time mEPSCs that are not completely blocked by APV (Wu et al., 1996). This implies that the A/N ratio increases from 0 to some non-zero value as cells mature. While mEPSCs in early postnatal rat auditory cortex do not lack an AMPA receptor-mediated component altogether, it does increase with age while the NMDA receptor component declines (Bellingham et al., 1998), suggesting an increasing A/N ratio.

The development of the A/N ratio has been investigated primarily in the context of silent synapses by comparing minimal stimulation failures at depolarized and hyperpolarized holding potentials. Consistent with the finding from this chapter that this ratio is held constant during ON/OFF sublamination in the LGN, it has been reported that the silent synapses persist during early development in another part of the thalamus, the ventroposterior nucleus (Golshani and Jones, 1998). In contrast, the A/N ratio measured in this way has been shown to increase over early development due to an initial absence of AMPA receptors in the hippocampus (Durand et al., 1996; Isaac et al., 1997; Liao et

al., 1999; Petralia et al., 1999), visual cortex (Rumpel et al., 1998), and somatosensory cortex (Golshani and Jones, 1999).

One would expect that, given an indication of silent synapses, sEPSC frequency would be greater at depolarized than at hyperpolarized holding potentials. Suprisingly, previous investigations of silent synapses do not test for this difference. I find no increase in sEPSC frequency at +40 mV although transmission failures are reduced. Due to rather low sEPSC frequencies, the window of time over which I collected sEPSCs (two minutes) may not have been sufficient to detect a very small increase in their frequency. This fact in conjunction with the relatively modest decrease in the failure rate, approximately 20%, suggests quite a small number of silent synapses[3].

These results reinforce the assessment of retinogeniculate synapses as remarkably

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[3] Given that I make the case that the number of silent synapses is small, an issue here is quantifying "small". Recent literature references a method in Wu et al. (1996) for determining the percentage of silent synapses based on failure rates at hyperpolarized and depolarized potentials. Wu et al. calculate the number of silent synapses so:

$$F_h = (1-P)^{nAN} \qquad (1)$$

$$F_d = (1-P)^{nAN + nN} \qquad (2)$$

$$\frac{n_N}{(n_{AN} + n_N)} = 1 - \frac{\ln F_h}{\ln F_d} \qquad (3)$$

where P is the probability of transmitter release, n_{AN} and n_N are the number of AMPA + NMDA synapses and the number of NMDA-only synapses, respectively; F_h is the number of failures at hyperpolarized, and F_d at depolarized, potentials.

The equation assumes a constant probability, P, for all synapses. However the probability of transmitter release is quite variabile (e.g., Rosenmund et al., 1993; Murthy et al., 1997). Using the characteristics of release probability demonstrated by Murthy et al., (1997; P = Γ(2,11.1)), Eq. 3 can significantly underestimate or overestimate the number of silent synapses to the degree that n_{AN} differs from n_N. So that $n_N = 1$, $n_{AN} = 10$ gives a different answer than $n_N = 10$, $n_{AN} = 100$.

Given the inadequacy of Eq. 3, I've chosen to characterize the number of silent synapses as "small" because their numbers are not evident as changes in the frequency of spontaneous events.

stable during a period of intense anatomical reorganization of their input. More specifically, the data presented here demonstrates that two additional candidate mechanisms for mediating changes in synaptic efficacy, the development of sN-EPSCs and the A/N ratio, are not recruited during ON/OFF sublamination. As I suggested in Chapter Four, it is possible that the synapses made by "incorrectly" placed axons prior to ON/OFF sublamination are replaced by synapses with properties that are indistinguishable from their forebears. Alternatively, it is possible that anatomically misplaced retinogeniculate axons make very few synapses with inappropriate target cells.

Figure Legends

Figure 1. Testing for the ratio of AMPA to NMDA receptor activation. (A) The schematic illustrates the methodology used in these experiments. Using both spontaneous and evoked EPSCs, comparisons were made between EPSCs that consisted of both AMPA and NMDA components (Control, +40 mV) and either EPSCs that consisted of AMPA components only (Control, -60 mV) or EPSCs that consisted of NMDA components only (CNQX, +40 mV). (B) Examples of recordings in the conditions outlined by the schematic in (A). At a holding potential of –60 mV, at which NMDA receptor activation is impeded by magnesium blockade, sEPSCs are completely blocked by the AMPA receptor antagonist CNQX. At a holding potential of +40 mV, at which the magnesium blockade of NMDA receptors is diminished, sEPSCs persist, but are blocked by the NMDA receptor antagonist D-AP5. (C) Examples of sEPSCs and the fitted curves used for measurement of their magnitude and kinetics at positive and negative holding potentials.

Figure 2. The charge transfer of sN-EPSCs is similar prior to and subsequent to ON/OFF sublamination. Histograms of sEPSC areas and example current traces from (A) a P14 and (B) a P31 neuron. Histograms for all properties measured were skewed toward larger values and showed considerable variability. The lower traces for each neuron are averages of 10 consecutive sEPSCs.

Figure 3. The properties of sN-EPSCs are stable throughout ON/OFF sublamination. (A) The areas of sN-EPSCs do not change significantly during this period. Their values

are an order of magnitude larger (with a slightly lower driving force) than AMPA receptor-mediated sEPSCs (Chapter Four). (B) The IEIs, (C) rise times, and (D) half-widths of sN-EPSCs maintain their initial values during retinogeniculate axon reorganization.

Figure 4. The sensitivity of sEPSC amplitudes and rise times to CNQX is constant throughout ON/OFF sublamination. (A) Amplitudes are slightly reduced in the presence of CNQX. The ratio of median sEPSC amplitudes in control and CNQX conditions recorded from the same cell is significantly different from unity (see text for details), but similar in both younger and older cells. (B) Average sEPSCs from a P16 cell in both control (larger trace) and CNQX (smaller trace) conditions. (C) Similarly, optic tract-evoked EPSCs are no more sensitive to CNQX at the end of sublamination than prior to it. (D) Average evoked EPSCs from a P14 cell in both control (larger trace) and CNQX (smaller trace) conditions.

Figure 5. On average, no fewer synapses are activated at a holding potential of –60 mV than at +40 mV. (A) The frequency of sEPSCs does not increase at positive holding potentials in either younger or older cells. (B) Example traces illustrate that while AMPA+NMDA receptor-mediated sEPSCS are significantly larger, their frequency is not different from that of AMPA receptor-only sEPSCs. (C) Stimulation failures are significantly reduced at +40 mV in most cells. Indeed, in particular cells there was evidence of single fibers without functional AMPA receptors. (D) Example current traces of minimally evoked EPSCs at positive and negative holding potentials.

Figure 6. The A/N ratio is highly variable and mostly stable throughout ON/OFF sublamination. (A) The A/N ratios of sEPSC amplitudes, (B) evoked EPSCs amplitudes, (C) the frequency of sEPSCs, and (D) the percentage of transmission failures at positive and negative holding potentials is held constant throughout this period.

Figure 1

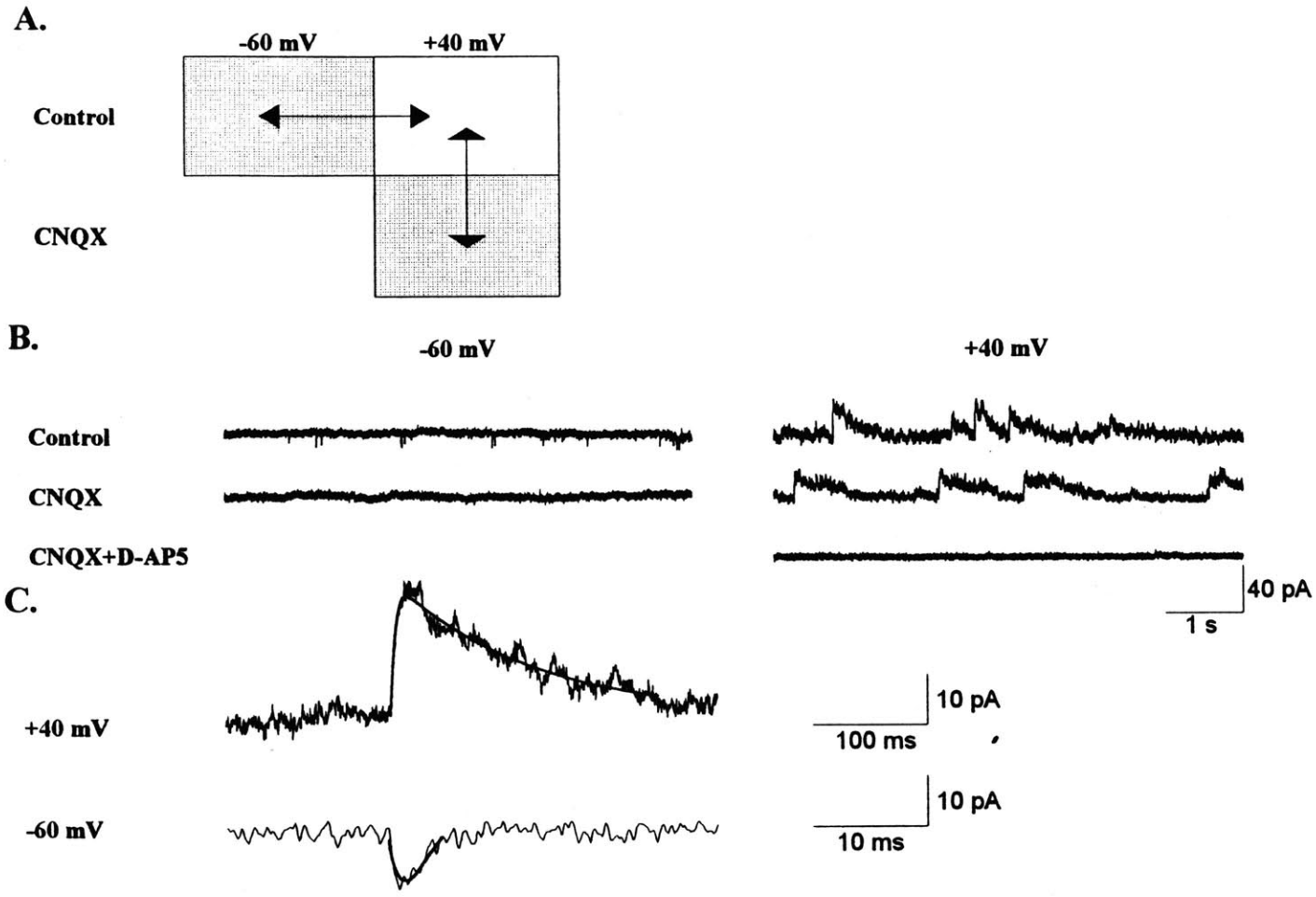

A.
-60 mV
+40 mV
Control
CNQX
B.
-60 mV
+40 mV
Control
CNQX
CNQX+D-AP5
40 pA
1 s
C.
+40 mV
-60 mV
10 pA
100 ms
10 pA
10 ms

Figure 2

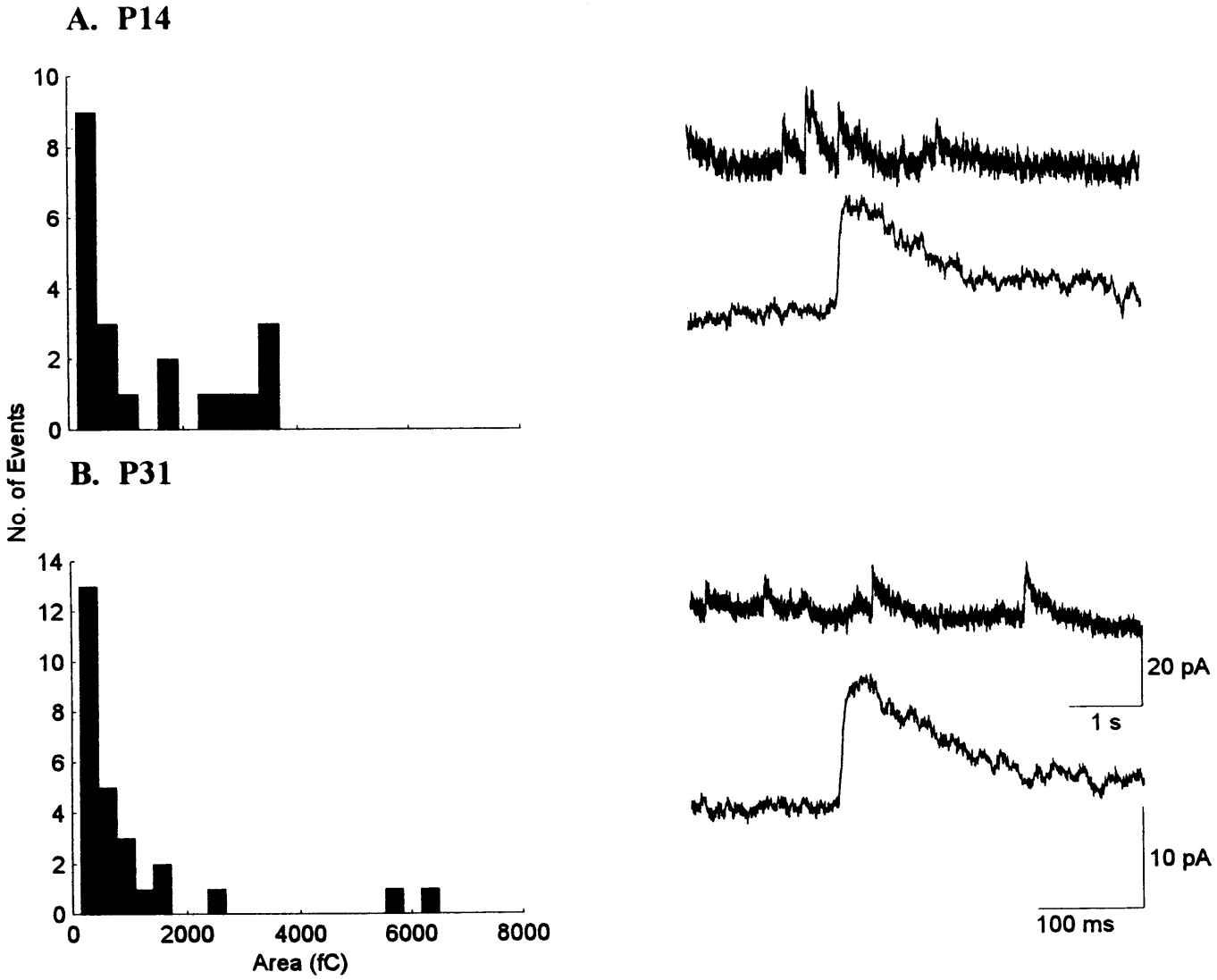


Figure 3

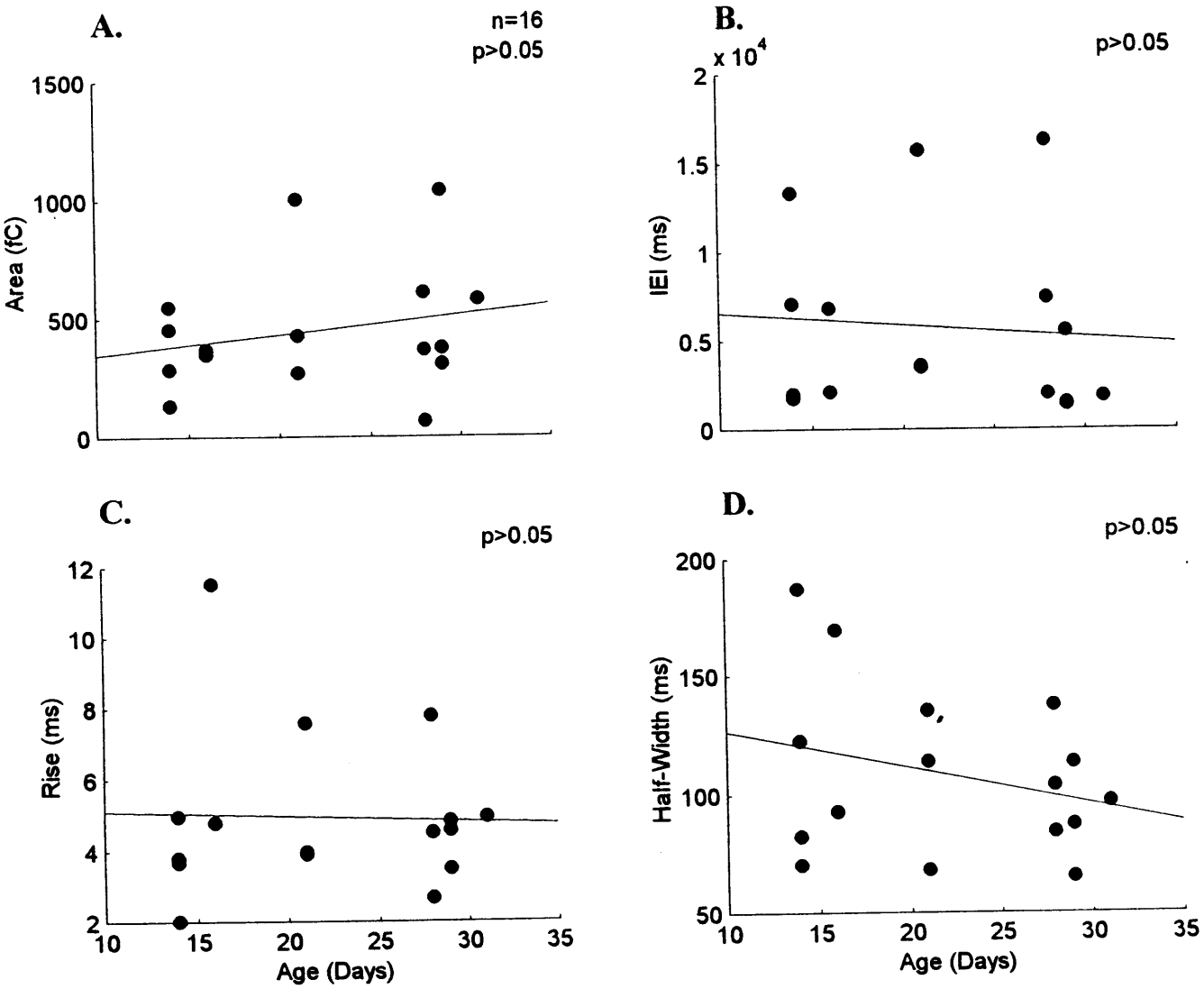

A.
n=16
p>0.05
Area (fC)
1500
1000
500
0
B.
x 10^4
p>0.05
IEI (ms)
2
1.5
1
0.5
0
C.
p>0.05
Rise (ms)
12
10
8
6
4
2
10
15
20
25
30
35
Age (Days)
D.
p>0.05
Half-Width (ms)
200
150
100
50
10
15
20
25
30
35
Age (Days)

Figure 4

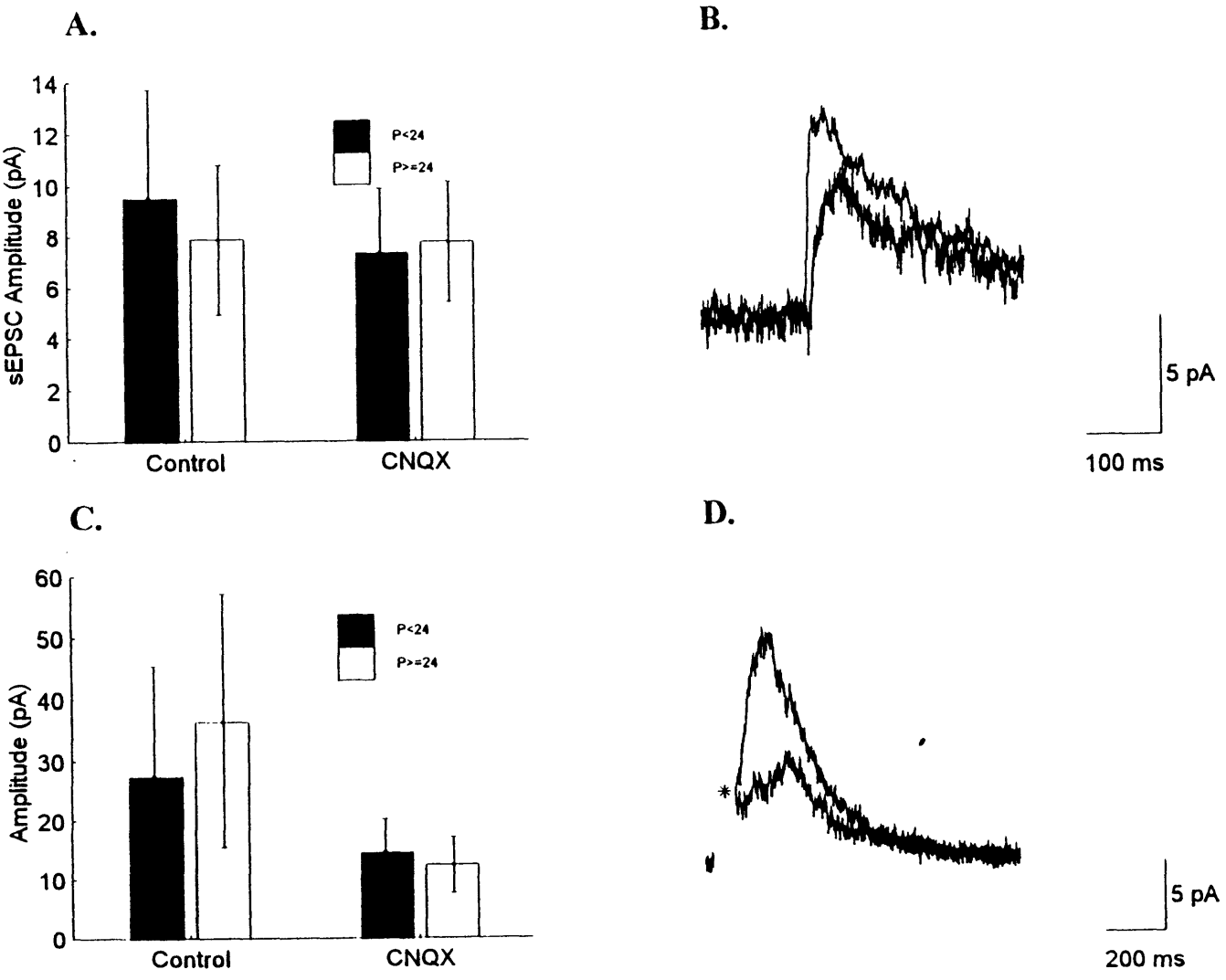

A.
B.
C.
D.
sEPSC Amplitude (pA)
Amplitude (pA)
P<24
P>=24
Control
CNQX
5 pA
100 ms
200 ms

Figure 5

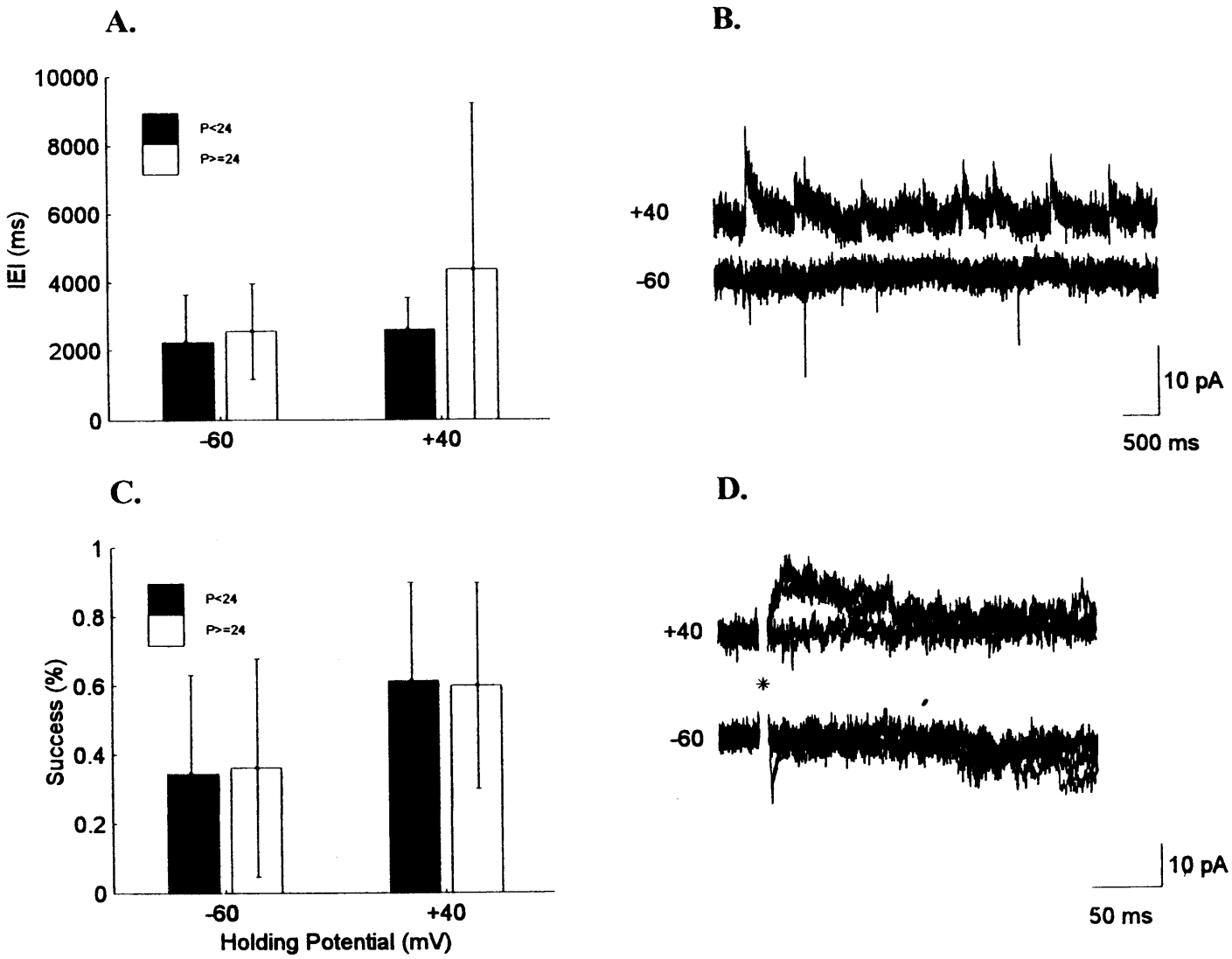
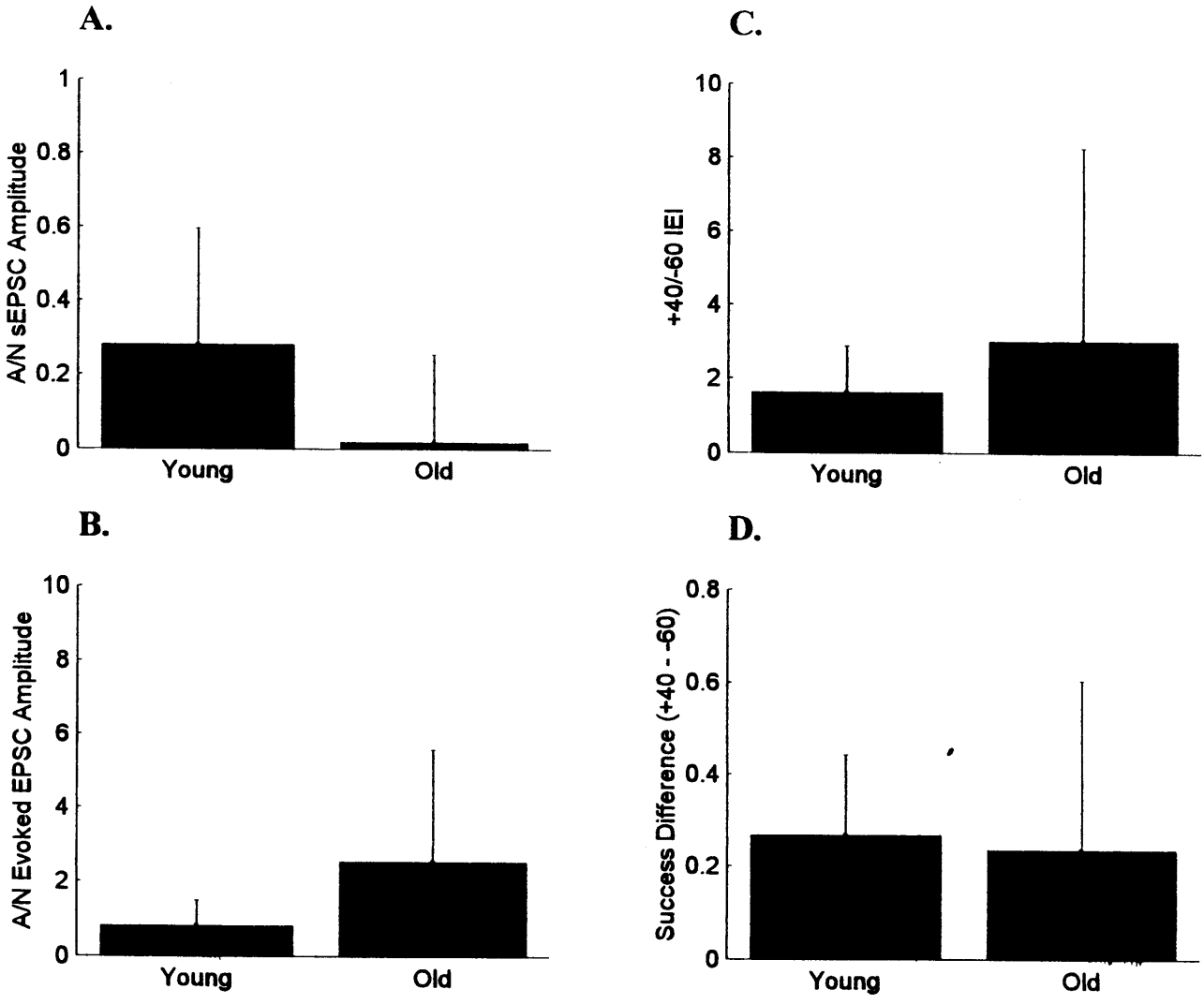

A.
10000
8000
6000
4000
2000
0
IEI (ms)
P<24
P>=24
-60
+40
B.
+40
-60
10 pA
500 ms
C.
1
0.8
0.6
0.4
0.2
0
Success (%)
P<24
P>=24
-60
+40
Holding Potential (mV)
D.
+40
-60
10 pA
50 ms

Figure 6


A.
A/N sEPSC Amplitude
1
0.8
0.6
0.4
0.2
0
Young
Old
C.
+40/-60 IEI
10
8
6
4
2
0
Young
Old
B.
A/N Evoked EPSC Amplitude
10
8
6
4
2
0
Young
Old
D.
Success Difference (+40 - -60)
0.8
0.6
0.4
0.2
0
Young
Old

Chapter Six: The Development of Retinal Axon Input onto Relay Cells in the Ferret Lateral Geniculate Nucleus during the Development of ON/OFF Sublamination

Abstract

During the third and fourth postnatal weeks in ferrets, retinal projections to the lateral geniculate nucleus (LGN) segregate into sublaminae that receive inputs from either ON-center or OFF-center afferents. However, the functional development of retinogeniculate axon input, either singly or as a group innervating a particular LGN relay cell, during this period is not well understood. That is, how many retinal fibers innervate a LGN cell as pattern formation proceeds and what is the effectiveness of innervation by a single fiber? I used minimal and increasing stimulation techniques during this period of dramatic activity-dependent growth and reorganization in order to address this issue. Single retinogeniculate axons were isolated by low-intensity stimulation of the optic tract and multiple retinogeniculate axons innervating the same cell were identified by gradually increasing optic tract stimulation and observing changes in the size of excitatory postsynaptic currents (EPSCs). In general, single fiber EPSCs (sfEPSCs) are stable during ON/OFF sublamination—their amplitudes, rise times and half-widths of sfEPSCs are held constant. Interestingly, while sfEPSCs typically have modest amplitudes, they nonetheless contribute significantly to cell depolarization. On average, sfEPSCs are not much larger than single-synapse EPSCs, but have slower rise times and longer half-widths. In the majority of cells, increasing the intensity of optic tract stimulation led to large increases in EPSC size (relative to the variability in size at low stimulus intensity). In younger cells, the increase is step-wise, indicative of a progressive recruitment of retinal axons. In older cells, the increase in EPSC size is often gradual, with EPSCs showing very slow rise times and long half-widths indicative of non-retinal axon recruitment. Considering the retinal EPSCs alone, there is a progressive reduction in the maximal number of steps evoked in LGN cells during the period of sublamination. The recordings also reveal an inverse correlation between the number of EPSC steps and the size of the sfEPSC. These results suggest that while the number of putative retinal axons innervating a LGN cell declines during sublamination, the axon remaining is elaborating its functional input.

Chapter Six: The Development of Retinal Axon Input onto Relay Cells

Introduction

Early in development, the retinogeniculate pathway's structure is significantly different from what it is in the mature animal. In the ferret, retinogeniculate axons from the two eyes segregate during development to form eye-specific laminae (Linden et al., 1981). Subsequently, axons from ON-center and OFF-center retinal ganglion cells that initially span an entire eye-specific lamina, segregate from one another to form sublaminae (Stryker and Zahs, 1983; Hahm et al., 1991). This reorganization of the retinogeniculate axons is dependent on normal neural activity (Hahm et al., 1991; Cramer et al., 1996; Cramer and Sur, 1997) as are other developmental events in the mammalian visual system (for reviews see Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Hohnke and Sur, 1999b).

While it is clear that blocking neural activity disrupts the normal development of precise connectivity, it is less clear what the subsequent links in the chain are that translate neural activity into morphological changes. However, activity-dependent development of connections in the visual system has components that seem to be shared with long-term potentiation, or LTP, of synapses in the CA1 region of the hippocampus (Shatz, 1990; Goodman and Shatz, 1993; Cramer and Sur, 1995; Hohnke and Sur, 1999b). For example, both phenomena require patterned electrical activity, but may also require NMDA receptor activation, nitric oxide production and neurotrophin signaling. Thus, LTP appears to be closely related to activity-dependent development and may underlie it (Bear et al., 1987; Constantine-Paton, et al., 1990; Kandel and O'Dell, 1992; Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Constantine-

Paton and Cline, 1998). That is, functional synaptic contacts in appropriate and inappropriate areas undergo differential changes in efficacy that signal axon branches to elaborate and stabilize in the former case and withdraw in the latter.

How might the role of LTP during axon reorganization be assessed? In Chapter Four I determined that no increase in AMPA-mediated synaptic transmission, one possible manifestation of LTP (Kauer et al., 1988; Muller et al., 1988; Davies et al., 1989; Isaac et al., 1995; Liao et al., 1995), occurred during ON/OFF sublamination. In Chapter Five I found the same to be true for NMDA-mediated synaptic transmission. Here I examine an additional possibility. Alternatively, LTP could result from induced synaptogenesis (for reviews see Thoenen, 1995; Bonhoeffer, 1996; Ghosh, 1996) and result in an increased effectiveness of a single retinogeniculate axon to bring its target cell to action-potential threshold. Consistent with this possibility, ON and OFF axons are known to progressively elaborate their connections in their appropriate sublayer (Roe et al., 1989; Hahm et al., 1991). Similarly, there is a progressive increase in thalamocortical and intracortical connectivity during visual cortex development (Katz and Shatz, 1996) and a developmental increase in the connectivity between cell pairs in the hippocampus (Hsia et al., 1998).

I also addressed the question of whether functional retinogeniculate axon branches are, in fact, pruned away during this period. While there is no evidence demonstrating that overly large retinogeniculate axons are making functional synaptic contacts in tissue from which they later withdraw (but see Shatz and Kirkwood, 1984; Campbell and Shatz, 1992), both anatomical evidence from the LGN and analogy with

other areas of the nervous system suggest that retinogeniculate axons are pruned away during early development (Purves and Lichtman, 1980; Sur et al., 1984; Sretavan and Shatz, 1986). Additionally, evidence suggests that this pruning is competitive. For example, when input is deprived from one eye during early development, neurons in the primary visual cortex that normally respond to stimulation of either eye, respond only to stimulation of the non-deprived eye (Hubel and Wiesel, 1970; Hubel et al., 1977). Similarly, blockade of patterned activity in a ferret retina leads to an expansion of LGN territory innervated by axons from the non-manipulated eye (Penn et al., 1998, but see Cook et al., 1996). Lastly, results from the neuromuscular junction demonstrate that axon branch pruning is a competitive process accompanied by changes in quantal content (Colman et al., 1997; Laskowski et al., 1998; Gan and Lichtman, 1998).

I find a reduction in the number of fibers providing fast, retinogeniculate-like input at the same time that the number of slow, non-retinal-like fibers is increasing. When the putative retinogeniculate fibers are considered separately, there is an inverse correlation between the number of EPSC steps and the size of the sfEPSC. These results suggest that while the number of putative retinal axons innervating a LGN cell declines during sublamination, the axon remaining is elaborating its functional input.

Methods

In this set of experiments recording pipettes contained either the potassium-based solution or the cesium-based solution. In some cases the cesium-based solution contained 5 mM QX-314. There were no group differences in the synaptic currents

recorded with the three solutions[1]. 10 μM CNQX disodium, 100 μM D-AP5, 500 μM MCPG were used to block AMPA, NMDA, and metabotropic gluatamate receptors, respectively. 50 μM BMI was present in all recordings to block $GABA_A$ receptors.

Afferents were stimulated as described in Chapter Two. Electrical stimuli, ranging between 3 and 7,500 pC were delivered at 0.2 to 0.5 Hz. Appropriate stimulus intensities were based on those used in previous investigations of minimal or single-fiber stimulation (Raastad et al., 1992; Stevens and Wang, 1995)[2] and increasing stimulation (e.g., Redfern, 1970). For minimal stimulation, a stimulus intensity was sought that a) resulted in both EPSCs and failures of synaptic transmission, b) produced constant EPSC latencies, and c) did not produce a significantly different response than those intensities just less than or great to it (that is, the minimal response was stable over a range of stimulus intensities). These intensities were typically just greater than the maximum at which no synaptic transmission occurred. For increased stimulation, a stable, minimal EPSC was established and the stimulus intensity was increased through a range of values. In some cases the onset of the EPSC was embedded in the stimulus artifact while the peak response (amplitude) was unambiguous. These responses were excluded from calculations of rise time and half-width. For purposes of approximation, the charge of an EPSC was calculated as the amplitude times the half-width. Because in some cases steps in EPSC amplitudes were small relative to the variability of EPSC size, I performed

[1] Also see Chapter Four which presents a quantitative discussion of the equivalence of sEPSCs recorded in these different conditions.
[2] See Chapter Two for a discussion of the issues surrounding the interpretation of minimal stimulation.

statistical comparisons between EPSC amplitudes of successive epochs for each increase in stimulus intensity. Changes occurring at the $p < 0.05$ level (Mann-Whitney U test) were considered steps.

Results

Single Fiber Stimulation

Single retinogeniculate axons were isolated by low-intensity stimulation of the optic tract. In most cases, single fiber isolation was stable across gradual increases in stimulus intensity and only after multiple increases in stimulus intensity did EPSC size increase (Figure 1). Single fiber EPSCs (sfEPSCs) were analyzed in 28 cells from animals between P14 and P31. I recorded 457 sfEPSCs from younger animals (14 cells from 9 animals) and 484 sfEPSCs from older animals (14 cells from 11 animals). The stimulus intensities that were chosen elicited sfEPSCs on 57% (± 22%) of the trials.

Distributions of amplitudes[3] for younger and older sfEPSCs are stable during ON/OFF sublamination. Histograms of sfEPSC amplitudes for amplitudes below 50 pA, which account for 75% and 89% of younger and older sfEPSCs, respectively, are similar (Figure 2). The distributions of the remaining sfEPSCs for the two groups, while being

[3] In this set of experiments amplitudes were used to measure the magnitude of sfEPSCs and EPSCs. In Chapter Three and Chapter Four I found that R_a tends to correlate with age, which in turn confounds developmental analysis of R_a-sensitive EPSC parameters such as amplitude. In this set of experiments, however, accurate calculation of EPSC areas was prohibited in a large number of cases, primarily due to their extremely short onset-latency with respect to the stimulus artifact. That is, EPSC onset often began within the duration of the stimulus artifact. Fortunately, in the present set of experiments R_a did not

centered on different amplitudes (Figure 2A, B inset), come from a small number of cells and so do not contribute significantly to any difference in the median sfEPSC amplitudes between the two groups (Figure 3A). Specifically, median amplitude in the younger animals was 18 pA (28 ± 26 pA) and median amplitude in the older animals was 14 pA (68 ± 122 pA, $p > 0.05$). While sfEPSC amplitudes recorded from a particular cell showed a good deal of variability, the coefficient of variation (CV) observed in recordings from younger animals (0.2, 0.3 ± 0.2) was not significantly changed in older animals (0.2, 0.2 ± 0.1, $p > 0.05$; Figure 3B). Additionally, no correlation was observed between age and amplitude ($rs = 0.0$, $p > 0.05$) or age and amplitude CV ($r_s = 0.2$, $p > 0.05$).

Neither were the rise times and half-widths of sfEPSCs significantly changed after the reorganization of retinogeniculate axons (Figure 3C, D). In the subset of cells for which rise time and half-widths could be reliably measured (see Methods), the median rise time in the younger animals (1.0, 1.4 ± 1.4 ms; $n = 6$) was the same as in the older animals (0.9, 1.2 ± 3.0 ms; $n = 10$, $p > 0.05$) and rise times were not correlated with age ($r_s = -0.2$, $p > 0.05$). Likewise, half-widths in the younger animals (4.9, 6.6 ± 7.4 ms; $n = 13$) were not significantly different from those in older animals (5.3, 8.5 ± 8.0 ms; $n = 14$, $p > 0.05$), nor was there a correlation between age and half-widths ($r_s = 0.2$, $p > 0.05$).

While sfEPSCs typically have modest amplitudes, they nonetheless contribute significantly to cell depolarization. In a subset of recordings I measured the change in

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correlate with age in either the minimal stimulation experiments ($r_s = 0.03$, $p > 0.05$) or the increasing

charge (ΔQ) required to generate an action potential using two separate measurements. For the first measure I determined the cell membrane's capacitance (C_m) and calculated the difference in resting voltage (ΔV_m) required to generate an action potential. Threshold membrane voltage (V_t) in younger animals (-42, -40 ± 7 mV) was not different from that in older animals (-41, -42 ± 5 mV). Solving the equation $\Delta V_m = \frac{\Delta Q_a}{C_m}$ for ΔQ_a gives the change in charge required to achieve ΔV_m. I calculated the approximate charge contributed by an sfEPSC (see Methods) and normalized by ΔQ_a giving the contribution of an sfEPSC to action potential generation (Figure 4A). The median value of $\frac{\Delta Q}{\Delta Q_a}$ in the younger animals was (0.2, 0.8 ± 1.8) and did not change significantly in the older animals (0.1, 0.1 ± 0.2, $p > 0.05$). For the second measure, I calculated the current injection (I_{inj}) required to generate a single action potential from a constant resting potential (-60 mV) and the input resistance (R_i). Consequently, $\Delta Q_b = \tau I_{inj}$ represents the change in charge required to depolarize the cell membrane to within $1-\frac{1}{e}$ of action potential threshold where τ is the membrane time constant $(R_i C_m)$[4]. I_{inj} was not different in younger animals (25.0, 25.8 ± 16.6 pA) than in older animals (15.0, 29.3 ± 28.8 pA, $p < 0.05$). Additionally, like $\frac{\Delta Q}{\Delta Q_a}$, the median value of $\frac{\Delta Q}{\Delta Q_b}$ in younger animals (0.4, 2.1 ± 5.4)

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stimulation experiments ($r_s = 0.39$, $p > 0.05$).

[4] See Footnote 3 in Chapter Three.

was unchanged in older animals (0.5, 2.6 ± 5.5, $p > 0.05$). Neither $\frac{\Delta Q}{\Delta Q_a}$ ($r_s = -0.1$) nor $\frac{\Delta Q}{\Delta Q_b}$ ($r_s = 0.2$) showed any correlation with age ($p > 0.05$).

Generally, sfEPSCs are slower and larger than single-synapse EPSCs[5]. In younger animals, sfEPSC rise times are 112% greater, and half-widths 150% greater, than reported values for sEPSCs from similarly aged animals (Chapter 4). Likewise, in older animals, sfEPSC rise times and half-widths were 102% and 79% greater, respectively, than those for sEPSCs from similarly aged animals (Chapter 4). More specifically, there was notable variation in the relationship between sfEPSCs and sEPSCs. In both age groups I observed sfEPSCs that were in some cases very similar in shape and size to sEPSCs recorded from the same cell and in other cases many times larger. In most younger animals (n = 3), sfEPSCs had amplitudes (7.6, 8.6 ± 4.0 pA) on the order of those for sEPSCs from the same cell. In one case median sfEPSC amplitude (73.2 pA) was many times larger than the median sEPSC amplitude (11.9 pA) recorded from the same cell. Similarly, in older animals (n = 2), sfEPSCs were either very similar in size to sEPSCs recorded from the same cell or many times larger. In all of these cases sfEPSCs are noticeably wider than sEPSCs from the same cell, as is true generally (Figure 5A,B). The relationship between sfEPSCs and sEPSCs implies that sfEPSCs also often have longer half-widths than emEPSCs given that there is no difference between sEPSCs and emEPSCs (Chapter 4).

Increasing Stimulation

Multiple retinogeniculate axons innervating the same cell were identified by gradually increasing optic tract stimulation and observing changes in EPSC size. Most frequently, increasing stimulation elicited either no change or discreet "jumps" in EPSC size in both younger and older animals (Figure 6) which were interpreted as the recruitment of at least one (and probably only one) additional innervating fiber (e.g., Redfern, 1970). Increasing stimulation was analyzed in 21 cells from animals between P14 and P31. I recorded 3,169 EPSCs from younger animals (11 cells from 8 animals) and 2,887 EPSCs from older animals (10 cells from 8 animals).

In the majority of cells (n = 13), increasing stimulation eventually lead to large increases in EPSC size relative to the variability in size at a given stimulus intensity (Figure 7A). However, in some cases (n = 8) EPSCs increased much more gradually such that plotting EPSC amplitude against stimulus intensity generated a more continuous curve than that seen in the majority of cases (Figure 7B). The EPSCs showing this behavior had dramatically slower rise times and longer half-widths than EPSCs that increased abruptly with increasing stimulation (right traces, Figure 7A,B). Both the gradual increase in EPSC size, indicating innervation by many axons, and their slower kinetic properties suggest non-retinal input. Non-retinal innervation of LGN relay cells is an order of magnitude greater than retinogeniculate innervation (Sherman and Koch, 1986, 1998; Erisir et al., 1997) and trigger slow-activating, long-lasting EPSCs

[5] In the text and in Figure 5 I compare sfEPSCs and sEPSCs recorded from the same cell. The

while retinogeniculate input does not (McCormick and Prince, 1987; McCormick and Von Krosigk, 1992; Zhou et al., 1994; Godwin et al., 1996).

An examination of the location of the recording electrode relative to the stimulating electrode suggested the possibility of non-retinal fiber activation. In older cells, when the stimulating electrode was distant from, or off of the projection line of retinogeniculate fibers, stimulation elicited slow-activating, long-lasting EPSCs (Figure 8A). In the cases where fast rise time, short half-width EPSCs were recorded in older cells, the cells were either close to the stimulation site or distant, but on the projection line while all EPSCs in younger cells had fast rise times and short half-widths. To test for the possibility of non-retinal, non-inhibitory[6] activation, I blocked NMDA-, AMPA-, $GABA_A$- and $GABA_B$-activation and observed whether some transmission persisted. In the presence of that blockade I observed significant remaining EPSCs in a cell with gradually increasing, slow and long EPSCs (Figure 7C, bottom traces) while in a cell with abruptly increasing, fast and short EPSCs, CNQX blocked all transmission (Figure 7C, top traces). This suggested to me either cortical or brain-stem input activation.

To test more directly for corticogeniculate activation I stimulated corticogeniculate fibers traversing the perigeniculate nucleus (PGN; Figure 8B). In younger cells (n = 4) stimulating in the PGN tended not to result in synaptic activation,

characteristics of those sEPSCs are reported in Chapter Four.

[6] Ideally, one could simply rely on the fact that the reversal potential is around –60 mV for $GABA_A$-mediated currents and around –80 mV for $GABA_B$-mediated currents in order to rule out inhibitory currents. In this case, with a cell voltage-clamped at –60 mV, $GABA_A$-mediated PSCs would be non-existent and $GABA_B$-mediated PSCs would have the expected opposite sign. However, at least in the case of a KGlu base, the reversal potential for inhibitory events can be significantly affected by intracellular

but did elicit fast rise time, long half-width EPSCs in one (25%) of the recordings. In older cells (n = 7) fast rise time EPSCs were elicited in 43% of the recordings and slow rise time EPSCs occurred in one (14%) recording. Given the predominance of fast rise time EPSCs in those cases in which any EPSCs were elicited from PGN stimulation (80%), the slow rise time EPSCs elicited by optic tract stimulation are unlikely to result from corticogeniculate inputs. Indeed, when all glutamatergic and GABAergic activity is blocked (10 μM CNQX + 50 μM D-AP5 + 500μM MCPG + 50 μM BMI + intracellular cesium), slow EPSCs persist (n = 2). Finally, optic tract stimulation elicited EPSCs in 100% of recordings during which PGN stimulation was attempted (n = 11). PGN stimulation was equidistant from the recording electrode and at stimulation intensities greater than any used during the increasing stimulation experiments. The fact that high-intensity PGN stimulation did not spread to activate confirmed retinogeniculate inputs suggests that increasing optic tract stimulation did not recruit corticogeniculate fibers.

In general, the number of EPSC steps in response to increasing stimulation does not change during ON/OFF sublamination, though there is a slight trend toward a greater number of steps with age (Figure 9A). Relatively few steps were observed in the younger animals (3, 3.9 ± 3.0) and this did not change significantly in the older animals (4.0, 5.8 ± 4.1, $p > 0.05$). Similar ranges of stimulus intensities were used to probe cell innervation in both younger and older animals. I noted both the stimulation intensity used to evoke the minimal EPSC, S_{min}, and the maximum stimulation intensity used during a particular

solutions.

recording, S_{max}. Neither S_{min} nor S_{max} were significantly different for the younger animals (S_{min}: 40.0, 59.6 ± 82.5 pC; S_{max}: 200.0, 973.5 ± 2,193.9 pC) compared with the older animals (S_{min}: 16.7, 28.1 ± 26.9 pC, $p > 0.05$; S_{max}: 102.7, 130.2 ± 95.3 pC, $p > 0.05$). More importantly, the normalized maximum stimulus intensity, $\frac{S_{max}}{S_{min}}$, for younger animals (6.3, 16.1 ± 29.2) was not significantly different from that for older animals (5.7, 6.0 ± 3.4, $p > 0.05$; Figure 9C).

Given the suggestion of two populations of EPSC shapes mentioned above, those with fast rise times and short half-widths and those with slow rise times and long half-widths, I measured those parameters to determine how they were distributed throughout the population of recordings. Analysis of the rise times and half-widths of the EPSCs indicated that older cells with moderate and large numbers of EPSC steps are limited to those with slow rise times (> 2 ms) and long half-widths (> 7 ms). Indeed, all older cells with greater than one EPSC step had EPSCs of the slow variety as did the oldest cells of the younger group; none of the youngest cells had slow EPSCs (Figure 9B,D).

This analysis led me to reevaluate the development of sfEPSCs. Here, too, the population of cells could be divided roughly into those show sfEPSCs with long half-widths (> 5 ms) and those with shorter half-widths. Figure 10A shows that for cells in which both minimal and increasing stimulation were performed, the same relationship holds between the no. of EPSC steps and age for sfEPSCs as exists for EPSCs. That is, in older cells short half-width sfEPSCs occurred in cells that subsequently, upon increasing stimulation, showed few EPSC steps. Interestingly, the sfEPSC of these older cells (n = 4) not only had shorter half-widths, but had much larger amplitudes (36, 35 ±

22 pA) than the other, long half-width sfEPSCs (10, 11 ± 3 pA, n = 8, $p < 0.05$) from older cells (Figure 10B,C). Additionally, for both young and old, short half-width sfEPSCs, there was a strong inverse correlation between sfEPSC amplitude and the number of subsequent EPSC steps revealed through increasing stimulation ($r_s = -0.6$, n = 10, $p < 0.05$, Figure 10D).

Discussion

The results presented here suggest that individual retinogeniculate axons maintain a stable relationship with their target cells during ON/OFF sublamination. This stability is especially notable given that I also present data suggesting that those retinogeniculate axons are being functionally pruned during this period. These data add to the discussion of the normal, functional development of retinogeniculate axons (Ramoa and McCormick, 1994b; Chapter Four).

Single Fiber Stimulation

I find that sfEPSCs are remarkably stable during ON/OFF sublamination given the dramatic growth and reorganization that retinogeniculate axons are undergoing throughout this period. In contrast, minimal EPSP amplitudes at the rat CA3-CA1 synapse in the hippocampus increase significantly between the third and fifth postnatal weeks (Dumas and Foster, 1995), probably due to an increased connectivity between cell pairs (Hsia et al., 1998). This difference may be due to differences between systems that undergo significant axonal rearrangement and those that do not or to differences between species and developmental stage.

On the whole, the sfEPSCs I report on here have modest amplitudes, being not much larger than single-synapse EPSCs, but have slower rise times and longer half-widths. This finding suggests that the probability of transmitter release, at least at the end of ON/OFF sublamination, is quite low—retinogeniculate fibers in the adult cat make hundreds of contacts onto relay cells (Wilson et al., 1984; Hamos et al., 1987). A decreasing probability of transmitter release is also suggested by my study of the development of sEPSCs during ON/OFF sublamination (Chapter Four). I found little change in the frequency of sEPSCs during a time when retinogeniculate axons are elaborating in their appropriate sublaminae. Presumably retinogeniculate axons are adding synaptic contacts onto their target cells during this period. In the cat, the number of retinogeniculate contacts onto LGN relay cells increases by about 25% from birth to eight weeks (Kalil and Scott, 1979; Mason 1982a), although this data is difficult to relate to postnatal ferrets.

While modest, sfEPSCs contribute significantly to cell depolarization. This finding is consistent with results demonstrating that the excitatory effect from one, or at most a few, retinal ganglion cells in the cat correlate with most of an LGN cell's action-potentials (Mastronarde, 1987). Additionally, I find that, in general, the relationship between the input provided by a single retinogeniculate axon and the target cell's action-potential threshold is stable throughout ON/OFF sublamination. Taken together with the stability of sfEPSCs, this finding is in accordance with results showing that action-potential thresholds in cells in the ferret LGN have reached stable values by the beginning of ON/OFF sublamination (Ramoa and McCormick, 1994a and Chapter

Three).

Increasing Stimulation

Competition among axons for synaptic contact onto a target cell implies a reduction in convergence. While the anatomical evidence that different types of retinogeniculate axons structurally segregate from one another is quite strong (Rakic, 1977; Linden et al., 1981; Shatz, 1983; Stryker and Zahs, 1983; Hahm et al., 1991), there is no evidence demonstrating that an LGN relay cell is functionally contacted by a larger number of retinogeniculate axons before ON/OFF sublamination than after.

The data presented here demonstrate a reduction in the number of fibers providing fast rise-time, short half-width input. This finding is consistent with anatomical data that suggests that pruning occurs throughout the nervous system. During the development of the segregated layers in the cat LGN, side branches emerge and disappear (Sretavan and Shatz, 1986) and X-cell axon arbors contract (Sur et al., 1984). In the adult, X-cell axon arbors, while contacting only a few LGN relay cells (Mastronarde, 1987), span a distance encompassing up to 43 neurons [(Hamos et al., 1987), Y axons are somewhat larger (Bowling and Michael, 1984; Sur et al., 1987)], suggesting that there is ample opportunity for axons to have made contacts with cells with which it subsequently has none.

Perhaps more importantly, evidence from the neuromuscular junction, autonomic ganglia, nucleus magnocellularis, and the cerebellum indicate that a reduction in the number of fibers innervating a muscle fiber or neuron is a general feature of a developing nervous system (Purves and Lichtman, 1980; Purves and Lichtman, 1985). In the nucleus

magnocellularis, for example, a decrease in the number of preterminal branches is accompanied by a reduction in the average number of axons innervating a neuron (Jackson and Parks, 1982). It is possible that the similar emergence and disappearance of side branches in the LGN (Stretavan and Shatz, 1986) provide transient innervation to LGN relay cells.

I find a subpopulation of slow rise-time, long-half width input fibers toward the end of ON/OFF sublamination. The insensitivity of these EPSCs to extracellular CNQX, D-AP5, and BMI and intracellular cesium suggest a non-retinal, non-inhibitory conductance. Corticogeniculate, but not retinogeniculate, synapses activate an excitatory mGluR on relay cells that lead to slow and long EPSCs (McCormick and Von Krosigk, 1992). More specifically, corticogeniculate inputs activate mGluR5s on LGN relay cell dendrites while retinal inputs activate mGluR1s present on F2-type terminals of interneurons (Godwin et al., 1996). Indeed, ultrastructural evidence also suggests that retinogeniculate and corticogeniculate inputs may differ physiologically. Retinogeniculate terminals have a final area ten times greater than, and a final length equal to, cortical inputs (compare Kalil et al., 1986 and Weber and Kalil, 1987) and so presumably will have different amounts of postsynaptic machinery available to them. However, a kinetic contribution to shape difference is unlikely, both because of the relative proximity of retinogiculate and corticogeniculate inputs (Hamos et al., 1987; Weber and Kalil, 1987) and the fact that LGN relay cells are likely to be electrotonically compact (Bloomfield et al., 1987). Relay cells in the LGN are also innervated by noradrenergic, serotonergic, and cholinergic inputs from the brainstem. Noradrenergic

leads to a very long-lasting (> 1 minute) depolarization and serotonergic inputs generally exert an inhibitory influence and so are also unlikely to mediate the responses in question (Sherman and Koch, 1998 and references therein). The fact the response was not blocked by the metabotropic glutamate receptor antagonist, MCPG, then, suggests that the activated inputs may be cholinergic fibers from the parabrachium. Expression of choline acetyltransferase and acetylcholinesterase in the ferret peaks during the time of ON/OFF sublamination (Hitz, 1998).

It is important to establish that the populations of innervating cells and target cells are stable during axon reorganization in the LGN. Retinal ganglion cell (RGC) birth is completed by P3 (Johnson and Casagrande, 1993) and RGC numbers reach adult values by P6 (Henderson et al., 1988; Johnson and Casagrande, 1993), approximately one week prior to the onset of ON/OFF sublamination. LGN cell birth is completed by E30 (Johnson and Casagrande, 1993).

These data also add to the discussion of the role of LTP in the activity-dependent development of synaptic connectivity (Bear et al., 1987; Constantine-Paton, et al., 1990; Kandel and O'Dell, 1992; Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Constantine-Paton and Cline, 1998). No clear consensus exists on what the mechanisms of LTP maintenance are or whether they are developmentally regulated. Consequently, assaying the role of LTP in activity-dependent development requires an examination of candidate mechanisms in systems undergoing this type of plasticity and also of developmental differences in LTP induction. Here I have taken on part of the first task.

LTP might be manifested as an increased effectiveness of a single retinogeniculate axon to bring its target cell to action-potential threshold. The implication of neurotrophic factors in LTP (for review see Lu and Figurov, 1997) suggest that, at least in the late phase of LTP (Korte et al., 1998), synaptogenesis may be involved in the maintenance of LTP (see also Thoenen, 1995; Bonhoeffer, 1996; Ghosh, 1996). Additionally, ON and OFF axons are known to elaborate in their appropriate layer (Hahm et al., 1991) and there is a developmental increase in the connectivity between cell pairs in the hippocampus (Hsia et al., 1998). Consequently, one would expect single retinogeniculate fibers that have been stabilized through this type of LTP to trigger larger EPSCs in their target cells. Indeed, I found that, when considering the short half-width sfEPSCs, there was a significant, inverse correlation between the number of EPSC steps produced in response to increasing stimulation and the amplitude of the sfEPSC.

These results suggest that while some retinogeniculate input is being pruned and other input is elaborating, on average the contribution of a single axon is "normalized" toward some acceptable level. However, it is also possible that LTP, acting through increased synaptogenesis, is involved, in a sub-population of fibers that produce fast rise-time, short half-width sfEPSCs resembling what would be expected from retinogeniculate input. One might expect that in this case the efficacy of individual synapses decreases with the increased innervation (Liu and Tsien, 1995). However, I have found that no such decrease in synaptic efficacy occurs in this system. Nor does there appear to be an increase in the frequency of spontaneous EPSCs (Chapter Four and Chapter Five) suggesting little net gain of functional synapses. Consequently, it is likely that the

increase in sfEPSC amplitude with age, results from an elaboration of retinogeniculate synapses from a single fiber that is commensurate with the loss from withdrawn fibers.

Figure Legends

Figure 1. Single retinogeniculate axons can be isolated by stimulation of the optic tract at low stimulus intensities. Stimulus intensity is adjusted so that both failures of transmitter release (open circles) and minimal EPSCs are detected (closed circles) and then increased slightly to rule out action potential activation failures (see Chapter Two). Single fiber isolation is stable across gradual increases in stimulus intensity (solid line, right y-axis) until additional fibers are recruited and EPSC size increases. Shown at the top are average EPSCs for the underlying set of trials at a particular stimulus intensity.

Figure 2. Single fiber EPSCs in the LGN are stable during ON/OFF sublamination. Histograms of sfEPSC amplitudes (not including failures) and example sfEPSCs from (A) a P15 and (B) a P30 neuron. Amplitudes less than or equal to 150 pA are shown in the histograms for easier comparison between the two age groups. Younger cells show a number of sfEPSCs between 50 and 150 pA while older cells show a population of sfEPSCs between 300 and 500 pA (B, inset). However, there is no significant change in median sfEPSC amplitude during ON/OFF sublamination ($p < 0.05$, see text for details). The stimulus artifact has been replaced with an asterisk.

Figure 3. Development of sfEPSC properties of relay cells in the LGN during ON/OFF sublamination. (A) Median sfEPSC amplitudes and (B) amplitude variability do not change significantly during this period ($p < 0.05$). Likewise, (C) the sfEPSC rise times and (D) widths are variable, but stable throughout the period that retinogeniculate axons are reorganizing ($p < 0.05$).

Figure 4. Single fiber EPSCs contribute significantly to cell depolarization. The approximate charge of the median sfEPSC (ΔQ) relative to the change in charge required to bring the cell to action potential threshold (ΔQ_a) remains stable throughout ON/OFF sublamination (A, $p < 0.05$, see text for details). (B) Likewise, the membrane time constant, τ, has reached adult values by P14. In an example from a P24 cell (C), the median sfEPSC provides 164% of ΔQ_a (top trace, right). The current injected into the cell is illustrated by the rectangular pulses (top trace, left) and resulted in sub- and super-threshold depolarization of the cell membrane (bottom trace).

Figure 5. Single fiber EPSCs are similar in amplitude to single synapse EPSCs, but have longer half-widths. Examples from a (A) P15 and a (B) P31 cell show that average sEPSCs (left, upper traces) have shapes and sizes that are similar to, but narrower than sfEPSCs (right, lower traces) from the same cell. Accompanying histograms show population data for half-widths. The first ten EPSCs from each recording were pooled. Examples from a different (C) P15 and (D) P31 cell illustrate that, like sEPSCs, unitary *retinal* EPSCs resemble sfEPSCs, but are narrower.

Figure 6. Increasing stimulation reveals discreet increases in EPSC size. Scatter plots and example traces from a (A) P16 and a (B) P30 cell show steps in EPSC size resulting from gradual increases in stimulus intensity.

Figure 7. Slow EPSCs increase in amplitude more gradually than fast EPSCs. (A) In the majority of cells increasing stimulation reveals discreet increases in EPSC size, but (B) in a subset of cells EPSC size increases continuously with gradual increases in stimulus

intensity. (C) These EPSCs are characterized by slow rise times and long half-widths and have components that are not mediated by CNQX, d-AP5, or BMI sensitive receptors (lower trace). Fast EPSCs are, however, completely blocked by CNQX (C, upper trace).

Figure 8. Recording sites are indicated by letters throughout the interiors of the schematics of a P14 (lighter gray) and a P28 (darker gray) horizontal LGN slice; corresponding stimulation sites lie along the periphery of the slice. Arrows show projection lines. (A) Slow rise time, long half-width EPSCs tended to be recorded from older cells that were relatively distant and not centered on the projection line from the stimulation site (circles). Note that the two recordings from older cells in which fast rise time, short half-width EPSCs were recorded (squares) were either close to the stimulation site [G] or distant, but on the projection line [N]. All EPSCs in younger cells had fast rise times and short half-widths. (B) Stimulation of corticogeniculate fibers tended to result in either no synaptic activation or the activation of fast rise time EPSCs (squares). Corticogeniculate stimulation tended not to elicit synaptic responses in younger cells [A,B,D], but in one case [C] did elicit fast EPSCs (dashed square).

Figure 9. The number of fast EPSC steps in response to increasing stimulation decreases during ON/OFF sublamination. (A) A scatter plot of the number of EPSC steps versus age shows the addition of a population of cells toward that end of the period of retinogeniculate axon reorganization that shows an increased number of steps relative to the beginning of ON/OFF sublamination. However, analysis of rise and width times reveals that older cells with moderate and large numbers of EPSC steps are limited to those with slow (> 2 ms) rise times (B) and long (> 7 ms) half-widths (D). The

normalized maximum stimulus intensity for each cell does not correlate with the number of EPSC steps observed in that cell (C).

Figure 10. Short half-width sfEPSC amplitude increases with age. (A) As with EPSCs during increasing stimulation, sfEPSCs could be divided roughly into those with long half-widths (> 5 ms) and those with shorter half-widths. The same relationship holds between the no. of EPSC steps and age for sfEPSCs as exists for EPSCs. (B) Short half-width sfEPSC from older cells had much larger amplitudes than the other, long half-width sfEPSCs from older cells (* = $p < 0.05$). (C) Example traces of long- (upper trace) and short half-width (lower trace) sfEPSCs from older cells. (D) More generally, there was a strong inverse correlation between sfEPSC amplitude and the number of subsequent EPSC steps revealed through increasing stimulation.

Figure 1

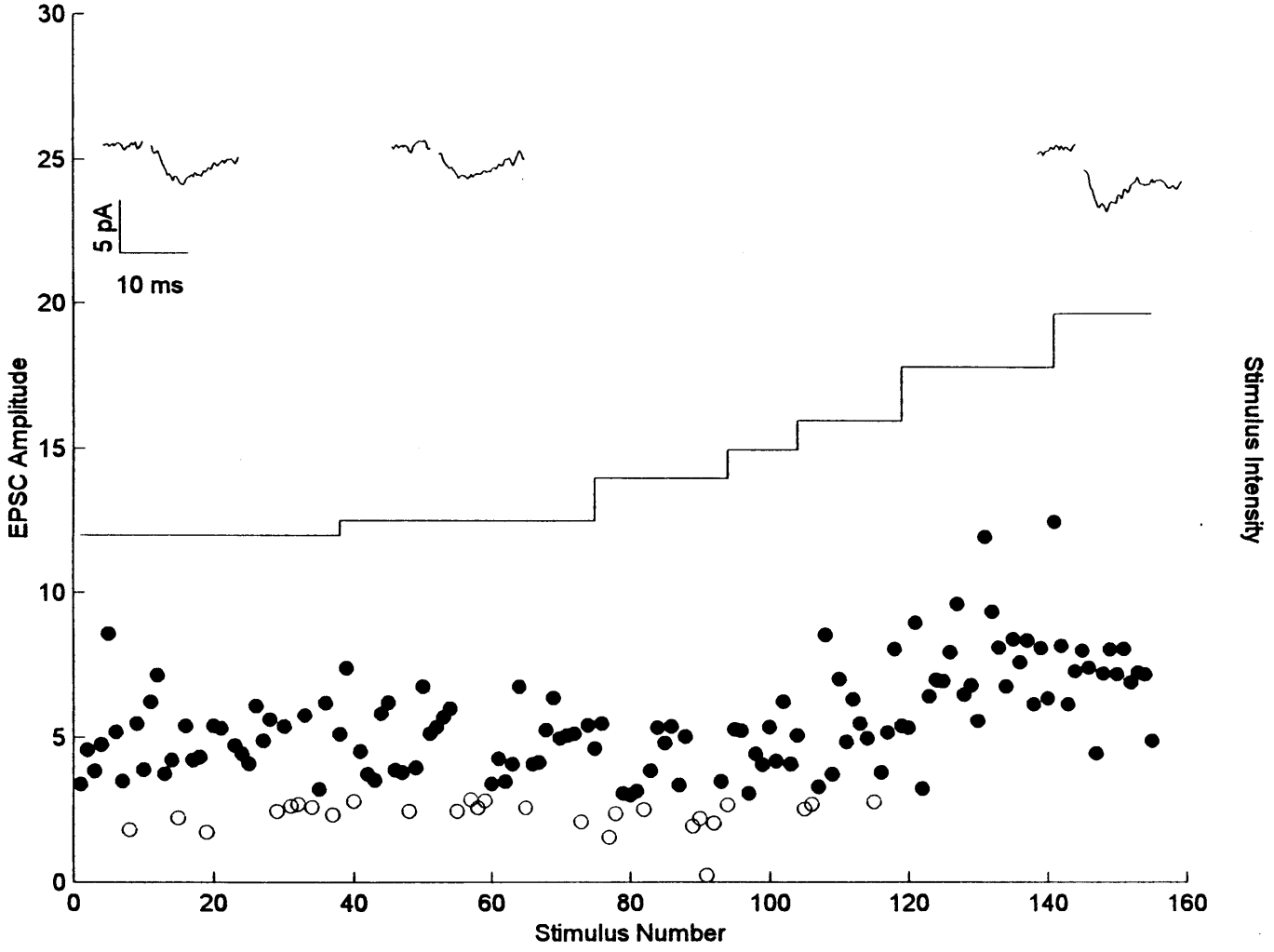

30
25
20
15
10
5
0
5 pA
10 ms
EPSC Amplitude
Stimulus Intensity
0
20
40
60
80
100
120
140
160
Stimulus Number

Figure 2

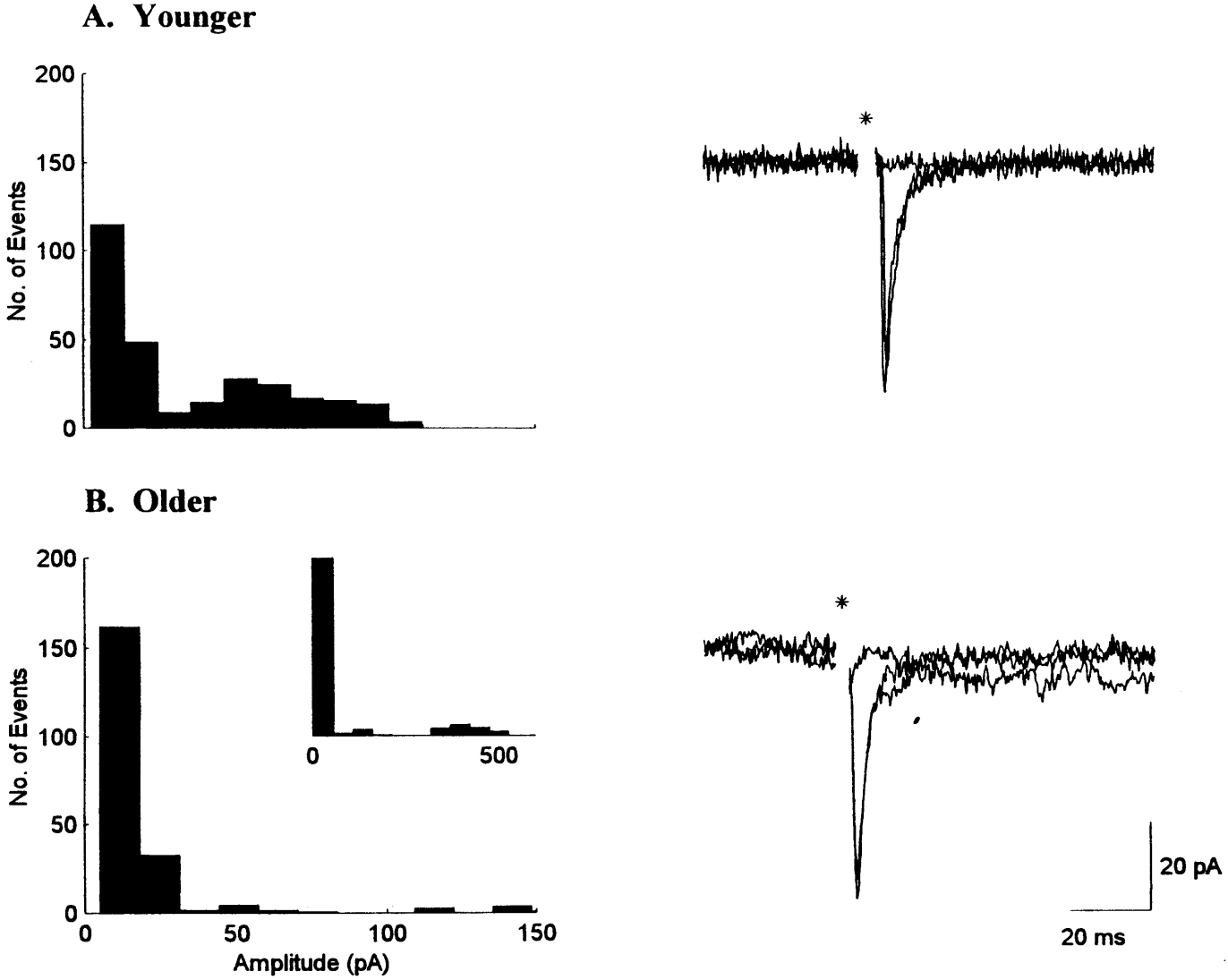

A. Younger
200
150
100
50
0
No. of Events
*
B. Older
200
150
100
50
0
No. of Events
0
500
0
50
100
150
Amplitude (pA)
*
20 pA
20 ms

Figure 3

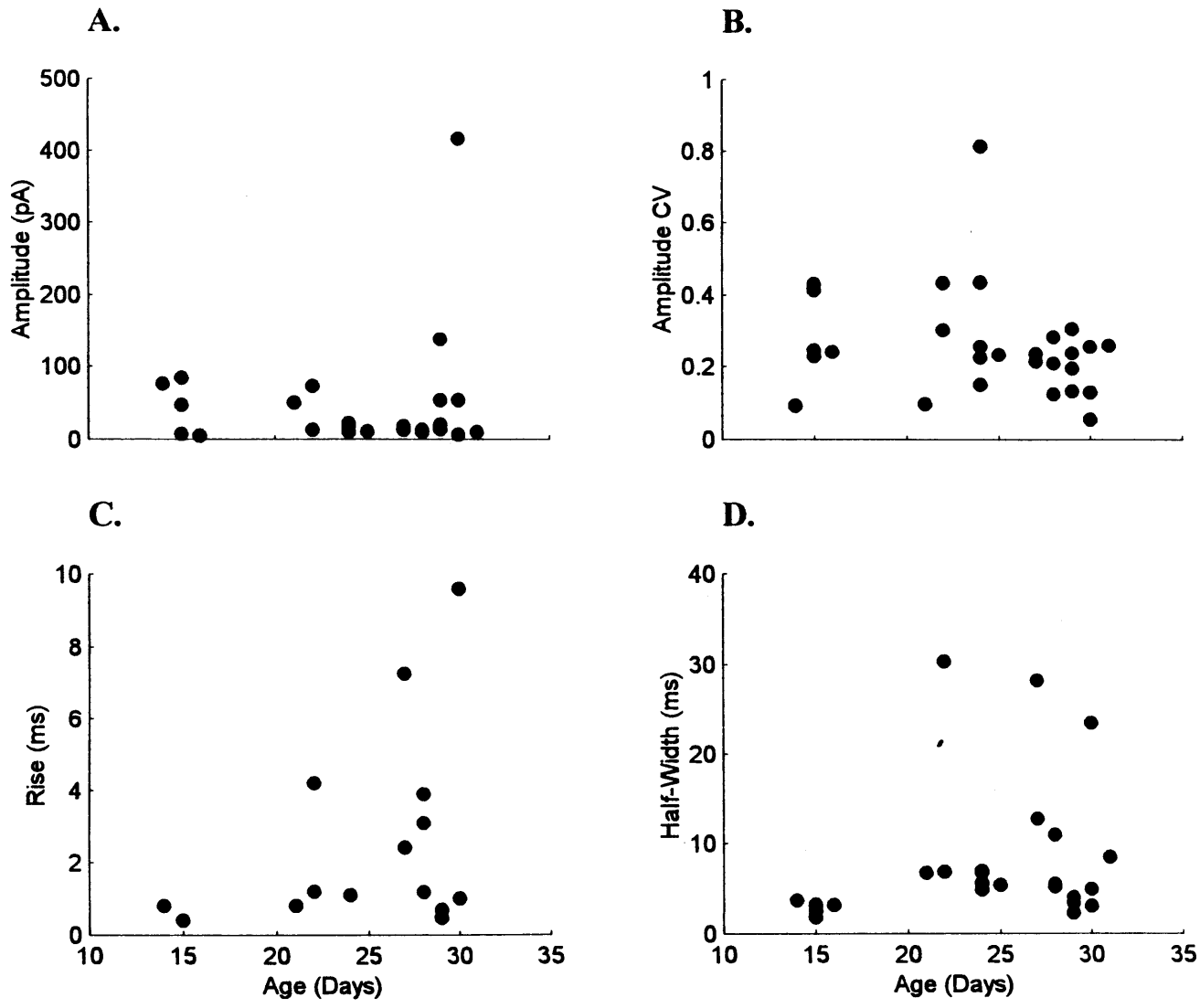

A.
B.
C.
D.
Amplitude (pA)
Amplitude CV
Rise (ms)
Half-Width (ms)
Age (Days)
Age (Days)

Figure 4

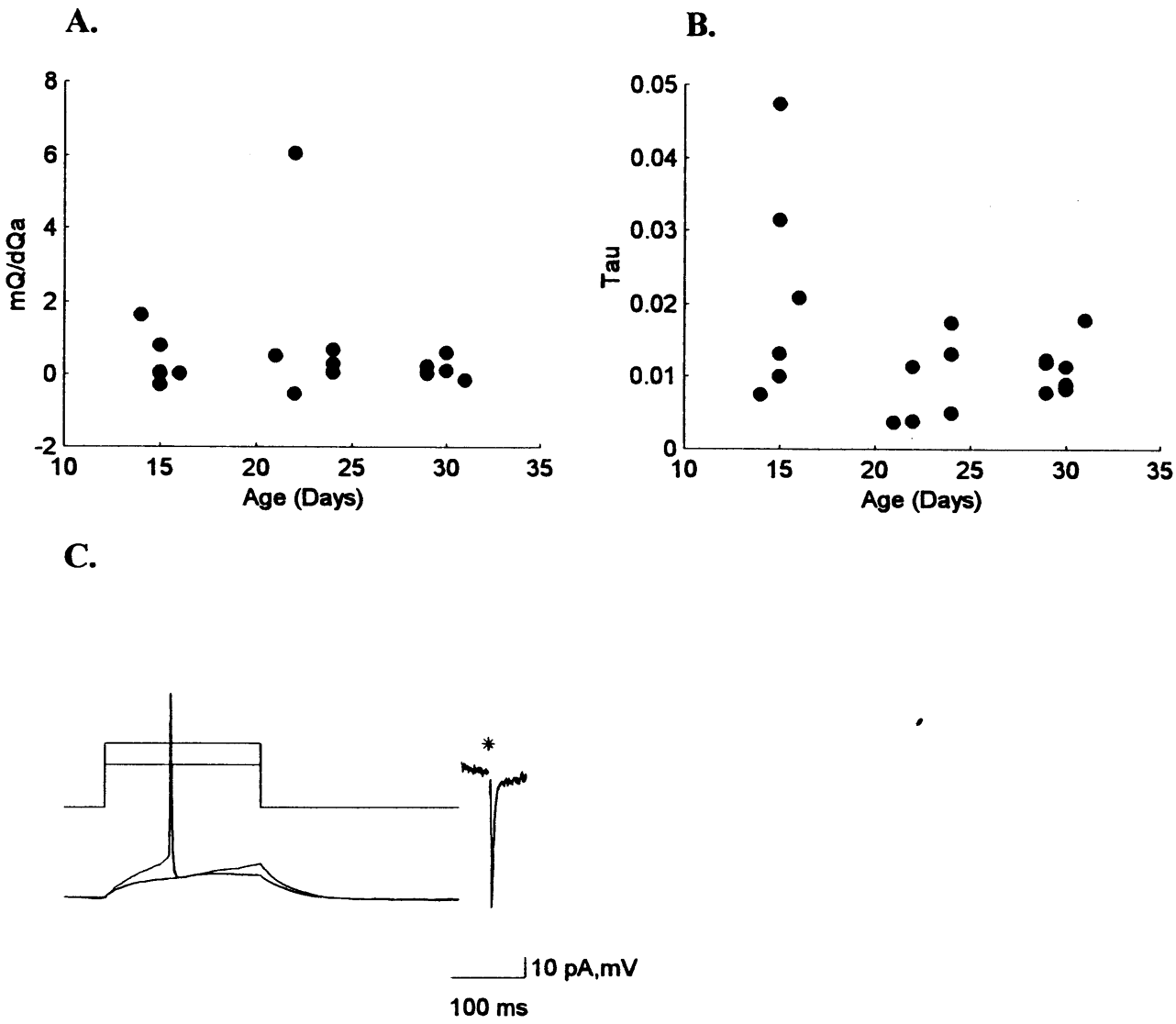

A.
mQ/dQa
8
6
4
2
0
-2
10
15
20
25
30
35
Age (Days)
B.
Tau
0.05
0.04
0.03
0.02
0.01
0
10
15
20
25
30
35
Age (Days)
C.
10 pA,mV
100 ms

Figure 5

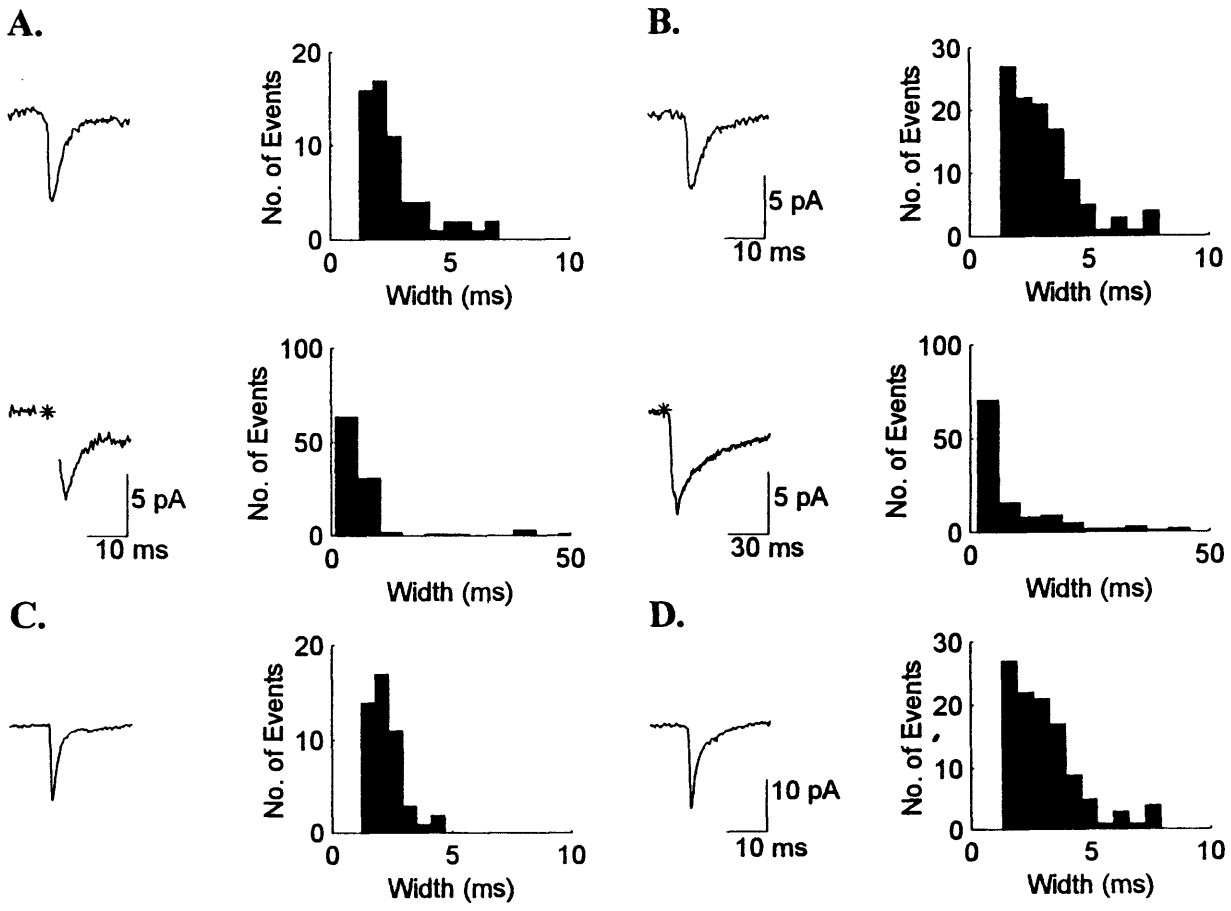


Figure 6

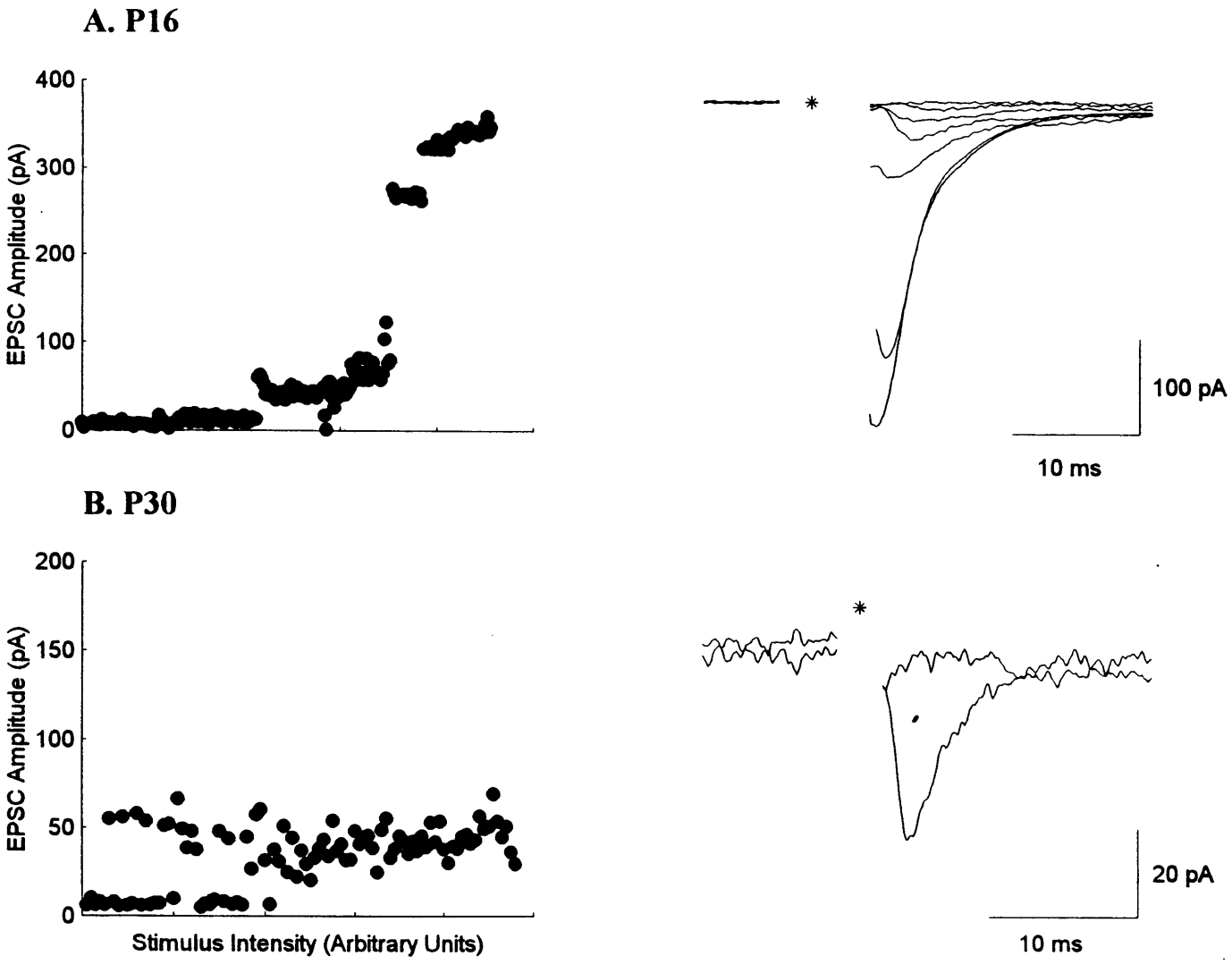

A. P16
400
300
200
100
0
EPSC Amplitude (pA)
100 pA
10 ms
B. P30
200
150
100
50
0
EPSC Amplitude (pA)
Stimulus Intensity (Arbitrary Units)
20 pA
10 ms

Figure 7

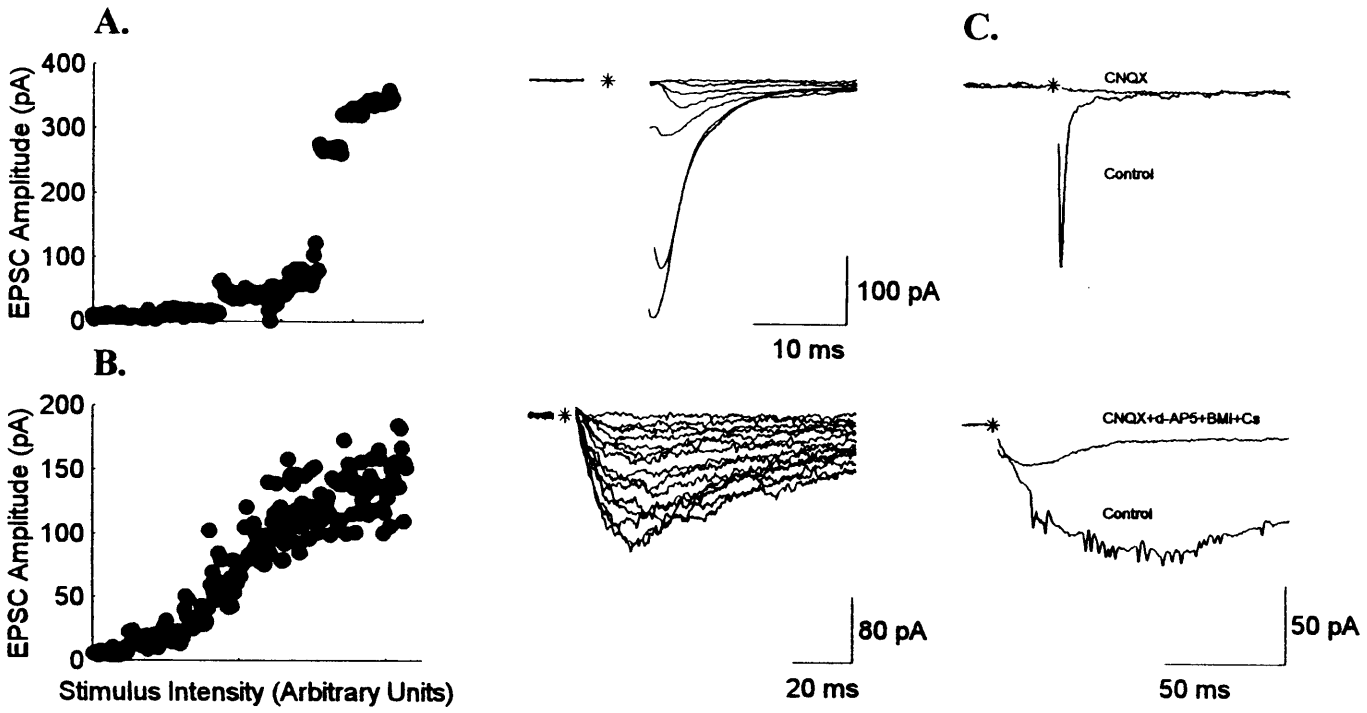

A.
EPSC Amplitude (pA)
400
300
200
100
0
100 pA
10 ms
C.
CNQX
Control
B.
EPSC Amplitude (pA)
200
150
100
50
0
Stimulus Intensity (Arbitrary Units)
80 pA
20 ms
CNQX+d-AP5+BMI+Cs
Control
50 pA
50 ms

Figure 8

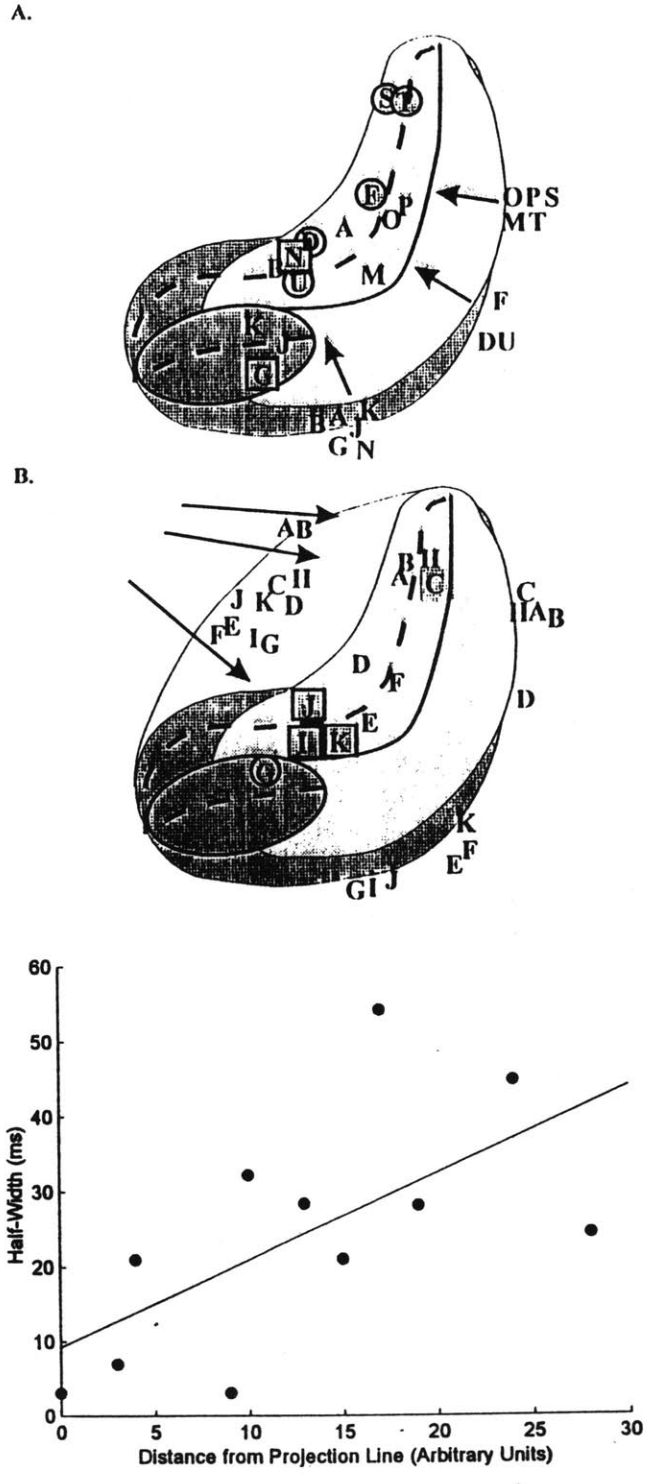

A.
OPS
MT
F
DU
B.
AB
C
D
K
GI J
E F
60
50
40
30
20
10
0
Half-Width (ms)
0
5
10
15
20
25
30
Distance from Projection Line (Arbitrary Units)

Figure 9

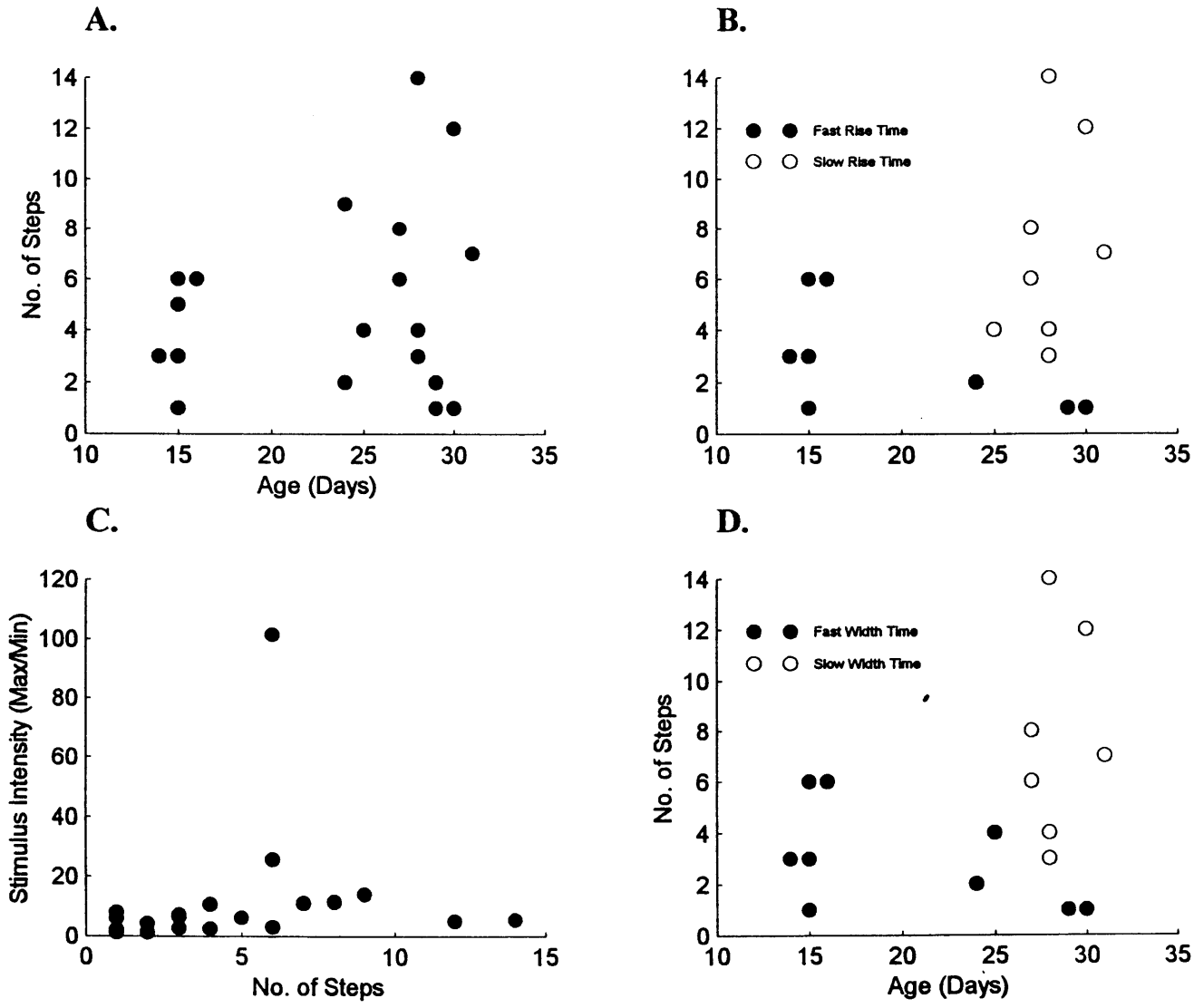
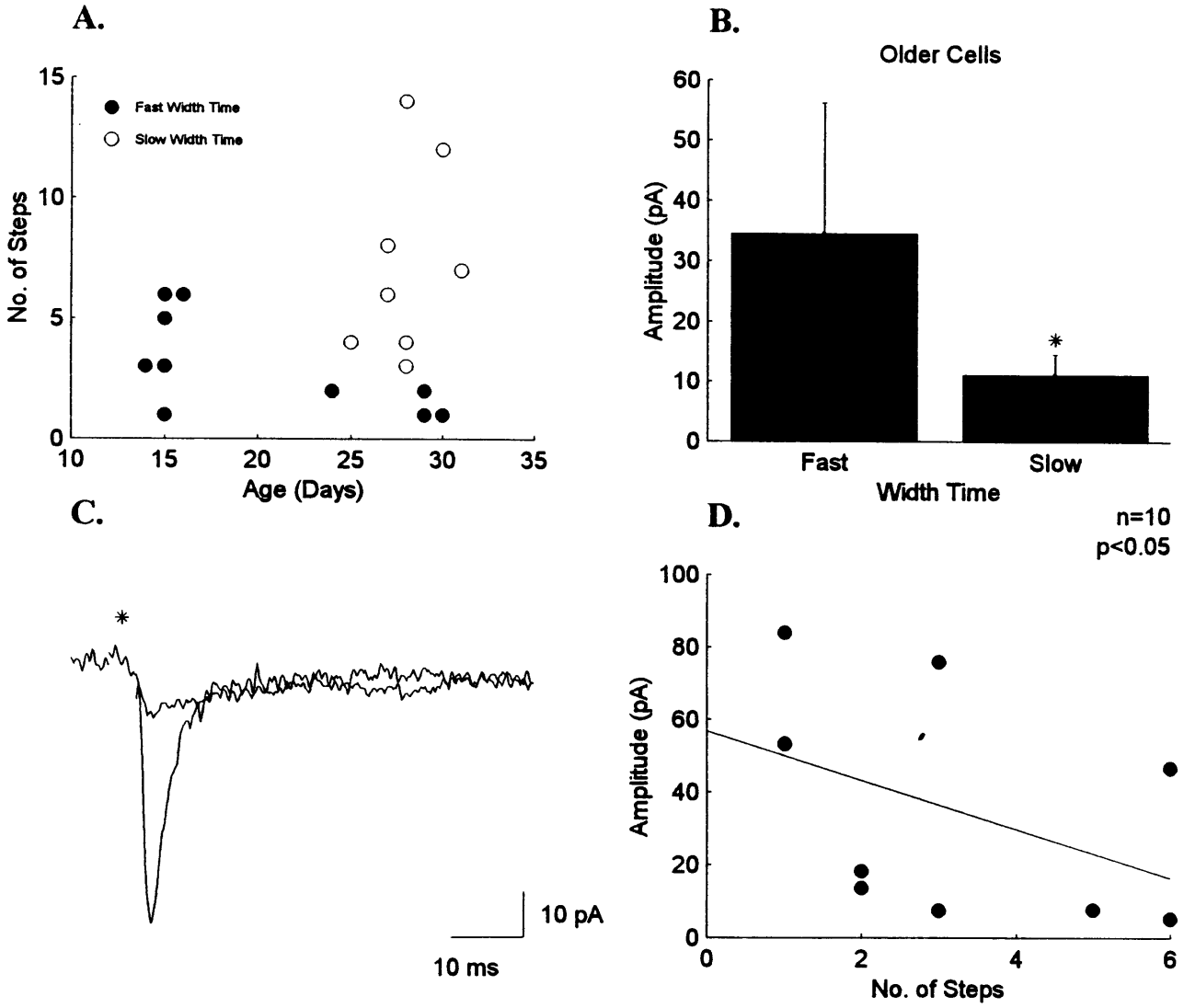

A.
No. of Steps
Age (Days)
B.
Fast Rise Time
Slow Rise Time
C.
Stimulus Intensity (Max/Min)
No. of Steps
D.
Fast Width Time
Slow Width Time
No. of Steps
Age (Days)

Figure 10


A.
Fast Width Time
Slow Width Time
No. of Steps
Age (Days)
B.
Older Cells
Amplitude (pA)
Fast
Slow
Width Time
C.
10 pA
10 ms
D.
n=10
p<0.05
Amplitude (pA)
No. of Steps

Chapter Seven: Summary and Conclusions

Summary

In the introduction, I presented one of the central questions in developmental neurobiology: How are the trillions of precise connections between neurons in the developing brain established? The answer, for many regions of the nervous system, is that appropriately patterned neural activity appears to instruct the last stage, the refinement, of neural connectivity.

Chapter one presented an overview of research on activity-dependent development: its appearance relative to activity independent events, its molecular components, the regulation of periods of heightened activity-dependent development, and the possible role of LTP during these times. Then I introduced the mammalian visual system, in general, and the ferret LGN in particular, as a model for examining activity-dependent development of both neural connectivity and synaptic efficacy. I described the normal development of the LGN and the result of blocking the neural activity of its retinal input. I also described the significant effects of disrupting the molecular components, NMDA receptors and nitric oxide production, which are also required for many forms of LTP.

Chapters three through six presented the data collected in four sets of experiments designed to test the hypothesis that long-term modifications of synaptic strength are involved in the stabilization and/or withdrawal of appropriate and inappropriate neural

connections, respectively. In the first, I presented a body of data that I gathered on the development of the electrophysiological properties of relay cells in the LGN, focusing on the period of ON/OFF sublamination.

In the second set of experiments, I tested for the presence of the most commonly reported form of LTP; namely, potentiation mediated by postsynaptic AMPA receptors. The results of those experiments demonstrate that, on average, the efficacy of the AMPA-mediated response of individual synapses onto relay cells in the LGN is stabilized throughout ON/OFF sublamination. More specifically, the AMPA-mediated efficacy of retinal inputs holds steady while the axons that give rise to them are being dramatically rearranged. Additionally, neither the frequency nor the variability of these inputs changes significantly throughout this time. These results suggest that either no significant change in the number of synapses (i.e., no withdrawal or stabilization by sprouting) is occurring during ON/OFF sublamination or "inappropriate" synapses are not physiologically distinguishable from the "appropriate" synapses that replace them.

The third set of experiments investigated conductances that are active at depolarized membrane potentials. In addition to the possibility that these conductances, those mediated by NMDA receptors, are potentiated at some time during ON/OFF sublamination, I explored the possibility that the ratio of NMDA receptor-mediated activity to AMPA receptor-mediated activity is modified. That is, I explored the possibility that silent synapses preferentially occur prior to periods of activity-dependent reorganization of neural connectivity. As with AMPA receptor-mediated sEPSCs, both sN-EPSCs and the A/N ratio are stable over the period of ON/OFF sublamination.

The last set of experiments explored the development of the convergence of retinal axons onto relay cells in the LGN. While the anatomical evidence that retinogeniculate axons are undergoing pronounced reorganization is quite clear, there is no evidence to suggest that a significant number of these axons actually make functional contacts in areas from which they later withdraw. Is there, in fact, evidence for a reduction in the convergence of retinogeniculate axons onto relay cells during ON/OFF sublamination? In short, the answer appears to be yes. The number of fibers producing fast, narrow EPSCs in relay cells is fewer at the beginning of postnatal week five than at the beginning of postnatal week three. At the same time, however, there is an appearance of fibers that produce slower, wider EPSCs that are likely to derive from non-retinal inputs. Interestingly, the median size of the EPSCs produced by the stimulation of a single retinogeniculate fiber is not much larger than those generated by the spontaneous activation of a single synapse. Additionally, the number of synaptic inputs required to depolarize the cell to action potential threshold is held constant throughout ON/OFF sublamination.

Conclusions

The results presented in this thesis complicate the hypothesis that the development of neural connectivity is guided by long-term changes in synaptic efficacy (Constantine-Paton et al., 1990; Kandel and O'Dell, 1992; Goodman and Shatz, 1993; Cramer and Sur, 1995; Katz and Shatz, 1996; Constantine-Paton and Cline, 1998). If such enduring changes were involved in establishing appropriate synapses early in development, then

one would expect synaptic efficacy to be increased after periods of activity-dependent development. However, as demonstrated in chapter three by the maintenance of the relative action potential threshold, in chapter four by the stability of the AMPA receptor-mediated response of single, retinal synapses, and in chapter five by the constant AMPA/NMDA ratio there is little evidence for such an increase. However, the increase in short half-width sfEPSC amplitude reported in chapter six suggests that increases in the strength of single fibers as a whole are a consequence of axonal reorganization in this system.

It is possible, however, that an increase in synaptic efficacy is involved in activity-dependent development, but that it occurs in a larger context of homeostasis in which changes in synaptic efficacy are normalized to a constant level. For example, an initial increase in synaptic efficacy in early development may be followed by an increase in the innervation of a target cell which subsequently decreases synaptic efficacy (Liu and Tsien, 1995). This particular scenario would result in an increase in the frequency of sEPSCs (assuming no change in the probability of transmitter release) which does not occur during the development of ON/OFF sublaminae (Chapter Four and Chapter Five). Nonetheless, the point remains that the temporal resolution of the experiments in this thesis (one to a few days) may be insufficient to detect short-term changes in synaptic efficacy that may serve as a blueprint for neural connectivity. The hypothesis under question in this thesis, however, postulates *long-term* changes in synaptic efficacy. Again, in the ferret LGN, this does not appear to be the case. Given the considerable anatomical and physiological changes that are occurring in relay cells throughout

ON/OFF sublamination (Sutton and Brunso-Bechtold, 1991; Rocha and Sur, in preparation; White and Sur, 1992; Ramoa and McCormick, 1994a)[1], the stability in synaptic efficacy demonstrated in this thesis suggests instead that synaptic input to a neuron is normalized toward some acceptable level.

Recently, there have been additional demonstrations of homeostatic plasticity, that is, mechanisms that stabilize the properties of neural circuits. For example, at the neuromuscular junction in *Drosophila*, genetic manipulations that reduce the number of synaptic contacts results in an up-regulation of synaptic efficacy (Davis and Goodman, 1998). A converse mechanism also appears to exist. In cultured hippocampal neurons, greater innervation is correlated with a down-regulation of mEPSC size (Liu and Tsien, 1995). Synaptic efficacy can also be scaled as a function of firing frequency. In cultured cortical neurons synaptic strength is increased after a decrease, and decreased after an increase, in the firing rate (Turrigiano et al., 1998). In this same system, homeostasis can also be maintained by activity-dependent changes in the intrinsic excitability of neurons. When cell cultures are deprived of activity, their firing rate given a particular current injection increases (Desai et al., 1999).

These investigations of homeostatic plasticity provide a balance to the investigations of Hebbian plasticity in activity-dependent development (for review see Turrigiano, 1999). It has long been recognized that a purely Hebbian mechanism is unstable. That is, correlated firing leads to an increase in synaptic strength, which, in

[1] See also Chapter Three.

turn, leads to an increase in correlated firing resulting in additional increases in synaptic strength—and this cycle is continued *ad infinitum*. The mechanisms of homeostatic plasticity described above provide a counter-force to this runaway positive feedback and the results presented in this thesis likely reflect the results of those mechanisms *in vivo* during a time of intense, activity-dependent development.

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