Cellular and Molecular Analysis of Neuronal Structural Plasticity in the Mammalian Cortex

by

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ABSTRACT

Despite decades of evidence for functional plasticity in the adult brain, the role of structural plasticity in its manifestation remains unclear. *cpg15* is an activity-regulated gene encoding a membrane-bound ligand that coordinately regulates growth of apposing dendritic and axonal arbors and the maturation of their synapses. Here we compare *cpg15* expression during normal development of the rat visual system, with that seen in response to dark rearing, monocular retinal action potential blockade, or monocular deprivation. Our results show that: (1) *cpg15* expression in visual cortex correlates with the electrophysiologically mapped critical period for development of eye-specific preference in the primary visual cortex. (2) Dark rearing elevates adult levels of expression. (3) A component of *cpg15* expression is activity-dependent after the peak of the critical period. (4) At the peak of the critical period, monocular deprivation decreases *cpg15* expression more than monocular TTX blockade. And (5) *cpg15* expression is robust and regulated by light in the superficial layers of the adult visual cortex. This suggests that *cpg15* is an excellent molecular marker for the visual system's capacity for plasticity and predicts that neural remodeling normally occurs in the extragranular layers of the adult visual cortex. To examine the extent of neuronal remodeling that occurs in the brain on a daily basis, we used a multi-photon based microscopy system for chronic *in vivo* imaging and reconstruction of entire neurons in the superficial layers of the rodent cerebral cortex. Here, we show the first unambiguous evidence of dendrite growth and remodeling in adult neurons. Over a period of months, neurons could be seen extending and retracting existing branches, and in rare cases adding new branch tips. Neurons exhibiting dynamic arbor rearrangements were GABA positive non-pyramidal interneurons, while pyramidal cells remained stable. These results are consistent with the idea that dendritic structural remodeling is a substrate for adult plasticity and suggest that circuit rearrangement in the adult cortex is restricted by cell type-specific rules.

Thesis Supervisor: Elly Nedivi
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Contents

But the functional specialization of the brain imposed on the neurones two great lacunae; proliferative inability and irreversibility of intraprotoplasmic differentiation. It is for this reason that, once development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centres the nerve paths are something fixed, ended, immutable. Everything may die, nothing may be regenerated.
It is for the science of the future to change, if possible, this harsh decree.

–Santiago Ramon y Cajal

Chapter 1

Introduction

Developmental and adult plasticity

Within the astounding complexity of the brain, fundamental yet unknown mechanisms sub serve learning, memory, perception, behavior, reason, and consciousness. How the outside world influences the brain likely changes the mind. The capacity of the central nervous system (CNS) to modify connections in response to activity is termed plasticity. Plasticity is a prominent feature of CNS development (Constantine-Paton et al., 1990; Shatz, 1990; Goodman and Shatz, 1993), and in the adult, putatively underlies learning and memory (Bailey and Kandel, 1993; Bliss and Collingridge, 1993) and adaptive reorganization of primary sensory maps (Merzenich et al., 1988; Kaas et al., 1990; Allard et al., 1991; Kaas, 1991; Gilbert and Wiesel, 1992; Recanzone et al., 1993; Zarzecki et al., 1993; Armstrong-James et al., 1994; Diamond et al., 1994). Anatomical and physiological studies of developmental plasticity in the visual system have provided valuable insight into how activity drives the final patterning of neuronal connections (Constantine-Paton et al., 1990; Shatz, 1990; Katz and Shatz, 1996).

The critical period for susceptibility to environmental influences is one of the most influential models to emerge from studies of visual system development. In highly binocular animals such as cats, primates, and ferrets, electrophysiological plasticity during the critical period for development of eye specific preference in the primary visual cortex is accompanied by activity-driven segregation of geniculocortical afferents into ocular dominance columns (Hubel et al., 1977; Shatz and Stryker, 1978; Issa et al., 1999). There exist, however, many instances where no apparent anatomical plasticity is detectable when physiological plasticity clearly exists. Electrophysiological studies of the rodent visual system show that cortical neurons develop well

defined functional properties relating to eye preference and orientation selectivity (Parnavelas et al., 1981; Maffei et al., 1992; Fagiolini et al., 1994; Gordon and Stryker, 1996; Antonini et al., 1999) and are capable of undergoing shifts in eye preference during a critical period in development (Drager, 1978; Fagiolini et al., 1994; Gordon and Stryker, 1996; Guire et al., 1999; Frenkel and Bear, 2004); however, they are not anatomically segregated according to these properties akin to the feline and primate visual system. Since there is no segregation into eye-specific zones, anatomical changes in response to monocular deprivation (MD) during the critical period can be seen only with single-cell labeling and reconstruction (Antonini et al., 1999).

The capacity for change in response to manipulation of patterned visual input is normally restricted to a defined age window during postnatal development (Hubel and Wiesel, 1970; Hubel et al., 1977). However, in kittens or rats dark-reared (DR) past the end of the normal critical period, subsequent manipulation of visual input results in significant plasticity of cortical binocular connectivity measured by electrophysiological recordings (Cynader and Mitchell, 1980; Mower et al., 1981; Fagiolini et al., 1994; Guire et al., 1999), but not by altered segregation of ocular dominance columns (Mower et al., 1985). The complexity of interpreting dark rearing experiments underscores our poor understanding of the molecular mechanisms underlying critical period plasticity.

Physiological plasticity without large-scale anatomical rearrangements, as found during the critical period in species without ocular dominance columns, or in the superficial cortical layers of adult animals of all species, normal or DR, suggests that the capacity for rearrangement of thalamocortical afferents in layer 4 is not always the most appropriate measure of plasticity. Recent studies show that organization of the extragranular layers precedes the establishment of

cortical columns and serves to guide the anatomical remodeling in layer 4 (Ruthazer and Stryker, 1996; Crair et al., 1997; Crair et al., 1998; Trachtenberg et al., 2000). In fact, since plasticity of the cortical extragranular layers persists in the adult beyond the critical period for anatomical rearrangement in layer 4 (Daw et al., 1992; Diamond et al., 1994; Glazewski and Fox, 1996; Rioult-Pedotti et al., 1998; Feldman, 2000), remodeling of layer 4 may not be a requisite for plasticity, but in some species may only serve to set a foundation for further progressive refinement at higher levels of the cortical circuitry (Trachtenberg et al., 2000). The most attractive aspect of this interpretation is that it unifies the adult plasticity seen in extragranular layers with developmental plasticity during the critical period.

Little is known about the cellular mechanisms underlying developmental activity-evoked synaptic remodeling, and how it relates to plasticity in the adult. During development inappropriate connections are withdrawn, while appropriate connections develop elaborate terminal arbors (Guillery, 1972; Sretavan and Shatz, 1986; Antonini and Stryker, 1993). Here, an appropriate connection is defined as one where pre- and postsynaptic activity is correlated, also termed a Hebbian synapse (Stryker and Strickland, 1984; Fregnac et al., 1988; Shulz and Fregnac, 1992). The Hebbian hypothesis provides a theoretical basis for linking developmental and adult plasticity. Hebbian characteristics of synapses in layer IV of visual cortex during the critical period, are similar to those of synapses in the CA1 region of the adult hippocampus that are capable of undergoing long-term potentiation, a form of synaptic plasticity associated with explicit forms of learning and memory (Morris et al., 1986; Bach et al., 1995).

The capacity of the developing and adult cortex to detect coincident activity is in large part derived from the N-methyl-D-aspartate (NMDA)-type glutamate receptor (Kleinschmidt et al., 1987; Fox et al., 1989; Bear et al., 1990). When neighboring fibers are activated

synchronously, or the same fiber fires in rapid succession, depolarization of the postsynaptic membrane releases the Mg^{2+} block from NMDA channels, allowing Ca^{2+} entry (Bliss and Collingridge, 1993). Ca^{2+} influx through the NMDA receptor activates second messengers, including cAMP and a variety of kinases (Bliss and Collingridge, 1993). One effect of second messengers is activation of transcription factors that induce expression of downstream genes (Goelet et al., 1986; Sheng and Greenberg, 1990). Beyond the NMDA receptor and the second messenger pathways triggered via this receptor, the ensuing processes that actually modify cellular properties to achieve synaptic reorganization are still unresolved. Identification and characterization of the downstream effector genes induced by neuronal activity and transcription factor activation is crucial in elucidating the cellular processes that produce long-term plasticity.

Candidate plasticity genes (CPGs)

To isolate activity-regulated effector genes, Nedivi and colleagues used a highly sensitive subtractive and differential cloning procedure (Nedivi et al., 1993). This screen isolated over 360 candidate plasticity genes (CPGs). Sequence analysis shows that 120 of the cloned CPGs encode known proteins, approximately 70 correspond to expressed sequence tags, while more than 100 are novel. The known CPGs can be classified into distinct functional categories; for example, transcription factors, proteins participating in second messenger pathways, growth factors and structural proteins. The second largest category is that of membrane-, vesicle- and synapse-related proteins. The large representation by structural and synaptic proteins suggests that terminal restructuring and synaptic remodeling are likely to play a crucial role in functional modification of adult synapses.

One of the novel genes isolated in this screen was *candidate plasticity gene 15* (*cpg15*) (Nedivi et al., 1996). *cpg15* expression is regulated by light adult cat and rat visual cortex (Corriveau et al., 1999; Lee and Nedivi, 2002) and by whisker activity in the somatosensory barrel cortex (Harwell et al., 2005). *cpg15* encodes a small protein putatively attached to the cell surface by a glycosylphosphatidylinositol anchor (Nedivi et al., 1998). Recently researchers found that a soluble secreted form of *cpg15* functions as a survival factor for early embryonic cortical progenitors (Putz et al., 2005). In the developing *Xenopus* retinotectal system, *cpg15* promotes elaboration of dendritic and axonal arbors, and synaptic maturation (Nedivi et al., 1998; Cantallops et al., 2000). Thus, *cpg15* is an activity-regulated gene encoding a protein that coordinately regulates growth of apposing dendritic and axonal arbors and the maturation of their synapses, and its localization and regulation in the postnatal developing visual system are consistent with a role in activity-dependent plasticity.

Two-photon microscopy

In 1990, Denk, Webb and co-workers introduced two-photon excitation microscopy (Denk et al., 1990), a promising technology for imaging deep in tissue with minimal photodamage (Denk et al., 1994). This innovation relies on the fact that fluorophores can be excited by the simultaneous absorption of two photons each having approximately half the energy required for the excitation transition. Since the two-photon excitation probability is significantly less than the one-photon probability, it occurs at appreciable rates only in regions of high temporal and spatial photon concentration. High spatial photon concentration can be achieved by using a high numerical aperture objective to focus a laser beam to a diffraction-

limited spot. High temporal concentration of photons is made possible by high peak power mode-locked lasers.

In general, two-photon excitation allows 3-D biological structures to be imaged with resolution comparable to confocal microscopes but with a number of significant advantages: (1) Conventional confocal techniques obtain 3-D resolution by using a detection pinhole to reject out-of-focal-plane fluorescence. In contrast, two-photon excitation achieves a similar effect by limiting the excitation region to a sub-micron volume at the focal point. This capability of limiting the region of excitation instead of the region of detection is critical. Photodamage of biological specimens is restricted to the focal point. Since out-of-plane chromophores are not excited, they are not subject to photobleaching. (2) Two-photon excitation wavelengths are typically red-shifted to about twice the one-photon excitation wavelengths. The significantly lower absorption and scattering coefficients at these wavelengths ensure deeper tissue penetration. (3) The wide separation between the excitation and emission spectra ensures that the excitation light and the Raman scattering can be rejected without filtering out fluorescence photons. This sensitivity enhancement improves the detection signal to background ratio. Another major problem of deep-tissue imaging is that tissue is highly scattering for visible light, resulting in degradation of resolution and contrast when using normal light and confocal microscopy. Two-photon microscopy, although not immune to the scattering properties of tissue, is better suited for deep-tissue imaging.

Today, two-photon microscopy has found many applications in neurobiology. This technique has been used to study motor neuron function in the Zebrafish spinal cord (O'Malley and Kao, 1996). Its use in mammalian neurobiology has been applied *in vitro* to studies of dendritic spines in brain slices, exploring their structural plasticity (Engert and Bonhoeffer, 1999;

Maletic-Savatic et al., 1999), the role of calcium signaling in their function (Denk et al., 1995; Yuste and Denk, 1995; Svoboda et al., 1996; Mainen et al., 1999; Yuste et al., 1999; Sabatini and Svoboda, 2000), and trafficking of AMPA receptors to their surface (Shi et al., 1999). *In vivo* studies of dendritic spines and calcium signaling (Svoboda et al., 1997; Helmchen et al., 1999; Svoboda et al., 1999; Chen et al., 2000; Lendvai et al., 2000; Ohki et al., 2005) have recently been extended to long-term monitoring of dendritic spine (Grutzendler et al., 2002; Trachtenberg et al., 2002; Majewska and Sur, 2003; Holtmaat et al., 2005; Zuo et al., 2005a; Zuo et al., 2005b; Majewska et al., 2006) and axon terminal (De Paola et al., 2006; Stettler et al., 2006) structure and dynamics.

Summary

Here, we show that *cpg15* expression in the rodent visual system reflects not just levels of activity, but the system's capacity for plasticity. This suggests *cpg15* may be a good molecular marker for plasticity. We used *in vivo* two-photon microscopy to directly test predictions generated from the expression pattern of *cpg15*. We asked the question: if *cpg15* promotes process outgrowth in the rodent cortex as it does in the developing *Xenopus* retinotectal system, why is *cpg15* robustly expressed and transcriptionally regulated in the superficial layers of the putatively 'hard-wired' adult visual cortex?

We found that neurons in the superficial layers of the adult visual cortex normally exhibit structural plasticity at the level of dendritic arbors, some of which may be driven by *cpg15*. Pyramidal neurons are relatively stable at basal levels of activity, whereas GABAergic non-pyramidal neurons undergo process retraction, elongation and branch tip addition. These results are consistent with the idea that structural remodeling may be substrate for adult plasticity and

suggest that circuit rearrangement in the adult cortex is greater than previously assumed and is subject to cell type-specific rules.

Chapter 2

Extended plasticity of visual cortex in dark-reared animals may result from prolonged expression of *cpg15*-like genes

Abstract

cpg15 is an activity-regulated gene encoding a membrane-bound ligand that coordinately regulates growth of apposing dendritic and axonal arbors and the maturation of their synapses. These properties make it an attractive candidate for participating in mammalian visual system plasticity. Here we compare *cpg15* expression during normal development of the rat visual system with that seen in response to dark rearing, monocular retinal action potential blockade, or MD. Our results show that onset of *cpg15* expression in visual cortex is coincident with eye opening, and increases until the peak of the critical period at 4 weeks postnatal (P28). This early expression is independent of both retinal activity and visual experience. After P28, a component of *cpg15* expression in the visual cortex, lateral geniculate nucleus (LGN), and superior colliculus (SC) develops a progressively stronger dependence on retinally driven action potentials. Dark rearing does not affect *cpg15* mRNA expression in the LGN and SC at any age, but significantly affects its expression in visual cortex from the peak of the critical period and into adulthood. In dark-reared rats, the peak level of *cpg15* expression in visual cortex at P28 is lower than in controls. Rather than showing the normal decline with maturation, these levels are maintained in dark-reared animals. We suggest that the prolonged plasticity in visual cortex seen in dark-reared animals may result from failure to down-regulate genes like *cpg15* that could promote structural remodeling and synaptic maturation.

Introduction

The critical period for susceptibility to environmental influence is one of the central concepts to emerge from studies of visual system development. The capacity for change in response to manipulation of patterned visual input is normally restricted to a defined age window during postnatal development (Hubel and Wiesel, 1970; Hubel et al., 1977). However, in kittens or rats DR past the end of the normal critical period, manipulation of visual input results in significant plasticity of cortical binocular connectivity measured by electrophysiological recordings (Cynader and Mitchell, 1980; Mower et al., 1981; Fagiolini et al., 1994; Guire et al., 1999). This prolonged functional plasticity is not accompanied by a prolonged plasticity measured by anatomical methods. Segregation of ocular dominance columns when visualized by autoradiography of geniculocortical afferents is incomplete in DR cats and is not altered by later visual manipulations (Mower et al., 1985). The complexity of interpreting dark rearingexperiments highlights our poor understanding of the molecular mechanisms underlying critical period plasticity.

Electrophysiological studies of the rodent visual system show that cortical neurons develop well defined functional properties relating to eye preference and orientation selectivity, although they are not anatomically segregated according to these properties as seen in cat and monkey visual cortex (Parnavelas et al., 1981; Maffei et al., 1992; Fagiolini et al., 1994; Gordon and Stryker, 1996; Antonini et al., 1999). Similar to cats and monkeys, cells in the rodent primary visual cortex are capable of undergoing shifts in eye preference during a critical period in development (Drager, 1978; Fagiolini et al., 1994; Gordon and Stryker, 1996; Guire et al., 1999). Anatomical studies show that in mice, MD during the critical period affects arbor growth of thalamocortical afferents (Antonini et al., 1999). These studies, in combination with new mouse genetic manipulation technologies, argue that the rodent visual projection can be a

valuable experimental system for examining the cellular and molecular mechanisms of developmental plasticity.

candidate plasticity gene 15 (*cpg15*) was isolated in a screen for seizure-induced genes in the rat hippocampus dentate gyrus (Nedivi et al., 1993). *cpg15* expression is regulated by light in the adult rat visual cortex (Nedivi et al., 1996), and its localization and regulation in the developing cat visual system are consistent with a role in activity-dependent plasticity (Corriveau et al., 1999). *cpg15* overexpression in the developing Xenopus optic tectum induces exuberant growth of tectal cell dendritic arbors (Nedivi et al., 1998), accompanied by enhanced retinal axon arbor growth and retinotectal synapse formation (Cantallops et al., 2000). *cpg15*'s effects on these different aspects of circuit formation are all non-cell autonomous, consistent with its glycosylphosphatidylinositol cell surface attachment (Nedivi et al., 1998; Cantallops et al., 2000).

cpg15's potential role in dendritic and axonal arbor restructuring and synaptic maturation led us to explore the effect of dark rearing on its expression and regulation. We monitored *cpg15* mRNA levels in visual structures of the rat, before, during, and after the cortical critical period for development of eye specific preference. We compared the effects of dark rearing with those of retinal action potential blockade and MD. Our results suggest a mechanism whereby the visual cortex of DR animals maintains a capacity for delayed plasticity through elevated levels of genes like *cpg15* that can enhance local circuit remodeling and new synaptic stabilization. These results may provide a clue to the intriguing discrepancy seen in DR animals between the capacity for change as measured by anatomical versus electrophysiological methods.

Results

Developmental profile of *cpg15* expression

To investigate a role for *cpg15* in the activity dependent phase of rat visual system development, *in situ* hybridizations with a *cpg15* probe were conducted on sections through visual structures starting at P10. LGN, SC, and visual cortex were monitored before and after eye opening, throughout the critical period for development of eye specific preference in the binocular zone of primary visual cortex, and in the adult. At birth and up to P10, expression of *cpg15* is undetectable in the neocortex (Nedivi et al., 1996). At P10, four days before eye opening, low levels of *cpg15* mRNA was detectable at the apex of the cortical hemispheres (Fig. 2-2). General onset of *cpg15* expression in visual cortex occurred coincident with eye opening at two weeks postnatal with highest expression in layers 2/3, 4 and 6. Subsequently, *cpg15* mRNA expression levels gradually increased, peaking at four weeks postnatal (P28), the height of the physiologically characterized critical period for development of eye specific preference in visual cortex. Subsequently, *cpg15* mRNA levels declined to a lower basal adult level. These results show that *cpg15* levels in rat visual cortex correspond well with the electrophysiologically mapped critical period.

Effect of dark rearing on *cpg15* expression

To test whether regulation of *cpg15* expression was sensitive to visual input, rats were deprived of visual experience by dark rearing to various developmental ages. *cpg15* mRNA levels were compared in DR and age-matched controls. Dark rearing did not alter the time of onset or the early increase in levels of *cpg15* expression in visual cortex (Fig. 2-2). At P28, *cpg15* mRNA levels in DR animals plateaued, but at a significantly lower level of expression than age-matched controls (Figs. 2-2 and 2-3a). This level of expression persisted in older DR animals, so that after eight weeks of dark rearing, *cpg15* expression levels were significantly

higher than in their age-matched controls, where *cpg15* expression normally declined after P28 (Figs. 2-2 and 2-3a). Differences in *cpg15* mRNA levels between normal and DR animals were not seen in the LGN or SC (data not shown). The enhanced expression levels of *cpg15* in visual cortex, seen with dark rearing, may be a molecular indicator of the prolonged plasticity specific to this region.

Short exposure to light can reportedly trigger the end of the delayed plasticity seen in DR animals (Mower et al., 1983; Philpot et al., 2001). To test if late onset of visual experience affects *cpg15* expression, animals were DR to eight weeks then exposed to light for 6, 12, 24 hours, 7 days, or 14 days. *In situ* hybridizations show that with exposure to light of up to 12 hours *cpg15* expression remains significantly higher than normal (Fig. 2-4). Following 24 hours of exposure to light, DR animals showed *cpg15* mRNA expression levels comparable to those found in normally raised adults (Fig. 2-4). These experiments indicate that brief visual experience in DR adults can reset *cpg15* expression to normal levels. This down-regulation of expression may be a molecular representation of the light induced end of the delayed plasticity in DR animals.

Effect of retinal action potential blockade on *cpg15* expression

To investigate whether *cpg15* mRNA expression in visual cortex is dependent on action potential driven activity from the retina, the Na^+ channel blocker TTX was applied to the left retina of normally raised rats for three day periods, starting at different developmental times. Age-matched control animals were treated with citrate buffer applied to the left retina. *cpg15* mRNA levels in visual cortex contralateral to the TTX treated eye were compared with those in visual cortex contralateral to the citrate treated eye in control animals. There was no change in

the onset of *cpg15* expression in response to monocular retinal activity blockade, and there was no significant influence on cortical levels of *cpg15* mRNA during early postnatal development (Fig. 2-5). Mean levels of *cpg15* expression at 2, 3, and 4 weeks postnatal were not significantly different between the visual cortex contralateral to the blocked eye and visual cortex contralateral to citrate controls (Fig. 2-3b). By contrast, following the peak of the critical period and into adulthood, monocular retinal action potential blockade decreased *cpg15* expression in visual cortex contralateral to the blocked eye (Figs. 2-3b and 2-5).

Monocular TTX blockade also decreased *cpg15* expression in the contralateral LGN and SC starting at three weeks postnatal. For both LGN and superficial layers of the SC contralateral to the treated eye, the effect of TTX blockade became more pronounced with age (LGN not shown; for SC see Figures 2-5 and 2-6). These experiments indicate that in all visual structures tested, developmental regulation of *cpg15* expression is divided into two phases. Early *cpg15* expression is independent of retinally driven action potentials. During the critical period for development of eye specific preference in visual cortex, an activity-dependent component of *cpg15* expression emerges, and the effect of retinal action potential blockade becomes progressively more pronounced with age.

Effect of MD on *cpg15* expression

Studies have shown that total blockade of retinal activity produces a smaller shift in ocular dominance plasticity than that produced by MD (Rittenhouse et al., 1999). To investigate whether the effect of monocular activity blockade on *cpg15* expression is less extreme than that of MD, we monocularly deprived normally raised rats for three-day periods, starting at different developmental times. *cpg15* levels in visual cortex and SC contralateral to the sutured eye were

compared to the visual cortex and SC in normal animals. Similar to both DR and TTX treated animals, there was no change in the onset of *cpg15* expression in the visual cortex of monocularly deprived rats (Fig. 2-3c). During the following 4 weeks of development, *cpg15* expression in the visual cortex contralateral to the monocularly deprived eye was significantly less than in normal animals. Unlike animals whose retinally driven action potentials are blocked by TTX, at the peak of the critical period (P28) monocularly deprived animals exhibit levels of *cpg15* expression significantly less than controls. With maturation the effect of MD becomes less significant, and at 8 weeks postnatal *cpg15* expression in the deprived visual cortex is no longer different from controls. This trend is opposite to the effect of TTX, which becomes more significant as the animals mature. As in DR animals and in contrast to animals with retinal activity blockade, MD did not affect *cpg15* levels in the contralateral SC.

Effect of early visual experience on adult *cpg15* expression

Previous studies suggest that early periods of visual experience are sufficient to trigger closure of the critical period despite later visual deprivation (Mower et al., 1983; Mower and Christen, 1985). To determine the window of early visual experience required for development of normal adult regulation of *cpg15* expression in visual cortex, animals were raised in a normal 12/12 hour light/dark cycle to 2, 3, 4 or 5 weeks postnatal, then transferred to a dark environment until adulthood at 8 weeks. Animals were sacrificed without seeing any further light or following 24 hours of re-exposure to light, in order to assess their ability to regulate *cpg15* expression. In normally raised adult rats *cpg15* mRNA expression in visual cortex is down-regulated following a two-week period in the dark. After re-exposure to light for 24 hours, such dark-adapted rats will up-regulate *cp15* levels (Nedivi et al., 1996) (Figs. 2-7h and 2-8h). In contrast, rats raised in

darkness to eight weeks show abnormally high levels of *cpg15* expression in visual cortex and upon exposure to 24 hours of light, levels of expression are decreased (Figs. 2-4, 2-7a and 2-8a). These two extremes were compared with animals exposed to visual experience at restricted windows during development. Animals exposed to visual experience for two or three weeks prior to being placed in the dark to adulthood, displayed regulation of *cpg15* expression essentially the same as in DR animals (Figs. 2-7b, 2-7c, and 2-8b, 2-8c). Animals reared with normal visual experience for the first four or five postnatal weeks and then placed in darkness until 8 weeks of age, regulate *cpg15* expression in visual cortex similar to normal animals (Figs. 2-7d, 2-7e, and 2-8d, 2-8e). These results suggest that one week of visual experience at the outset of the critical period is sufficient to confer normal patterns of adult *cpg15* regulation. To test this prediction, animals were raised to eight weeks in darkness except for a week of light either between weeks 3 and 4 or weeks 4 and 5. *cpg15* expression in visual cortex was assayed at 8 weeks. *cpg15* mRNA levels in animals allowed just one week of visual experience during the critical period are strikingly similar to those seen in normally raised animals and are dramatically induced upon re-exposure to light for 24 hours (Fig. 2-8f and 2-8g). Our results show that a restricted window of visual experience of one week during the critical period is sufficient to determine the adult patterns of *cpg15* regulation in visual cortex.

Discussion

The experiments described here address the temporal and spatial expression patterns of *cpg15* in the developing visual system of the normal rat and in response to visual manipulations. *cpg15* expression was monitored in relation to the critical period for development of eye specific preference in visual cortex. We compared expression during normal development with that seen in response to dark rearing, monocular retinal action potential blockade or MD. The regulation of

cpg15 expression patterns suggests that it may serve as a molecular indicator of the potential for visual system plasticity.

Activity-independent and activity-dependent phases of *cpg15* expression

We found that in the rat visual system the onset of *cpg15* mRNA expression in visual cortex occurs at two weeks postnatal. *cpg15* expression levels gradually rise and peak two weeks later, in the midst of the electrophysiologically mapped cortical critical period for shifts in eye preference. Following its peak expression, *cpg15* mRNA levels in visual cortex decline to a lower basal level in adults, concomitant with critical period closure at approximately six weeks (Fagiolini et al., 1994; Gordon and Stryker, 1996). Although onset of *cpg15* expression is coincident with eye opening, it is unaffected by dark rearing, blockade of retinally driven action potentials, or MD. This is consistent with previous studies of *cpg15* expression in the cat visual system demonstrating that early *cpg15* expression in visual cortex is activity-independent (Corriveau et al., 1999). The initial timing of *cpg15* expression is therefore likely to be controlled by a developmentally regulated activity-independent mechanism. These findings are consistent with electrophysiological and optical imaging studies demonstrating that the basic structure of cortical maps is innate and develops in the absence of visual experience (Crair et al., 1998).

These studies also show that experience is necessary at a later stage of development for the refinement of ocular selectivity and maintaining responsiveness (Crair et al., 1998). We find that after the peak of the critical period, as levels of *cpg15* in visual cortex begin to decline, a component of *cpg15* expression dependent on retinally driven action potentials becomes evident and is progressively more pronounced with age. Activity-dependent regulation of *cpg15* expression in the SC and LGN can be detected a week earlier than in visual cortex, perhaps

related to earlier maturation of these visual structures. Our results indicate that in all visual structures regulation of *cpg15* expression is biphasic. Early *cpg15* expression is independent of retinally driven action potentials. With maturation during the critical period, an activity-dependent component of *cpg15* expression emerges, and the effect of retinal action potential blockade becomes progressively more pronounced with age. Activity-dependent regulation of *cpg15* arises relatively late in development and may represent an adult feature of visual system plasticity.

Effect of visual experience on *cpg15* expression

The aspects of *cpg15* regulation that we found to be dependent on normal visual experience were its peak levels in visual cortex at P28 and its subsequent down-regulation following the closure of the critical period. Although *cpg15* levels in visual cortex of DR rats are indistinguishable from those in control rats within the first 3 postnatal weeks, the expression at P28 is lower than controls. Rats DR beyond P28 maintain the same peak level of *cpg15* expression through adulthood, levels that are significantly higher than in their control counterparts. There is a crossover of the *cpg15* expression profiles in normal and DR rats, such that at the peak of the critical period expression is higher in normal animals, while after the critical period *cpg15* is higher in DR animals. This crossover can also be seen in the developmental profiles of susceptibility to MD in normal and DR cats (Mower, 1991). In rodents, there have been no studies that examine the effects of dark rearing on susceptibility to MD during the height of the critical period. Studies at later ages show that similar to cats, DR rats also retain a prolonged capability to respond to MD, even at P90 (Guire et al., 1999).

In respect to dark rearing, we show here two additional cases in which *cpg15* regulation closely parallels plasticity as measured by susceptibility to MD. Electrophysiological studies have demonstrated that a short exposure to light can trigger the end of the delayed plasticity that results from dark rearing (Mower et al., 1983; Philpot et al., 2001). Similarly, early visual experience in DR kittens attenuates the effects of later dark rearing so that there is no delayed plasticity (Cynader, 1983). These results show that the effect of dark rearing can be negated by a short period of light in DR adults, or with sufficient early visual experience. We find that a 24-hour exposure to light returns *cpg15* levels in DR rats to those found in normally raised adults, and that one week of visual experience during the critical period is sufficient to confer normal adult patterns of *cpg15* regulation. This suggests that visual experience is not persistently required during development for normal maturation of visual system function. Rather, exposure to patterned vision for at least a week during the critical period can irreversibly trigger the molecular machinery required for maturation, and will likely result in normal adult responses to visual manipulations. Since, in the absence of visual experience *cpg15* expression is abnormally high in the adult, the molecular trigger for maturation may involve a general down-regulation of plasticity genes such as *cpg15*.

Differences in the effect of dark rearing on *cpg15* from effects of activity blockade and MD

The effect of binocular elimination of patterned vision on *cpg15* expression during the critical period is profoundly different from the effect of retinal action potential blockade or MD. The effect of dark rearing is exclusive to visual cortex, and causes a prolonged up-regulation of *cpg15* expression starting at the peak of the critical period and into adulthood. Retinal action potential blockade at the same developmental times causes a decrease in *cpg15* expression in the

LGN and SC as well as visual cortex. The result, whereby the effect of dark rearing on *cpg15* expression is restricted to visual cortex, corresponds with the observation that the delayed plasticity seen in DR cats is not manifested in the LGN (Mower et al., 1985; Mower and Christen, 1985).

During the critical period, down-regulation of *cpg15* expression in visual cortex by MD is more severe than that caused by retinal activity blockade. This is consistent with electrophysiological studies showing that MD during the critical period produces a greater shift of ocular dominance in visual cortex than total blockade of retinal activity (Rittenhouse et al., 1999). A possible explanation is that during MD the residual activity from the retina actively depresses efficacy of synaptic connections driven by the deprived eye (Bear and Rittenhouse, 1999; Rittenhouse et al., 1999). Despite the fact that a monocularly deprived eye is generating some activity as opposed to total loss of activity generated by retinal action potential blockade, during the critical period *cpg15* levels are lower in visual cortex of MD animals than in animals after retinal TTX blockade. This could reflect depression of cortical synaptic activity driven by the deprived eye.

Taken together, this indicates that regulation of *cpg15* expression does not correspond directly to levels of activity, but rather, seems to reflect a propensity for functional plasticity.

Effect of dark rearing on expression of visually responsive genes

It has been proposed that mechanisms underlying adult plasticity during learning and memory, or long-term potentiation and long term depression, also play a key role in developmental plasticity (Kandel and O'Dell, 1992; Goodman and Shatz, 1993; Constantine-Paton and Cline, 1998; Nedivi, 1999). For this reason, many genes isolated or characterized on

the basis of their response to activity in the adult have been investigated in the context of developmental plasticity in the visual system. These include both regulatory genes that encode transcription factors as well as effector genes that can directly affect neuronal morphology and function (reviewed in (Nedivi, 1999)). Multiple genes show a transcriptional response to dark rearing, although the type of response varies. While GAP43, CaMKII and GAD all show the same increase in expression shown by *cpg15* in response to dark rearing past the critical period (Neve and Bear, 1989), junB, zif/268, and BDNF show the opposite response and are down-regulated (Rosen et al., 1992; Lein and Shatz, 2000). In contrast to light-independent onset of *cpg15* expression, dark rearing prevents the normal onset and transcriptional increase of Homer, zif/268, and BDNF in visual cortex (Worley et al., 1990; Brakeman et al., 1997; Capsoni et al., 1999; Lein and Shatz, 2000). Subsequent exposure to light causes their rapid induction (Worley et al., 1990; Brakeman et al., 1997; Capsoni et al., 1999; Lein and Shatz, 2000). The transcriptional regulation of this latter group provides an accurate 'molecular readout' of activity, while regulation of *cpg15* together with GAP43, CaMKII and GAD corresponds more closely with the capacity for plasticity.

Summary

During Xenopus visual system development, CPG15 concurrently regulates multiple aspects of retinotectal circuit formation (Cantallops et al., 2000). CPG15 promotes tectal cell dendritic arbor growth, stabilizes retinal axon arbors, and promotes maturation of retinotectal synapses (Nedivi et al., 1998; Cantallops et al., 2000). Our finding that DR rats fail to down-regulate *cpg15*, raises the possibility that perhaps the residual plasticity measured electrophysiologically in these animals reflects an extended capacity for local synaptic

remodeling. The prolonged plasticity seen in DR animals may result from failure to down-regulate genes like *cpg15* that could promote structural remodeling and synaptic maturation.

Methods

Animal manipulations and tissue isolation

All animal work was approved by the Massachusetts Institute of Technology Committee on Animal Care, and conforms to NIH guidelines for the use and care of vertebrate animals. Wistar-Kyoto rats (Taconic, Germantown, NY) were housed either in a room with a 12/12 hour light/dark cycle or in a room sealed from visible light. A 15-watt safelight shielded by a #2 Kodak dark-red filter (Kodak, Rochester, NY) was used for daily care and maintenance of animals housed in the darkroom (approximately 30 minutes per day). At various points during the experiment Polaroid photographic paper placed in the dark room was monitored for exposure. In cat, intermittent safelight exposure can be sufficient to prevent the apparent extension of the critical period by dark rearing. To test whether safelight exposure affected dark rearing of rats, a group of animals handled with an infrared viewing system (950 nm) was raised in the dark to 3, 4, 5, and 8 weeks (each group n=3). The levels of *cpg15* expression in rats handled with intermittent safelight exposure were not significantly different from those handled under infrared conditions, and both were significantly different from their age-matched controls at 4 weeks and 8 weeks (data not shown). The difference in sensitivity to safelight exposure may reflect the relatively poor vision of albino rats as compared to cats.

Rats were reared under normal conditions (12/12-hour light/dark), DR from birth, or dark-adapted at different developmental time points. DR and age-matched control animals were sacrificed postnatally at progressive times, starting at postnatal day 10 (P10) and at 1 week

intervals from P14 (the approximate day of eye opening) to 8 weeks (P10, each group n=2; P14, P21, 5 weeks, 6 weeks, each group n=4; 4 weeks DR n=4, controls n=3; 7 weeks controls n=3; 8 weeks DR n=3, controls n=5). A parallel set of DR rats were removed from the darkroom at the same intervals, and exposed to light for 24 hours before sacrifice (n=3 for each time point). A group of DR 8 week old rats were exposed to light for 6H, 12H, 24H, 7 or 14 days (n=2-3 for each time point). Dark-adapted adult rats were placed in the dark for two weeks and sacrificed in the dark with a parallel set sacrificed following re-exposure to 24 hours of light (n=3 each group). Rats dark-adapted to adulthood were raised normally to 2, 3, 4, or 5 weeks then placed in the dark until 8 weeks and sacrificed in the dark or following re-exposure to light for 24 hours (n=3 per group per time point). Two additional groups of rats were allowed a 1 week window of visual experience (between weeks 3 and 4 or between weeks 4 and 5) during dark rearing. At 8 weeks, these animals were sacrificed in the dark (each group n=3) or following a 24 hour exposure to light (Lt3-4, n=3; Lt4-5, n=2).

Monocular blockades of retinal action potentials were done essentially as previously described (Prusky and Ramoa, 1999). A strip of Elvax containing either tetrodotoxin (TTX) or citrate buffer was surgically implanted into the vitreous of the left eye of each animal for 3 days of sustained release, starting at P11, P18, P25, P32, P39 and P53 (TTX, n=3; citrate, n=2). Briefly, 200 mg of washed Elvax beads (DuPont, Wilmington, DE) were dissolved in methylene chloride. The dissolved Elvax was mixed with 20 μl of 1% Fast Green in DMSO and either 20 μl of 0.3 M TTX in citrate buffer (CalBiochem, La Jolla, CA), or 20 μl of 18.6 mM citrate buffer (Sigma, St. Louis, MO). The methylene chloride was slowly evaporated over the course of one day at -70°C and for five subsequent days at -20°C. The Elvax was then sectioned into 180-micron thick disks by cryostat and stored at -80°C. Before surgeries, the Elvax was washed in

70% ethanol for 30 minutes and twice in sterile PBS for 30 minutes. For younger animals, the Elvax was cut into approximately 2 x 0.75 mm strips. For older animals the Elvax strips were approximately 4 x 1 mm. The strips were carefully inserted into the vitreous following a small incision at the sclera's edge. One eye in each animal was treated with either TTX or citrate Elvax while the other eye remained intact. Anesthesia was maintained by halothane/O_2 via mask. Following Elvax implantation, the rats recovered from anesthesia under observation. The effectiveness of activity blockade was monitored daily by assaying for consensual pupil response to bright white light under light halothane/O_2 anesthesia.

MD by eyelid suture was initiated at P18, P25, P32, P39 and P53 (for each time point, n=3). Under Ketamine/Xylazine (80/10 mg/kg) anesthesia, the area surrounding the left eye was cleaned with Betadine and isopropyl alcohol. The lid margins were trimmed and the eye flushed with sterile PBS. Two to three horizontal mattress sutures using 6.0 Ethilon (Johnson & Johnson, Somerville, NJ) closed the length of the apposed lids. Ophthalmic ointment (Fougera, Melville, NY) was applied and the animals were monitored for recovery. For the P14 time point (n=3), rather than suture the unopened eye, tissue adhesive (Vetbond; 3M, St. Paul, MN) was applied at P11 to prevent possible eye opening. Following three days of TTX blockade or MD, animals were sacrificed by guillotine decapitation, brains were removed immediately, trimmed and positioned for coronal sectioning before being frozen on powdered dry ice and stored at -80°C.

In situ hybridization

Ten micron coronal sections through anterior visual cortex were sectioned by cryostat, thaw mounted on Superfrost/plus microscope slides, dried, fixed in 4% paraformaldehyde, washed in PBS, dehydrated in ethanol, air-dried, then stored desiccated at -80°C. Before

hybridization, slides were pretreated (at room temperature, unless otherwise stated) with 0.2 M HCl (20 min.); DDW (5 min.); 2x standard saline citrate (30 min. at 70°C); DDW (5 min.). The next prehybridization treatments, from pronase (Sigma, St. Louis, MO; Type XIV) to air-drying slides for 1 hour, were conducted as described (Hogan et al., 1994). RNA probes were synthesized with an RNA transcription kit (Stratagene, La Jolla, CA) and ^{35}S-UTP (Amersham Pharmacia Biotech, Piscataway, NJ; 800 Ci/mmol), using linearized *cpg15* cDNA as a template. Hybridizations were done as previously described (Nedivi et al., 1996). Posthybridization wash conditions: 3 hours at 50°C, in 50% formamide, 1x salt solution (Hogan et al., 1994), with 10 mM DTT; 15 min. at 37°C in TNE (10 mM Tris pH 7.5, 0.5 M NaCl, 1 mM EDTA); 30 min. at 37°C in TNE containing RNase A (Sigma, St. Louis, MO; 20 μg/ml); 30 min. at 37°C in TNE; and finally overnight at 50°C in 50% formamide, 1x salt solution. Slides were dehydrated with 0.3M NH_4Ac in ethanol, air-dried, and processed for autoradiography as described (Hogan et al., 1994) using NTB-2 emulsion (Kodak, Rochester, NY) diluted 1:1 with 2% glycerol, and exposed for 3-5 days at 4°C.

Quantitative data analysis

Darkfield images of 2-4 sections from each brain were acquired into Photoshop 5.0 with a Diagnostics Instruments Spot2 digital camera mounted on a Nikon Eclipse E600 using a 1x/0.04 Plan UW objective (MVI, Avon, MA). Images were saved as grayscale TIFFs and imported into NIH Image (version 1.62). Each section contained, in addition to visual cortex and SC, the medial geniculate body (MGB) of the thalamus, an auditory sensory area (Fig. 2-1). Areas were defined by Nissl staining of alternate sections. Mean pixel density measurements were taken from four areas on each section: the visual cortex, SC, background, and MGB. Pixel

density was measured on a 0 to 255 scale where 255 is white. The background served as a zero-labeling negative control, while the MGB served as a positive control with a high level of labeling that is unaffected by visual manipulations. Visual cortex and SC measurements were normalized on a scale of 0 to 1 interpolating between the background (0) and MGB (1) values. The background mean pixel density was first subtracted from the mean pixel densities in the visual cortex, SC, and MGB yielding the net mean pixel densities for each area. The net mean pixel density in the visual cortex or SC was then divided by the net mean pixel density from the MGB in the same section. Statistical significance was determined by unpaired Student's *t*-test.

Figure 2-1. *In situ* hybridization on a coronal section through rat visual cortex using a *cpg15* probe.

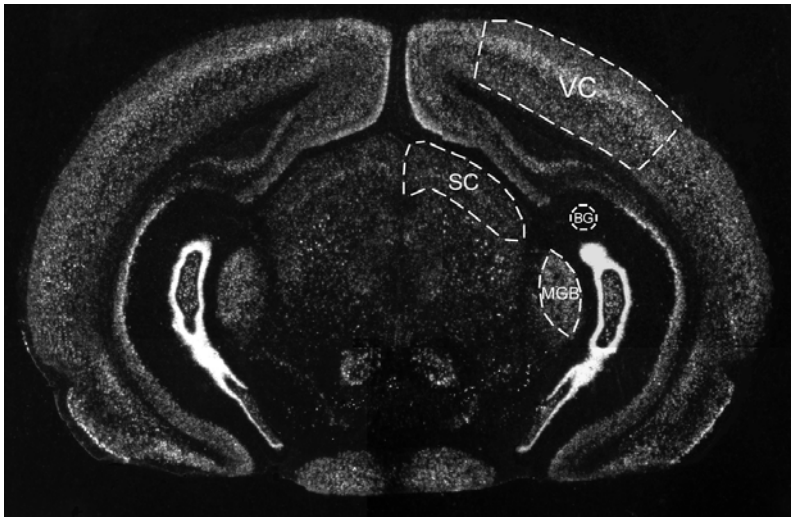


Figure 2-2. Developmental time course of *cpg15* expression in the visual cortex of normal and DR rats.

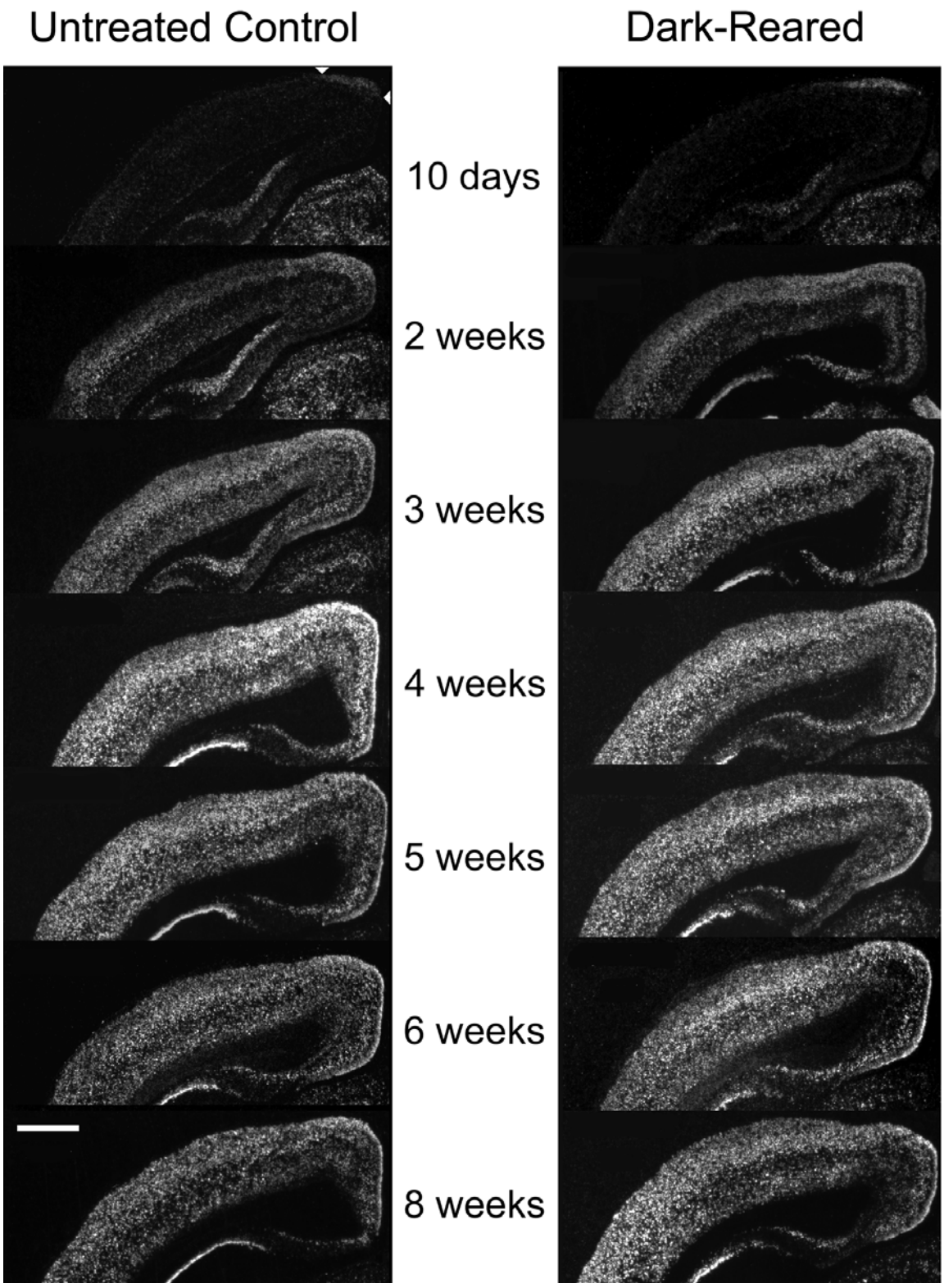


Figure 2-3. Quantification of *cpg15* expression in visual cortex of control, DR rats, rats after retinal activity blockade, or after MD.

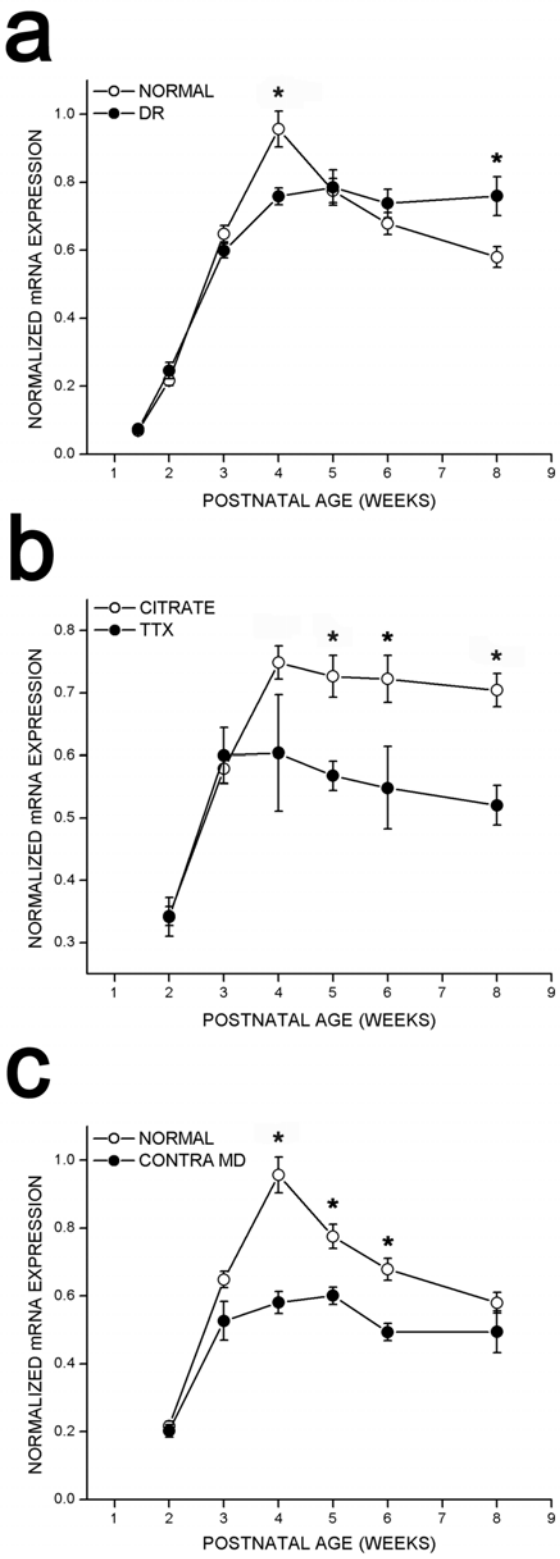


Figure 2-4. Light triggers down-regulation of *cpg15* expression in DR rats.

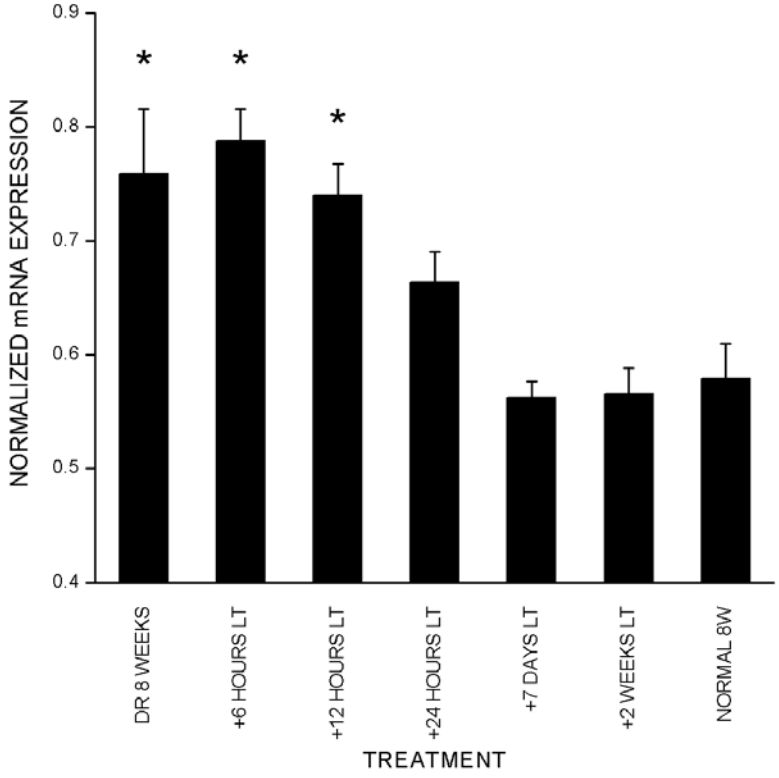


Figure 2-5. *cpg15* expression in visual cortex after 3 days of monocular TTX blockade initiated at different developmental times.

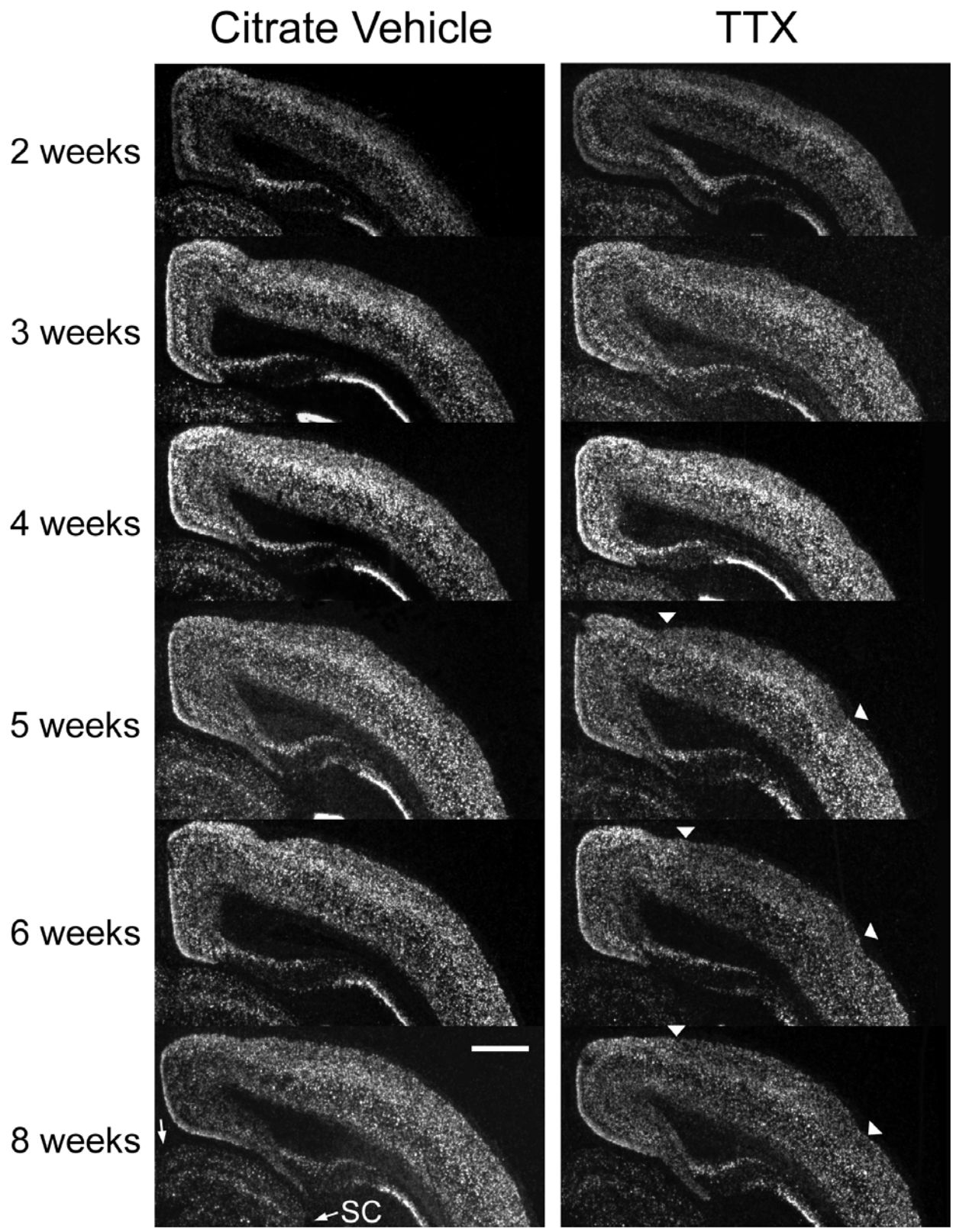


Figure 2-6. Quantification of *cpg15* expression in superior colliculus after 3 days of monocular TTX blockade initiated at different developmental times.

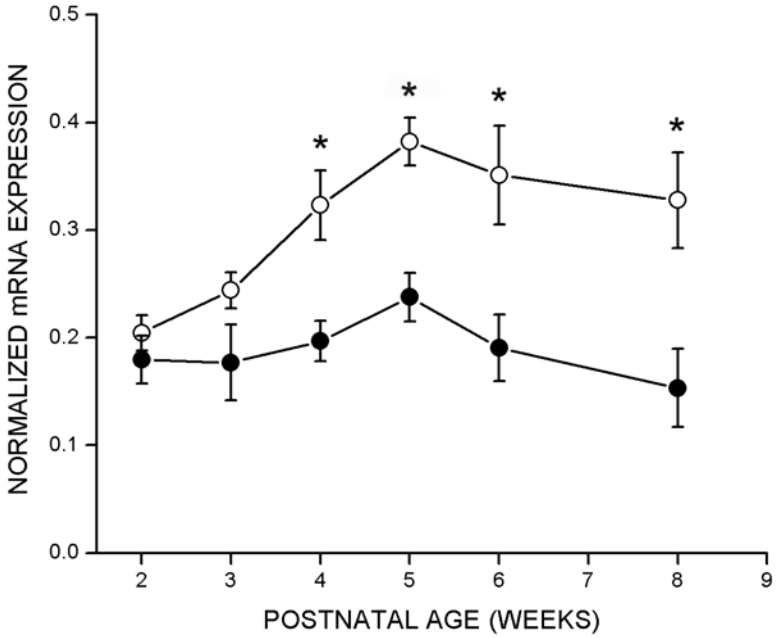


Figure 2-7. Early visual experience confers normal adult patterns of *cpg15* regulation.

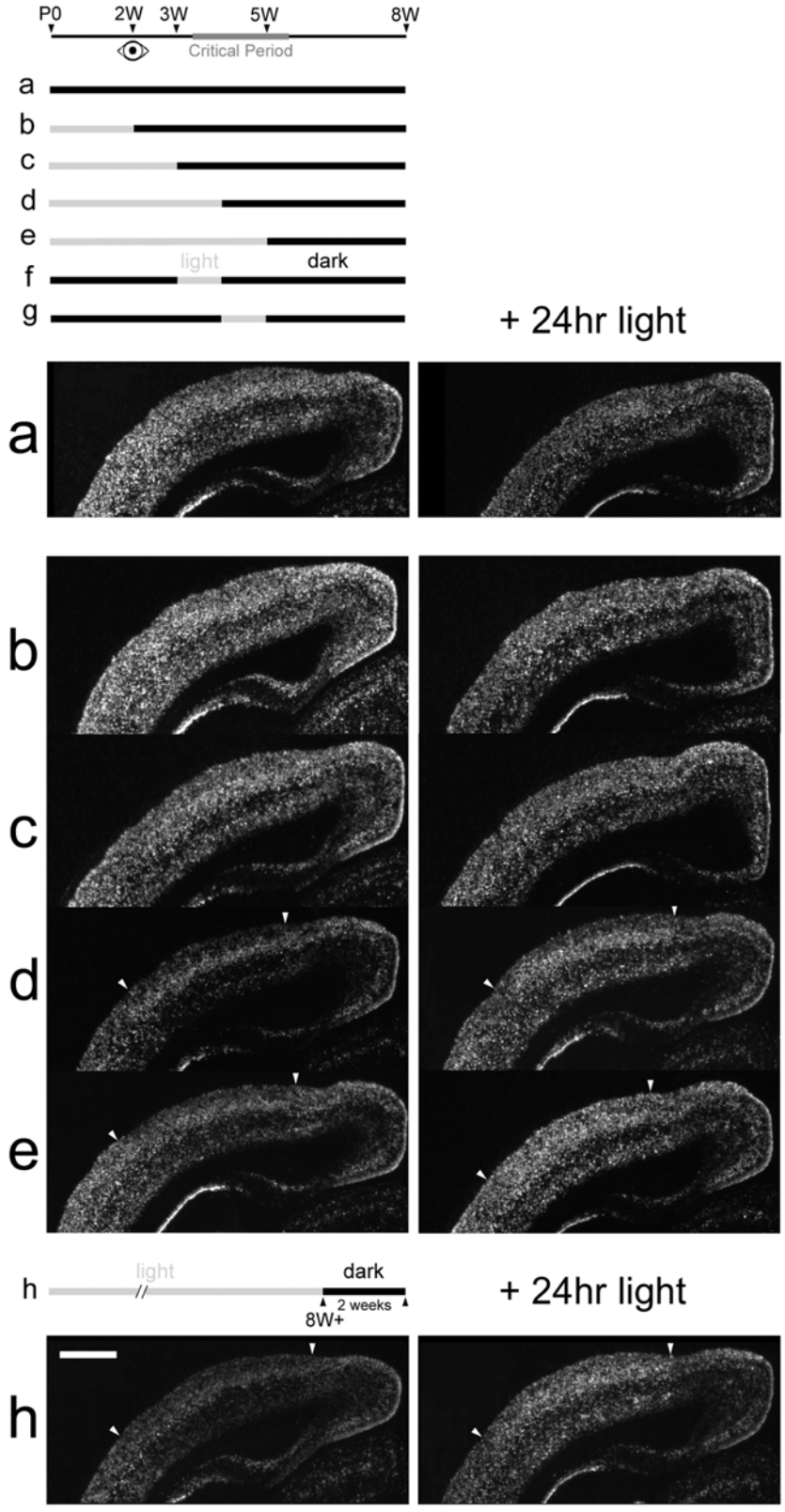


Figure 2-8. One week of visual experience during the critical period is sufficient to confer normal adult patterns of *cpg15* regulation.

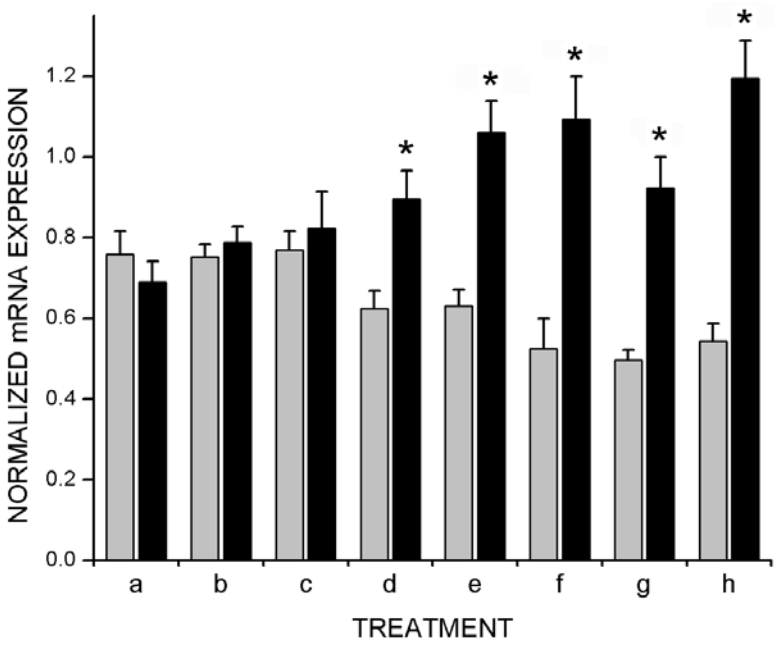


Figure Legends

Figure 2-1. *In situ* hybridization on a coronal section through rat visual cortex using a *cpg15* probe. Regions relevant for quantification are outlined. Mean pixel densities were measured in four areas on each section: the visual cortex (VC), superior colliculus (SC), medial geniculate body (MGB), and in an area of the section with zero labeling for background (BG). The background is used as the zero-point while the MGB served as a positive control with a high level of labeling that is unaffected by visual manipulations (see Methods).

Figure 2-2. Developmental time course of *cpg15* expression in the visual cortex of normal and DR rats. Representative dark-field photomicrographs of *in situ* hybridizations for *cpg15* mRNA. Coronal hemi sections through visual cortex of normal animals (left), and DR animals (right) are shown at the designated postnatal ages. The onset of *cpg15* expression in neocortex for both normal and DR animals is coincident with eye opening at 2 weeks. Normally, *cpg15* expression peaks at 4 weeks and then declines to a lower basal adult level. In DR animals, *cpg15* levels remain elevated past 5 weeks. Arrowheads point to the earliest *cpg15* expression at medial apex of neocortex. Some individual panels (6W, DR6W, 4W, DR4W, DR3W, and DR2W) were normalized with respect to MGB for assembly of the montage. Scale bar,1 mm.

Figure 2-3. Quantification of *cpg15* expression in visual cortex of control, DR rats, rats after retinal activity blockade, or after MD. In all cases, after background subtraction, the net average pixel value in visual cortex was normalized by the net average pixel value of the MGB (see methods for detail). **a**, Effect of dark rearing on *cpg15* expression in visual cortex. Data from

four *in situ* experiments are shown, of which Figure 2-2 is representative. Filled circles represent DR animals, unfilled circles represent untreated control animals, and error bars represent SEM. Points marked with * are significantly different between normal and DR animals (at 4 weeks p = 0.001, at 8 weeks p = 0.016, unpaired Student's *t*-test). **b**, Effect of 3 days monocular TTX blockade initiated at different developmental times on *cpg15* expression in visual cortex. Data from three *in situ* experiments are shown, of which Figure 2-5 is representative. Filled circles represent visual cortex contralateral to TTX blockade, unfilled circles represent visual cortex of control animals implanted with a citrate control in the contralateral eye, and error bars represent SEM. Points marked with * are significantly different between visual cortex of control and TTX treated rats (at 5 weeks p = 0.0012, at 6 weeks p = 0.032, at 8 weeks p = 0.0008, unpaired Student's *t*-test). **c**, Effect of 3 days MD by eyelid suture initiated at different developmental time points on *cpg15* expression in visual cortex. Filled circles represent visual cortex contralateral to the sutured eye, unfilled circles represent visual cortex of untreated control animals, and error bars represent SEM. Points marked with * are significantly different between visual cortex of control and monocularly deprived rats (at 4 weeks p = 0.003, at 5 weeks p = 0.006, at 6 weeks p = 0.007, unpaired Student's *t*-test).

Figure 2-4. Light triggers down-regulation of *cpg15* expression in DR rats. Quantification of *cpg15* expression in visual cortex of animals DR to 8 weeks and subsequently exposed to light (Lt) for designated times. Data from two *in situ* experiments are shown. In each section the net average pixel value in visual cortex is normalized by the net average pixel value of the MGB. Abnormally high levels of *cpg15* expression in animals DR to 8 weeks decline to normal adult levels within 24 hours of exposure to light. Error bars represent SEM. * mark significant

differences compared to normal 8 week animals (DR8W p = 0.016, DR8W+6hl p = 0.009, DR8W+12hl p = 0.0086, unpaired Student's *t*-test).

Figure 2-5. *cpg15* expression in visual cortex after 3 days of monocular TTX blockade initiated at different developmental times. Dark-field photomicrographs of *in situ* hybridizations for *cpg15* mRNA in coronal sections through the visual cortex contralateral to the treated eye of either control animals (left) implanted with the citrate vehicle or TTX-treated animals (right), at the designated ages. Starting at 5 weeks postnatal, retinal TTX blockade decreases levels of *cpg15* expression in visual cortex (see cortical area between arrowheads). Starting at 4 weeks postnatal, retinal TTX blockade causes a down-regulation of *cpg15* expression in the SC (see area between arrows marked SC). Some individual panels (C4W, TTX4W, TTX3W) in this figure were normalized with respect to MGB for assembly of the montage. Scale bar, 1 mm.

Figure 2-6. Quantification of *cpg15* expression in superior colliculus after 3 days of monocular TTX blockade initiated at different developmental times. Data from three *in situ* experiments are shown, of which Figure 2-5 is representative. After background subtraction, the net average pixel value in superficial layers of the SC was normalized by the net average pixel value of the MGB. *cpg15* expression in the SC is depressed by retinal TTX blockade soon after eye opening. Filled circles represent SC contralateral to TTX blockade, unfilled circles represent SC contralateral to citrate control, and error bars represent SEM. Points marked with * are significantly different between SC of control and TTX treated rats (at 4 weeks p = 0.026 at 5 weeks p = 0.0003 at 6 weeks p = 0.027, at 8 weeks p = 0.011, unpaired Student's *t*-test).

Figure 2-7. Early visual experience confers normal adult patterns of *cpg15* regulation. Representative dark-field photomicrographs of *in situ* hybridizations for *cpg15* mRNA on coronal hemi sections through visual cortex. Animals were raised to different ages with normal visual experience, then transferred into a dark environment until 8 weeks (left), followed by a 24 hour re-exposure to light (right). Animals raised normally to 4 or 5 weeks of age (**d**, **e**), then DR to 8W, show the normal decline in *cpg15* levels with maturation as well as the normal adult regulation (**h**) of *cpg15* expression by light (arrowheads delineate visual cortex). Animals raised normally to 2 and 3 weeks of age (**b**, **c**), then DR to 8W, similarly to animals DR from birth (**a**), fail to down-regulate *cpg15* as adults and do not re-induce *cpg15* in response to light. Panels were not normalized for montage assembly. Scale bar, 1 mm.

Figure 2-8. One week of visual experience during the critical period is sufficient to confer normal adult patterns of *cpg15* regulation. Quantification of *cpg15* expression in visual cortex of rats with early visual experience. Measurements and calculations were done as described in Figure 2-3. The treatment legend is identical to Figure 2-7. A one week-window of light during the critical period, either between weeks 3 and 4 (**f**) or weeks 4 and 5 (**g**), confers normal adult patterns of *cpg15* regulation. Grey bars represent *cpg15* expression levels without re-exposure to light, black bars represent levels following 24 hours of light exposure, and error bars represent SEM. Points marked with * are significantly different between animals sacrificed in the dark or following 24 hours of light exposure (at **e** and **h**, $p < 0.0001$; at **f**, $p = 0.0049$; at **g**, $p = 0.0022$; at **d**, $p = 0.009$, unpaired student's *t*-test).

Chapter 3

Dynamic remodeling of dendritic arbors in GABAergic interneurons of adult visual cortex

Abstract

Despite decades of evidence for functional plasticity in the adult brain, the role of structural plasticity in its manifestation remains unclear. To examine the extent of neuronal remodeling that occurs in the brain on a daily basis, we used a multi-photon based microscopy system for chronic *in vivo* imaging and reconstruction of entire neurons in the superficial layers of the rodent cerebral cortex. Here, we show the first unambiguous evidence of dendrite growth and remodeling in adult neurons. Over a period of months, neurons could be seen extending and retracting existing branches, and in rare cases adding new branch tips. Neurons exhibiting dynamic arbor rearrangements were GABA positive non-pyramidal interneurons, while pyramidal cells remained stable. These results are consistent with the idea that dendritic structural remodeling is a substrate for adult plasticity and suggest that circuit rearrangement in the adult cortex is restricted by cell type-specific rules.

Introduction

Hubel and Wiesel's groundbreaking work in the 1960-1970's defined a critical period in development when manipulating visual inputs causes dramatic functional and structural changes in layer 4 of primary visual cortex (Hubel and Wiesel, 1970; Hubel et al., 1977; LeVay et al., 1980). Since their finding that large scale rearrangement of thalamic afferents in visual cortex is restricted to a developmental critical period, the adult brain has been considered relatively 'hard-wired' and limited in its capacity for structural change. Functional reorganization of primary

sensory maps in the adult brain (reviewed (Buonomano and Merzenich, 1998)), even across long distances (Pons et al., 1991), was explained as unmasking of existing connections and was not considered to require outright growth (Ramachandran et al., 1992). Although there are indications that adult cortex is capable of anatomical change in response to peripheral manipulation, particularly in the superficial layers (Darian-Smith and Gilbert, 1994; Florence et al., 1998), the scale of change is small compared to the critical period, and is difficult to detect against the general variance in the size and shapes of cortical neurons. Moreover, such changes are seen only in response to external perturbation, leaving it unclear whether arbor remodeling normally occurs in the adult cortex on a daily basis, and to what extent.

With the advent of new technologies to time-lapse image neuronal morphology *in vivo* (Denk et al., 1990), the issue is now being revisited. Repeated *in vivo* imaging of apical dendrites extending from layer 5 pyramidal neurons into the superficial layers has been used to investigate dendritic spine dynamics in both somatosensory and visual cortex (Grutzendler et al., 2002; Trachtenberg et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005a). Less attention has been paid to potential changes in the overall structure of dendritic arbors. In fact, it has been suggested that little if any structural plasticity occurs in the apical dendrites of layer 5 pyramidal neurons in the adult somatosensory cortex (Trachtenberg et al., 2002), or in the apical dendrites of mitral and tufted cells in the adult olfactory bulb (Mizrahi and Katz, 2003). No study has directly addressed the potential for structural dynamics in a cross section of neurons that reflects the diversity of neocortical cell types. More specifically, the non-pyramidal neurons of the neocortex have yet to be the focus of investigation by *in vivo* imaging studies, despite their important role in adult cortical plasticity and in reorganization of cortical maps (Jacobs and Donoghue, 1991; Jones, 1993).

Here, we investigate the dendritic arbor dynamics of pyramidal and non-pyramidal neurons in the superficial layers of the adult visual cortex *in vivo*. Our results show that while dendritic branches of layer 2/3 pyramidal cells remain stable, non-pyramidal interneurons in these layers are dynamic, exhibiting a range of structural changes on a weekly basis.

Results

Repeated *in vivo* imaging of cortical neurons

Studies suggest that intracortical connections in the supragranular layers of the neocortex, and in particular axonal sprouting, may be a locus of adult structural plasticity (Darian-Smith and Gilbert, 1994). To directly test the prediction that neurons in layer 2/3 are capable of structural change and to assess the extent of dendritic structural dynamics in the adult cortex, we chronically imaged neuronal morphology in the intact rodent brain. To allow long-term visualization of neuronal structure *in vivo*, cranial windows were bilaterally implanted over the visual cortices of *thy1*-GFP-S mice (Feng et al., 2000) between 4-6 weeks of age. These mice express green fluorescent protein (GFP) in a sparse pseudo-random subset of neocortical neurons. Imaging began at least 2 weeks after surgery to allow for recovery and optical clarification of the cranial windows. Brains were screened for optically accessible GFP positive neurons using wide-field fluorescence, and neuronal location was noted using local landmarks in the brain's surface vasculature. Individual GFP labeled neurons in layers 2/3 of visual cortex of anesthetized adult mice were then time-lapse imaged using a custom made two-photon microscope (Kim et al., 1999). To include as many neuronal branch tips as possible within the imaging volume, nine slightly overlapping volumes were imaged in a 3 x 3 array through z-x-y translation of an automated motorized stage. Individual image planes were stitched together to create a montage of adjoining x-y sections for a given depth from the pial surface. In an attempt to provide a

comprehensive view of adult structural plasticity, data collection was initially not restricted to any particular cell type. Six pyramidal cells and 8 non-pyramidal cells from 13 animals were time-lapsed imaged for 4 to 10 weeks (Fig. 3-1) and 4-dimensional morphometric analysis was carried out by quantitative comparison of dendritic branch tip length (BTL) as a function of time. Branch tips that were not imaged clearly for multiple imaging sessions or whose termination was unclear were excluded from analysis.

Pyramidal cells are stable over time

Imaged pyramidal cells exhibited typical small pyramidal morphologies with a spiny apical dendrite and a skirt of spiny basal dendrites emanating from the lower half of a pyramid-shaped cell body. An example is shown in Figure 3-2 and Supplementary Movie 3-1. The cell body of this pyramidal neuron, ‘dow’, was located 180 microns below the pial surface. It had a total of 57 dendritic branch tips on its apical dendrite and 5 primary basal dendrites. We monitored 28 of the 57 branch tips over 9 weeks. Examination of individual branch tips revealed no overt sign of structural change (Fig. 3-2a-c). Similar examination of all cells in the pyramidal population did not identify any change in apical and basal dendritic branches. These data suggest that under normal conditions the dendritic branches of layer 2/3 pyramidal neurons in visual cortex are relatively stable in the adult, and are consistent with previous studies reporting dendritic branch stability in other cortical areas (Trachtenberg et al., 2002; Mizrahi and Katz, 2003).

Dynamic remodeling of non-pyramidal neurons

We next examined layer 2/3 non-pyramidal neurons. Figure 3-3 shows maximum-intensity z-projections (MZPs) of image planes close (± 15 microns) to the cell body of a non-pyramidal neuron 'nmr' with a bitufted dendritic morphology revealing its highly complex local arborization (Fig. 3-3a and Supplementary Movie 3-2). The cell body's center of mass was 118 microns below the pial surface. This neuron had 4 primary dendrites with a total of 49 branch tips. 28 of the 49 branch tips were monitored for 4 weeks. Four of the 28 branch tips exhibited variations in length over 4 weeks. Two examples are shown where branch tips visibly elongated in the x-y plane (Fig. 3-3b-f). Branch tip #20 elongated by approximately 16 microns over 4 weeks in (Fig. 3-3b-c, f). Concurrently, branch tip #15 increased in length by approximately 10 microns (Fig. 3-3d-f). Both branch tips emanate from the same primary dendrite whose dendritic branch length accounts for 62% the total monitored dendritic length of the neuron. These results demonstrate that dendritic arbors of neurons within the adult neocortex are capable of growth.

A different non-pyramidal neuron, 'paz', residing 78 microns below the pial surface, is shown in Figure 3-4. 2-D projections of the 3-D traces show a moderately branched interneuron with a bitufted dendritic morphology (Fig. 3-4a). This cell had 7 primary dendrites with 47 branch tips. 29 of 47 dendritic branch tips were monitored for 7 weeks. Virtually all the branches were stable, however two branches exhibited remodeling, one of which was so large that it exceeded the imaging volume. Time-lapse images revealed that within as little as 2 weeks, this branch tip more than doubled its length and exited the imaging volume (Fig. 3-4). Although at 13 weeks postnatal we were unable to follow the process to its termination, we measured a net extension of > 92 microns from the branch tip at 11 weeks to its location at the edge of the imaging volume two weeks later. The axon of this neuron projected from the cell body in the

opposite direction of this changing dendritic branch tip. This dramatic increase in BTL indicates that neurons in adult visual cortex have the capacity for large scale remodeling.

Although many non-pyramidal cells are spine-free, we also observed and imaged sparsely spinous non-pyramidal neurons. These cells typically exhibited multipolar dendritic morphologies (Fig. 3-5a). The interneuron 'zen' shown in Figure 3-5 had 3–7 primary dendrites with 61 dendritic branch tips and 7 spines on 2814 microns (averaged over 5 weeks) of monitored dendrite. The cell body's center of mass was 100 microns below the pial surface. The few spines on this neuron exhibited motility (Fig. 3-5b-c) as previously described for spines on pyramidal neurons (Grutzendler et al., 2002; Trachtenberg et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005a). Six branch tips showed changes in length (Fig. 3-5i). For example, branch tip #50 shown in Fig. 3-5 (b, d) elongated by approximately 7 microns (Fig. 3-5i). In this cell we also observed a couple of rare de novo branch tip additions. The initial addition of one new branch was first seen at 13 weeks postnatal and could be observed elongating to approximately 22 microns by 14 weeks postnatal (Fig. 3-5e-f, i). Also in the proximity of the branch addition, we observed the retraction of a putative axon of unknown origin (Fig. 3-5e-f). A second branch tip addition was concurrently seen on an independent dendrite (Fig. 3-5g-h), extending approximately 9 microns from 11 weeks to 15 weeks postnatal (Fig. 3-5i).

Every one of the layer 2/3 non-pyramidal cells imaged showed at least 1 and as many as 7 changing dendritic branch tips. On average, approximately 14% of the monitored branch tips on non-pyramidal interneurons showed structural rearrangement of which 3% were new branch tip additions, 2% were loss of existing branch tips, and of the remainder approximately half were elongations and half were retractions. Remodeling in some cases was incremental, but could also occur in short temporal bursts. Changes were never observed in primary, first-order, dendritic

branches; but were otherwise not restricted by branch order. These data demonstrate that even without peripheral perturbations, branch tips of non-pyramidal cells in the superficial layers of the adult neocortex exhibit elongation, retraction and branch tip addition.

Dynamically remodeling neurons express GABA

Neocortical interneurons are a diverse population with distinct morphological, physiological and molecular sub-types (Feldman and Peters, 1978; Peters and Kara, 1985; White and Keller, 1989; Kawaguchi and Kubota, 1997; Markram et al., 2004). Three of the eight imaged non-pyramidal neurons were unequivocally identified in coronal sections after post-imaging immunohistochemistry based on morphology and location. All of these cells were immunopositive for GABA (Fig. 3-6a-c), while pyramidal cells were GABA negative (Fig. 3-6d-e).

DAPI staining located the border between layer 1 and layers 2/3 at ~80 microns below the pial surface (Supplementary Fig. 3-1a-d), consistent with previous findings (Paxinos and Franklin, 2001), thus placing the imaged non-pyramidal cell bodies within layer 2/3 or at the layer 1-2/3 border (Supplementary Fig. 3-1). In an attempt to further classify their subtype, we also probed the sections for parvalbumin, somatostatin and cholecystokinin (CCK), but found the imaged neurons negative for all three (data not shown). This was not surprising given the low representation of these subtypes in layers 1-2/3 of visual cortex in both GFP and non-GFP labeled GABA positive interneurons (Supplementary Fig. 3-1e). From 158 GFP positive non-pyramidal cells in 7 animals 92% (SEM = 2.1%) were GABA positive. Since virtually all the non-pyramidal interneurons in the superficial layers of visual cortex are GABA positive, it is likely that the 5 imaged neurons that were not successfully identified in the sectioned brains were

also GABAergic, strongly suggesting that dendritic arbor remodeling in adult neurons occurs predominantly in inhibitory GABAergic interneurons.

Discussion

Although the capacity for change in the adult brain is limited compared to development, the adult cerebral cortex does maintain a degree of plasticity (reviewed (Buonomano and Merzenich, 1998)). Electrophysiological recordings and anatomical analysis both suggest that potential sites for this plasticity are the horizontal connections within the superficial cortical layers (Kaas et al., 1990; Gilbert and Wiesel, 1992; Darian-Smith and Gilbert, 1994; Darian-Smith and Gilbert, 1995). Additional evidence comes from molecular studies demonstrating that in the superficial layers of adult striate cortex visual input transcriptionally regulates genes involved in process outgrowth (Tsui et al., 1996; Corriveau et al., 1999; Lein and Shatz, 2000; Lee and Nedivi, 2002). Together these data led us to hypothesize that neurons in the superficial layers of the adult cortex can undergo arbor remodeling without extreme peripheral perturbation. To test this hypothesis we imaged the dendritic arbors of neurons in visual cortex of adult mice over several months. Our findings show that in layers 2/3 the dendritic structure of pyramidal neurons is stable, while inhibitory interneurons undergo dendritic arbor remodeling.

Sixty-two percent of the non-pyramidal dendrites were included in the analysis, while 42% of pyramidal dendrites were successfully monitored. Given that 35 of the 259 monitored non-pyramidal branch tips changed, if the two cell types were equivalently dynamic, then the probability of a branch tip change event can be estimated as 35/259 = 0.135. In 124 monitored pyramidal branch tips we would expect to have observed at least one branch tip change of this type with probability $1-10^{-8}$ (see Appendix). Therefore, the probability that we missed an event

due to sampling issues is 10^{-8}. The fact that we did not observe any changes in the pyramidal branch tip group allows us to reject the null hypothesis that the probabilities of change for the two groups are the same in favor of the more plausible alternative that the two groups have significantly different dynamic properties. Arguing against the possibility that missed events could be accounted for by a sampling bias is the fact that when comparing the size distributions of sampled branch tips for the pyramidal and non-pyramidal population there is a similar sampling of processes in the 40-120 micron range (Supplementary Figure 3-2), the size range where most changes occur within the non-pyramidal population (Supplementary Figure 3-3).

Although there are excellent studies in the field that promote the view that adult neocortical structure is stable, to date, all focus on pyramidal cell morphology and most focus on spine dynamics (Grutzendler et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005a). Our data does not contradict, but rather, complements these studies. While our results are consistent with previous reports on stability of the apical dendrites on layer 5 pyramidal cells (Trachtenberg et al., 2002), we do not exclude the possibility that pyramidal neurons undergo any arbor remodeling, especially in response to perturbations of the sensory periphery (Hickmott and Steen, 2005). Rather, we suggest that under normal conditions their structural rearrangements are less pronounced than those seen in layer 2/3 interneurons.

In felines and primates, the physiological plasticity manifested by cortical cells during the critical period for development of eye specific preference is accompanied by clear activity-driven segregation of geniculocortical afferents into ocular dominance columns. Yet, there are many instances where physiological plasticity can be seen in the superficial cortical layers of adult animals without such a clear anatomical ‘readout’ (Mower et al., 1985; Crair et al., 1998; Trachtenberg et al., 2000). The scale of structural change during circuit refinement in the adult

may be below the threshold for visualization with the anatomical methods used to monitor layer 4 afferent segregation. It is understandable that the structural remodeling described here was previously undetected by classical anatomical methods relying on sample statistics, as the changes are on the order of tens of microns (small relative to the entire dendritic arbor); occur on a subset of dendritic branches, in a subset of cell types; and can occur sporadically in bursts of remodeling. In addition, when dendritic branches of the same neuron both grow and retract the total net change may be negligible (~1% of the total monitored dendritic branch length). A technology where the same neurons can be vitally imaged over days and weeks in the intact brain allows discrimination of small scale structural dynamics, opening to reinterpretation the apparent disconnect between functional plasticity measured electrophysiologically in the extragranular layers and the absence of measurable anatomical change. Our results using chronic *in vivo* imaging demonstrate that there is an intrinsic capacity for structural remodeling in the superficial layers of the adult neocortex. Unlike development when changes in dendritic arbors are widespread, changes in the adult are localized to a small subset of processes. The change in these individual processes on the scale of 20-90 microns, however, may be large enough to influence receptive field properties. The changing tips are specific to a certain neuronal subtype, suggesting cell type-specific rules to this remodeling. It has yet to be shown that this minority of 'plastic' dendritic processes are ones that underlie the functional reorganization of adult cortical maps measured electrophysiologically. As there is previous evidence for horizontal axonal sprouting in the adult striate cortex following retinal lesions (Darian-Smith and Gilbert, 1994), and in somatosensory cortex following whisker trimming (De Paola et al., 2004), another area for further investigation would be the structural plasticity of axonal arbors in the long-range pyramidal cells of layers 2/3, and how they relate to dendritic changes.

It is a key unresolved question in the field, if and what kind of structural changes are expected in adult plasticity and how best to detect and analyze them. If averaged across the entire arbor, the changes in BTL of non-pyramidal neurons correspond to approximately 6% of the monitored dendritic branch length. However, individual branch tips changed by 16-456% (not including new branch tip additions), with average elongations and retractions of 16 microns. Net changes ranged from -5% to +8% of the average monitored branch length. Since changes are localized, it may be preferable for both detection and analysis methods to be implemented on a process by process basis rather than by conventional approaches that rely on statistical averaging across all the processes of a cell or an entire cell population. In conventional analyses, averaging across the entire arbor would bias against events with potential functional importance if they occurred in a minority of dendrites. Even when viewed on a process by process basis, clearly changing branch tips can be difficult to identify when looking at the absolute change in BTL (Fig. 3-7). For example branches on cells like ‘nmr’ and ‘zen’ (shown in Figures 3-3 and 3-5 respectively) that are unambiguously changing may be scored as false negatives. Thus, an alternative analysis that better represents the data scored by direct observation is clearly needed. We found that scaling the changes in length by the average length of the dendritic branch tip enhanced our ability to detect changes and represented salient changes in structure better than a non-scaled analysis. Advances in such quantitative analysis of morphometric measures over time will determine how to best represent structural change of neurons in the adult cortex.

20%-30% of the neurons in the neocortex are non-pyramidal interneurons and most adult neocortical interneurons are considered inhibitory, using GABA as a neurotransmitter (Feldman and Peters, 1978; Peters and Kara, 1985; White and Keller, 1989; Kawaguchi and Kubota, 1997; Markram et al., 2004). A diverse population of non-pyramidal interneurons preferentially

populate the superficial layers of the neocortex and are characterized by their morphological, electrophysiological, molecular, and targeting properties (Peters and Kara, 1985; Markram et al., 2004). Most mature non-pyramidal interneurons lack dendritic spines, and most project locally, usually arborizing within a cortical column or projecting horizontally across columns, but rarely projecting to distant brain regions (Fairen et al., 1984). Inhibitory interneurons are thought to have an important role in modulating excitatory circuitry by depressing, blocking, or sculpting the temporal response properties of excitatory neurons (Singer, 1996; McBain and Fisahn, 2001). During development, inhibitory circuitry is crucial for the onset of critical period ocular dominance plasticity (Hensch et al., 1998; Hanover et al., 1999; Huang et al., 1999; Fagiolini and Hensch, 2000) (reviewed (Hensch, 2004)), plasticity of somatosensory cortex (Foeller and Feldman, 2004), and refinement of visual receptive fields (Dan and Poo, 2004; Tao and Poo, 2005). By shortening stimulus-evoked spike trains in immature neurons, GABAergic activity can decrease the temporal asynchrony of uncorrelated inputs (Feldman, 2000). In addition, interneurons can coordinately synapse onto nearby excitatory pyramidal cells in a developing network, locally synchronizing their spike timing. Both shortening prolonged discharge and orchestration of spike timing could enhance the ability of target neurons to participate in spike timing-dependent plasticity (Dan and Poo, 2004; Foeller and Feldman, 2004). Interestingly, in adult monkeys MD modifies the expression of GABA and GAD in primary visual cortex in an eye specific manner (Hendry and Jones, 1986), suggesting that GABAergic transmission is sensitive to activity-dependent plasticity in the adult. Our data indicating that the structural plasticity of interneurons is continuous through adulthood raises the intriguing possibility that local remodeling of inhibitory connections may underlie adult cortical plasticity. This finding

would have important implications for models of cortical functional circuitry and its activity-dependent modulation.

Materials and Methods

Animal surgery

thy1-GFP-S mice (Feng et al., 2000) were anesthetized with 2.5% Avertin (I.P., 0.015 ml/g) and anesthesia was monitored by breathing rate and foot pinch reflex. The skull overlying both visual cortices (Paxinos and Franklin, 2001) was carefully removed leaving behind the dura and 5 mm diameter circular glass cover slips (No. 1) were positioned over the openings and sealed in place with bone cement (Palacos R). Following surgery, mice were given lactated ringers solution (S.C., 0.015 ml/g) and Buprenix (S.C., 0.3 mg/ml 2x's daily for 5 days) as an analgesic and returned to individual cages for recovery under observation. Surgeries were performed at 4-6 weeks postnatal to allow at least two weeks recovery before imaging.

In vivo two-photon imaging

In vivo two-photon imaging was achieved using a custom-built microscope and acquisition software (Kim et al., 1999) modified for *in vivo* imaging by including a custom-made stereotaxic restraint affixed to a stage insert for the motorized stage (Prior Scientific). While designed to run at high acquisition rates, for these experiments a conventional scanning rate was used to increase signal intensity by locking the polygonal mirror and using both raster-scanning mirrors. The light source for two-photon excitation was a commercial Ti:Sapphire laser (Mira, Coherent) pumped by a 10-W solid state laser delivering 150 fs pulses at a rate of 80 MHz with the power delivered to the objective (with transmittance between 20-30%) ranging from ~100-

250 mW depending on imaging depth. The excitation wavelength was set to ~890 nm, with the excitation signal passing through a 40x/0.8 NA water immersion objective (Achroplan, Zeiss) and collected after a barrier filter by a photomultiplier tube. Due in part to the sparse labeling of cells in the superficial layers of the *thy1*-GFP-S neocortex, the same cells could be identified and re-imaged for up to 3 months using local fiduciary landmarks of the brain's surface vasculature.

Image acquisition and analysis

Adult mice (8-19 weeks postnatal) previously implanted with cranial windows were anesthetized with 2.5% Avertin (I.P., 0.015 ml/g). Anesthesia was monitored by breathing rate and foot pinch reflex and additional doses of anesthetic were administered during the imaging session as needed. The head was positioned in a custom made stereotaxic restraint affixed to a stage insert for a motorized stage (Prior Scientific). Nine slightly overlapping volumes in a 3 x 3 array were imaged through z-x-y translation of a motorized stage (z-spacing ~1.5 microns). Due to variations in head position across imaging sessions, cells of interest were not always centered in the imaging volume. These shifts in registration slightly affected image borders, so that a fraction of dendritic branch tips were excluded at any given imaging session. Since the direction of shift is random, there was no intentional bias in exclusion of tips for a particular neuron or for a particular imaging session and the same exclusion rules applied to all neurons. However, the longer process radius of the pyramidal cell dendrites potentially biased against their sampling (see discussion regarding this point). Raw scanner data was processed in Matlab (Mathworks) and ImageJ (National Institutes of Health). Individual image planes were stitched together (VIAS 2.1, http://www.mssm.edu/cnic/tools.vias.html) such that each is a 3 x 3 montage of adjoining x-y sections at a given depth from the pial surface. 4D (x, y, z, t) stacks were traced and analyzed

blind to age using Object Image (http://simon.bio.uva.nl/object-image.html) (Ruthazer and Cline, 2002) and Neurolucida (MicroBrightField, Inc). 3D surface reconstructions were generated using Imaris (Bitplane AG).

The analysis included 124 branch tips from 6 pyramidal cells in 6 animals and 259 branch tips from 8 non-pyramidal cells in 7 animals at least 65 microns from the pial surface (2 non-pyramidal cells: 'ttr' and 'ttc' were from the same animal) ranging in age (at the time of imaging) from 8-19 weeks postnatal (Fig. 3-1). Cells were arbitrarily named with a 3 letter code. Branch tip length (BTL) was measured as the linear arc length from well-defined distal ends to the first encountered branch point. Axons were not included in the skeletal tracings. Dendritic and axonal branches were distinguished by morphology. Axons were typified as tubular, thin (sometimes less than the point spread function of the microscope) processes, often studded with varicosities every few microns. Dendrites were distinguished by thicker diameters (generally > 2 microns), smooth gradually tapering processes, and characteristic branching patterns. We determined an estimate of the measurement error using the pyramidal and non-pyramidal branch tips that did not show change over time. Because the time-lapse data is for a given branch at a given time point, we cannot measure the same branch tip for a given time point multiple times; thus we used repeated measures on the unchanging branch tips over time to provide an upper bound on the true measurement error we would have observed if we had made multiple measurements at the same time. The average SEM for the monitored pyramidal cell branch tips was 1.9 microns and for non-changing non-pyramidal branch tips was 1.1 microns. The larger error in pyramidal branch tips can be attributed to the increased measurement error of long branch tips and their extension over multiple stitching boundaries.

Immunohistochemistry

Previously imaged mice were heavily anesthetized with 2.5% Avertin (I.P., 0.030 ml/kg) and their brains processed for immunohistochemistry essentially as described (Chattopadhyaya et al., 2004). Sections were first incubated with GABA (rabbit polyclonal antibody; 1:5000; Sigma, St. Louis, MO) followed with Alexa555 conjugated goat IgG secondary antibodies (1:400; Molecular Probes, Eugene, OR). Alternatively, sections were first incubated with parvalbumin (monoclonal antibody; 1:1000; Sigma, St. Louis, MO) and somatostatin (rabbit polyclonal antibody; 1:1000; Chemicon) followed with appropriate Alexa555 and 647-conjugated goat IgG secondary antibodies (1:400; Molecular Probes, Eugene, OR). After visualization, sections were unmounted in PBS and reprocessed using CCK (monoclonal antibody #9303; 1:1000; CURE/Digestive Disease Research Center, Vaglahs, CA) and GABA (rabbit polyclonal antibody; 1:500; Chemicon) followed with appropriate secondary antibodies. Imaged cells were identified by location, morphology, and local landmarks. Images were acquired with a confocal (Olympus Fluoview) or an upright epi-fluorescence scope (Nikon) using a 20x/N.A. 0.5 (Olympus) 20x/N.A. 0.75 (Nikon) or 40x/N.A.1.30 (Nikon) objective.

Figure 3-1. Summary of imaging sessions displayed by age.

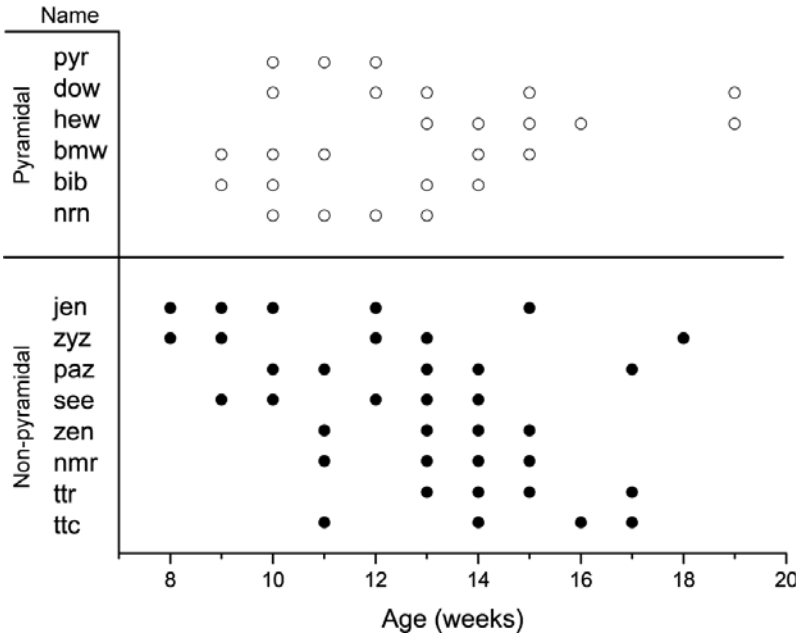


Figure 3-2. Dendritic arbors of pyramidal neurons are stable.

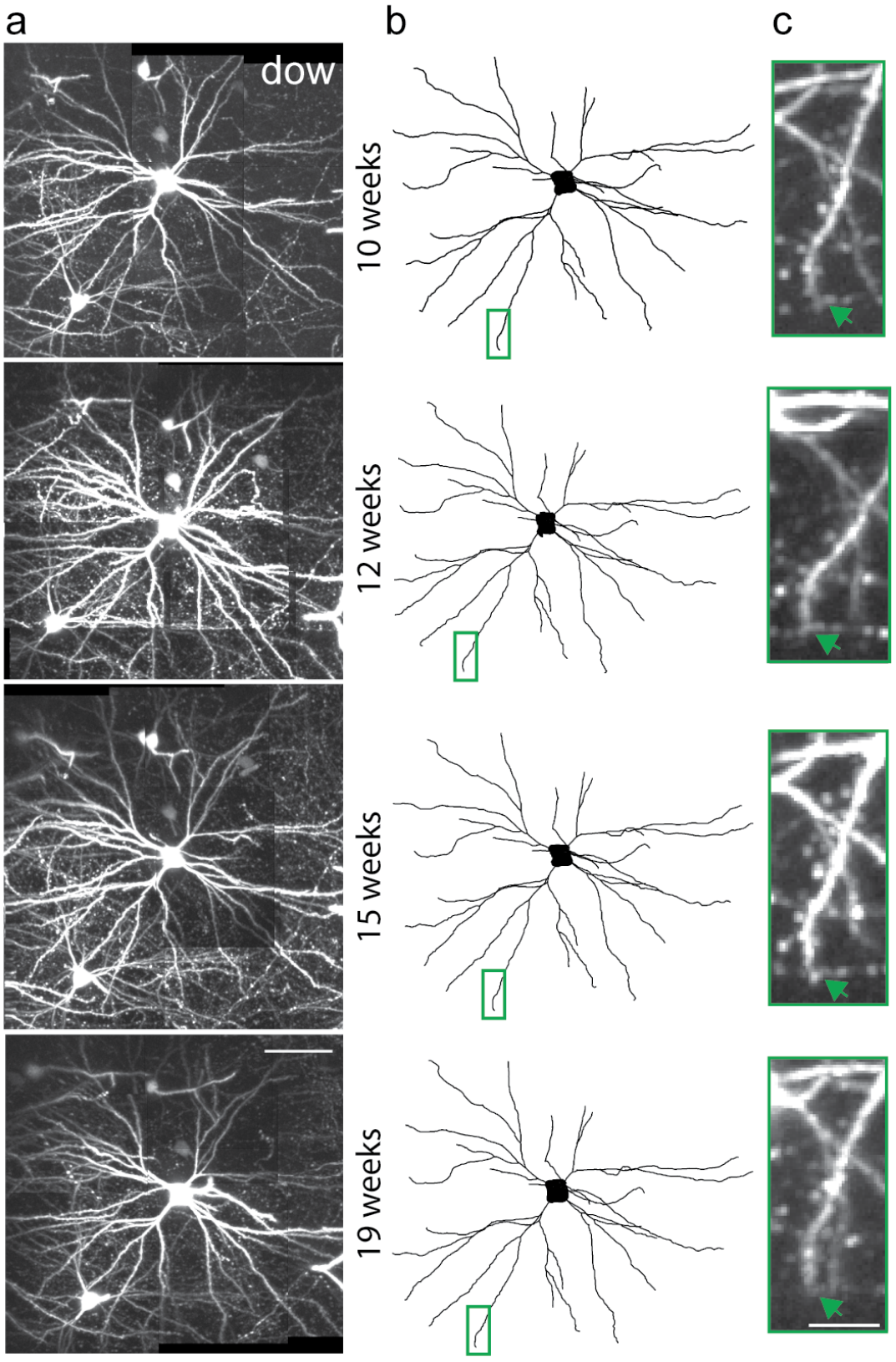


Figure 3-3. Dendritic growth in multiple branches of a non-pyramidal neuron.

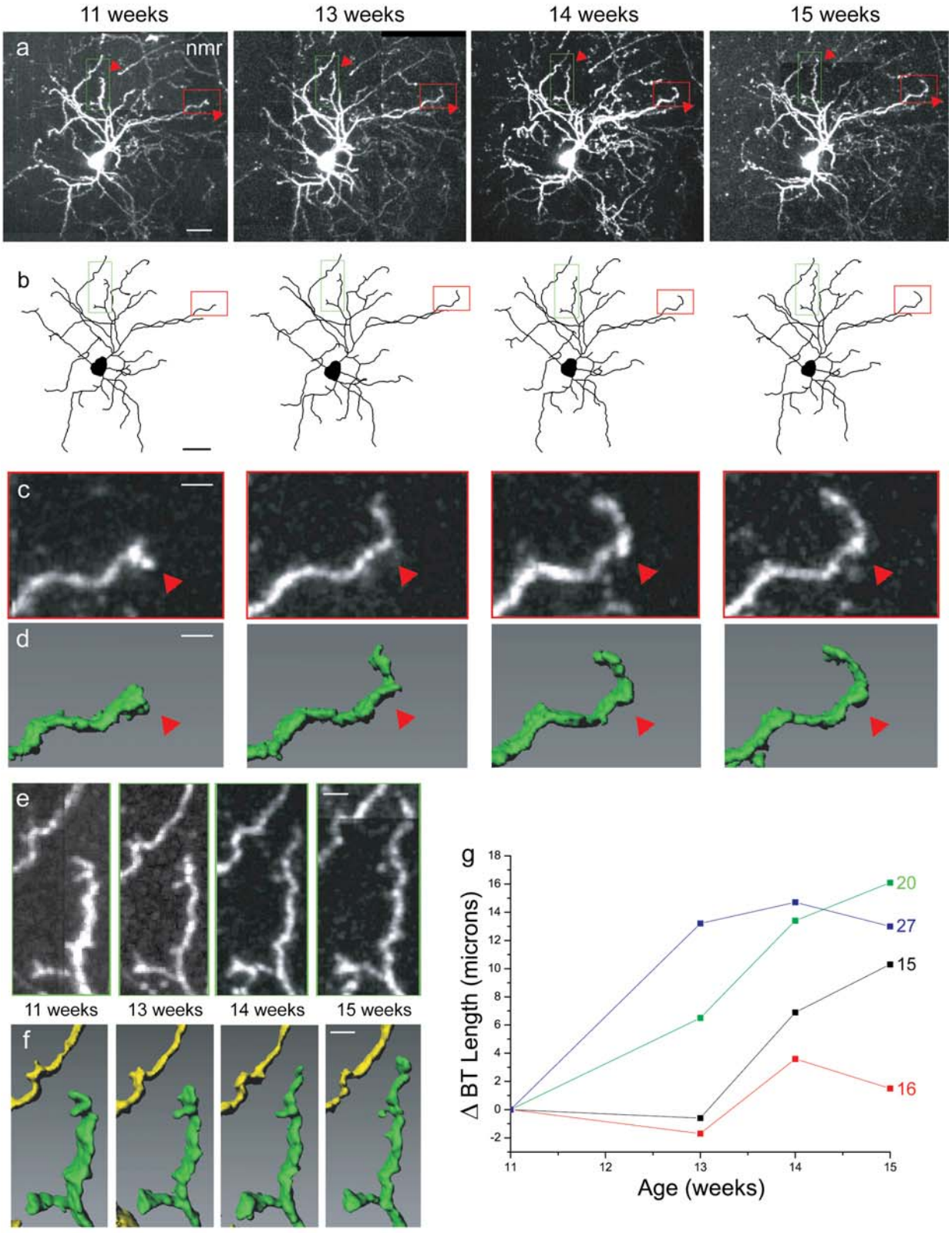


Figure 3-4. Large-scale dendritic branch growth in a non-pyramidal neuron.

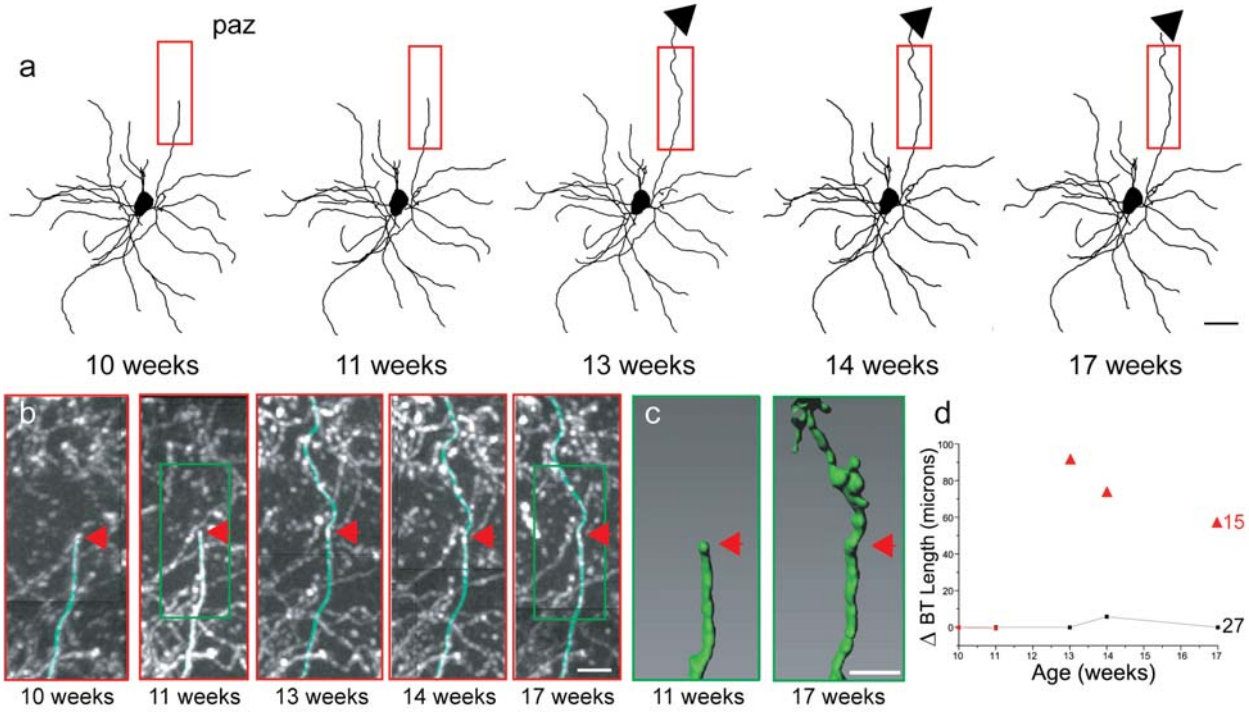


Figure 3-5. Branch extensions, retractions, and de novo branch tip addition in a non-pyramidal neuron.

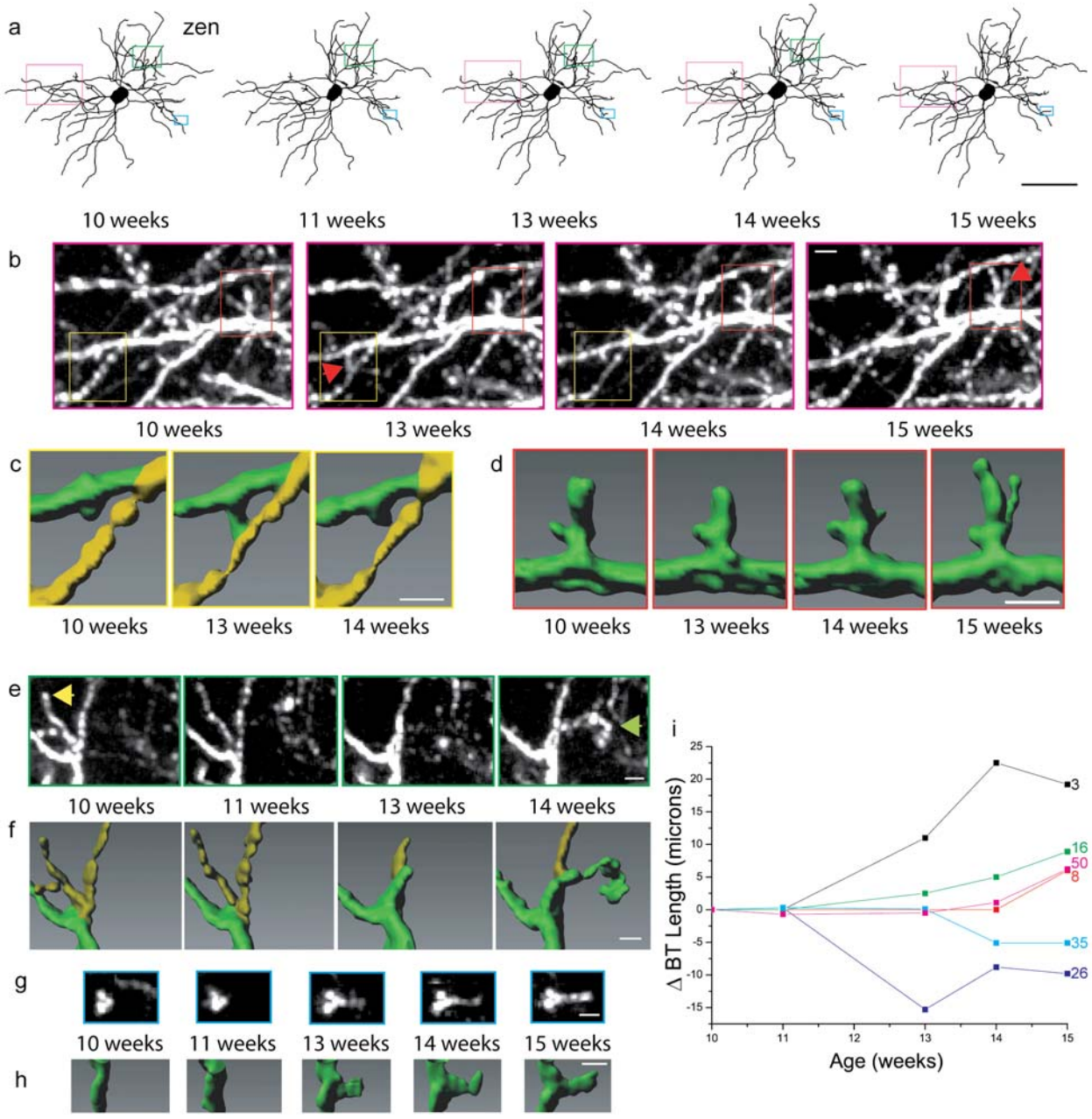


Figure 3-6. Remodeling adult non-pyramidal neurons in the superficial layers of the visual cortex are inhibitory GABAergic interneurons.

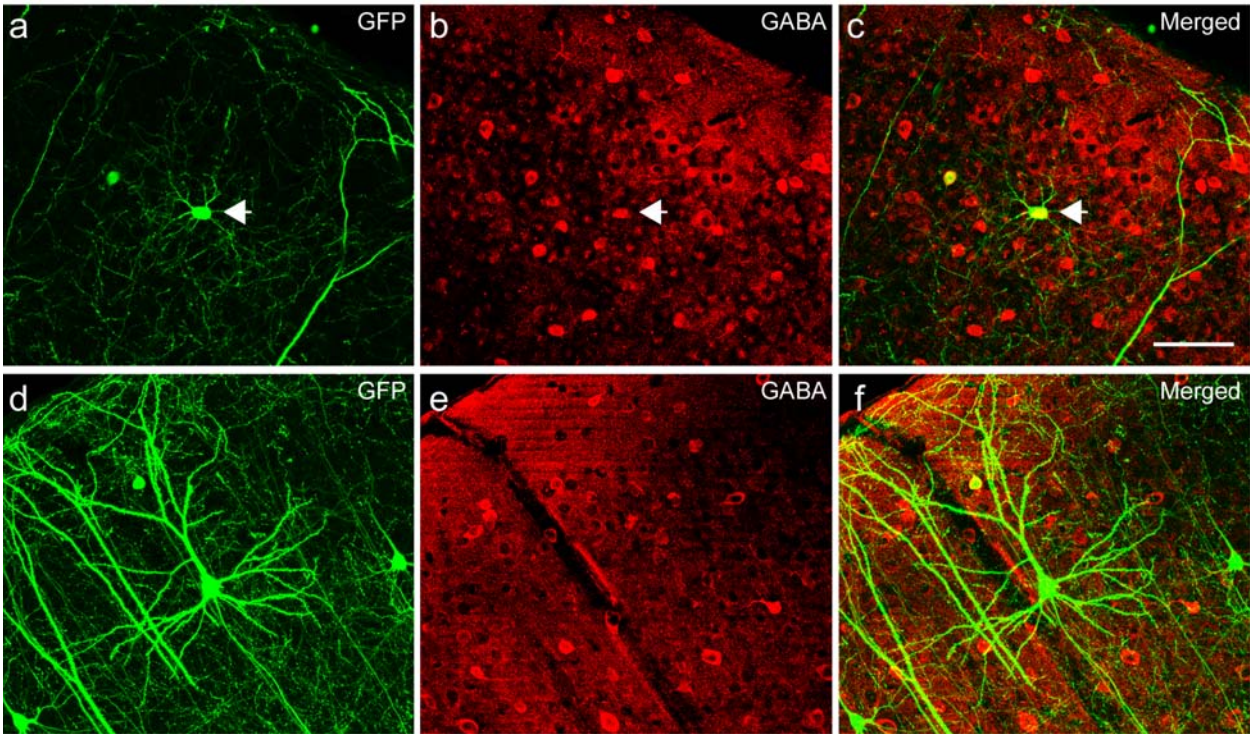
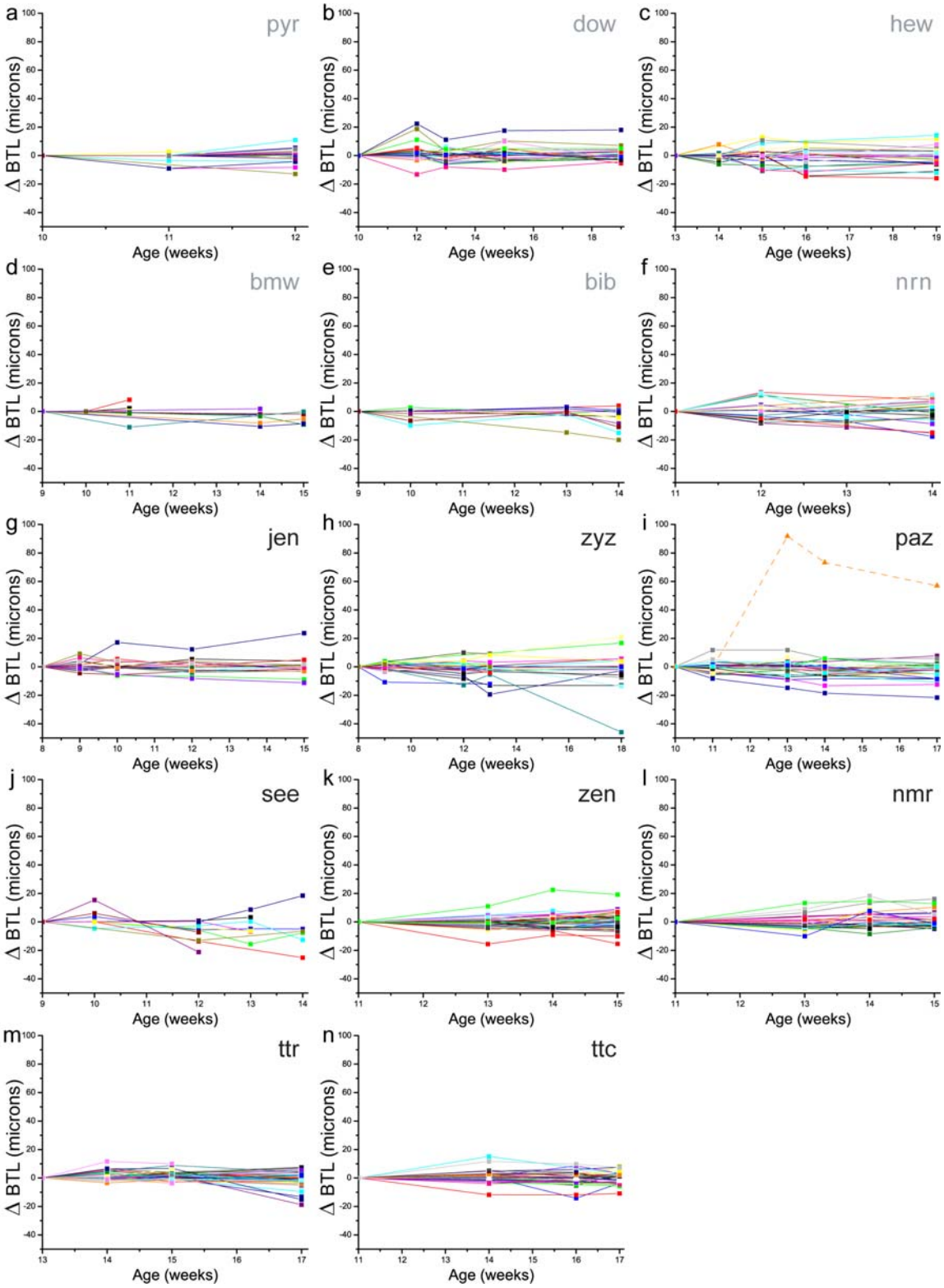


Figure 3-7. a-n, The change in BTL is plotted for each individual monitored branch tip of every imaged cell (3 letter code top right).



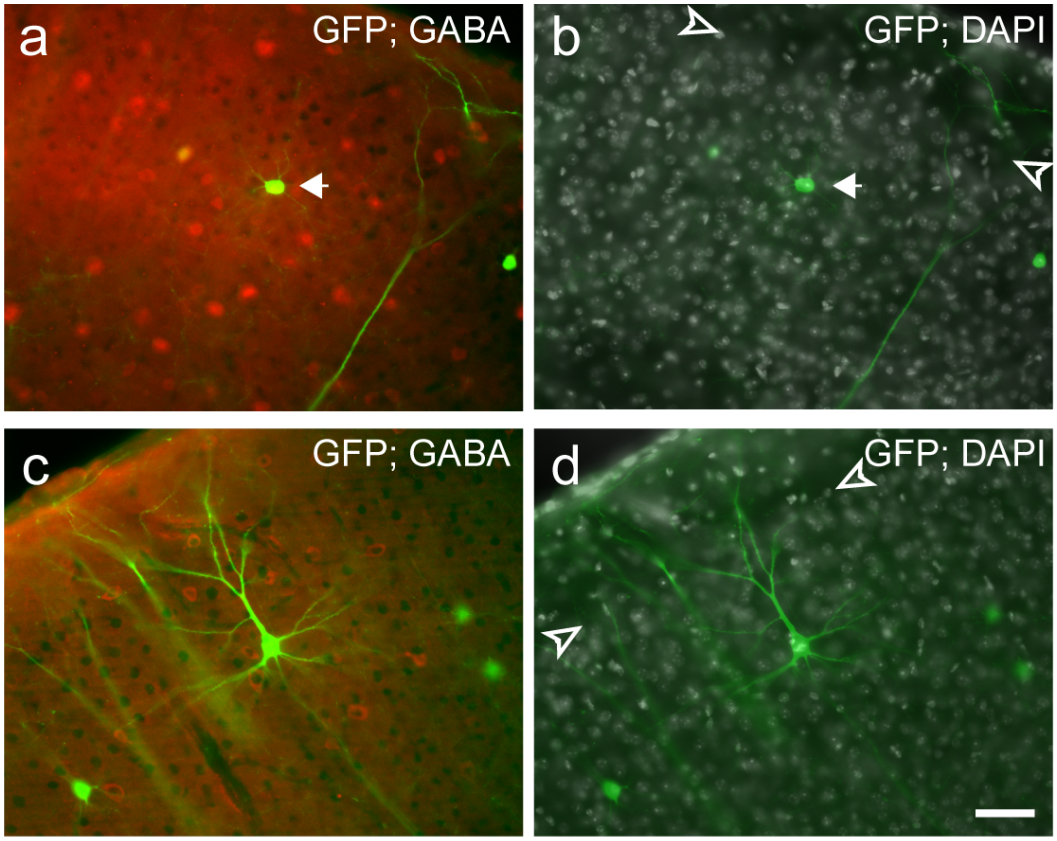
Supplementary Movie 3-1. Z-stack of pyramidal cell 'dow' descending at 1.5 micron steps.

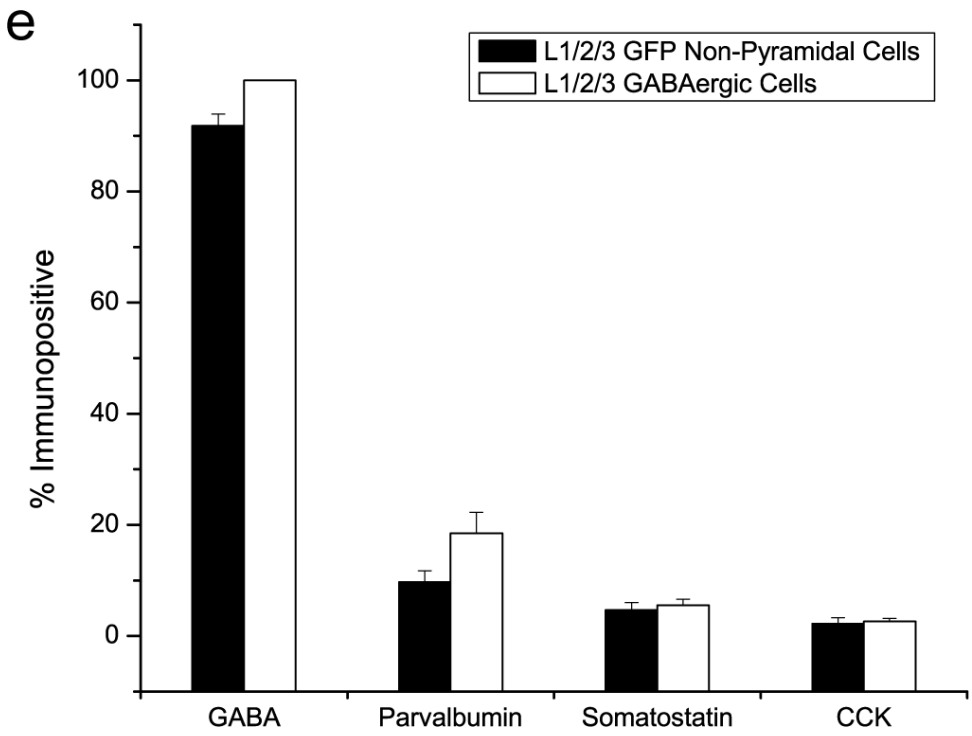
http://biology.plosjournals.org/archive/1545-7885/4/2/supinfo/10.1371_journal.pbio.0040029.sv001.wmv

Supplementary Movie 3-2. Z-stack of cell non-pyramidal cell 'nmr' descending at 1.5 micron steps.

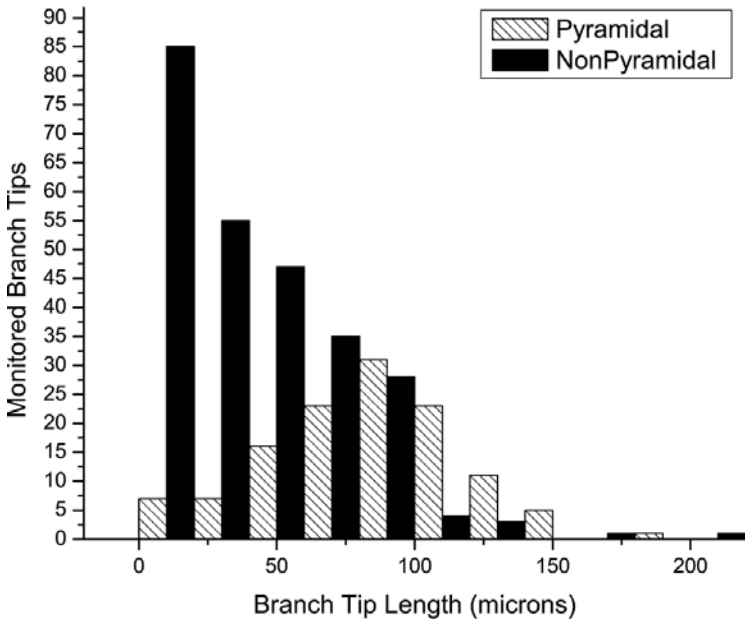
http://biology.plosjournals.org/archive/1545-7885/4/2/supinfo/10.1371_journal.pbio.0040029.sv002.wmv

Supplementary Figure 3-1. Layer localization of imaged neurons and distribution of GABAergic subtypes.





Supplementary Figure 3-2. Histogram of the number of monitored branch tips for different branch lengths of pyramidal and non-pyramidal cells.



Supplementary Figure 3-3. Histogram of the number of remodeling events in non-pyramidal neurons as a function of distance from the cell soma.

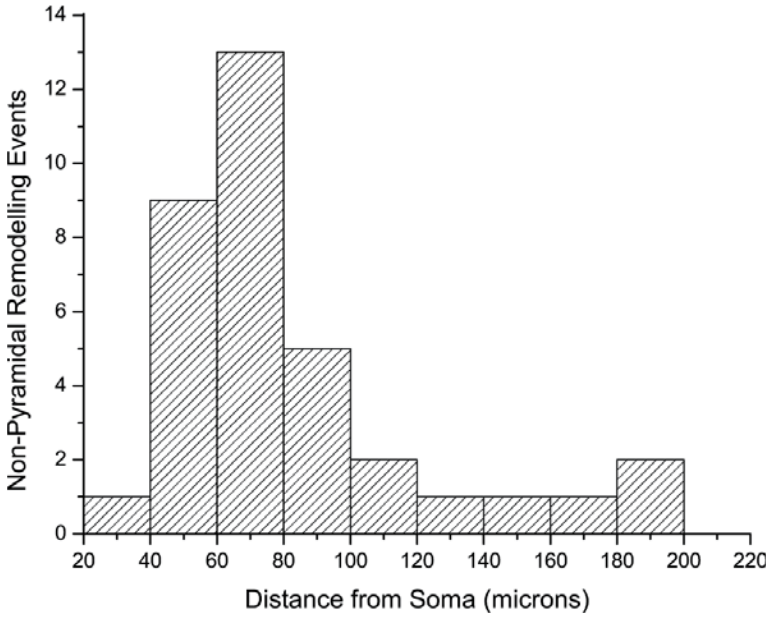


Figure Legends

Figure 3-1. Summary of imaging sessions displayed by age. Each neuron was named by an arbitrary three letter code. Empty circles represent pyramidal and filled circles represent non-pyramidal neuron imaging sessions.

Figure 3-2. Dendritic arbors of pyramidal neurons are stable. **a**, Maximum-intensity z-projections (MZPs) near the cell body of the pyramidal cell 'dow' acquired over 9 weeks. **b**, Two-dimensional projections of three-dimensional skeletal reconstructions of 'dow'. **c**, High-magnification view of branch tip (green arrow) in region outlined by green box in (**b**). Scale bars: (**a**-**b**), 50 microns; (**c**), 10 microns.

Figure 3-3. Dendritic growth in multiple branches of a non-pyramidal neuron. **a**, MZPs near the cell body of the (~118 microns deep) non-pyramidal cell 'nmr' acquired over 4 weeks. Two examples of dendritic branch growth are indicated by red arrowheads. **b**, Two-dimensional projections of three-dimensional skeletal reconstructions of the non-pyramidal neuron 'nmr'. **c**, High-magnification view of one growing branch tip (#20) (red box in **a** and **b**). Red arrowhead marks the approximate distal end of the branch tip at 11 weeks. **d**, Three-dimensional isosurface reconstructions of branch tips in (**c**). **e**, High-magnification view of branch tip (#15) (green box in **a** and **b**). **f**, Three-dimensional isosurface reconstructions of branch tips in (**e**). **g**, Plot of change in branch tip (BT) length of dynamic branch tips as a function of age. Number to the right denotes branch tip number. Scale bars: (**a**-**b**), 25 microns; (**b**-**e**), 5 microns.

Figure 3-4. Large-scale dendritic branch growth in a non-pyramidal neuron. **a**, Three-dimensional skeletal reconstructions of the non-pyramidal neuron ‘paz’ from images acquired over 7 weeks. Note that a growing branch tip exceeded the imaging volume after 11 weeks and black arrowheads denote its postulated continuation. **b**, MZPs of region outlined by red box in (**a**). The branch tip (#15) elongates radially in the x-y axis and away from the pial surface in the z-axis. Non-specific labeling in the MZP is exacerbated by the summation of additional z-stacks due to elongation on the z-axis. The traced branch of interest is highlighted by a green overlay. Red arrowheads mark the approximate distal end of the branch tip at 11 weeks. **c**, Three-dimensional isosurface reconstructions of the region of the branch tip outlined by a green box in (**b**). **d**, Plot of change in branch tip (BT) length of dynamic branch tips as a function of age. Triangles denote the minimum length of the branch tip as it exceeds the border of the imaging volume. Number to the right denotes branch tip number. Scale bar: (**a**), 25 microns; (**b**, **c**), 10 microns.

Figure 3-5. Branch extensions, retractions, and de novo branch tip addition in a non-pyramidal neuron. **a**, Three-dimensional skeletal reconstructions of the sparsely spinous non-pyramidal neuron ‘zen’ from images acquired over 5 weeks. **b**, High-magnification MZP view of region outlined by purple box in (**a**). Red arrowheads indicate examples of structural remodeling. Three-dimensional isosurface reconstructions show c, the elongation and retraction of a spine towards an axon (yellow overlay). **d**, structural change in a cluster of branch tips (#50 far right) (red box in **b**). **e**, Higher-magnification MZP view of region outlined by green box in (**a**). Examples of process retraction and branch (#3) addition are labeled with yellow and green arrowheads respectively. **f**, Three-dimensional isosurface reconstructions of (**e**) with axon in

yellow overlay. **g**, MZP view of region outlined by cyan box in (**a**) shows branch tip (#16) addition on a different dendrite. **h**, Three-dimensional isosurface reconstructions of (**g**). **i**, Plot of change in branch tip (BT) length of dynamic branch tips as a function of age. Number to the right denotes branch tip number. Scale bars: (**a**), 50 microns; (**b**-**h**), 5 microns.

Figure 3-6. Remodeling adult non-pyramidal neurons in the superficial layers of the visual cortex are inhibitory GABAergic interneurons. **a**, Confocal image stack of a coronal section containing the chronically imaged non-pyramidal neuron 'ttr' is visualized by GFP staining (green, filled arrows) and is **b**, immunopositive for GABA (visualized in red) overlay of GFP and GABA shown in (**c**). **d**, Confocal image stack of a coronal section containing the chronically imaged pyramidal neuron 'dow' is **e**, immunonegative for GABA (visualized in red) overlay of GFP and GABA shown in (**f**). Scale bar (**a**-**f**), 100 microns.

Figure 3-7. **a**-**n**, The change in BTL is plotted for each individual monitored branch tip of every imaged cell (3 letter code top right). **a**-**f**, pyramidal cells, **g**-**n**, non-pyramidal cells. Triangles and dashed lines denote the minimum length of the branch tip as it exceeds the border of the imaging volume.

Supplementary Movie 3-1. Z-stack of pyramidal cell 'dow' descending at 1.5 micron steps.

Supplementary Movie 3-2. Z-stack of cell non-pyramidal cell 'nmr' descending at 1.5 micron steps.

Supplementary Figure 3-1. Layer localization of imaged neurons and distribution of GABAergic subtypes. **a**, **c**, Neurons shown in Figure 3-6 were in layers 2/3 as shown by DAPI staining **b** and **d** (border of layer 1 and layers 2/3 delineated by empty arrowheads). Scale bar (**a-h**), 50 microns. e, GFP labeling of non-pyramidal cells in layers 1 and 2/3 shows a representative distribution of GABAergic, parvalbumin, somatostatin and CCK immunopositive cells. Solid bars represent % of layer1/2/3 GFP labeled non-pyramidal cells immunopositive for a given marker and empty bars represent % of all GABA positive cells immunopositive for the same markers. Error bars represent SEM.

Supplementary Figure 3-2. Histogram of the number of monitored branch tips for different branch lengths of pyramidal and non-pyramidal cells.

Supplementary Figure 3-3. Histogram of the number of remodeling events in non-pyramidal neurons as a function of distance from the cell soma.

Chapter 4

Conclusion

Assuming the brain generates the mind how the brain achieves such a feat has been a fundamental question of humanity for centuries (Locke et al., 1818; Descartes, 1984). Information from the outside world is putatively encoded and transformed into perceptual representations that may be processed, stored, and recalled within the brain. For over a century the *neuron doctrine* has shaped modern neuroscience (Ramon y Cajal, 1990; Ramon y Cajal 1995). Precisely how individual neurons participate in representations is still unclear, but it is thought that populations of neurons acting in cohort engender representations (Gilbert, 1992; Wilson and McNaughton, 1993; Singer, 1995). How representations change likely depends on the cellular players in the network and ultimately by the cellular and molecular mechanisms that shape them.

Studies into the development of cortical circuits and their subsequent plasticity have served to richly advance our understanding of structure and function of the brain. Early in development, genetic programs orchestrate a first-pass wiring of the CNS (Katz and Crowley, 2002; Ruthazer, 2005). Activity, both spontaneous and driven by sensory experience, refines immature circuits into mature circuitry (Constantine-Paton et al., 1990; Shatz, 1990; Katz and Shatz, 1996). Mechanisms by which activity drives the changes resulting in functional and anatomical plasticity are currently the provenance of molecular and cellular neurobiology. Neuronal activity regulates gene expression whose gene products shape connectivity and the efficacy of synaptic transmission (Corriveau, 1999; Nedivi, 1999). The isolation and characterization of activity-dependent genes (Nedivi et al., 1993; Qian et al., 1993; Yamagata et al., 1993; Lyford et al.,

1995; Tsui et al., 1996; Brakeman et al., 1997; Nedivi et al., 1998; Cottrell et al., 2004) has led to molecular insights into the underpinnings of experience-dependent plasticity, guiding our understanding and predictions about how the adult brain works. Our findings on *cpg15* expression and adult structural plasticity have tremendous implications for the understanding of cortical functional circuitry from, molecular interactions to computational modeling.

cpg15 and the visual system

cpg15's potential role in dendritic and axonal arbor restructuring and synaptic maturation led us to explore the effect of visual experience on its expression and regulation. Here, we compared *cpg15* expression during normal development of the rat visual system with that seen in response to dark rearing, MD, or monocular retinal action potential blockade. Our results show that the effect of eliminating patterned visual activity on *cpg15* expression is profoundly different from the effect of retinal action potential blockade. The effect of dark rearing is exclusive to visual cortex, and causes a prolonged up-regulation of *cpg15* expression starting at the peak of the critical period and into adulthood. Retinal action potential blockade at the same developmental times causes a decrease in *cpg15* expression that can be seen in the LGN and *SC* as well as visual cortex. The transcriptional regulation of *cpg15* during development differs from that of other activity-regulated genes, such as BDNF (Lein and Shatz, 2000) and Homer (Brakeman et al., 1997). While the transcriptional regulation of these latter genes provides an accurate molecular indicator of activity, regulation of *cpg15* does not only reflect levels of activity, but corresponds more closely with the capacity for plasticity. In addition, our finding that DR rats fail to down-regulate *cpg15* raises the possibility that the residual plasticity measured electrophysiologically in these animals reflects an extended capacity for local synaptic

remodeling. The prolonged plasticity seen in DR animals may result from failure to down-regulate genes like *cpg15* that could promote structural remodeling and synaptic maturation.

Adult plasticity

Our ability to learn as adults, and the continual adaptation of primary sensory areas, reflects a general capacity for change in the adult brain. Yet, despite decades of evidence for such functional plasticity, the existence and role of structural plasticity remains controversial (Calford et al., 2005; Smirnakis et al., 2005a; Smirnakis et al., 2005b). For over a century the adult brain has been considered 'hard-wired', with limited ability to rearrange or rewire circuits. Ramon y Cajal argued that adult brains were structurally invariant because the complex architecture of brains from different adults appeared similar (Ramon y Cajal, 1959). The dogma was buttressed by Hubel and Wiesel's finding that the susceptibility for anatomical change of geniculocortical afferents in response to manipulation of visual input is normally restricted to an early developmental time window (Hubel and Wiesel, 1970; Hubel et al., 1977). Armed with a prediction from *cpg15* expression, we monitored the structure of individual cells in the superficial layers of the living adult brain for several months through a window inserted in the skull. We showed the first unambiguous evidence for remodeling of adult brain cells at the level of arbors and suggest that circuit rearrangement is restricted by cell type-specific rules.

Recently, using *in vivo* two-photon microscopy, researchers have examined the structural plasticity of dendritic spines on adult pyramidal neurons with two seemingly contradictory results. Trachtenberg and colleagues found a large turnover of spines, where approximately half turnover within days (Trachtenberg et al., 2002). In contrast, Grutzendler and colleagues found that 96% of spines were stable for over a month (Grutzendler et al., 2002). One explanation for

the difference is that visual cortex may be less structurally plastic than somatosensory cortex (Holtmaat et al., 2005; Majewska et al., 2006), where electrophysiological plasticity persists through adulthood (Diamond et al., 1993). Here, we show evidence of dendritic arbor growth and retraction and of branch tip additions in the adult visual cortex. Interestingly, the neurons exhibiting dendritic arbor rearrangements were non-pyramidal, GABA positive interneurons. Consistent with previous reports (Trachtenberg et al., 2002), we found that pyramidal dendritic arbors were relatively stable. The structural plasticity of GABAergic interneurons is relatively small, but significant, and could become large over the lifetime of the animal or if driven by plasticity inducing paradigms. Our data indicate that the structural plasticity of interneurons is continuous through adulthood, and raises the intriguing possibility that remodeling of local inhibitory connections may underlie adult cortical plasticity.

Plasticity of inhibitory circuitry

Given that excitatory pyramidal neurons account for the vast majority (~70-80%) of neurons in the cortex and are considered the principal feed forward output of cortical circuits, it may not be surprising that most efforts have focused on their characterization. But, the preponderance of interneurons in higher animals suggested to Ramon y Cajal that interneurons may be responsible for higher level brain function (Ramon y Cajal, 1926; Yuste, 2005). Now, mounting evidence suggests that inhibitory circuitry is a significant player in experience-dependent plasticity (Jacobs and Donoghue, 1991; Jones, 1993). During development, cortical blockade of GABAergic transmission with bicuculline diminishes shifts in ocular dominance toward the non-deprived eye (Ramoa et al., 1988), whereas hyperactivating GABAergic inhibition by muscimol shifts cortical responses toward the deprived eye (Reiter and Stryker,

1988). Elegant experiments in genetically engineered mice have demonstrated that developmental maturation of inhibition regulates critical period timing (Hensch et al., 1998; Hanover et al., 1999; Huang et al., 1999; Fagiolini and Hensch, 2000; Hensch, 2005).

The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) is thought to be important for neuronal structural plasticity (Martin and Kandel, 1996), and in the prefrontal cortex of adult rodents it is expressed by interneurons, but not by pyramidal cells (Varea et al., 2005). An additional level of adult plasticity necessitating structural remodeling is the migration and integration of neuronal stem cells into mature functional circuits. Interestingly, PSA-NCAM is also a marker of adult neuronal stem cells (Seki and Arai, 1993) and virtually all neuronal stem cells born in the adult subventricular zone that eventually integrate into functional circuits in the olfactory bulb are GABAergic interneurons (Gheusi et al., 2000). And, although controversial, evidence of adult neocortical neurogenesis suggests that newborn neurons migrating into the neocortex are exclusively GABAergic interneurons (Dayer et al., 2005). This presents another possible hypothesis, that the remodeling interneurons we observed were adult generated stem cells; but to address this we would need to know when and where they were born (Xu et al., 2004; Butt et al., 2005).

Why should the dendritic arbors of adult pyramidal cells be structurally stable, but interneurons labile? Whereas the structural plasticity of dendritic spines may provide pyramidal cells sufficient capacity for altering connectivity, perhaps due to of the lack of spines on interneurons, they require structural rearrangement at the arbor level to enact changes in connectivity. Should interneurons act to shape the firing patterns of pyramidal cell populations (Paulsen and Moser, 1998; Klausberger et al., 2003), alterations in population coding may require long-range changes in connectivity. Like glutamatergic synapses, GABAergic synapses

undergo activity-dependent plasticity (Gaiarsa et al., 2002). The expression of GABA, GAD, and $GABA_A$ receptors are regulated by sensory deprivation in primate primary visual cortex (Hendry and Jones, 1986, 1988; Hendry et al., 1994). GABA is regulated by deafferentation in the monkey somatosensory cortex (Garraghty et al., 1991) and is reduced in human sensorimotor cortex within an hour of reversible forearm ischemia (Levy et al., 2002). Inhibitory synapse number is increased ~3 fold by 24 hours of single-whisker stimulation in adult rodent barrel cortex (Knott et al., 2002). In the adult owl, GABA mediated inhibition serves as a gate to multiple stored representations of auditory space (Zheng and Knudsen, 1999), where, with incremental training adult owls are able to surmount first-order constraints on plasticity (Linkenhoker and Knudsen, 2002). Taken together, such data suggest that GABAergic inhibitory circuitry is an integral component of adult plasticity.

Circuit models

Constrained by the dogma that the adult brain is structurally invariant, much experimental focus has been placed on the ability of existing connections to alter synaptic weights, but not circuit connectivity (Chklovskii et al., 2004). In both development and in the adult, changes in synapse efficacy may be the initial stages of broader, persistent changes in circuitry. Our data is consistent with inhibitory GABAergic interneurons dynamically acting to associate stable excitatory feed forward projection neurons generating flexible, high-capacity, distributed neural networks (Singer, 1995). A model for developmental versus adult modes of plasticity would include the structural plasticity of excitatory projection neuron arbors waning early in development, during so called ‘critical periods’. Then, in the adult, the prevailing plasticity is of modulatory neuronal cell types such as local inhibitory interneurons, which would

underlie normal adult plasticity by dynamically associating varying populations of the more stable projection neurons.

How might local inhibitory interneurons mechanistically act to produce adult functional plasticity? One model invokes global unmasking and remasking of horizontal connections by changes in inhibition (Merzenich et al., 1983b; Merzenich et al., 1983a; Jacobs and Donoghue, 1991; Foeller and Feldman, 2004), where, upon deafferentation, previously driven principal neurons are silenced, relieving local feed forward lateral inhibition to unmask weak neighboring connections. Recovering responsiveness would expand to activate new territory until homeostatic mechanisms (Turrigiano et al., 1998; Turrigiano, 1999) refocus inhibition around newly potentiated receptive fields. The demonstration that GABAergic neurons structurally remodel is consistent with reorganization of adult cortical receptive fields. Multiple phases of this model may be testable. Increasing GABAergic inhibition by infusion of muscimol or benzodiazepine should block the initial unmasking of weak neighboring responses and prevent functional plasticity by the continued masking of latent connections. In the future, when more is understood about the molecular mechanisms underlying interneuron plasticity, we should be able to block the structural plasticity of interneurons which should prevent reorganization of receptive fields, but not the initial unmasking of latent responses.

A local circuit model where GABAergic interneurons are more actively instructive is based on models of spike timing dependent plasticity, where potentiation and depression rely on the precise timing and order of pre- and postsynaptic spiking at millisecond resolution (Bi and Poo, 1998; Dan and Poo, 2004). In this case, inhibitory interneurons would dynamically act to sculpt the activity of pyramidal cells by driving the association of feed forward projections. For example, fast-spiking parvalbumin positive basket cells principally target pyramidal cell somas

and could act to prevent action potential back-propagation, whereas chandelier cells often target axon initial segments and thus are in an ideal position to regulate pyramidal cell output (McBain and Fisahn, 2001; Markram et al., 2004). By shortening stimulus-evoked spike trains and limiting spontaneous discharge, inhibitory interneurons could decrease the temporal asynchrony of uncorrelated activity (Feldman, 2000) and thus synchronize the spike timing of local pyramidal cells. Recent evidence shows fast-spiking interneurons facilitated by autaptic transmission create precisely timed circuit output (Bacci and Huguenard, 2006). Dynamically rearranging interneuron connectivity would alter populations of synchronized principal cells and facilitate spike timing dependent plasticity of neuronal ensembles. Again, in this model, disabling interneuron remodeling should prevent changes of circuit output. Though details of the cortical microcircuit architecture remain unclear, it would also be interesting to dissect the role of individual interneuron subtypes by subtype-specific disruption of interneuron activity and plasticity.

Future directions

Using our understanding of *cpg15* function and its developmental and adult expression pattern, we were able to make testable predictions on when and where neuronal structural rearrangements occur in the mammalian visual cortex. The direct demonstration that neuronal remodeling occurs in the normal adult visual cortex contributes to the shift away from dogma of structurally invariant adult circuits. Furthermore, the demonstration that GABAergic interneurons are normally more structurally plastic than pyramidal neurons is consistent with inhibitory local circuitry acting as an associative network that is dynamic in the adult and could change the response of more structurally stable pyramidal neurons (Singer, 1995). Our current

and future investigations aim to further understand the functional significance of the structural remodeling in adult interneurons and the cellular and molecular mechanisms that underlie it.

Loss of function and gain of function analyses are classical approaches to characterize a protein's function. To directly test the requirement for *cpg15* in the remodeling and synaptic maturation associated with developmental and adult plasticity, our laboratory has generated *cpg15* deficient mice with fluorescent neurons. We are currently using chronic *in vivo* two-photon microscopy to monitor neurons in *cpg15* knockout animals to determine the role of *cpg15* in the structural dynamics of neurons in the adult visual cortex. Given its function, we predict that neuronal structural remodeling would be less dynamic in *cpg15* mutants. Additionally, we are addressing how activity affects the remodeling of neurons in the superficial layers of the adult neocortex. Here, we are exploiting the experimental tools provided by the rich history of the visual system. Response to monocular and binocular deprivation and dark adaptation will determine how structural remodeling of adult neurons in visual cortex is a function of visual experience and activity. These experiments will provide insight into the function of interneuron remodeling and the role of *cpg15* in adult structural plasticity.

Appendix

Analysis of the Dendritic Change Propensities for Non-pyramidal and Pyramidal Neurons

How likely is it that we missed observing changing branch tips in the pyramidal neurons if at least one had occurred when we monitored 124 pyramidal neuron branch tips and we assume the probability of a change was equal to that estimated for the non-pyramidal neuron branch tips p = 35/259?

To answer this question, we assume that the propensity to change for branch tips of the pyramidal neurons is the same as for the branch tips of the non-pyramidal neurons. That is, our null hypothesis is that the two types of neurons have the same propensity to changes their dendritic branch tips. We want to calculate how likely is our observation of no branch tip changes observed among the pyramidal neuron branch tips if the two branch tip change probabilities are same. This is equivalent to asking how likely is it that we missed observing all the possible change events in branch points of the pyramidal neurons. Therefore, we estimate the probability of a pyramidal neuron changing from the non-pyramidal neurons as

$$p = 35/259 = 0.\overline{135}. \tag{S.1}$$

The desired probabilities in this problem can be computed from the binomial distribution which defines the probability of observing exactly k changing events for a given N, the number of monitored pyramidal branch tips, and a given p, the probability of a branch tip changing. This is given as (Ross, 1997)

$$\Pr(\textit{of exactly } k \textit{ changes}) = \left(\frac{N!}{k!(N-k)!}\right) p^k (1-p)^{N-k} \tag{S.2}$$

where $k! = 1 \cdot 2 \cdot 3 ... \cdot k.$ The question to now ask is, What is the probability that we should have seen at least one changing event for the pyramidal neuron branch tips if they had the same change probability p as the non-pyramidal neurons? This is

$$\begin{aligned}\Pr(\textit{of at least one change event}) &= \sum_{j=1}^{\infty} \Pr(\textit{of } j \textit{ changing events}) \\ &= 1 - \Pr(\textit{zero changing events}).\end{aligned} \tag{S.3}$$

Since we monitored 124 pyramidal branch tips then,

$$\begin{aligned}\Pr(\textit{of at least one change event}) &= 1 - \Pr(\textit{zero changing events}). \\ &= 1 - \left[\left(\frac{N!}{N!0!}\right) p^0 (1-p)^{N-0}\right] \\ &= 1 - \left[\left(\frac{124!}{124!0!}\right) 0.135^0 (0.865)^{124}\right] \\ &= 1 - \left[1 \times 1 \times 1 \times 10^{-8}\right] \\ &\approx 1\end{aligned} \tag{S.4}$$

This calculation tells us that if the propensity for the pyramidal branch tips to change were equal to the propensity of the non-pyramidal neurons to change, then the probability of seeing at least one change in the pyramidal branch tips would be one. Thus, we should have seen a pyramidal branch tip change event with near total certainty. Stated another way, the probability of missing this event is the probability of observing any other event except this one. Since this event is the event that at least one branch tip changed, the probability of not observing or missing this event is $\Pr(\textit{zero changing events}) = 10^{-8}$ (Eq. S.4).

The fact that we did not observe any change events in the pyramidal branch tip group allows us to reject the null hypothesis that the change probabilities for the two groups are the same in favor of the more plausible alternative that the two groups have significantly different dynamic properties.

We could not use the standard Poisson approximation to the binomial rare events computation in these analyses because the expected number of occurrences, i.e., 17 events, was too high to satisfy the conditions for this approximation to be accurate.

To be complete, we consider two other cases. First, if for the pyramidal neurons p>0.135, then $\Pr(\textit{zero changing events}) < 10^{-8}$. Second, if $0.05 < p < 0.135$ $\Pr(\textit{zero changing events}) < 2\times10^{-3}$. Hence, for a change probability as small as 0.05 the probability of observing no branch tip change events in a sample of 124 pyramidal branch tips is less than 0.002. Alternatively stated, the probability that you should have observed at least one of these change events is still, in this worse, case 0.998. This remains near certainty.

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