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Factors that control ocular dominance plasticity in the rat visual cortex

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INTRODUCTION

THE VISUAL SYSTEM: A PARADIGMATIC MODEL FOR STUDYING PLASTICITY

Plasticity is the ability of the brain to reorganize its connections (at both the structural and functional level) in response to changes in sensory experience. Plasticity is fundamental for the development of neuronal circuitry, enables the brain to adapt to its environment and plays a crucial role in normal brain functions such as learning and memory. Experience-dependent refinements are particularly prominent during well defined periods in early life, the so called critical periods (CPs). During CPs neural circuits display a heightened sensitivity to certain environmental stimuli and proper experience is required to set in motion a cascade of functional and anatomical events in the brain, which ultimately consolidate synaptic connections into their final wiring patterns.

Understanding the mechanisms involved in the development and plasticity of connections is an issue of great interest for neuroscientists. This topic has important implications not only for elucidating how neural circuitry is formed, but also for identifying therapeutic approaches to developmental disorders or strategies to promote recovery after injuries in adulthood, when the brain is normally less plastic.

The visual cortex has long been an established model for the study of experience-dependent plasticity because of the relatively easy manipulation of visual inputs. In particular ocular dominance (OD) plasticity triggered by monocular eyelid suture is a classic paradigm to study experience-dependent changes in neural connectivity. Classic experiments have been performed in cats and primates, while rodents have become popular quite recently due to the advent of gene manipulating techniques and to the possibility of combining physiology with biochemical and molecular analysis.

Anatomy of the visual system

The basic anatomical organization of the visual system is highly conserved amongst mammals. The sensory structures are represented by the eyes: light enters the eye by first passing the cornea and finally reaching the very back of the eye, the retina. The retina is responsible for converting light into neural signals that can be relayed to the brain. The retina is a very specialized sensory structure, consisting of a group of

different types of neurons whose role is to collect light, extract basic information and pass the pre-processed image to visual structures in the brain.

These cell types are photoreceptors, bipolar cells, horizontal cells, amacrine cells, and ganglion cells. They are arranged within the retina in precise layers. Axons from the ganglion cells bundle together to form the optic nerves. Fibers from the nasal half of each retina turn towards the opposite side of the brain in a point called optic chiasm, while the fibers from the temporal half of each retina do not cross. Past the chiasm, retinal ganglion cell axons run within the two optic tracts.

Retinal inputs terminate within two major subcortical visual structures, the superior colliculus (SC) and the dorsal geniculate nucleus (dLGN), a portion of the thalamus. Retinal connections are topographically organized: neighboring ganglion cells project to nearby target neurons setting up a retinotopic map in the dLGN. The dLGN is the structure that relays input to visual cortex. In primates and humans, the dLGN contains six layers, each of which receives inputs from one eye only. Indeed, retinal axons coming from the two eyes terminate in adjacent but not overlapping eye-specific layers that are strictly monocular (Hickey and Guillery, 1974).

Projections of neurons in dLGN reach the primary visual cortex, or V1, in the occipital portion of the brain. The adult visual cortex, like all neocortices, consists of six cellular layers between the pial surface and the underlying white matter and contains a complete representation of the contralateral visual hemifield. The majority of inputs from dLGN terminate in layer IV, then neurons in layer IV relay their information to layers II/III, that in turn communicate to layer V-VI. In carnivores and primates, inputs of each eye reach layer IV into alternating stripes, the ocular dominance (OD) columns (LeVay et al., 1975). The columnar systems of the visual cortex communicate together by means of long-range horizontal connections. These connections allow individual cells to integrate information from a wide area of cortex (Gilbert, 1992). Many mammals have binocular vision, and their visual cortical neurons can respond to stimulation of both eye, even if the response to one eye can be predominant (eye preference). Hubel and Wiesel (1962) recorded cells from cat visual cortex and classified them according to their relative response to the stimulation of the two eyes (Ocular Dominance, OD). They indicated with the class 1 the cells that respond only to the stimulation of the contralateral eye, with class 7 the cells driven exclusively by the ipsilateral eye and with class 4 the cells equally driven

by the two eyes. The other classes correspond to cells with an intermediate degrees of dominance of each eye (Hubel and Wiesel, 1962).

Primary visual cortex contains two main types of neurons: pyramidal cells are projection neurons, while non-pyramidal cells represent local interneurons. There are several types of pyramidal cells and interneurons characterized by distinct morphology, physiological properties, and synaptic connectivity patterns.

On a functional level, visual cortical neurons share important response properties which were described for the first time by Hubel and Wiesel (1962). Neurons are selective for several spatial and temporal variables of the visual stimulus such as orientation, direction and velocity of movement, spatial and temporal frequency (Maffei and Fiorentini, 1973; Fregnac and Imbert, 1984).

Once basic processing of visual space has occurred in V1, the visual signal goes to secondary visual cortex, V2, which surrounds V1. Primary and secondary visual areas are connected with associative areas as well as with other sensory cortices.

The rat visual system

The rat, a nocturnal rodent, has a refined and effectively functioning visual system. Its eyes are laterally positioned and therefore the binocular field is relatively small.

In relation to eyes position, the proportion of uncrossed optic axons amounts to only 3% of all the retinal ganglion cells and most ganglion cells project to the contralateral side of the brain. In albinos the ipsilateral projection is even smaller (about 1.5%; Lund, 1965; Dreher et al., 1985; Ahmed et al., 1996). The largest retinal projection terminates in the superior colliculus (SC); 40% of ganglion cells project to the dorsal LGN (dLGN) that lacks the lamination typical of other mammals. Indeed, in rodents the dLGN contains two patches, each receiving eye-specific input (Godement et al., 1984; Reese, 1988). The inner core is ipsilateral, surrounded by an outer shell representing the contralateral patch.

In rodent primary visual cortex, no clear anatomical indication of ocular dominance columns can be found, and afferents serving the two eyes converge on the same postsynaptic target cells at the level of layer IV (Antonini et al., 1999). However, Thurlow and Cooper (1988) found hints of a patchy organization of ipsilateral and contralateral inputs in the visual cortex of the rat, using a functional mapping by

means of deoxyglucose (Thurlow and Cooper, 1988). This was confirmed by Caleo and coworkers (1999) with electrophysiological recordings (Caleo et al., 1999a).

The physiological properties of the visual cortical neurons of the rat are immature at eye opening (postnatal day 15, P15) and develop gradually during the first month of postnatal life. For example, properties like selectivity for orientation and for the direction of movement are absent at P17 and increase progressively to reach adult values at around P30 (Fagiolini et al., 1994). Binocularity is also immature at P17 and the OD distribution gradually takes the adult shape (Fagiolini et al., 1994).

It has been estimated that in the lateral segment of V1 (mapping the central part of the visual field) 80% of cortical neurons are binocular (Fagiolini et al., 1994; Caleo et al., 1999a; Caleo et al., 1999b; Di Cristo et al., 2001; Caleo et al., 2007). This percentage is surprisingly high considering the very low amount of ipsilateral projection in rat visual system. Indeed, based on this last evidence the input from the ipsilateral eye should be small and the fraction of binocularly driven cells be consequently much lower than 80%. Despite of this, a previous work by Montero et al. (1973) showed that the inputs from ipsilateral eye are not so restricted as expected. Indeed, the area of the visual cortex over which gross potentials can be evoked by stimulation of the ipsilateral eye is quite extensive (Montero, 1973). Understanding how binocular responses are determined in the rodent primary visual cortex has important implications for the studies of OD plasticity.

One possibility is that a contribution to binocularity is provided by the Corpus Callosum which links retinotopically corresponding positions of the two hemispheres (Jacobson, 1970; Cusick and Lund, 1981; Mizuno et al., 2007).

The corpus callosum: a role in cortical binocularity?

Anatomy and physiology of the Corpus Callosum

The corpus callosum (CC) is the largest and one of the most important connection systems of the brain because it provides the main link between the neocortical areas of the two cerebral hemispheres. It is an evolutionary innovation restricted to placental mammals because it is absent in the brain of non-mammalian vertebrates as well as of non-placental mammals.

Many studies have assigned to the CC an important role in integrating and unifying the activities of the two hemispheres and in the development of hemispheric asymmetry (Bloom and Hynd, 2005). The nature and function of the CC has long

been investigated not only for its interesting role in interhemispheric cooperation, but also for the evidence that alterations in this structure are frequent in psychiatric and developmental disorders. For example, abnormalities in the size of the CC have been found in patients diagnosed with schizophrenia, autism, mental retardation, Down's syndrome, Attention Deficit Hyperactivity Disorder, developmental dyslexia and developmental language disorders (Bloom and Hynd, 2005). Despite the amount of research devoted to the CC, the basic nature and physiology of interhemispheric integration is not fully understood. The primary visual cortex is a good model to address this issue essentially for two reasons. First, visual perception requires interaction between the two hemispheres. Indeed, each hemisphere receives information from the contralateral half of the visual field. Second, the physiological properties of V1 cells are well known and extensively characterized.

Visual callosal connections mature late in humans, at around one month of age in cats and at P15 in rodents. The CC first enlarges caudally then develops rostrally. Similarly myelinization occurs slowly and with a caudal-rostral development (Bloom and Hynd, 2005).

In primary sensory areas, interhemispheric projections link essentially homotopic zones. As mentioned above, the cortical representation of the visual field is split along the vertical midline, with the left and the right hemifields projecting to separate hemispheres. There is a central region of overlap that is represented in both hemispheres, and amounting to 1 degree (deg) of visual angle in humans (foveal region) and to about 10 deg in rodents. The functional continuity of the visual field is re-established by interhemispheric connections that reciprocally connect cortical zones where the representation of the vertical midline of the visual field is located. Indeed, anatomical and electrophysiological studies in cat show that callosal connections form a dense stripe along the border of areas 17 and 18 (Payne, 1994). Neurons in this boundary have receptive fields mapping the vertical midline, together with a smaller portion of the ipsilateral hemifield (Blakemore et al., 1983; Payne, 1990; Payne and Siwek, 1991; Payne, 1994). Recordings from split-chiasm cats in which the genicolo-cortical and transcallosal pathway can be differently activated by stimulation of one or the other eye, reveal that the inputs of both pathways converging onto a given target neuron are remarkably similar in terms of receptive field size and orientation selectivity (Berlucchi and Rizzolatti, 1968; Milleret et al.,

1994). Furthermore, the "callosal" and "genicolo-cortical" receptive fields lie in corresponding points of the two halves of the visual field in close contact with the vertical meridian, forming together a receptive area that crosses the vertical meridian (Berlucchi and Rizzolatti, 1968).

In rodents, the projection field of callosal axons seems to be wider with respect to cats or higher mammals (Olavarria and Van Sluyters 1985; Lewis and Olavarria, 1995; Houzel and Milleret, 1999). Indeed, the entire mediolateral extent of striate cortex contains callosal cells; however, callosal terminals are still quite concentrated at the border between area V1 and V2 (Cusick & Lund, 1981; Mizuno et al., 2007; Olavarria and Van Sluyters, 1983). The spatially organized pattern of callosal projections is characterized by a dual connectivity scheme. As in cat, GFP-labeled callosal axons from one hemisphere project densely to a narrowly restricted region at the border between areas 17 and 18 in the contralateral hemisphere, in which they terminated in layers II–III and V (Mizuno et al., 2007; Fig. 1.1). Here callosal connections link cortical loci sharing the same receptive fields along the vertical meridian, as in higher species. Experiments with retrograde tracers have revealed a second pattern of callosal projections. In the medial-most region of the area 17 there are callosal afferents that link symmetric cortical regions mapping symmetric positions of the extreme periphery of the visual field (Houzel et al., 2002). These projections are likely to be involved in processing requiring large-scale integration of features across the entire visual field such as the detection of symmetric shapes.

Callosal connections also play a role in certain binocular functions such as depth perception (Berardi et al., 1988). Experiments in cats, monkey and humans have also revealed the spatial and temporal characteristics of the visual information transmitted through the callosum. At least in adults, the callosum behaves as a low pass filter. Indeed, high spatial and temporal frquencies are attenuated. The sensitivity to contrast is also reduced (Berardi et al., 1988).

The population of cortical neurons projecting to the corpus callosum (less than 10% of all cortical neurons) has been characterized genetically, biochemically, morphologically and electrophysiologically. Most callosally projecting neurons can be classified as pyramidal neurons, although their phenotypic variability is considerable (Vercelli and Innocenti, 1993). The few non-pyramidal neurons which project to the corpus callosum in adult animals are spiny stellate, but also smooth

stellate and fusiform cells which use the synaptic transmitter gamma-aminobutyric acid (GABA) and are presumably inhibitory to their targets. Reported percentages of GABAergic neurons in total populations of callosally projecting neurons vary from 3-5% in rats (Gonchar et al., 1995), and 8% in cats (Fabri and Manzoni, 2004).
Consistent with these percentages, electrophysiological evidence in experimental animals indicates that most of callosal fibers are mainly excitatory to their direct target neurons in the cortex (Matsunami and Hamada, 1984; Karayannis et al., 2007). Callosal fibers activate monosynaptically corticofugal pyramidal neurons, as well as local non-pyramidal neurons, including spiny excitatory and aspiny inhibitory interneurons. Through these last inhibitory GABAergic interneurons the callosal input can inhibit pyramidal neurons in the opposite hemisphere (Carr and Sesack, 1998; Karayannis et al., 2007). Callosal fibers use the glutamate transmitter for monosynaptic excitation (Cisse et al., 2004; Ziskin et al., 2007), while the disynaptic inhibition from callosal inputs is mediated by both $GABA_A$ and $GABA_B$ receptors (Kawaguchi, 1992; Chowdhury et al., 1996; Chowdhury and Matsunami, 2002).

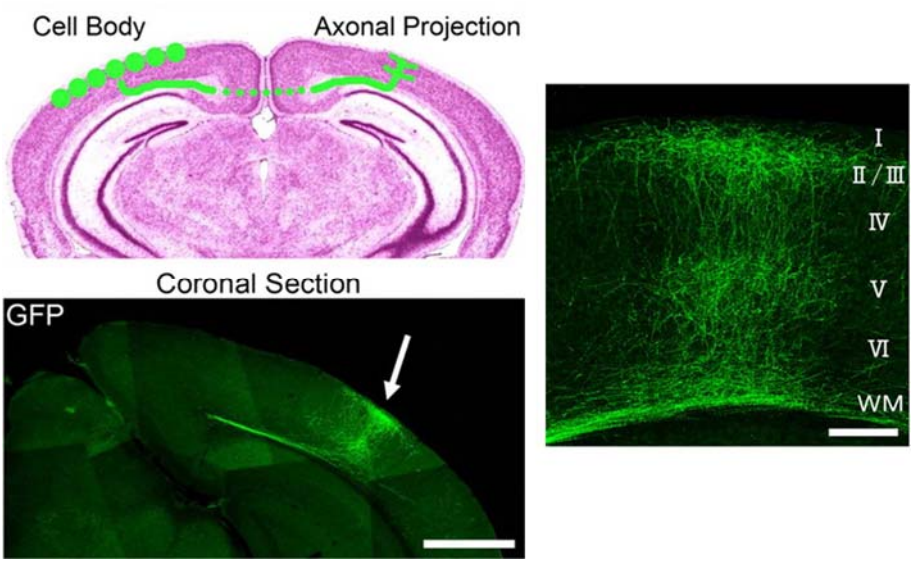


Figure 1.1 Distribution of interhemispheric axon projections in the mouse visual cortex visualized using GFP. GFP-labeled callosal axons projected densely to a restricted region of the contralateral cortex, at the border between areas 17 and 18 (arrow). Scale bar: 1 mm. The right panel represents the layer-specific innervation patterns of callosal axons in the visual cortex. Callosal axons terminate in layers II-III and V. Scale bar: 200 μm (Mizuno et al., 2007).

Role of callosal connections in cortical binocularity: a matter of controversy

As I discussed above, the transcallosal pathway of visual cortex has been implicated in a variety of functions, including fusion of the two visual hemifields (Hubel and Wiesel, 1967), extension of receptive fields across midline (Antonini et al., 1983) and depth sensitivity (Berardi et al., 1988). In addition, the corpus callosum seems to be involved in further binocular tasks such as interocular alignment (Elberger, 1979). To perform these functions we may expect that the binocularity of neurons in the area 17/18 border region is strongly influenced by callosal input. The contribution of callosal input to cortical binocularity has been studied by cutting the optic chiasm (split chiasm preparation) or the optic tract on one side, by transection of the corpus callosum and by cooling the area 17/18 border of one hemisphere. The results obtained from these studies are controversial and the role of callosal connections in cortical binocularity is far to be clear.

One of the first evidence of the influence of callosal connections on binocular response comes from experiments by Berlucchi and Rizzolatti (1968). They reported that some neurons at the 17/18 border could be driven binocularly after midsagittal transection of the optic chiasm. This result clearly demonstrates a callosal input to these cells, since visual information from the contralateral eye could only come through the callosum (Berlucchi and Rizzolatti, 1968).

Other studies reported that after cutting the optic tract on one side in the cat, some neurons in the visual cortex ipsilateral to the lesion could still be activated by visual stimulation trough the callosal connections (Choudhury et al., 1965; Vesbaesya et al., 1967). Conversely, others reports indicated that callosal input is modest in the deafferented visual cortex of similarly operated cats (Yinon et al., 1982; Podell et al., 1984).

A direct demonstration of the importance of callosal connections for binocularity in primary visual cortex arises from induced lesions of the corpus callosum in cats. Payne and colleagues (1980) showed that removal of callosal input significantly reduces the numbers of binocularly driven simple and complex cells (Payne et al., 1980) in cat visual cortex. Similar results were reported by Yinon et al. (1992) that demonstrated a reduction in cortical binocularity after CC transaction in cat and kitten (Yinon et al., 1992). Moreover, inactivation by cooling of the contralateral hemisphere gave rise to a substantial increase in the proportion of monocular cells

with a consequent destruction of binocularity (Blackmore et al., 1983). All these findings are in contradiction with those of other groups reporting no changes in binocularity after callosotomy in adult cats (Minciacchi and Antonini, 1984; Elberger and Smith, 1985). In one of this studies it has been found that alterations in binocularity occurred only when section of the callosum was performed during an early phase of development (Elberger and Smith, 1985).

The discrepancies between these different reports likely arise as a consequence of technical aspects, including age at which the callosal section is performed, and time elapsed between surgery and recording.

In rodents, the studies on the role of callosal connections in binocularity are also controversial (Drager, 1975; Diao et al., 1983; Coleman et al., 2009).

Recent work by Coleman et al. (2009) argues aganist an involvement of the CC in binocularity. Using morphometric measures, they suggested that OD in the primary visual cortex of the mouse can be solely accounted by the relative density of feed-forward geniculocortical inputs from the two eyes. Indeed, the contralateral eye pathway exhibits a higher degree of convergence on geniculate neurons than the ipsilateral eye pathway (Coleman et al., 2009).

Conversely, electrophysiological experiments based on inactivation of callosal connections suggested an important role for interhemispheric connections in determining cortical OD. Diao et al. (1983) recorded single units in primary visual cortex of adult albino rats before and after inactivation by cooling of the contralateral hemisphere. After cooling ipsilateral responses were reduced and the cumulative OD distribution shifted towards the contralateral eye (Diao et al., 1983).

It is important to stress that a clarification of the role of callosal connections in binocularity is fundamental for the interpretation of studies on OD plasticity. Indeed, rodents have become the most popular model for studies of cortical plasticity at the physiological and molecular level. One of the aims of the present thesis is exactly an assessment of the role of the CC in the construction of normal OD and in OD plasticity.

EXPERIENCE-DEPENDENT PLASTICITY IN THE VISUAL CORTEX

Before eye opening, the initial formation and the development of anatomical and physiological features of the visual system are controlled by intrinsic factors like genetic programs and spontaneous activity (Crowley and Katz, 1999, 2002; Sur and Rubenstein, 2005). Successive aspects of brain development require experience-dependent activity to reach complete maturation (Zhang and Poo, 2001; Sengpiel and Kind, 2002). Indeed, total absence of sensory input leads to a delay in the maturation of the visual cortex. In animals reared in darkness (Dark Rearing) from birth, cortical neurons display immature properties such as reduced orientation and direction tuning, larger receptive field sizes, and lower visual acuity (Fregnac and Imbert, 1978; Timney et al., 1978; Benevento et al., 1992; Fagiolini et al., 1994; Pizzorusso et al., 1997; Gianfranceschi et al., 2003). A total lack of visual experience also affects neuronal structure: dendritic spine are increased in size, changed in morphology, and reduced in density (Wallace and Bear, 2004). Some developmental processes seem to be restored once the animals are exposed to light (Buisseret et al., 1978, 1982). For example, spine size is recovered by light exposure while spine density remains abnormally small (Wallace and Bear, 2004). Deprivation of vision during development also perturbs the regulation of age-specific gene sets suggesting that experience is implicated in the expression of genes required for brain maturation (Majdan and Shatz, 2006).

Visual experience is particularly crucial during the critical period (CP). During this period of heightened plasticity, experience can produce permanent and extensive modifications of cortical organization.

Critical period for ocular dominance plasticity

A classic paradigm to study experience-dependent changes in neural connectivity is ocular dominance (OD) plasticity: the rapid changes in visual cortex circuitry which result from unbalanced inputs from the two eyes (Tropea et al., 2009).

Hubel and Wiesel (1963) first demonstrated that in cat cortical neurons had an eye preference, and cells driven by the same eye were grouped together, originating the columns of ocular dominance. Blocking input from one eye by lid suture (monocular deprivation, MD) during development leads to a loss of physiological responses to that eye and to a dramatic increase in the number of neurons responding

preferentially to stimuli presented to the open eye (Hubel and Wiesel, 1963). This ocular dominance shift results as a consequence of the heightened plasticity present in cortical circuits during CP. Indeed, susceptibility to MD changes with age: it begins 5-10 days after onset of vision, it is most robust during CP, then it declines and it is absent or minimal in the adult age (Wiesel and Hubel, 1963a; Fagiolini et al., 1994; Gordon and Stryker, 1996). Age-dependent OD shift induced by alteration of visual experience has been reported in all mammals studied like monkeys (Horton and Hocking, 1997), ferrets (Issa et al., 1999) and rats (Maffei et al., 1992; Fagiolini et al., 1994). Interestingly, critical period duration is tightly correlated with average life expectancy. The concept of an early CP for the effect of MD is well established for carnivores, primates, and for the rat. The mouse seems to display OD plasticity also outside the classical CP depending on the type of anesthesia and the method used to assess OD. However, an age-dependent decline of OD is also observed in mice and fully adult mice are not sensitive to MD (Lehmann and Lowel, 2008).

In all mammals examined MD leads to anatomical and functional effects. Anatomical changes comprise an expansion of territories driven by open eye, and a subsequent reduction of those driven by deprived eye (Katz and Shatz, 1996). Moreover, geniculate neurons receiving input from deprived eye are shrunken (20-25%) and those driven by open eye are hypertrophic (10-15%) (Sherman and Spear, 1982). Studies by Stryker and colleagues showed that anatomical changes at thalamocortical level occur days after detection of the functional effects. Indeed, an OD shift is already detectable after a short period of MD (1-3 days), while changes in thalamocortical arborization are visible only after 4 days of MD (Antonini and Stryker, 1993, 1996; Antonini et al., 1998). Moreover, experiments by Trachtenberg and coworkers (2000) showed that functional OD shift occurs first in the supragranular layers and then they guide changes at the geniculocortical synapse. Rapid OD plasticity in the upper layers of the cortex is accompanied by similarly rapid anatomical changes in the long-range horizontal connections between OD columns in these layers (Trachtenberg and Stryker, 2001). This evidence suggested that horizontal connections could represent a structural correlate for functional OD shift.

In mouse, as in higher mammals, MD promotes growth of the open eye's geniculocortical connections and arrest of growth of deprived arbors (Antonini et al.,

1999). Interestingly, mouse OD shift has been found in all layers but the shift was more pronounced in extragranular layers than in layer IV, with the greatest shift in layer V. This finding suggests that in the mouse, as in other species, intracortical as well as geniculocortical synapses undergo plasticity with MD (Gordon and Stryker, 1996).

In addition to the shift in OD, MD impairs the animal's behaviour by reducing visual acuity of the deprived eye and affecting stereoscopic vision (Medini and Pizzorusso, 2008). Remarkably, physiological responses in the deprived retina and thalamus remain completely unaffected (Wiesel and Hubel, 1963b; Sherman and Stone, 1973). An imbalance in binocular vision during childhood affects visual acuity also in humans leading to a pathological condition designated amblyopia or "lazy eye" (Medini and Pizzorusso, 2008). Amblyopia is clinically important because it is the most frequent cause of vision loss in infants and young children, occurring naturally in about 2-4% of the population (Levi, 2006).

Synaptic mechanisms of ocular dominance plasticity

The shift in ocular preference induced by MD has been originally thought to be the outcome of a process of activity-dependent competition between the synaptic terminals driven by the two eyes for connection with the postsynaptic neuron. This idea was supported by the fact that binocular lid suture was not effective to alter OD columns in mammals (Wiesel and Hubel, 1965; Sherman and Spear, 1982; Gordon and Stryker, 1996). In favour of a competitive view, Chapman and coworkers showed that an imbalance in the electrical activities of the two retinas is sufficient to shift OD also in visual deprivation conditions (Chapman et al., 1986). In addition, reversible blockade of intrinsic cortical activity by intracortical infusion of tetrodotoxin (TTX) or muscimol has a remarkable impact on OD shift (Reiter et al., 1986; Reiter and Stryker, 1988). Interestingly, the results obtained with these two manipulations are extremely different. Activity blockade by TTX infusion completely prevents the OD shift following MD (Reiter et al., 1986). By contrast, the administration of the inhibitory neurotransmitter agonist muscimol, that selectively blocks postsynaptic cells discharges, causes an OD shift in the direction of the less active, deprived eye (paradoxical shift; Reiter and Styker, 1988). One possible explanation of this result is that the activity of deprived thalamocortical terminals is

now better correlated with that of the inhibited postsynaptic cell, therefore leading to synapse strengthening. Thus, the correlation between pre and postsynaptic activity appears to be very important for the expression of OD plasticity.

Binocular competition was originally related to heterosynaptic mechanisms by which open eye inputs drive down the synaptic efficacy of the deprived inputs (Miller et al., 1989; Harris et al., 1997). Indeed, active geniculate neurons from the open eye become functionally and structurally strengthened because they compete better for postsynaptic space than less active afferents from deprived eye. It has been suggested that inputs from the two eyes compete for the acquisition of a neurotrophic factor from target structures (Maffei et al., 1992). An alternative view is that OD plasticity is due to different homosynaptic mechanisms, whereby inputs from the two eyes are affected separately by MD (Smith et al., 2009). For example, in rodents, modifications in the monocular zone of the visual cortex (where no competition can occur) that resemble those taking place in the binocular region have been demonstrated after contralateral MD (Pham et al., 1999; Heynen et al., 2003).

Homosynaptic mechanisms

In support of the homosynaptic view recent data suggest that the OD shift is the result of two forms of synaptic plasticity: an initial depression of deprived eye inputs and only later a potentiation of responses from the open eye (Fig. 1.2).

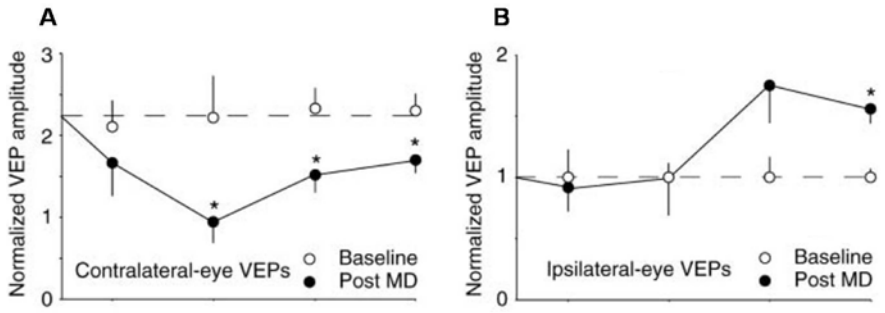


Figure 1.2 Two responses to monocular deprivation. Absolute VEP amplitudes (normalized to average baseline ipsilateral-eye VEP) before (open symbols) and after (filled symbols) various periods of visual deprivation. (A) Depriving the contralateral eye of vision by lid closure causes a rapid depression of contralateral-eye VEP amplitude that reaches statistical significance after 3 days of MD and remains significant at 5 and 7 days. (B) Depriving the contralateral eye of vision by lid closure

leads to a delayed potentiation of the ipsilateral-eye VEPs, significant after 7 days (Frenkel and Bear, 2004).

It is worth noting that the quality of visual experience through the open eye is not changed during MD, therefore its response potentiation has to be ascribed to modification in cortical circuitry. This aspect of OD plasticity is theoretically predicted by the influential Bienenstock, Cooper and Munro (BCM) model (Bienenstock et al., 1982). According to this theory, the reduction in overall cortical activity caused by closing the contralateral eyelid decreases the value of the modification threshold, θ_m thereby facilitating potentiation of correlated inputs (reviewed by Bear 2003; Smith et al., 2009).

Several lines of evidence indicate that homosynaptic depression occurs only at active synapses. Indeed, lid suture was more efficient in shifting OD towards open eye than blockade of all residual retinal activity by intravitreal TTX (Frenkel and Bear, 2004). In particular, retinal silencing prevents depression of the deprived eye inputs but enhances potentiation of responsiveness to the open eye (Rittenhouse et al., 1999).

In the last years researchers have tried to elucidate the cellular and molecular mechanisms underlying this bidirectional kinetics of OD plasticity. To this end strengthening and weakening of inputs from the eyes has been related to the mechanisms involved in long term potentiation (LTP) and long term depression (LTD) at central synapses.

Various line of evidence strongly suggest that LTD-like mechanisms influence depression of deprived-eye responses. Brief MD at the peak of the critical period induces the AMPA receptor internalization and the same phosphorylation pattern of glutamate receptor 1 (GluR1) subunit that occurs after LTD induction *in vitro* (Heynen et al., 2003). Interestingly MD occluded further LTD, causally linking LTD-like mechanisms in the loss of responsiveness observed after MD (Heynen et al., 2003). Another study by Bear's group showed that in MD mice, blocking the internalization of GluR2, that is necessary for LTD, deprived eye depression and ocular dominance shift are prevented (Yoon et al., 2009). This effect is restricted to layer IV suggesting that LTD is layer specific (Crozier et al., 2007; Yoon et al., 2009).

Other lines of evidence support the idea that long-term potentiation (LTP) of the synapses driven by the open eye is important for OD plasticity. For instance, αCaMKII activity is required for both LTP *in vitro* and OD plasticity *in vivo* (Kirkwood et al., 1997; Taha and Stryker, 2002). Moreover, one form of LTP (white matter-layer II-III) in the visual cortex is developmentally regulated with a decline over time that mirrors that of the critical period (Kirkwood et al., 1996). Other forms of NMDA-dependent LTP (layer IV- layer II-III) are present in visual cortical slices of adult rat.

Further suggestion comes from the recently discovered phenomenon of stimulus-selective response potentiation: in juvenile mice, the magnitude of visually driven thalamo-cortical responses in layer IV increases following repeated presentation of an oriented stimulus and this potentiation is dependent on NMDAR activation. Moreover, it has been shown that GluR1 delivery to synapses, that is crucial for LTP, is required for visual experience-dependent plasticity (Frenkel et al., 2006).

Remarkably the capability of use-dependent potentiation remains relatively intact in the adult visual cortex, as shown by the fact that potentiation of visually driven responses has been described *in vivo* in the adult rodent visual cortex after tetanic stimulation of the dLGN (Heynen and Bear, 2001). Important findings in this field come from Sawtell and coworkers (2003). They found that in adult mouse depriving the dominant contralateral eye of vision leads to a persistent NMDA receptor-dependent potentiation of the weak ipsilateral eye (Sawtell et al., 2003). Moreover these data are one of the first demonstration that adult mouse visual cortex has a greater potential for experience-dependent plasticity than previously appreciated. It is important to note that the proprieties of OD plasticity vary significantly with age. In adulthood the rapid depression of deprived eye response is absent while delayed potentiation of the open eye inputs continues to occur. According to these data, in slices of mouse visual cortex LTP can be elicited beyond P35, but NMDAR-dependent long-term depression (LTD) cannot (Kirkwood et al., 1997). Furthermore LTD occlusion and AMPA receptor modifications are not observed in adult animals subjected to MD (Heynen et al., 2003). These evidences suggest that the capability to depress a deprived input is developmentally regulated.

Other studies indicate that LTD- and LTP- like processes may not be sufficient to fully describe naturally occurring plasticity observed *in vivo*. For example, a mutation that disrupts LTD dependent on metabotropic glutamate receptor (mGluR) does not alter the normal OD shift in response to MD (Renger et al., 2002). Furthermore, OD plasticity is blocked by overexpression of the protein phosphatase calcineurin, the only known Ca^{2+}/calmodulin-activated protein phosphatase in the brain, but LTD appears normal in these animals (Yang et al., 2005). Brain-derived neurotrophic factor (BDNF) prevents LTD in V1, but BDNF-overexpressing mice are sensitive to MD at least during an early phase of postnatal development (Huang et al., 1999; Jiang et al., 2003). Finally, GAD65 knockout (KO) mice, which lack OD plasticity, show no deficit in induction of LTP or LTD in layer II/III of mouse binocular visual cortex (Hensch et al., 1998). Thus, it is still unclear whether MD effects can be entirely modeled by the solely LTD/LTP mechanism. Indeed, several alternative hypotheses have been formulated to explain the phenomenology of OD plasticity.

Role of inhibition

Considering that neuronal circuits in the brain are intricately interconnected, changes of thalamocortical inputs are not sufficient to totally explain the process of OD plasticity. In the complicate neuronal network of the visual cortex, inhibitory interneurons have been emerged as a key regulator of neuronal plasticity (Hensch, 2004).

During last years, it has become clear that inhibition not only is a 'brake' for excitation but also has an important role in sculpting the pattern of electrical activity (Berardi et al., 2003). Indeed, GABAergic transmission in mammalian forebrain has been implicated in sharpening the temporal signaling in neurons (Pouille and Scanziani, 2001). Thanks to inhibition a postsynaptic target neuron can detect an imbalance of activity between afferents and can consequently be engaged within the plastic process.

In neocortex inhibitory connections are developed later than excitatory connections (Blue and Parnavelas, 1983). It is possible that, excitatory and inhibitory circuit elements reach an optimal balance once in life during which activity-dependent plasticity may occur.

Drastic pharmacological perturbations of excitatory-inhibitory balance, such as hyperexcitation by bicuculline (Ramoa et al., 1988) or total silencing by TTX (Reiter et al., 1986) disrupt OD plasticity. However, these results do not explain how changes in the relative amounts of excitation and inhibition interfere with plasticity.

Taking advantage of gene-targeting technology, Hensch and co-workers demonstrated a decisive role for excitatory-inhibitory balance. Mice carrying a deletion of the 65-kDa isoform of glutamic acid decarboxylase (GAD65), the GABA biosynthetic enzyme, exhibit a significant reduction of stimulated GABA release and show no shift in responsiveness toward the open eye following brief MD (Hensch et al., 1998). A direct physiological consequence of excitatory-inhibitory unbalance in GAD65 KO mice is the observation of a prolonged discharge, that is the tendency to continue to fire even after stimuli have passed the cell's receptive field. This prolonged discharge resemble that seen in wild type mice before CP, when intrinsic inhibition is weak and OD plasticity is absent. When CP starts this discharge drops down. Thus, ocular dominance plasticity and prolonged discharge are tightly co-regulated by inhibition (Fagiolini and Hensch, 2000). Notably, in GAD65 KO mice mechanisms of synapse modification *in vitro*, as LTP and LTD, are not impaired, demonstrating no general deficit in activity-dependent plasticity (Hensch et al., 1998). Normal OD plasticity in GAD65 KO mice can be rescued if GABA transmission is enhanced in the visual cortex by means of diazepam (Hensch et al. 1998). Remarkably, rescue of plasticity is possible at any age in GAD65 KO mice, which indicates that the critical period is dependent on the proper level of inhibitory transmission (Fagiolini and Hensch., 2000). According to this view, the onset of the critical period can be accelerated by prematurely enhancing inhibition with benzodiazepines just after eye opening (Fagiolini and Hensch, 2000; Iwai et al., 2003). In addition in transgenic mice overexpressing brain-derived neurotrophic factor (BDNF) development of GABA mediated inhibition is accelerated and this results in early opening and closure of the critical period (Huang et al., 1999).

Not all GABA circuits are involved in critical period regulation. Among the many types of GABA-positive interneuron (they account for nearly 20% of cortical neurons), parvalbumin-positive (PV) interneurons seem to be the most important players (Fig. 1.3). Indeed, maturation of parvalbumin-positive interneurons parallels critical period onset (Del Rio et al. 1994) and both events are accelerated by BDNF

overexpression (Huang et al., 1999). Interestingly, only GABA type A ($GABA_A$) receptor circuits have been found to drive cortical plasticity. In particular, $GABA_A$ receptors carrying the α1 subunit appear to be crucial in CP regulation (Fagiolini et al., 2004). These receptors are preferentially enriched at somatic synapses opposite to terminals of PV large basket cell.

With age, large PV cells are preferentially enwrapped in perineuronal nets (PNN) of extracellular matrix (ECM). When these are disrupted, perisomatic inhibition of their targets is reduced (Saghatelyan et al., 2001), and ocular dominance shifts can be induced by monocular deprivation, even in adulthood (Pizzorusso et al., 2002).

Taken together, these results indicate that during development there are two functional threshold of inhibition in the visual cortex: the first one is necessary to trigger plasticity and the second one causes the end of critical period. Pharmacological reduction of inhibitory transmission by infusion of mercaptopropionic acid (MPA, an inhibitor of GABA synthesis) or Picrotoxin (a $GABA_A$ receptor antagonist) into the rat visual cortex effectively reactivates OD plasticity in adulthood (Harauzov et al., 2010). Moreover, also other manipulations resulting in reductions of cortical inhibition promote adult plasticity (He et al., 2006; Sale et al., 2007; Maya Vetencourt et al., 2008).

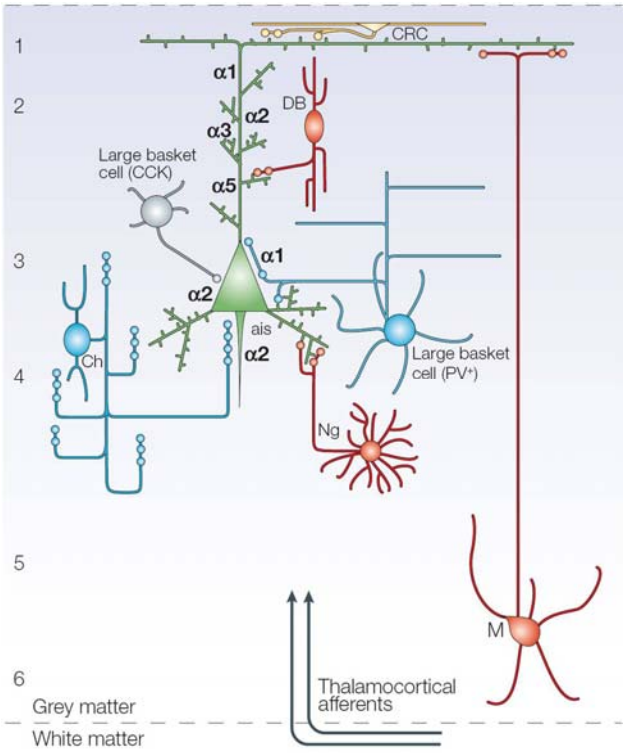


Figure 1.3 Heterogeneity of local GABA circuits in the neocortex. Many subtypes of GABA releasing inhibitory interneuron can be identified in the neocortex on the basis of morphology, connectivity, expression of calcium-binding proteins or neuropeptide content. Moreover, specific contacts are preferentially enriched in specific $GABA_A$ receptor α-subunits. The perisomatic localization of different subunits is shown next to the central pyramidal neuron. All subunits are found diffusely along the dendrite. *ais*, axon initial segment; CCK, cholecystokinin expressing; *Ch*, chandelier cell; CRC, Cajal–Retzius cell; DB, double bouquet cell; M, Martinotti neuron; N, neurogliaform neuron; PV+, parvalbumin positive (Hensch 2005).

Recently the relative contribution of excitation and inhibition in OD plasticity has been addressed with new approaches. By recording from synaptically coupled pairs of neurons in layer IV, it has been shown that brief MD increases the strength of inhibitor $GABA_A$ mediated-synaptic responses (Maffei et al., 2006). These data propose that this novel form of long-term potentiation of inhibition (LTPi) contribute to the deprivation-induced loss of visual responsiveness in the rodent visual cortex (Maffei et al., 2006). It has also been suggested that this inhibitory plasticity is fundamental in modulating cortical circuits refinement during development and may regulate the onset of OD plasticity (Maffei et al., 2010). Other studies, by measuring the early consequences of MD in different classes of cortical neurons *in vivo*, showed that responses of inhibitory cells are also modified by deprivation (Gandhi et al., 2008; Yazaki-Sugiyama et al., 2009; Kameyama et al., 2010). In particular, Yazaki-Sugiyama and co-workers identified Fast Spiking (FS) interneurons (PV positive large basket cells), as a substrate for the early phase of the OD plasticity process. Intracellular recordings showed that these cells normally exhibit binocular response which following MD first shift paradoxically towards the deprived eye and only later towards the open eye (Yazaki-Sugiyama et al., 2009). These results suggest that the initial enhanced activation of FS interneurons by deprived-eye inputs may trigger an inhibitory suppression of their own responses in the visual cortex (Yazaki-Sugiyama et al., 2009).

A completely different view of the role of inhibition in the expression of OD plasticity has been provided by Khibnik and coworkers (2010). They induced an OD shift with 3 days of MD and then pharmacologically removed the influences of intracortical inhibition and excitation, leaving only afferent thalamocortical synaptic inputs. They found that OD shift induced by brief MD is not influenced by

intracortical inhibition, being fully expressed by modification of excitatory talamocortical inputs. They concluded that inhibition precedes and perhaps influences modification of exitatory synapses but play a marginal role in OD plasticity (Khibnik et al., 2010).

Homeostatic plasticity

The role of other forms of plasticity in OD plasticity has begun to be investigated only recently. Homeostatic synaptic plasticity has emerged as an important complement to Hebbian forms of plasticity in the activity-dependent refinement of synaptic connectivity (Turrigiano and Nelson, 2004; Davis, 2006; Turrigiano, 2008). The term 'homeostatic' refers to a form of plasticity that acts to stabilize the activity of a neuron or neuronal circuit against perturbations, such as changes in cell size or in synapse number or strength, that alter excitability (Turrigiano, 2007). Examples of homosynaptic mechanisms described in these years include: activity-dependent regulation of intrinsic neuronal firing properties (Zhang and Linden, 2003); pre and post–synaptic forms of excitatory synaptic plasticity, such as synaptic scaling, that adjust the strength of all excitatory synapses of a neuron up or down to stabilize firing (hence the term "synptic scaling"; Turrigiano and Nelson, 2004); balancing of excitation and inhibition within neuronal networks (Maffei et al., 2004); compensatory changes in synapse number (Wierenga et al., 2006). All these changes seem to act in order to restore the neuronal firing rates to normal levels after perturbation.

From all these findings three principal models by which neurons implement homeostatic plasticity have been postulated. A cell-autonomous mechanism where individual neurons sense their own activity, through, for instance, changes in Ca^{2+} influx, and then adjust all of their synaptic strength up or down to keep this value relatively constant. A synapse-specific mechanism, in which local synaptic signaling induces compensatory changes in presynaptic and/or postsynaptic function. For example, depolarization induced by glutamate receptors activation might negatively change the number of glutamate receptors on the postsynaptic cell or generate a retrograde signal that blocks vesicle release from the presynaptic terminal. Finally, changes in network activity could lead to altered release of a diffusible 'activity signal' that negatively regulates synaptic function.

The first reports of synaptic homeostasis at central synapses suggested that neurons respond to changes in activity by scaling up or down the strength of all of their synapses through a simple change in the accumulation of postsynaptic glutamate receptors (O'Brien et al., 1998; Turrigiano et al., 1998). For example, treatment of neocortical cultures with TTX increases the amplitude but not the frequency of miniature excitatory postsynaptic currents (mEPSC) (Lissin et al., 1998; O'Brien et al., 1998; Turrigiano et al., 1998). Other *in vitro* studies showed that also changes in presynaptic functions, such as alterations in release probability and in number of release sites, are involved in homeostatic plasticity (Murthy et al., 2001; Burrone et al., 2002; Thiagarajan et al., 2005). Chronic activity blockade in cortical cultures increases the expression of vesicular glutamate transporter vGLUT1, whereas hyperactivity reduces vGLUT expression (De Gois et al., 2005; Erickson et al., 2006).

Despite the large number of studies, the nature of the activity signal that controls synaptic scaling is still debated. Indeed, it is currently unknown whether the activity signal relevant for synaptic scaling is postsynaptic changes in firing, presynaptic changes in release, or local dendritic changes in receptor activation and/or Ca^{2+} influx. Synaptic scaling has been observed at cortical synapses also *in vivo* following sensory deprivation and is developmentally regulated. It has been proposed that synaptic scaling is important in regulating cortical excitability during activity-dependent development (Desai et al., 2002; Maffei et al., 2004; Goel et al., 2006).

An important issue in homeostasis is how changes in activity are signaled to neurons or synapses. It has been proposed that several activity-dependent molecular mechanisms are involved. Documented examples are BDNF, cytokine tumor-necrosis factor α (TNFα) and the immediate-early gene product Arc (Rutherford et al., 1998; Shepherd et al., 2006; Stellwagen and Malenka, 2006; Kaneko et al., 2008a). BDNF enhances mEPSC amplitude onto excitatory neurons (Copi et al., 2005) but its effect seems to be depend on brain region and developmental stage. TNFα originates from glia and seems to regulates AMPA receptors on neurons in a network-level homeostatic process (Turrigiano, 2007). Finally, Arc overexpression decreases AMPA-receptor mediated transmission and prevents the increase in mEPSC amplitude induces by chronic TTX (Shepherd et al., 2006).

Concerning visual cortex plasticity, a recent study reports evidence for homeostatic mechanisms using two-photon calcium imaging *in vivo* (Mrsic-Flogel et al., 2007). The authors investigated how MD shifts the magnitude of deprived and non-deprived eye responses in individual neurons. They found that the population of neurons driven only by the deprived eye unexpectedly showed strong responses after MD. These findings demonstrate that the weak input of deprived eye is not able to induce response depression, which instead seems to be dependent on the input of the open eye. In addition the proportion of monocular, closed eye-driven cells remained constant after MD and most neurons in monocular cortex increased their responsiveness. All these findings suggest that in the deprived visual cortex there are compensatory mechanisms that maintain firing rates within a certain range during MD.

Another recent work shows that the pharmacological or genetic blockade of TNFα signalling in visual cortex has no effect on the depression of the deprived eye input but prevents the gain of responsiveness to the non-deprived eye. This result demonstrate that synaptic scaling underlies the enhancement of responsiveness to the non-deprived eye (Kaneko et al., 2008a).

Some studies have addressed the importance of a form of synaptic plasticity designed spike-timing dependent (STDP) for the experience-dependent plasticity. STDP refers to the temporal order of pre- and postsynaptic spiking in eliciting long-term synaptic depression or potentiation (Celikel et al., 2004; Dan and Poo, 2004). It has been proposed that loss of responsiveness of deprived inputs is mediated by STDP (Celikel et al., 2004; Hensch, 2004; Kuhlman et al., 2010) but its role in OD plasticity is not fully clarified. Recently, it has been proposed that MD-induced OD plasticity observed in fast-spiking interneuron is consistent with a STDP rule (Yazaki-Sugiyama et al., 2009).

Molecular basis of OD plasticity

In parallel with the physiological mechanisms described above, a complex and interrelated molecular network sets the basis for OD plasticity. This network involves a large number of molecules whose expression is developmentally regulated

and differentially altered by visual experience. In this chapter I describe some of the molecular mechanisms associated with to visual cortex plasticity.

Neurotrophins

Neurotrophins (NTs) are a family of neurotrophic factors that include: nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). They support the survival and differentiation of neurons by binding to and activating tyrosine kinase receptors of the Trk family.

In 1990s pioneering studies by Maffei's group suggested that NTs are important players of plastic processes. The authors demonstrated that exogenous NGF administration prevents the loss of visual acuity and the shift of OD induced by MD in the rat visual system (Domenici et al., 1991; Maffei et al., 1992). Based on these finding, they put forward the idea that competition in OD plasticity might depend on the uptake of NT secreted by cortical neurons in an activity-dependent manner and retrogradely transported by geniculate neurons. Several other subsequent reports confirmed this hypothesis (Harris et al., 1997; McAllister et al., 1999; Pizzorusso et al., 1999). In particular it has been demonstrated that, with the exception of NT-3, all neurotrophins influence MD, but not all factors play an identical role on visual neuron properties (Lodovichi et al., 2000). Indeed, NT-4 and NGF prevent the shift induced by MD without affecting spontaneous or visually-driven activity (Gillespie et al., 2000; Lodovichi et al., 2000). In contrast, BDNF is less effective in preventing OD shift, but it alters both spontaneous and visually-evoked activity of cortical neurons.

Other studies, using a complementary strategy based on antagonizing the action of endogenous NTs, demonstrated that NTs are important for normal visual cortical development and plasticity (Berardi et al., 1994; Domenici et al., 1994; Cabelli et al., 1997). For example, inactivation of NGF signaling by specific antibodies alters visual acuity and binocularity of cortical neurons, induces a shrinkage of geniculate neurons (Berardi et al.,1994) and prolongs the sensitive period for MD in the rat (Domenici et al., 1994).

NT synthesis and release are regulated during development in an experience-dependent mode (Bozzi et al., 1995; McAllister et al., 1999). NTs can modulate electrical activity, at pre and postsynaptic level, increasing neurotransmitter release

and depolarizing neurons but they also act on gene expression (Sala et al., 1998; Kafitz et al., 1999; Lodovichi et al., 2000; Poo, 2001). Moreover, evidence from literature indicates that NTs stimulate dendritic growth and this action is activity-dependent (McAllister et al., 1995; McAllister et al., 1996). Regulation of synaptic plasticity by NTs in the cortex requires afferent electrical activity (Caleo et al., 1999a). Taken together these results indicate that activity and NTs interact reciprocally. Their interaction may provide a mechanism by which active neuronal connections are selectively enhanced.

Striking evidences for the role of NTs in regulating the experience-dependent plasticity came from studies in mice overexpressing BDNF. A precocious BDNF expression accelerates the development of visual acuity and the time course of the critical period. In addition, in these mice maturation of inhibition is accelerated suggesting a close link between BDNF and GABA circuits (Huang et al., 1999). Moreover, mice overexpressing BDNF display a normal functional development even when reared from birth in total darkness (Dark Rearing). Indeed these mice display visual acuity, critical period for OD plasticity and inhibitory transmission identical to those of normal, light-reared mice (Gianfranceschi et al., 2003).

In rodents, BDNF expressed in the retina is transported anterogradely by retinal ganglion cells to the geniculate nucleus. Levels of BDNF in the retina not only influence development of the lateral geniculate nucleus and superior colliculus (Caleo et al., 2000; Caleo et al., 2003), but also have a role in OD plasticity (Mandolesi et al., 2005). Indeed, MD reduces BDNF expression in the deprived retina and intravitreal injection of BDNF into the deprived eye prevents the OD shift induced by MD (Mandolesi et al., 2005). However, other studies provide different insights on the role of BDNF in OD plasticity. Data from heterozygous knockout mice for BNDF demonstrate that a 50% reduction in the BDNF levels has no effect on OD plasticity (Bartoletti et al., 2002). Furthemore, Stryker and colleagues, using a genetic approach to inhibit TrkB signaling, show that OD shift in response to MD does not require TrkB activity (Kaneko et al., 2008b). However, these data do not exclude the important and well recognized role of NTs in visual cortex plasticity. Indeed, the actions of other NTs could compensate for the lack of modification in visual cortex plasticity observed in these mutant mice.

NMDA receptors

NMDA receptors (NMDARs) mediate excitatory synaptic transmission and their expression is widespread in the brain. They have the characteristic of being both transmitter and voltage-dependent, and permitting Ca^{2+} influx, they are related to the intracellular signaling implicated in plasticity. Evidence for NMDA receptor involvement in visual cortex plasticity comes from experiments showing that blockade of NMDA receptors prevented the effects of MD (Bear et al., 1990; Roberts et al., 1998; Sawtell et al., 2003). NMDA receptors are developmentally regulated and their expression is modified by electrical activity. This activity-dependent regulation seems to be mediated by epigenetic mechanisms (Lee et al., 2008).

In visual cortex, NMDA subunit composition varies over development from an increased expression of receptors containing the NR2B subunit to a progressive addition of the subunit NR2A, with a time course paralleling that of critical period (Roberts and Ramoa, 1999). Dark rearing (which delays CP closure and impairs cortical maturation) delays the expression of NR2A subunit, suggesting that NR2B/NR2A switch is involved in visual cortex development and CP regulation (Berardi et al., 2003). However, in transgenic mice with deletion of NR2A subunit the sensitivity to deprivation is weakened but restricted to the typical critical period (Fagiolini et al., 2003).

A recent study shows that GAD65KO mice not only have altered inhibition but also have alterations in NMDA receptor subunit composition and function. Remarkably, treatment with benzodiazepines, which rescues OD plasticity, increases NR2A levels. These results suggest that changes in either inhibition or excitation would engage mechanisms that converge to regulate NMDA receptors, thereby enabling plasticity (Kanold et al., 2009). Another suggestion of the possible mode by which NMDA receptor are implicated in plastic mechanism is provided by Bear's group (2009). The deprived-eye responses in NR2A KO mice are unchanged after brief MD (3 days), whereas the non-deprived eye responses dramatically potentiate (Cho et al., 2009). These data suggest that a reduction in the NR2A/NR2B ratio during monocular deprivation is permissive for the compensatory potentiation of non-deprived inputs (Cho et al., 2009).

Intracellular pathway and gene expression

Electrical activity, neurotrophins, and NMDA receptors control plasticity of cortical circuitry operating on three intracellular kinases: cAMP-dependent protein kinase (PKA; Beaver et al., 2001; Cancedda et al., 2003), extracellular-signal-regulated kinase (ERK; Di Cristo et al., 2001; Cancedda et al., 2003), α Ca2+/calmodulin dependent protein kinase II (α CAMKII; Taha et al., 2002). Besides these kinases, the phosphatase calcineurin seems to be involved in OD plasticity (Yang et al., 2005). Thus, OD plasticity may be regulated by the balance between kinases and phosphatases.

All these effectors are mutually interconnected by a complex network of interactions and have targets both in the cytoplasm and the nucleus. In particular, in the cytoplasm substrates crucial for synaptic transmission, neuronal excitability and morphological stabilization are target of phosphorylation, while in the nucleus, the targets are molecules engaged in gene expression (Berardi et al., 2003).

Long lasting modifications in neuronal circuits require gene expression and protein synthesis (Mower et al., 2002; Taha and Stryker, 2002). These mechanisms are necessary also for mediating the action of experience on visual cortex development. Visually-driven activity activate transcriptional factors, such as zif268 or CREB (Caleo et al., 1999b; Pham et al., 1999; Mower et al., 2002) but zif268 is not necessary for OD plasticity (Mataga et al., 2001) while activation of CREB is crucial for OD plasticity (Pham et al., 1999; Liao et al., 2002; Mower et al., 2002). CREB triggers the expression of genes essential for establishment and maintenance of plastic changes and which are under the control of the cAMP-response element (CRE) promoter (Pham et al., 1999; Mower et al., 2002). Studies based on a combination of DNA microarrays, RT PCR and immunohistochemistry show that several sets of genes are modulated by visual experience or deprivation (Majdan and Shatz, 2006; Tropea et al., 2006). For example, expression of a binding protein of insulin-like growth factor-1 (IGF1) is highly upregulated after MD, and exogenous application of IGF1 prevents the physiological effects of MD on OD (Tropea et al., 2006). Moreover, a recent microarray screen indicates that the calcium sensor cardiac troponin C (part of a complex that mediates calcium-dependent actin-myosin interaction) is elevated in visual cortex during the critical period, and is regulated by visual activity (Lyckman et al., 2008).

Recently, it has been proposed that Arc is an effector molecule important for OD plasticity. In visual cortex Arc expression starts only after eye opening and is activated by visual stimulation (Tagawa et al., 2005; Wang et al., 2006). In KO mice the total absence of Arc, renders visual cortical synapses insensitive to the effects of both experience and deprivation (McCurry et al., 2010).
In these years, the regulation of chromatin structure has emerged as one mechanism regulating visual cortex plasticity. Indeed, histone phosphoacetylation has been demonstrated to be involved in OD plasticity (Putignano et al., 2007). The histone phosphoacetylation is mediated by ERK and is developmentally regulated. In adult mice visual experience activate ERK but induction of histone phosphoacetylation and CREB-mediated gene expression are much lower than in juvenile animals. In line with this, a pharmacological-induced histone acetylation restores plasticity in the adult visual cortex (Putignano et al., 2007). Experience-dependent regulation of histone acetylation could be a way to regulate specific sets of genes important to consolidate plastic changes (Medini and Pizzorusso, 2008).

Recently, a novel factor has been added in the list of molecules involved in visual cortex plasticity. Sugiyama and colleagues (2008) have found that Otx2 homeoprotein, an essential morphogen for embryonic head formation, is reused later in life to regulate CP (Sugiyama et al., 2008). Otx2 is transported from the retina to the visual cortex where it is internalized by PV positive GABAergic interneurons. Intracortical delivery of the recombinant Otx2 protein in mice before the onset of the critical period shifts prematurely OD towards the open eye after MD. In contrast, in an Otx2 conditional KO mouse, MD during CP fails to induce OD plasticity. This plasticity defect could be rescued by diazepam treatment, consistent with an immature PV cell function in Otx2 KO mice (Sugiyama et al., 2008). Taken together these observations suggest that Otx2 is a molecular messenger that travels from retina to visual cortex to regulate time-course of CP by promoting the maturation of PV positive interneurons.

Extracellular environment

Extracellular and pericellular microenvironment contains important regulators of visual cortical plasticity. One of the first demonstration of the involvement of extracellular components in OD plasticity comes from the evidence that pharmacological inhibition of extracellular protease tissue plasminogen activator (tPA) attenuates the OD shift induced by MD (Mataga et al., 1996). tPA is an immediate early gene induced by electrical activity (Qian et al., 1993) and its proteolytic activity is increased during MD (Mataga et al., 2002). In keeping with these results, tPA KO mice display an impaired MD induced ocular-dominance shift that could be rescued by exogenous tPA (Mataga et al., 2002). A subsequent study indicated that the increase of tPA that occurs after MD is needed for structural plasticity of dendritic spines (Mataga et al., 2004). Thus, tPA, by proteolitically removing extracellular molecules inhibitory for plasticity, enables OD shift induced by MD. Part of the inhibitory action of the extracellular environment resides in components of the extracellular matrix (ECM) called chondroitin-sulfate proteoglycans (CSPGs). These molecules are organized in typical structures, the perineuronal nets (PNNs), around soma and dendrites of PV-positive neurons. Several studies indicate that the developmental increase of PNNs correlates with the end of the classical CP and that Dark Rearing delays the formation of PNNs in the visual cortex (Hockfield et al., 1990; Koppe et al., 1997; Pizzorusso et al., 2002). The inhibitory action of CSPGs for plasticity is demonstrated by the evidence that degradation of CSPGs in adulthood by the bacterial enzyme chondroitinase ABC is able to restore OD plasticity (Fig. 1.4) and to promote recovery from amblyopia (Pizzorusso et al., 2002; Pizzorusso et al., 2006). Interestingly, treatment with chondroitinase ABC increases spine density (Pizzorusso et al., 2006) suggesting that removal of CSPGs may provide a permissive environment for structural plasticity. It is worth noting that ECM proteolysis could also be regulated by particular rearing conditions. Enriched environment promotes amblyopia recovery and decreases PNNs number (Sale et al., 2007).

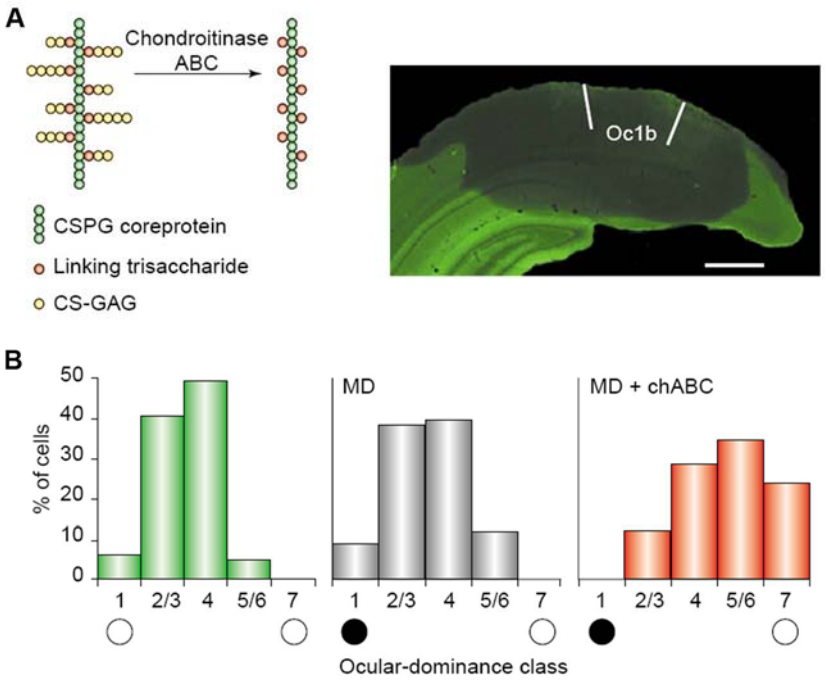


Figure 1.4 Relationship between chondroitin-sulfate proteoglycans (CSPGs) and adult visual cortical plasticity. (A) Treatment with chondroitinase ABC degrades the chondroitin-sulfate glycosaminoglycans (CS-GAGs) from CSPG. This degradation results in major disruptions to the macromolecular heterophilic interactions that hold the perineuronal net together (left). Immunostaining for the CSPG neurocan shows that the treatment of the adult visual cortex with chondroitinase ABC removes CSPGs from the whole binocular subfield of the adult visual cortex (area Oc1b) and from neighbouring cortical areas (right). (B) In adult rats (> P100), MD (black and white circles indicate the ocular-dominance class corresponding to the deprived and non-deprived eyes, respectively) does not cause a shift of ocular dominance. When adult rats were treated with chondroitinase ABC (chABC), monocular deprivation elicited a significant shift of ocular dominance towards the non-deprived eye (Berardi et al., 2003).

Another candidate for plasticity regulation has also been recognized in myelin-associated proteins. It is well known that these proteins exert an active inhibitory role in mechanisms of brain repair. Few years ago it has been demonstrated that myelinization not only inhibits recovery from injury, but can also promote the decrease of plasticity observed at the end of the CP. Indeed, maturation of intracortical myelination has been found to correlate with the end of the critical period (McGee et al., 2005). In addition the absence of either NogoA (a growth inhibitor associated to myelin) or its receptor NgR prevents the closure of the critical period and OD plasticity persists well into adulthood (McGee et al., 2005).

Recently, the paired immunoglobulin-like receptor B (PirB) has also been shown to have high affinity for NogoA and MAG (myelin-associated glycoprotein), whose signaling is inhibitory for axonal regeneration (Atwal et al., 2008). In line with this, PirB restricts ocular dominance plasticity in the visual cortex (Syken et al., 2006).
Polysialic acid (PSA) presented by neural cell adhesion molecule (NCAM) is another ECM factor involved in cortical plasticity. In particular, it has been shown that activity-dependent PSA expression regulates the maturation of GABAergic circuits and hence the time-course of CP plasticity. Indeed, premature removal of PSA in visual cortex induces a precocious maturation of perisomatic GABAergic innervations and anticipates the onset of CP plasticity (Di Cristo et al., 2007).

Callosal plasticity in the visual cortex

As described in the first section, an important input to visual cortex is represented by afferents from the opposite hemisphere running within the corpus callosum. In this paragraph I discuss how visual experience shapes callosal connections and how these connections are involved in visual cortex development.

Corpus callosum and visual experience

Like any other brain system, the callosal system can undergo plastic changes during its early formation and maturation as well as a result of physiological and pathological experiences during an entire life.
In experimental animals the plasticity of the callosal system has been the object of several studies with special regard for the visual cortex, where the formation of callosal connections has been shown to be significantly influenced by manipulations of the visual input (Stryker and Antonini, 2001).
Callosal axons are initially exuberant, but during development there is a partial elimination of callosal axon terminals (Innocenti and Caminiti, 1980). The development and the plasticity of the callosal connections of the visual cortex in early development depend on the spontaneous activity of callosal neurons (Mizuno et al., 2007) as well as on the influence of visual experience (Innocenti and Frost, 1980; Innocenti et al., 1985; Frost and Moy, 1989). The period of initial formation and extensive callosal axon arborisation is sensitive to manipulation of spontaneous neuronal activity. Indeed, the silencing of the spontaneous activity of callosal terminals by the induced expression of Kir2.1 (an inward rectifying potassium

channel) produces significantly decreased number of callosal axons in the visual cortex of neonatal mice before eye opening (Mizuno et al., 2007).

After eye opening the organization of callosal connections is driven by visual experience (Innocenti et al., 1985). In particular, vision seems to act in callosal projections rearrangement by modulating developmental elimination of the overabundant callosal axons (Innocenti, 1986; Mizuno et al., 2007).

By modulating visual experience early in life, it is possible to affect the development of callosal connections. Rearing animals in complete darkness from birth exaggerates the elimination of immature callosal projections (Frost and Moy, 1989). Furthermore, bilateral eyelid suture or bilateral enucleation decreases the number of callosally projecting neurons (Innocenti and Frost, 1980; Innocenti et al., 1985). In contrast, in kittens raised with convergent or divergent strabismus, monocular enucleation and monocular eyelid suture, callosal neurons acquire a more wide-spread distribution than in normal animals (Innocenti and Frost, 1979).

In adult cats, alteration of visual experience does not modify the cortical distribution of callosal neurons but can influence their functional properties. Indeed, few weeks of monocular deprivation leads to an increase of their receptive field size and to a loss of their orientation selectivity (Watroba et al., 2001).

Role of callosal connections in cortical maturation

The data described above indicate that sensory experience alters callosal afferents. In literature there are also some studies that indicate a role for the callosum in cortex development. In cats, visual functions are permanently altered by the section of the callosum during early development. Indeed, there is a decreased number of binocular cells and visual acuity is reduced (Elberger, 1984).

More recently Caleo and co-workers (2007) provided an interesting demonstration of the fundamental role for interhemispheric connections in the development and plasticity of the visual cortex (Caleo et al., 2007). They used the bacterial enzyme botulinum neurotoxin E (BoNT/E) to produce a unilateral, reversible blockade of neural activity in rat visual cortex during the critical period at the time of eye opening. Neurons in the injected cortex displayed a permanently reduced visual acuity, consistent with a deficit of acuity at the behavioral level, and an abnormally protracted susceptibility to MD. These effects extended equally to the contralateral,

uninjected side, whose neuronal activity was not directly affected by the neurotoxin (Fig. 1.5). The very similar developmental deficits observed ipsilateral and contralateral to the activity blockade indicate a fundamental role for callosal linkages in ensuring parallel maturation of the two hemispheres of the brain (Caleo et al., 2007).

In line with these results, mice KO for the transcription factor AP2γ and displaying a decreased number of callosal neurons in layer II-III of visual cortex also show a profound reduction in visual acuity and an abnormal binocularity (Pinto et al., 2009). These mice remained plastic beyond the normal CP. In a series of experiments, I found that 3 days of MD are effective in shifting OD in KO adult mice, while in wild type animals there is no such shift. Thus, the functional deficits found in AP2γ KO mice are reminiscent of a more immature state of the cortex. These results resemble the alterations observed after inactivation of one hemisphere during development (Caleo et al., 2007), hence reinforcing the idea that interhemispheric connections have an important role in functional maturation of the visual cortex.

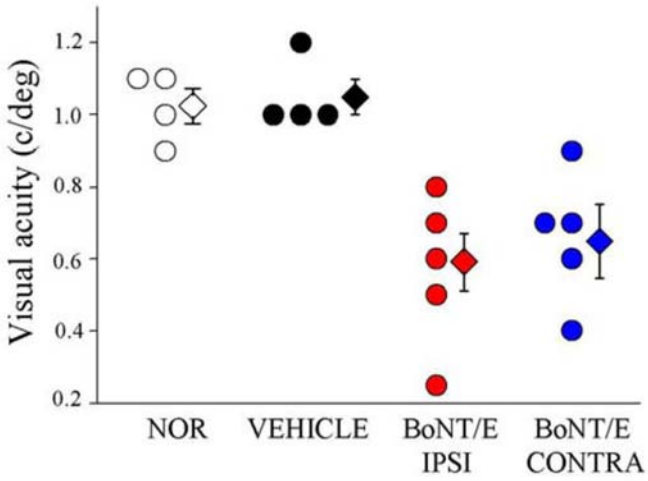


Figure 1.5 Bilateral impairments in spatial resolution in BoNT/E rats. Summary of visual acuities in all animal groups. Each circle represents one animal. Mean visual acuity (diamonds) is significantly reduced in both hemispheres of BoNT/E rats compared with that in normal or vehicle-injected animals. Error bars indicate SE. NOR, normal animals; CONTRA, Contralateral; IPSI, ipsilateral (Caleo et al., 2007).

Experience-dependent plasticity in adulthood

In contrast to the classic notion of a well defined CP for experience-dependent plasticity, in recent years, several studies have reported residual cortical plasticity in adult mice.

MD-induced OD plasticity has been demonstrated in adult mouse using different methods including intrinsic signal imaging (Hofer et al., 2006a), visual evoked potential (VEP; Sawtell et al., 2003) and the activity reporter gene *Arc* (Tagawa et al., 2005). Several methodological aspects of these recent studies can account for the discrepancy with previous results. In particular, the electrophysiological measure of OD was performed under different anesthetic regime and in awake animals: previous studies used barbiturate (Fagiolini and Hensch, 2000) while recent works used urethane (Pham et al., 2004) or no anesthesia (Sawtell et al., 2003).

As I mentioned before, Sawtell and coworkers (2003) demonstrated for the first time that OD plasticity occurring in adult mice uses different mechanisms than plasticity in younger mice. They measured in normal mice the ratio of VEPs measured after visual stimulation of the contralateral versus the ipsilateral eye. As expected, they found a strong contralaleral eye bias. After brief MD in adult mice (3 days, a period that is effective in shifting OD in juvenile mice), the contra-ipsi ratio was unchanged. However, longer MD (5 days) was able to induce a shift in OD. Then they daily analyzed VEPs amplitude in chronically implanted animals, to identify the mechanism. Five days after MD they found a potentiation of the absolute VEP amplitude elicited by stimulation of the ipsilateral, open eye. In juvenile mice both initial weakening of deprived eye response and delayed strengthening of open eye responses contribute to the shift in OD (Frenkel and Bear, 2004). The absence of the early depression of inputs from the open eye probably explains why short periods of MD are ineffective in shifting OD in adult mice. Other works suggest that depression of inputs is still possible in adult cortex. Indeed, in adult mice the initially weaker ipsilateral eye undergoes further weakening after ipsilateral eye closure (Tagawa et al., 2005; Hofer et al., 2006a). A recent work by Fischer and coworkers (2007) demonstrates that the adult visual cortex retains a remarkable potential for plasticity that persists throughout life, also in mature adulthood. Using VEP they found that OD plasticity is robust in young adults (four months) as well as in middle-aged mice (nine months). Furthermore, in these mice MD alters visual acuity, decreasing the

acuity of deprived eye and improving that of the open eye (Fischer et al., 2007). These data contrast to data obtained by optical imaging, showing that MD is no longer effective in shifting OD in fully adult mice (> four months of age; Lehmann and Lowel, 2008).

It is important to stress that plasticity in adulthood is qualitatively and quantitatively different with respect to juvenile animals and the potential for plasticity remains maximal during the critical period (Hofer et al., 2006b; Medini and Pizzorusso, 2008). Differently from mice, an OD shift can not be induced in rats past the critical period (Fagiolini et al., 1994; Guire et al., 1999). Strategies aimed to reactivate developmental levels of plasticity in the adult brain may also promote recovery from amblyopia in the visual system as well as from many forms of damage in other parts of the brain.

Promoting adult plasticity

Visual experience by itself and environmental manipulation can promote the capacity of mature circuits to undergo plastic modifications. Ten days of visual deprivation (dark rearing) in adult rats restore susceptibility to MD (He et al., 2006). Moreover, a period of dark exposure followed by reverse suture promotes recovery from amblyopia consequent to early eyelid suture (He et al., 2007). Interestingly, prior experience can facilitate adult OD plasticity. Indeed Hofer and coworkers found that inducing a brief OD shift by eye closure in juvenile or adult mouse visual cortex enabled stronger and more persistent OD change in response to a second MD performed several weeks later (Hofer et al., 2006a). Finally, environmental enrichment is sufficient to promote complete recovery from early MD (Sale et al., 2007).

Other rodent studies have exploited the properties of several treatments to reopen plasticity in adult visual cortex. As I discussed before, pharmacological degradation of the ECM reactivates OD plasticity (Pizzorusso et al., 2002) and promotes recovery from amblyopia (Pizzorusso et al., 2006). A pharmacological-induced histone acetylation by thricostatin equally enhanced adult plasticity (Putignano et al., 2007). In addition, chronic treatment with valproic acid or sodium butyrate (two different histone deacetylases inhibitors) leads to a complete recovery from amblyopia (Silingardi et al., 2010). Recently it has been shown that chronic administration of

the well-known antidepressant fluoxetine, a selective serotonin reuptake inhibitor (SSRI), reinstates OD plasticity in adulthood and promotes the recovery of visual functions in adult amblyopic rats (Maya Vetencourt et al., 2008).
Interestingly, chronic fluoxetine as well as enriched environment (Sale et al., 2007) reduces GABAergic transmission, and their rescue effect can be prevented by enhancing inhibition with diazepam. Accordingly, a pharmacological reduction of inhibitory transmission by intracortical infusion of MPA or Ptx effectively reopens OD plasticity in adult rat visual cortex (Harauzov et al., 2010). Thus, resetting of excitatory/inhibitory balance by a selective reduction of inhibition is a useful approach for stimulating plasticity.

EXPERIENCE-DEPENDENT STRUCTURAL PLASTICITY

So far I have mainly discussed how visual experience exerts its powerful influence on the function of neuronal circuits in the visual cortex. These functional changes are accompanied by extensive reorganization of neuronal structure. In particular, excitatory synaptic structures, such as dendritic spines, are particularly sensitive to experience. A total lack of visual experience early in life (Dark Rearing) induces profound changes in spine morphology and density, partially reversible by subsequent light exposure (Wallace and Bear, 2004). Similarly, several other forms of deprivation, such as monocular deprivation in visual cortex or whisker trimming in barrel cortex, influence spine motility, spine turnover, spine number and spine morphology (Lendvai et al., 2000; Trachtenberg et al., 2002; Majewska and Sur, 2003; Mataga et al., 2004; Oray et al., 2004; Hofer et al., 2009).
In general, dentritic spines are dynamic and motile entities with a remarkable capacity to change rapidly also under normal circumstances. They appear or disappear over days to months (Holtmaat et al., 2005) and they have rapid motility over seconds to minutes (Majewska and Sur, 2003). These characteristics make spines an optimal substrate for circuit plasticity. Changes in synaptic connectivity through the *de novo* growth and retraction of dendritic spines might contribute to functional changes in the brain. Importantly, circuit changes mediated by structural plasticity are thought to underlie aspects of long-term memory formation (Bailey and Kandel, 1993).

Understanding to what extent structural plasticity contributes to experience-dependent rewiring remains an important issue to address (Holtmaat and Svoboda, 2009).

Spine structure and dynamics

Spines are membranous protrusions from the neuronal surface. They consist of a head (volume 0.001–1 μm^3) connected to the neuron by a thin (diameter <0.1 μm) spine neck. They mainly arise from the dendrites but they can be found also in the soma or even in the axon hillock. More than 90% of excitatory synapses terminate on spines; the human brain contains $>10^{13}$ spines. They are structures specialized for synaptic transmission. Neurotransmitter receptors are largely restricted to the surface of the spine and concentrated close to the presynaptic element. This zone is indicated by the postsynaptic density (PSD), a membrane-associated disc of electron dense material.

The PSD consists of receptors, channels, and signaling systems involved in synaptic transmission and plasticity (see below). PSD shape and size are not fixed and may change with alterations in the level of synaptic activity (Desmond and Levy, 1986). The spine head contains the PSD and some other specialized structures such as actin microfilaments and smooth endoplasmic reticulum (SER). Polyribosomes are frequently encountered in spines, for example 82% of spines in the visual cortex have Polyribosomes. Dendritic spines are characterized by an elevated morphological diversity. The most commonly used nomenclature, introduced by Peters&Kaiserman-Abramof in 1970, divides spines into three main categories: *mushroom spines* have a large head and a narrow neck; *thin spines* have a smaller head and a narrow neck; and *stubby spines* without any constriction between the head and the attachment to the shaft. Successively another category was added, the *filopodium*, for its headless hairlike morphology, reminiscent of the axonal filopodium on the axonal growth cone, and, like the axonal version, found mostly during development (Skoff and Hamburger, 1974).

The ability to image dendritic spines in living preparations has revealed that they are extremely dynamic structures (Bonhoeffer and Yuste, 2002). Several groups have imaged dendritic spines in the developing (Lendvai et al., 2000; Grutzendler et al., 2002; Holtmaat et al., 2005) and adult neocortex *in vivo* (Grutzendler et al., 2002;

Trachtenberg et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005; Majewska et al., 2006; Hofer et al., 2009). Spine density and morphology change during development. Spine densities in the mouse neocortex are found to increase during the second and third week of life, followed by a period of net spine pruning and loss (Holtmaat et al., 2005; Zuo et al., 2005). This leads to fairly stable spine densities in the adult brain. Early spines are often very long, and many of these are filopodia. Length decreases with age until reaching mature values. In mature brain large thick mushroom spines are frequent and large spines tend to be more stable than thin spines or filopodia (Trachtenberg et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005). During development spines appear and disappear at a rapid rate and as the brain matures, spine turnover decreases. In adult brain, only a subpopulation of spines continues to turn over (Holtmaat et al., 2005; Zuo et al., 2005). These spines are short lived and appear/disappear over days. All the other spines are most stable persisting for months, probably even for the life of the animals (Grutzendler et al., 2002; Holtmaat et al., 2005; Zuo et al., 2005). The fraction of stable spines varies in different areas and layers of the cortex. Spines in visual cortex are more stable than those in somatosensory cortex (96% and 50% of total spines respectively) (Grutzendler et al., 2002; Trachtenberg et al., 2002; Holtmaat et al., 2005) and in layer II/III of the visual cortex, spines have a higher density and a lower percentage of short lived spines than in layer IV (Holtmaat et al., 2005).

Spine motility, the capacity to change rapidly shape and size, has been proposed to be important in synaptogenesis (Petrak et al., 2005). Some studies suggest that motility and the resulting changes in spine morphology regulate diffusion of the spines biochemical components and regulate spine calcium dynamics (Majewska et al., 2000). Thus motility may be involved in seeking out synaptic partners and in regulating transduction of signals that can then serve to stabilize the connections. Spine motility decreases as development proceeds (Lendvai et al., 2000; Majewska and Sur, 2003).

In conclusion, dendritic spines undergo extensive remodeling in juvenile age but are far more stable in adulthood. The adult cortex consists of fully functioning circuits so a certain level of structural and functional stability would be advantageous. Compared to developing visual cortex, experience-dependent modification of adult cortex is in general less prominent. The relationship between reduction in dendritic

spine dynamics and decline of experience dependent plasticity in adult age has not been clarified yet.

Dendritic spines and sensory experience

The idea that changes in the structure of the brain might occur as a consequence of experience was first proposed by Ramon y Cajal more than a century ago (Ramon y Cajal, 1893). Many successive studies have confirmed this predictive idea. Indeed, changes in spine density, morphology, and motility have been shown to occur with changes in neuronal activity and experience. A bright demonstration has been provided by Engert and Bonhoeffer (1999). Combining the stimulation pattern of LTP induction with two-photon imaging in hippocampal slice culture, they found that induction of LTP in a small dendritic region resulted in the emergence of new spines in the stimulated region. Thus, when the neurons change the efficacy of their connections, there are corresponding structural changes (Engert and Bonhoeffer, 1999). Similar effects on spines have been reported also after sensory stimulation. Indeed, raising rats in an enriched environment (Diamond et al., 1975; Leggio et al., 2005) or deflecting a whisker of a freely moving animal (Knott et al., 2002) leads to an increase in spine and synapse densities.

Many of the studies focused on the experience-dependent changes in spine dynamics have been done in mouse barrel cortex. As I discussed before, with respect to visual cortex, spine population in adult somatosensory cortex appears to be much more transient and less stable. In this system, deprivation by chessboard whisker trimming up-regulates spine turnover, further decreasing the number of stable spines, while overall spine density is unchanged (Trachtenberg et al., 2002). In a successive work it has been demonstrated that the fraction of new persistent spine increases after whisker trimming. This increase is accompanied by a parallel loss of other persistent spines, so the total spine number remains constant. Importantly, new persistent spines always form synapses (Holtmaat et al., 2006). In barrel cortex, trimming a subset of mystacial whiskers causes experience-dependent changes in receptive fields. For example, responses to deflection of the spared whiskers potentiate. This response potentiation is largest for layer V neurons close to the interface between deprived and spared cortical barrel columns. Recently, it has been found that the trimming-induced growth of new persistent spines is especially pronounced in this

border. In addition, in homozygote αCAMKII-T286A mice, which lack experience-dependent potentiation, whisker trimming failed to increase the growth of new persistent spines (Wilbrecht et al., 2010). These findings provide strong support for a role of spine growth in response potentiation.

Evidence for a role of spine growth in experience-dependent plasticity has also come from experiments in the adult mouse visual cortex (Hofer et al., 2009). As discussed above, in adult mouse the MD-induced Ocular Dominance shift is implemented primarily by the strengthening of the open eye responses (Sawtell et al., 2003). A monocular deprivation episode doubled the rate of spine formation, thereby increasing spine density in layer V of binocular visual cortex (Hofer et al., 2009). Also in this case new spine formation during MD seems to be strictly correlated with the potentiation of the responses. Interestingly, spine growth was specific to layer V cells, as in the somatosensory cortex, suggesting a circuit-specific structural plasticity.

In juvenile visual cortex, after four days of MD there is a significant spine loss in layer II/III, consistent with the initial strong reduction of responses elicited by the deprived eye (Mataga et al., 2004). Additional days of MD led to formation of new connections, in keeping with the subsequent strengthening of open eye inputs in juvenile mice (Mataga et al., 2004). Moreover, spine loss after MD is not observed in adulthood, consistent with the absence of depression of deprived eye inputs (Sawtell et al., 2003; Mataga et al., 2004). Taken together these results demonstrated that gain or loss of spines during MD closely parallels the functional consequences of MD and further sustain the idea of a causal link between structural changes and experience-dependent plasticity.

Not only spine turnover and spine density are influenced by sensory experience, several studies have shown that also spine motility changes in response to experience. Binocular deprivation up-regulates spine motility during the peak of the critical period, while after CP it slightly reduces spine motility (Majeska et al., 2003). Brief monocular deprivation induces rapid upregulation of spine motility in mouse visual cortex. This effect seems to be restricted to the critical period, as MD at later stages has a modest effect in reducing spine motility (Oray et al., 2004).

All the studies described above have found a link between structural and functional plasticity studying the effects on spines following alteration of neuronal activity or sensory experience. Another approach to study the relationship between structural and functional changes consists in inducing structural changes and then looking at the functional effects on neuronal circuits. For example, reducing dendritic spines and filopodia densities limits LTP induction in connections from layer IV to layer II/III of mouse visual cortex (Dahlhaus et al., 2008). To induce structural changes it is important to know the key molecular players that control spine dynamics.

Molecular mechanisms of spine plasticity

There are several spine signaling pathways involved in various postsynaptic physiological processes. Among these pathways, there are for example, those controlled by the second messenger Ca^{2+}, by the second messenger cyclic AMP (cAMP) and those regulated by metabotropic receptor and G proteins.

One of the postsynaptic physiological processes crucial for spine dynamics and synaptic plasticity is the regulation of actin cytoskeleton. Spines are enriched in actin (Matus et al., 1982) and spine plasticity is regulated by the rate of actin polymerization (Fischer et al., 1998). A pathway important in controlling actin polymerization involves the Rho family of small GTPases, a subgroup of the Ras superfamily of GTPases (Hall, 1998).

The Small Rho GTPases

The small Rho GTPase family consists of a large number of proteins including Rho, Rac, and Cdc42. These proteins are binary switches that cycle between a GDP-bound inactive and a GTP-bound active state. In response to various extracellular signals, this switch is turned on or off by regulatory proteins (Fig. 1.6). The regulatory proteins of the Rho GTPases include guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAP). Some of these regulators are localized in post synaptic densities (Kang et al., 2009). Several evidences indicate that they mainly act downstream to activation of NMDA and ephrin receptors. Moreover, the low-affinity neurotrophin receptor p75 has been shown to bind directly to Rho and to trigger its activity (Yamashita et al., 1999; Sin et al., 2002; Penzes et al., 2003; Govek et al., 2004; Kennedy et al., 2005). In their GTP bound states, Rho GTPases bind to a variety of effectors to elicit their biological functions. One important common

effector for Rac and Cdc42 is the p21-activated kinase (Pak) while for Rho the best-studied effector is the serine/threonine Rho kinase, ROCK. These effectors act on many substrates and most of them are implicated in the regulation of actin polymerization. Rho and Rac pathways have a common final target: the myosin light chain. Rho activation leads to increased myosin light chain phosphorylation (through ROCK) while Rac activation leads to a decrease in myosin light chain phosphorylation (through inhibition of myosin light chain kinase by Pak). As a consequence, Rho and Rac have a different effect on actin-based motility and this might explain the opposing effect of Rho and Rac observed in many occasions (see below).

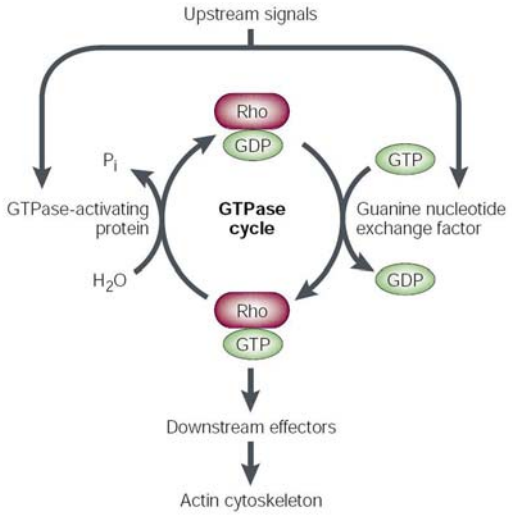


Figure 1.6 Rho GTPases as molecular switches. Upstream signals transduce signals to Rho GTPases through regulation of the activities of guanine nucleotide exchange factors (GEF) or GTPase-activating proteins (GAP), which facilitate switching on or switching off Rho GTPases. In their GTP-bound state, Rho GTPases bind to and activate their effectors to transduce the signal downstream (Luo, 2000).

Rho GTPases, regulating actin cytoskeleton, are involved in many neuronal development processes that require morphological changes, like neuronal migration and axon growth and guidance. In addition, they are implicated in a variety of other cellular processes, such as cell cycle regulation and gene transcription.

Rho GTPases were originally indentified as regulators of the actin cytoskeleton in non-neuronal cells, in which they cause growing (or retracting) of filopodia and lamellipodia (Hall, 1998). Successively, they have been shown to play an important role in dendritic remodeling of *Xenopus* retinal ganglion cells (Ruchhoeft et al., 1999) and tectal neurons (Li et al., 2002).

In a pioneering study of the role of the Rho family on dendritic spines Luo and coworkers engineered transgenic mice that expressed a constitutively active (CA)

form of Rac1 (Rac1-CA) in cerebellar Purkinje cells and found that these cells had a large number of small, supernumerary spines (Luo et al., 1996). Thus, Rho GTPases are involved in spinogenesis. In rat hippocampal slice cultures with maturing neurons tranfected biolistically, Rac1-CA again resulted in the overproduction of spines and membrane ruffling. In contrast, Rac1-DN (dominant negative) produced a reduction in spine density (Nakayama et al., 2000). These results indicate a key role of Rac1 in both the formation and maintenance of spines. In further experiments, using transfected neurons from rodent cerebral cortex and hippocampus, Tashiro et al. (2000) confirmed the Rac results, and additionally showed that RhoACA produced the opposite effect of Rac (Tashiro et al., 2000). Indeed RhoACA produced a reduction in the number of spines, resulting, in some cells, in their complete elimination. Conversely, inhibiting Rho by C3 transferase leads to an increases in spine density, a phenotype that resemble the one of neurons transfected with Rac1CA. Thus, Rac and Rho had differential effects on spine density and spine neck length (Fig. 1.7). Indeed, Rac promotes the development of new spines while Rho appears to block their formation and maintenance as well as their elongation (Tashiro et al., 2000).

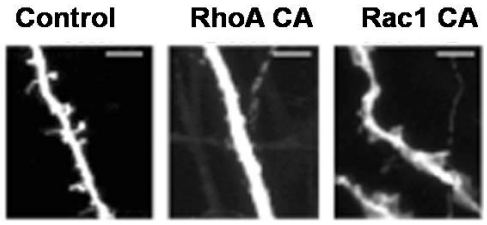


Figure 1.7 Activated RhoGTPase spine phenotypes in rat hippocampal CA1 pyramidal cells in slices expressing constitutively active RhoGTPaese mutant (CA). RhoA CA results in reduced spine protrusions, whereas Rac1 CA causes the formation of lamellipodia /veil like protrusions, likely composed of a multitude of very small, thin spines (Govek et al., 2004).

The different effect of Rho and Rac on dendritic and spine growth is sustained by many other important papers (Threadgill et al., 1997; Li et al., 2000; Sin et al., 2002; Govek et al., 2004). One of these, done in *Xenopus*, demonstrates that Rho GTPase activity is required for the enhancement of dendritic arbor development induced by visual stimulation. In particular, the visual stimulation-induced arbor development was completely blocked by expression of DN forms of Rac and Cdc42 and by

RhoACA (Sin et al., 2002). Moreover, it appears that NMDA activation decreases Rho activity while increasing Rac and Cdc42 activity. Thus, endogenous Rho GTPases activity is modulated by synaptic inputs to regulate dendritic morphology (Li et al., 2002; Van Aelst and Cline, 2004).

Further support to the role of the Rho GTPases family on dendritic protrusions was obtained by Wong and coworkers. They found that Rho decreases the motility of dendritic branchlets while Rac increases it (Wong et al., 2000).

It is worth note that Rac not only is important for spinogenesis but also for inducing the clustering of glutamate receptor in newly formed spines. Thus, Rac is required also to induce the proper formation of new spines (Wiens et al., 2005).

The importance of Rho GTPases for morphogenesis and maturation of dendritic spine and branches has been indirectly demonstrated by modulating the expression of Rho pathway regulators (Penzes et al., 2003; Buttery et al., 2006; Kang et al., 2009; Impey et al., 2010). For example, increased expression of α1-chimaerin, a RhoGAP that inactivates Rac, results in the pruning of dendritic spines and branches. By contrast, suppression of its expression resulted in increased process growth from the dendritic shaft (Buttery et al., 2006). It is interesting to note that mutations in the Rho pathway regulators have been implicated in Mental Retardation syndromes in humans (Luo, 2000). For instance, Oligophrenin-1, a protein with GAP activities for Rho, Rac, and Cdc42, is linked to X-linked mental retardation (Billuart et al., 1998). In addition, Mental Retardation is associated to abnormalities in spine shape and reduced spine densities. It has been demonstrated that knock down of Oligophrenin-1 levels in neurons in rat hippocampal slices significantly decreased spine length. This phenotype can be reproduced using RhoACA and rescued by inhibiting Rho- kinase (Govek et al., 2004). All these data indicate that small RhoGTPases may represent important targets for therapies aimed to ameliorate mental deficits. In addition, considering that spine pathology and alteration in Rho pathway have been observed in association with many brain disorders such as Alzheimer's disease, Parkinson's disease, Prion diseases, schizophrenia and epilepsy, RhoGTPases may be important molecular target also for such pathologies.

CNF1 activates RhoGTPases

CNF1 (cytotoxic necrotizing factor -1), is a bacterial protein toxin produced by *Escherichia coli* that exerts a very specific control of Rho GTPase activity (Flatau et al., 1997; Schmidt et al., 1997).

CNF1 was first described in 1983 by Caprioli and coworkers as a toxin capable of causing multinucleation ("cytotoxic") in cultured cells and necrosis in rabbit skin ("necrotizing") (Caprioli et al., 1983; Caprioli et al., 1984), but the necrotizing effect was very probably due to the presence hemolysin in the original preparation.

CNF1 structure, internalization and enzymatic activity

CNF1 toxin is a 114 KDa single-chain multidomain protein, containing a N-terminal receptor-binding domain and a C-terminal catalytic domain. The two domains are separated by an amino-acidic region consisting of two short membrane spanning helices H1 and H2, which are involved in membrane translocation (Fig. 1.8). CNF1 binds to the surface of cultured epithelial cells with high affinity (Contamin et al., 2000). The exact nature of CNF1 receptor is not totally clear. However, it has been proposed that the ubiquitously expressed laminin receptor (LRP) could be a receptor for CNF1 (Chung et al., 2003; McNichol et al., 2007). In addition, heparansulfate proteoglycan (HSPG) has been identified as a co-receptor for CNF1 (Blumenthal et al., 2007). After binding to its receptor(s), CNF1 enters endocytic vesicles by receptor-mediated endocytosis, which is independent of clathrin. Once inside vesicles, the toxin is routed to the endosomal compartment where its catalytic domain is transferred into the cytosol (Contamin et al., 2000). The cytoplasmic target of CNF1 is represented by Rho GTPases (Fig. 1.8). The enzymatic activity of CNF1 consists in the deamidation of a specific glutamine residue of Rho proteins, which is pivotal for GTPase activity. CNF1 modifies glutamine into glutamic acid and this impairs the action of GAPs. As a consequences, Rho proteins remain permanently activated in their GTP-bound state. The intervention of Ubiquitin-mediated proteasomal degradation can later restores the physiological levels of Rho-GTPases (Doye et al., 2002).

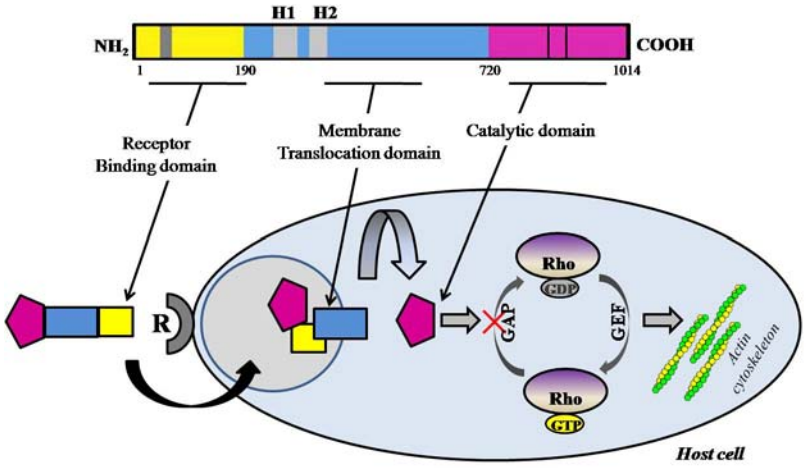


Figure 1.8 Mechanism of action of CNF1. On the top is illustrated the multidomain structure of CNF1. CNF1 binds to the membrane receptor R with its Receptor Binding domain. CNF1 enters endocytotic vesicles by receptor-mediated endocytosis. Once inside the vesicles, the Membrane Translocation domain transfers the Catalytic domain into the cytosol where CNF1 enzymatic activity on RhoGTPases takes place. CNF1 modifies one single aminoacid in the Rho molecules preventing GTP hydrolysis and thus leading to their permanent activation. Thereafter, activated Rho proteins modify actin cytoskeleton.

Effects of CNF1 treatment

By activating Rho proteins, CNF1 induces a remarkable reorganization of the actin cytoskeleton. Human epithelial cells treated with CNF1 show prominent stress fibers, membrane ruffles and filopodia (Fiorentini et al., 1988; Fiorentini et al., 1997). CNF1-treated cultured cells have an extreme flattening of the cell body and acquire a multinucleated phenotype, due to an impairment of cytokinesis (Falzano et al., 1993a).

Interesting results have been obtained following *in vivo* application of CNF1. Diana and coworkers (2007) demonstrated that intracerebroventricular injection of CNF1 in mice, leads to an early similar activaction of Rho and Rac. Ten days later there is a persistent activation of Rac but no longer activation of Rho, likely due to a faster degradation of activated Rho as compared to activated Rac. The net result of CNF1 administration is therefore an alteration of small GTPases signaling with a prominence of Rac signaling. This modulation of cerebral Rho and Rac by CNF1 results in rearrangements of actin in cortical neural cells and in a sustained enhancement of cognitive performances. Indeed, in primary neuronal cells treated with CNF1, the authors found an increase in dendritic surface particularly by spine-

like neo-formations. Interestingly, CNF1 treated mice display an enhanced neurotransmission, enhanced synaptic plasticity, and improved learning and memory in various behavioral tasks (Diana et al., 2007).

Recently, it has been reported that peripheral or central administration of CNF1 potently counteracted formalin-induced inflammatory pain in mice. This analgesic effect is due to CNF1 induced up-regulation of μ-opioid receptors (MORs), the most important receptors controlling pain perception. The CNF1 effects on inflammatory pain were associated with sustained Rac activation and the consequent actin cytoskeleton rearrangement (Pavone et al., 2009).

In conclusion, all these findings highlight the great potential of CNF1 as therapeutic tool for a broad range of brain pathologies. CNF1 may be exploited for the pharmacological control of inflammatory pain (Pavone et al., 2009) as well as for therapy of memory and cognitive disorders (Diana et al., 2007). In particular, the potential pharmacological importance of CNF1 is mainly evident for those pathologies where Rho GTPases signaling and spine morphology are consistently affected, such as Alzheimer's Disease and Mental Retardation.

AIM OF THE THESIS

The aim of this thesis is to investigate some important and unresolved issues in the field of experience-dependent plasticity.

Ocular dominance (OD) plasticity triggered by monocular eyelid suture (MD) is a classic paradigm to study experience-dependent changes in neural connectivity. OD plasticity is high during critical periods and declines in adulthood. Recently, rodents have become the most popular model for studies of OD plasticity. It is therefore important to understand how OD is determined in the rodent primary visual cortex. Cortical cells receive considerable inputs from the contralateral hemisphere via callosal axons, but the role of these connections in controlling eye preference remains controversial. In addition, it has been reported that the organization of callosal fibers is shaped by experience during the critical period, however the functional role of the corpus callosum in cortical plasticity has not been investigated so far.

Another important aspect of plasticity concerns experience-dependent structural changes. It is known that dendritic spines undergo extensive remodeling in juvenile age, but are far more stable in adulthood. It remains unclear, however, whether this reduction in dendritic spine dynamics sets the limit for experience-dependent plasticity in adult age.

My work is composed of three main parts. In a first study I investigated the contribution of callosal connections to OD in rat primary visual cortex during the critical period. I used two complementary experiments: (i) I electrophysiologically measured OD before and after acute silencing of the lateral geniculate nucleus ipsilateral to the recording site; (ii) I measured cortical binocularity before and after acute silencing of callosal connections. The results of these experiments show that the corpus callosum plays an important role in determining OD.

On the basis of these findings, in a second part of my thesis, I investigated the role of the callosum in OD plasticity. I performed an acute silencing of the corpus callosum (via muscimol injection in the contralateral hemisphere) in MD rats. The

data demonstrate that callosal inputs play a key role in functional weakening of less active connections during brain plasticity.

Finally, in a third study I have exploited the bacterial protein toxin CNF1, that regulates actin dynamics and dendritic spine plasticity via a persistent activation of Rho GTPases. I show here that CNF1 treatment increases length and density of dendritic spines, and reinstates OD plasticity in adulthood. These results suggest that promoting structural changes can restore experience- dependent functional plasticity in adult age.

MATERIALS AND METHODS

CNF1 preparation

CNF1 was obtained from the 392 *E. coli* ISS strain (kindly provided by V. Falbo, Istituto Superiore di Sanità, Rome, Italy) and purified as described previously (Falzano et al., 1993b). CNF1 was diluted to a final working concentration of 15 pM with sterile 20 mM TRIS-HCl buffer, pH 7.5. As control I used used either the vehicle solution (TRIS-HCl buffer) or a recombinant toxin (CNF1 C866S) in which the change of cysteine with serine at position 866 abrogates the enzymatic activity on Rho GTPases. The plasmid coding for CNF1 C866S was a kind gift of Prof. Lemichez (U627 INSERM, Nice, France) and the recombinant toxin was purified as described (Falzano et al., 1993b). CNF1 activity was tested on cultures of mouse NIH-3T3 fibroblasts. The toxin was considered active when treated cultures showed a phenotype like that reported previously (Falzano et al., 1993a). As compared to vehicle or CNF1 C866S treated cells, active CNF1 dramatically alters cell morphology: whitin 24 hours of CNF1 incubation cells become giant and multinucleated (Fig. 3.1). For my experiments I only used CNF1 preparations which activity was ensured by this test.

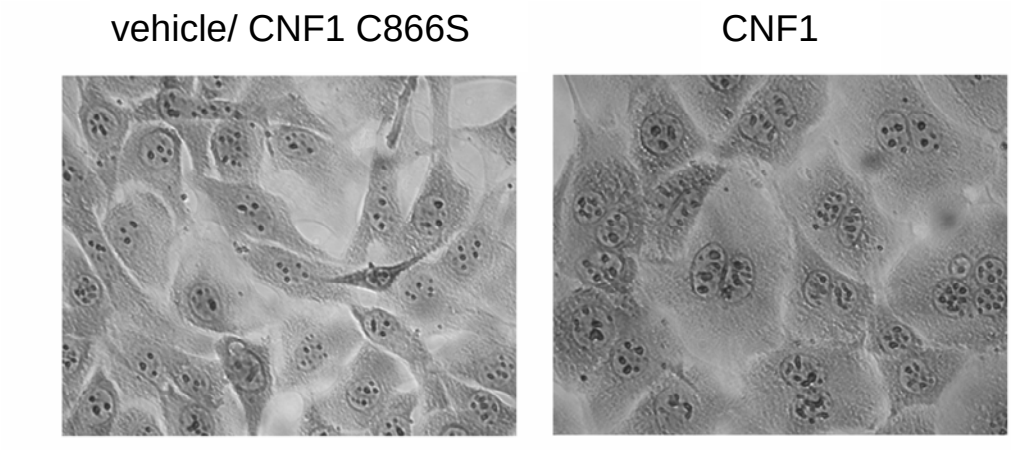


Figure 3.1 Phenotype of NIH-3T3 fibroblasts incubated 24 hours with vehicle (left) or with CNF1 (right). Vehicle or CNF1 C866S addition on culture medium has no effect on fibroblast phenotype while CNF1 treated cells become giant and multinucleated.

Animal treatment and surgical procedures

Long-Evans hooded female rats were used in this study. Animals were reared in a 12 hr light/dark cycle, with food and water available *ad libitum*. All experimental procedures conformed to the European Communities Council Directive n° 86/609/EEC.

I used 36 naïve animals during the critical period (age range: P26 – P31). An additional 20 rats were monocularly deprived for seven days starting at P20-P23. 55 adult rats (age > P90; naïve, n = 24; monocularly deprived, n = 31) were also used. Monocular deprivation was performed by eyelid suture under isoflurane anesthesia. MD animals were carefully inspected every day to make sure that the lid suture remained intact. The deprived eye was re-opened using thin scissors at the time of recording.

I performed microinjection of TTX (1 µl; 30 µM solution, Sigma) or saline into the dorsal geniculate nucleus (dLGN), microinjection of muscimol (1 µl; 30 mM solution, Sigma) and microinjection of CNF1 (3 µl of a 15 pM solution) or control solution into the cortex with a glass pipette (tip diameter, 40 µm) mounted on a micromanipulator. Coordinates for geniculate injection were as follows: 2.2 mm anterior and 3.3 mm lateral with respect to lambda, and 3.8 - 4.5 mm deep from the pial surface. For intracortical muscimol injections, the pipette was positioned 4.5 - 4.7 lateral and in correspondence with lambda, and the muscimol solution was slowly delivered at a depth of 0.6 – 1 mm from the pial surface (Restani et al., 2009). CNF1/vehicle injections were performed in three sites: 4.0 lateral and 1 mm anterior, 1mm posterior, and in correspondence with lambda. CNF1 was slowly delivered at a depth of 1-1.2 mm from the pial surface.

Electrophysiology

Geniculate recordings

Recordings were performed as described (Mandolesi et al., 2005). Rats were anesthetized with urethane (7 ml/kg; 20% solution in saline, i.p.; Sigma) and placed in a stereotaxic apparatus. Both eyes were fixed by means of adjustable metal rings surrounding the external portion of the eye bulb, and optic disk locations were projected onto a tangent screen to determine the vertical meridian. Body temperature during the experiments was constantly monitored with a rectal probe and maintained

at 37°C with a heating blanket. Electrocardiogram was also continuously monitored. After exposure of the cerebral surface, a glass micropipette (tip resistance, 2 MΩ) filled with 3 M NaCl was inserted into the brain at the appropriate stereotaxic coordinates (2.2 mm anterior and 3.3 mm lateral from lambda). The first evoked visual activity was usually encountered at a depth of 3.6 mm from the pial surface and had an audible ‘‘swish’’, characteristic of discharges from fibers of the optic tract. Receptive fields of single geniculate units were mapped onto a tangent screen by using an ophtalmoscope. I verified that cells were driven by the contralateral eye and had their RFs within 20º of the vertical meridian, and in the upper visual field. Computer-controlled visual stimuli were presented on a monitor with a mean luminance of 15 cd/m2. Visual stimuli consisted of sinusoidal gratings (spatial frequency 0.07 cycles/deg, contrast 90%, frequency of alternation 4 Hz). Signals were amplified 25.000-fold, bandpass filtered (300–5000 Hz), and conveyed to a computer for storage and analysis. The stimulus grating was presented at least 50 times, and responses were averaged to produce peristimulus time histograms (PSTHs). The dLGN was injected with TTX, and after a delay of 30 minutes we started to record again from the same site in the geniculate.

Visual cortex recordings

Rats were anesthetized with urethane and placed in a stereotaxic apparatus as described above. A portion of the skull overlying the binocular visual cortex was carefully drilled on both sides of the skull. In a first series of experiments, we mapped the effects of muscimol injection on cortical activity. Single units were recorded in a series of penetrations on one side. Then I injected muscimol in the binocular cortex. After 40 minutes I started to record again, having care to map the entire binocular area around the muscimol infusion site.

For the analysis of the callosal contribution to OD, in a first sets of experiments I recorded either VEP or single units. The ipsilateral dorsal lateral geniculate nucleus (dLGN) was then injected with saline or TTX. After a delay of 30 min, I started to record VEP and single units again from the cortex. At the end of the experiment, muscimol injection was performed into the cortex contralateral to the recording site, and VEPs were recorded starting from 40 min later. In a second sets of experiments I measured OD with single units recordings in näive or in MD (juvenile and adult),

rats and then I injected saline or muscimol into the cortex contralateral to the recording site. After 40 min I started to record single units again. All the recording were made in two- three penetrations per animal on one side. Care was taken to record at the same coordinates (4.5-4.7 mm lateral from lambda, in correspondence with the cortical representation of the vertical meridian) before and after TTX or muscimol injection in each animal.
For CNF1 experiments I analyzed OD in adult rats using either VEP or single units recorded from four- five penetrations per animal in one side.

For VEP recordings, the electrode was typically positioned at a depth of 100 μm within the cortex. In some animals, recordings were also performed at a depth of 400 μm and yielded the same results, consistent with a single major VEP dipole source in visual cortex (Restani et al., 2009). Transient VEPs were recorded in response to abrupt reversal (1 Hz) of a horizontal square wave grating (spatial frequency 0.07 c/deg, contrast 90%), generated by computer on a display (Sony; subtending 110x85 degrees of visual angle; mean luminance 15 cd/m2) by a VSG card (Cambridge Research System). Signals were amplified (10,000 fold), bandpass-filtered (0.1 – 500 Hz) and fed to a computer for storage and analysis. At least 50 events were averaged in synchrony with the stimulus contrast reversal. VEPs in response to a blank stimulus (0% contrast) were also frequently recorded to estimate noise. VEP amplitude was quantified for each eye by measuring the peak to trough amplitude, as described previously (Porciatti et al., 1999; Restani et al., 2009; Cerri et al., in press). Analysis of the amplitude of VEP responses was performed blind to experimental treatment.

Extracellular recordings of spiking activity were performed from supragranular layers (i.e. at a depth less than 800 μm from the cortical surface). The visual stimulus consisted of a computer-generated bar (contrast, 90%; thickness, 3°; speed, 25°/sec) presented on a monitor (Sony; mean luminance 15 cd/m2). Signals were amplified 25,000-fold, bandpass filtered (300-5000 Hz), and conveyed to a computer for storage and analysis. Action potentials were discriminated from background by a voltage threshold, that was set as 4.5 times the standard deviation of noise, as described (Caleo et al., 2007). Spontaneous activity and peak response were

determined from peristimulus time histograms (PSTHs; bin size = 33 msec) of the cell response to the stimulus, averaged over 20 consecutive stimulations as described (Lodovichi et al., 2000; Mandolesi et al., 2005; Caleo et al., 2007).

Peak response was evaluated as the peak firing rate (spikes per second) in the cell response to the stimulus.

Ocular dominance was evaluated according to the methods of Hubel and Wiesel (Hubel and Wiesel, 1962). Neurons in ocular dominance class 1 were driven only by stimulation of the contralateral eye; neurons in ocular dominance class 2/3 were binocular and preferentially driven by the contralateral eye; neurons in ocular dominance class 4 were equally driven by the two eyes; neurons in ocular dominance class 5/6 were binocular and preferentially driven by the ipsilateral eye; neurons in ocular dominance class 7 were driven only by the ipsilateral eye. For each animal, the bias of the OD distribution toward the contralateral eye [contralateral bias index (CBI)] was calculated as follows: $CBI = [(N_{(1)}-N_{(7)})+1/2\ (N_{(2/3)}-N_{(5/6)})/N_{TOT}]/2N_{TOT}$, where $N_{(i)}$ is the number of cells in class i, and N_{TOT} is the total number of recorded cells in a specific animal.

In CNF1 experiments to obtain a finer and statistically more robust comparison of OD distributions I computed the normalized OD score of single neurons (Rittenhouse et al., 1999) and plotted the cumulative distribution for each experimental group. OD score was computed on cells with complete PSTH analysis of peak and baseline spiking activity after closure of either eye. OD score was defined as {[Peak(ipsi)-baseline(ipsi)]–[Peak(contra)-baseline(contra)]}/{[Peak(ipsi)-baseline(ipsi)] +[Peak(contra)-baseline(contra)]}.

Pull-Down Assay

A total of 8 CNF1- and 8 vehicle-injected rats were examined. Visual cortex samples were dissected, frozen in dry ice and processed for a pull-down assay to determine levels of activated Rac1. The test is based on the ability of activated Rac1 to bind its effector PAK. Samples were homogenized in 50 mM Tris (pH 7.4), 1 mM EDTA (pH 8.0), 0.5% Nonidet P-40, 150 mM NaCl, 10% glycerol, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1 mM PMSF and processed for pull-down assay as previously described (Travaglione et al., 2005; Diana et al., 2007; Pavone et al., 2009). Tissues extracts were incubated with 50 mg of GST-PAK70-106 fusion protein bound to

glutathione-coupled Sepharose beads (Amersham Pharmacia) for 40 min at 4°C. Beads were washed three times in the lysis buffer and bound proteins were eluted in sample buffer, subjected to SDS/PAGE and immunoblotted as described (Travaglione et al., 2005; Diana et al., 2007; Pavone et al., 2009).

Dendritic spine analysis

Adult rats injected with CNF1 (n = 11) or vehicle solution (n = 10) were deeply anesthetized and perfused through the heart with 4% paraformaldehyde 10 days after injection. A block of visual cortex was sectioned in the coronal plane into 300 μm thick slices by using a vibratome (Leica). The lipophilic dye DiI (1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine; Invitrogen) was coated onto tungsten particles (diameter, 1.1 μm; Bio-Rad) according to Gan et al. (Gan et al., 2000). 1,1′-dioctadecyl-3,3,3′,3-tetramethylindocarbocyanine-coated particles were delivered to the slices by using a Helios Gene Gun System (Bio-Rad). A polycarbonate filter with a 3.0-μm pore size (Molecular Probes) was inserted between the gun and the preparation on a platform to remove clusters of large particles. Density of labeling was controlled by gas pressure (80 psi of helium). After labeling, slices were fixed in 4% paraformaldehyde. Labeled structures were analyzed with confocal microscope (63 X oil immersion objective; numerical aperture 0.9, 3X zoom). Two-four labeled pyramidal neurons per animal were randomly selected from layer II–III in the binocular zone of the visual cortex. Images of basal and apical dendrites were acquired, stacked (0.5-μm step), and then analyzed with Metamorph (Molecular Devices) blind to the treatment. All protrusions along each dendrite were counted and measured in length. Specifically, we measured the length of the spine neck (from the dendritic shaft to the beginning of the spine head). Spine density was calculated in the interval 0-100 μm from the neuronal soma. A total of 31 neurons/2,356 spines and 21 neurons/1,761 spines were analyzed in CNF1- and vehicle-treated rats, respectively.

Immunoblotting for vGLUT1 and GAD65/67

We injected CNF1 (n = 8) or vehicle solution (n = 8) in the visual cortex of > P90 rats. Their visual cortices were dissected 10 days after injection. Proteins were extracted (Viegi et al., 2002) with lysis buffer (1% Triton X-100, 10% glycerol, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.1 mM Na3VO4, 1 μg/ml

leupeptin, 1 μg/ml aprotinin, and 1 mM PMSF). Protein extracts (10 μg) were separated by electrophoresis and blotted, and filters were incubated with an antibody specific for the vGLUT1 (1:2000 dilution; Synaptic System) or with an antibody recognizing GAD65 (1:5000 dilution; Sigma). Blots were then reacted with HRP-conjugated secondaries (Bio-Rad, Hercules, CA) and developed by ECL (Amersham Biosciences, Little Chalfont, UK). Filters were also probed with anti-α-tubulin monoclonal antibody (1:10000 dilution; Sigma), which serves as an internal standard for protein quantification. Levels of vGlut-1 and GAD65/67 were determined as described previously (Antonucci et al., 2008; Mainardi et al., in press).

Anatomical analysis

Rats were perfused 10 days after injection of CNF1 (n = 8) or control solution (n = 4). A group of naïve, untreated rats was also included (n = 4). Serial cortical sections (one out of three, 40 μm thick, cut on a freezing microtome) were Nissl stained for the evaluation of cortical thickness. The remaining serial sections were immunostained with the neuron-specific anti-NeuN (mouse monoclonal, Chemicon) or with OX-42 (mouse monoclonal, Pharmingen) antibodies. Briefly, sections were blocked with 10% normal goat serum + 0.3% Triton X-100 in PBS and then incubated overnight with the primary antibodies diluted 1:500. On the following day, sections were reacted with a biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) followed by avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) and diaminobenzidine (DAB) reaction. Cortical thickness and neuronal and microglial density were measured in five coronal sections per animal using Stereo Investigator software (Microbrightfield, Colchester, VT). Analysis was performed blind to experimental treatment.

Cortical thickness: In each section, I measured the distance from the pial surface to white matter. These values were averaged to obtain cortical thickness in individual animals.

Density of NeuN- and OX-42 positive cells: NeuN- and OX-42-positive cells were counted in three-dimensional counting boxes (100 μm x 100 μm x 20 μm) positioned in layer II/III primary visual cortex. An average of 450 NeuN-positive and 340 OX-42-positive cells were counted for each experimental animal. Cell density was calculated by averaging values obtained from at least 15 counting boxes per animal.

Statistical Analysis

Statistical analysis was performed with SigmaStat (version 3.1). A paired t-test was used for all comparisons before/after TTX (or saline) injection. In single unit recording experiments, cells were assigned to one of five OD classes (see above). Therefore, I employed a χ^2 test (four degrees of freedom) to compare OD distributions before/after silencing of dLGN and before/after muscimol injection in the contralateral hemisphere in näive and in MD (both juvenile and adult) (Caleo et al., 2007; Restani et al., 2009). The statistical analysis of the peak response of single units was made with Kruskal-Wallis one way ANOVA (ANOVA on ranks) with Dunn's post hoc test.

For CNF1 experiments, differences between two groups were assessed with Student's t-test for data normally distributed, and with Mann-Whitney rank sum test for data non normally distributed. Statistical analysis of pull-down experiments was made with a paired t-test. Differences between three groups were evaluated with one way analysis of variance (ANOVA) followed by Holm-Sidak test. Cumulative frequency distributions were compared with the Kolmogorov-Smirnov test. Differences between OD histograms were assessed using a χ^2 test (four degrees of freedom).

RESULTS

CALLOSAL CONTRIBUTION TO OCULAR DOMINANCE IN RAT PRIMARY VISUAL CORTEX

I examined the role of callosal connections in binocularity of the visual cortex in naïve young rats (P26-P31) using two complementary experiments. In the first one I electrophysiologically measured OD before and after acute silencing (via stereotaxic TTX injection) of the lateral geniculate nucleus ipsilateral to the recording site. This protocol allowed me to isolate visual responses transmitted via the corpus callosum. In the second experiment I measured ocular dominance before and after the silencing of the callosal pathway, performed by muscimol injection into the visual cortex contralateral to the recording site.

TTX EXPERIMENT

Experimental protocol

The experimental protocol is summarized in Fig. 4.1A. I measured binocularity (via either VEP or single unit recordings) in the primary visual cortex of young rats before and after silencing of the lateral geniculate nucleus ipsilateral to the recording site. Blockade of visual afferent input from the geniculate was achieved via stereotaxic injection of TTX. This allowed me to isolate visual responses conveyed via the sole callosal route. As a control, in a series of animals I examined binocularity before and after infusion of saline into the ipsilateral dLGN. All recordings were made in correspondence with the cortical representation of the vertical meridian, where callosal afferents terminate most densely (Cusick and Lund, 1981; Mizuno et al., 2007). To make sure that administration of TTX completely silenced the thalamocortical pathway, I injected muscimol into the visual cortex contralateral to the recording site at the completion of each experiment. Injection of muscimol completely abolished visual responses in the opposite side, confirming that all residual cortical responses were due to callosal input activity (see Fig. 4.1B).

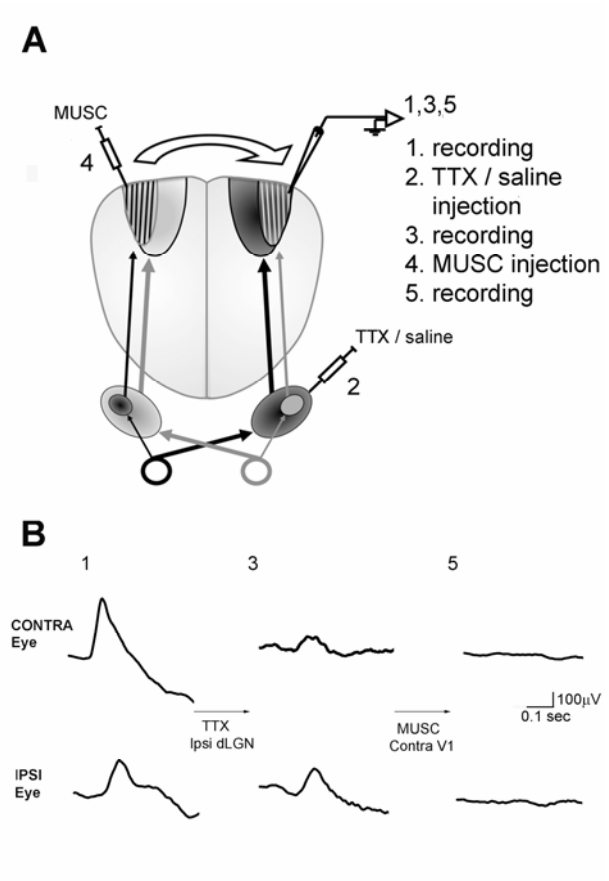


Figure 4.1 Experimental protocol. (A) Schematic diagram of the rat visual system and description of the experimental protocol. The striped areas indicate the binocular portion of the primary visual cortex in both hemispheres. Binocularity was recorded before and after injection of either saline or TTX into the ipsilateral dorsal lateral geniculate nucleus (dLGN). A further injection of muscimol (MUSC) was performed into the visual cortex (V1) contralateral to the recording side to block callosal input. (B) Representative examples of VEP responses for both eyes, before and after TTX administration to the ipsilateral dLGN (1,3) and after muscimol injection into contralateral V1 (5). TTX injection into the dLGN causes a strong decrease of the contralateral-eye VEP amplitude and a less marked reduction of the ipsilateral eye response (3). Subsequent muscimol injection into the contralateral visual cortex completely flattens visual responses from both eyes (5). Visual stimulus: square-wave grating alternating at 1 Hz, spatial frequency 0.07 c/deg, contrast 90%. CONTRA, contralateral eye; IPSI, ipsilateral eye.

TTX and muscimol injections silence activity within the geniculate and cortex

In a preliminary series of experiments, I controlled that injection of TTX and muscimol was effective in silencing activity within the geniculate and cortex. I performed these experiments by recording visual responses before and after local delivery of the blockers. I found that injections of TTX into the dLGN (targeted at the area mapping the central part of the visual field) rapidly abolished the spiking activity of geniculate cells. One of such experiments is shown in Fig. 4.2. Fig. 4.2A reports a typical progression of the receptive field (RF) centers of single geniculate units along a micropipette track; cells were stimulated with sinusoidal gratings of optimal spatial frequency and contrast (Fig. 4.2B, top). Such responses were completely abolished within 30 min of local delivery of TTX (Fig. 4.2B, bottom). Similarly, injection of muscimol was very effective in silencing activity within the cortex. Fig. 4.3 reports the results of a representative experiment in which we recorded single unit responses in a series of penetrations across the binocular portion of primary (V1) and secondary (V2) visual cortex. The mapping was performed in the same animal before and after muscimol injection (asterisk) at the V1/V2 border. I found that all the locations indicated in Fig. 4.3A became completely unresponsive following delivery of muscimol (Fig. 4.3C), and the blocking effect was apparent starting from 40 min after injection (Caleo et al., 2007; Restani et al., 2009). Some residual activity was still detectable after muscimol in the most medial part of V1 (i.e., at a distance > 1.8 mm from the injection site), corresponding to the monocular area (data not shown). Thus, visual responses in the entire binocular zone were eliminated by muscimol injection (Fig. 4.3).

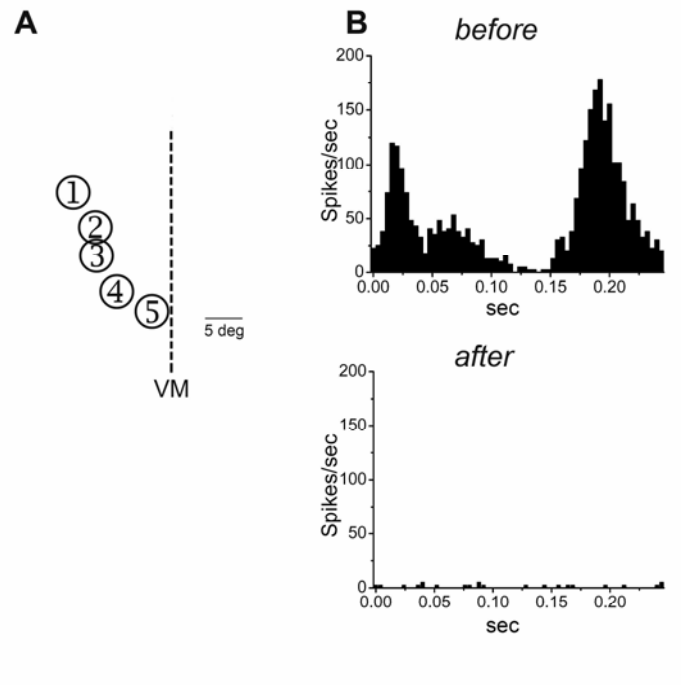


Figure 4.2 TTX injection silences geniculate activity. (A) Typical progression of the RF centers of single geniculate units along a electrode track. The circles indicate the RF centers, and the numbers indicate the order in which these neurons were found as the micropipette was moved dorsoventrally. The cells were recorded at a distance of 60-100 μm from each other. VM, vertical meridian. (B) Representative response of geniculate cells to the presentation of a sinusoidal grating (spatial frequency 0.07 cycles/deg, contrast 90%, temporal frequency of alternation 4 Hz), before (top) and after TTX injection into the geniculate (bottom). TTX administration completely blocks the spiking activity of geniculate units.

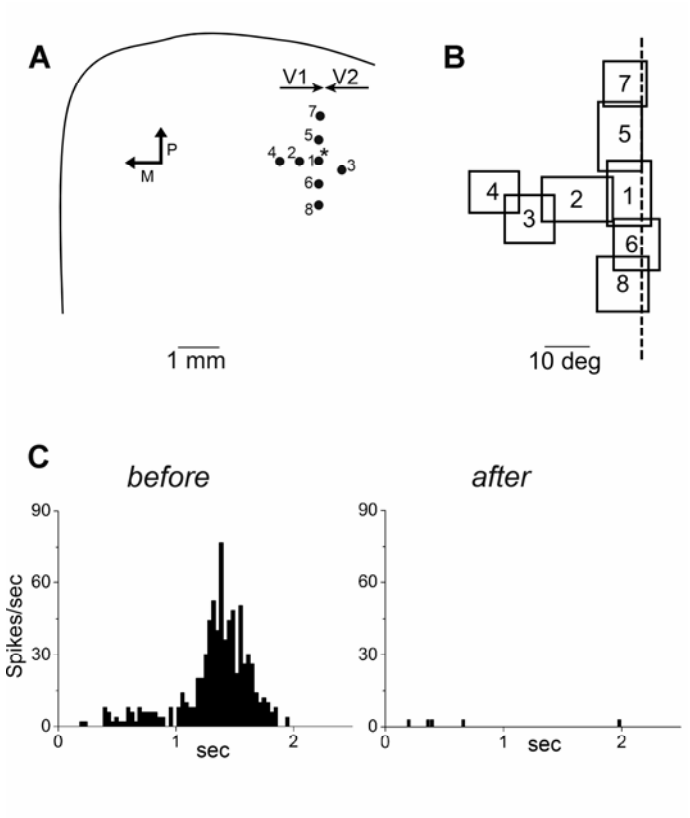


Figure 4.3 Silencing of the visual cortex after muscimol delivery. (A-B) Representative physiological mapping of the effects of muscimol in a P27 rat. Panel A is a diagram showing the location of a series of penetrations made in the visual cortex of one hemisphere. The muscimol injection sites is indicated by an asterisk, and the vertical line on the left corresponds to the midline. M, medial; P, posterior; V1, primary visual cortex; V2, secondary visual cortex. Receptive fields encountered in the penetrations shown in (A) are plotted in panel B. Note that when the electrode was moved into V2 (track #3), there was a shift in receptive field position away from the vertical meridian (dotted line). (C) Example of Peristimulus Time Histograms (PSTHs) recorded from cortical units before and after muscimol delivery. Visual response is abolished following application of muscimol. Similar findings were obtained at all locations shown in panel A. Visual stimulus: passage of a light-bar stimulus (contrast, 90%; thickness, 3°; speed, 25°/sec) across the visual field.

Contra/Ipsi VEP ratio before and after silencing of the geniculocortical pathway

I initially measured ocular dominance (OD) in primary visual cortex by calculating the contralateral-to-ipsilateral (C/I) VEP ratio, i.e. the ratio of VEP amplitudes recorded by stimulating the contralateral and ipsilateral eye. VEPs represent the integrated response of cortical neurons to patterned visual stimuli and are routinely used to assess changes in binocularity (Porciatti et al., 1999; Frenkel and Bear, 2004; Sale et al., 2007; Maya Vetencourt et al., 2008). VEPs are particularly useful since they allow to determine absolute levels of responses of the two eyes, in addition to

ratios (Frenkel and Bear, 2004; Restani et al., 2009). In normal young rats, I found that the C/I VEP ratio was about 2. Injection of saline solution into the ipsilateral geniculate had no significant effect on binocularity (C/I VEP ratio before vs. after saline, paired t-test, $P = 0.34$; Fig. 4.4A). In contrast, silencing the ipsilateral geniculate with TTX produced a dramatic reduction of visual responses, with the C/I ratio dropping down to 0.46 ± 0.07 (before vs. after saline, paired t-test, $P = 0.002$; see Fig. 4.1B and 4.4B). Thus, a significant shift of OD towards the ipsilateral eye was apparent following inactivation of the ipsilateral dLGN.

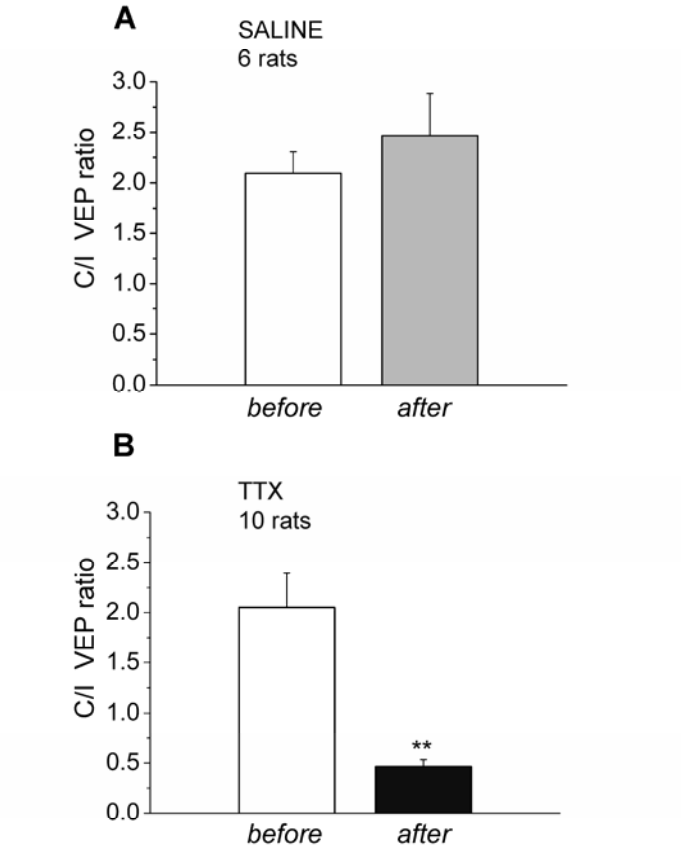


Figure 4.4 Cortical binocularity before and after silencing of the geniculocortical pathway. Contralateral to Ipsilateral (C/I) VEP ratio in normal young rats before and after injection of saline (A) or TTX (B) into the geniculate. Saline had no effect on binocularity (A, P = 0.34), while rats injected with TTX showed a dramatic reduction of the C/I ratio (B, P < 0.01). Data are mean ± SEM. Number of animals as indicated. **, paired t-test, P < 0.01.

VEP amplitude and latency for the contralateral and ipsilateral eye after silencing of the geniculocortical pathway

It was of interest to determine how the strength of each eye in driving cortical neurons was modified by silencing the thalamocortical pathway. The results were clear in demonstrating an overall decrease of visual responsiveness, with a much more dramatic reduction of contralateral eye strength following TTX (see representative VEPs in Fig. 4.1B). Quantification of absolute VEP amplitudes in the different animals indicated a very robust decrement of visual drive through the contralateral eye (contralateral eye, VEP amplitude, before vs. after TTX, paired t-test, $P = 0.002$; Fig. 4.5A). This is consistent with the idea that most of the influence of the contralateral eye on cortical neurons arrives via the direct geniculocortical route. Ipsilateral eye inputs were also reduced, but to a lesser extent, after TTX administration (ipsilateral eye, VEP amplitude, before vs. after TTX, paired t-test, $P = 0.006$; Fig. 4.5B). These data indicate that, in addition to the geniculocortical pathway, a significant input from the ipsilateral eye to cortical cells is provided via callosal connections. The silencing of the geniculocortical pathway also produced a dramatic increase of the latency of the visual responses in the visual cortex. In the recordings performed before TTX, I noted that the latency of the major positive VEP component (recordings at 100 μm depth, see Fig. 4.1B) was consistently greater for the ipsilateral than for the contralateral eye (ipsilateral eye: 156.8 ± 6.4 ms; contralateral eye: 135 ± 5.7 ms; paired t-test, $P = 0.002$; Fig. 4.5C). VEP latency increased for both eyes following TTX administration into the geniculate (contralateral eye, 135 ± 5.7 ms before TTX vs. 186.4 ± 6 ms after TTX, paired t-test, $P < 0.001$; ipsilateral eye, 156.8 ± 6.4 ms before TTX vs. 193.8 ± 9.4 ms after TTX, paired t- test, $P = 0.002$; Fig. 4.5C). This increase was more marked for the contralateral eye, and inactivation of the dLGN abrogated the difference in latency between the contralateral and ipsilateral eye (paired t-test, $P = 0.5$; Fig. 4.5C). The effect on latency was specific for TTX administration since control animals injected into the geniculate with saline solution showed no significant change in VEP latency (contralateral eye, paired t-test, $P = 0.10$; ipsilateral eye, paired t-test, $P = 0.14$).

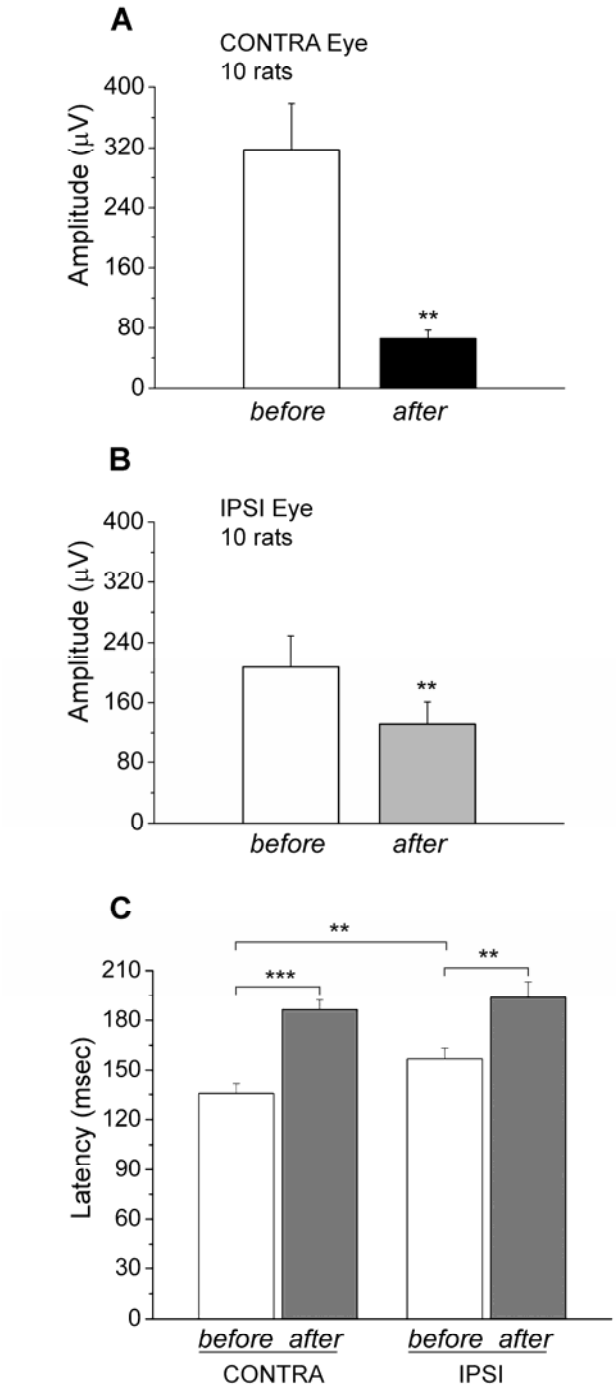


Figure 4.5 VEP amplitude and latency for the contralateral and ipsilateral eye after silencing of the geniculocortical pathway. (A, B) Quantification of absolute VEP amplitudes before and after injection of TTX into the ipsilateral geniculate. Following TTX injection, contralateral-eye VEP amplitude was dramatically decreased (A, $P < 0.01$). Ipsilateral-eye VEP amplitude was reduced but to a lesser extent (B, $P < 0.01$). Data are mean ± SEM. Number of animals as indicated. (C) Increase in VEP latency after TTX delivery. The graph reports the mean peak latency of VEP responses evoked by stimulation of the contralateral and ipsilateral eye in normal young rats before and after

injection of TTX into the geniculate. Before TTX, VEP latency was consistently greater for the ipsilateral than for the contralateral eye ($P < 0.01$). Silencing the geniculate with TTX produced a significant increase in VEP latency for both eyes ($P < 0.001$ for contralateral eye and $P < 0.01$ for ipsilateral eye) and abolished the difference in latency between the contralateral and ipsilateral eye ($P = 0.5$). Data are mean ± SEM. For each histogram, n = 10 rats. **, paired t-test, $P < 0.01$; ***, paired t-test, $P < 0.001$.

Binocularity of cortical units before and after silencing of the geniculocortical pathway

To determine the contribution of callosal afferents to cortical spiking activity, I also performed extracellular recordings of single unit discharges from the binocular visual cortex before and after inactivation of the ipsilateral dLGN. OD was quantitatively assigned to each unit according to a five point scale (Maffei et al., 1992; Lodovichi et al., 2000) and was based on the computer-calculated peak firing rate in response to stimulation of each eye with a light bar drifting into the receptive field (RF; Mandolesi et al., 2005; Caleo et al., 2007). The OD distribution recorded before TTX was biased towards the contralateral eye, as expected (Fig. 4.6A); administration of TTX clearly skewed the OD histogram towards the ipsilateral eye. The difference in the OD histograms before/after TTX was highly significant (χ^2 test, $P < 0.001$). The OD histogram of a single animal can be summarized by the contralateral bias index (CBI), that indicates the degree of dominance of the contralateral eye. CBI dropped from an average of 0.68 ± 0.02 before TTX to 0.33 ± 0.07 after TTX (paired t-test, $P = 0.024$; Fig. 4.6B). These data are in line with those obtained by VEP recordings and further reinforce the idea that callosal connections mainly carry visual input from the ipsilateral eye.

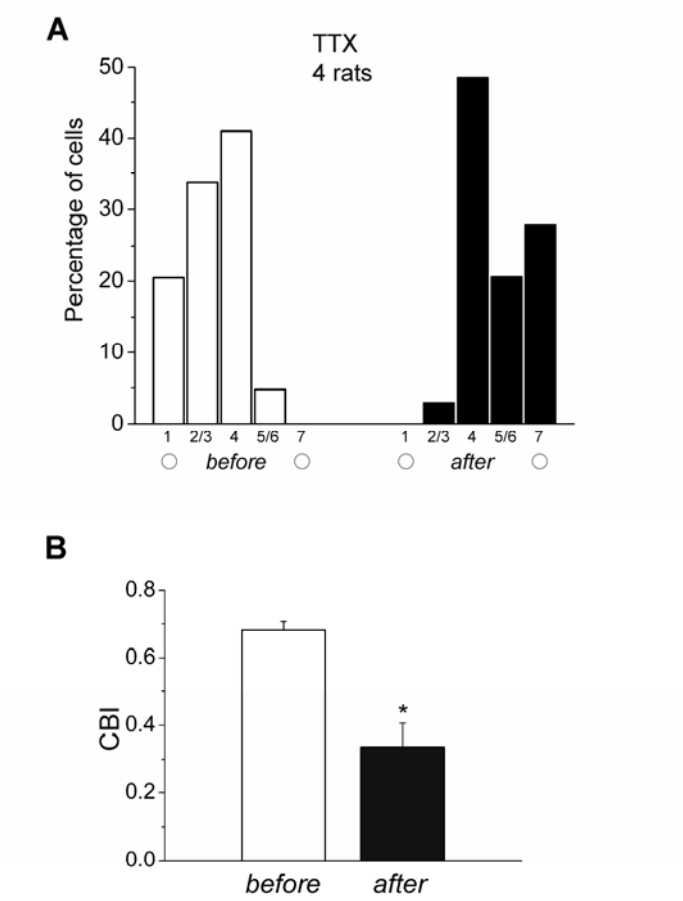


Figure 4.6 Binocularity of cortical units before and after silencing of the geniculocortical pathway. (A) OD distributions of normal young rats before and after injection of TTX into the geniculate. Silencing of geniculo-cortical pathway determined a clear increase in the number of units driven exclusively by the ipsilateral eye (χ^2 test, $P < 0.001$). Number of animals as indicated. Before TTX, n = 83 cells; after TXX, n = 68 cells. B) CBIs of all treated animals. Data are mean ± SEM. There is a significant decrement of contralateral bias after TTX (*, paired t-test, $P < 0.05$).

MUSCIMOL EXPERIMENT

Experimental protocol

I compared binocularity of cortical cells before and after silencing of the visual cortex contralateral to the recording site (Fig. 4.7A). The spiking activity of cortical neurons was recorded extracellularly in two-three penetrations in one hemisphere. Activity was recorded from superficial layers and in correspondence with the cortical representation of the vertical meridian (i.e., at the border between area 17 and 18), where callosal inputs terminate most densely (Cusick and Lund, 1981; Mizuno et al.,

2007). After recording, I injected either the GABAA agonist muscimol (1 ml; 30 mM solution) or saline into the contralateral hemisphere. Muscimol blocked activity in the infused side within 30 min (Fig. 4.7B). After this period I started to record from single units again in the opposite cortex (Fig. 4.7A).

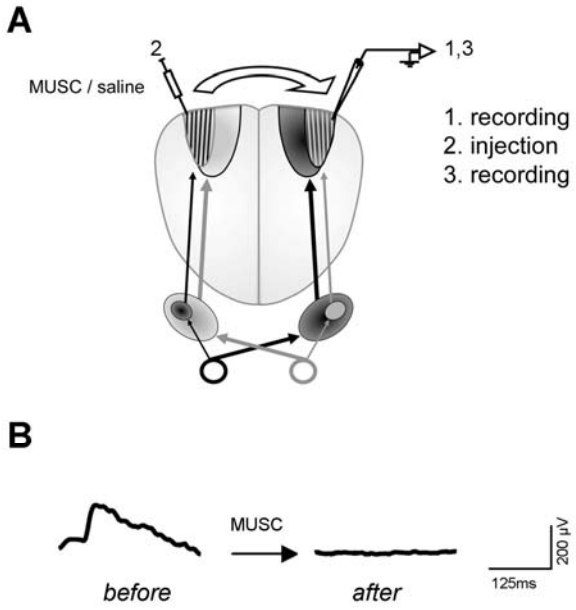


Figure 4.7 Experimental protocol (A) Schematic diagram of the rat visual system and description of the experimental protocol. The striped areas indicate the binocular portion of the primary visual cortex in both hemispheres. Binocularity was recorded before and after injection of either saline or muscimol (musc) into the contralateral cortex. (B) Representative examples of VEP recordings demonstrating blockade of injected visual cortex after muscimol (musc) delivery.

Cortical binocularity before and after silencing of callosal connections

I found that the OD distribution of cortical neurons significantly shifted toward the contralateral eye following muscimol, but not saline, injection. Saline infusion into the opposite side had no effect on binocularity, as shown by analysis of both OD distributions (χ^2 test, naive before saline versus after saline, $p = 0.48$; Fig. 4.8A) and contralateral bias index (CBI; postANOVA Holm-Sidak test, $p = 0.82$; Fig. 4.8C). Conversely, muscimol injection increased the proportion of class 1 cells and led to a corresponding decrease of binocular units (χ^2 test, naive before muscimol versus after muscimol, $p < 0.001$; Fig. 4.8B). Accordingly, CBIs were significantly higher

following muscimol (one-way ANOVA, $p < 0.001$; post hoc Holm-Sidak test, $p < 0.001$; Fig. 4.8C). I conclude that acute silencing of callosal input affects cortical OD.

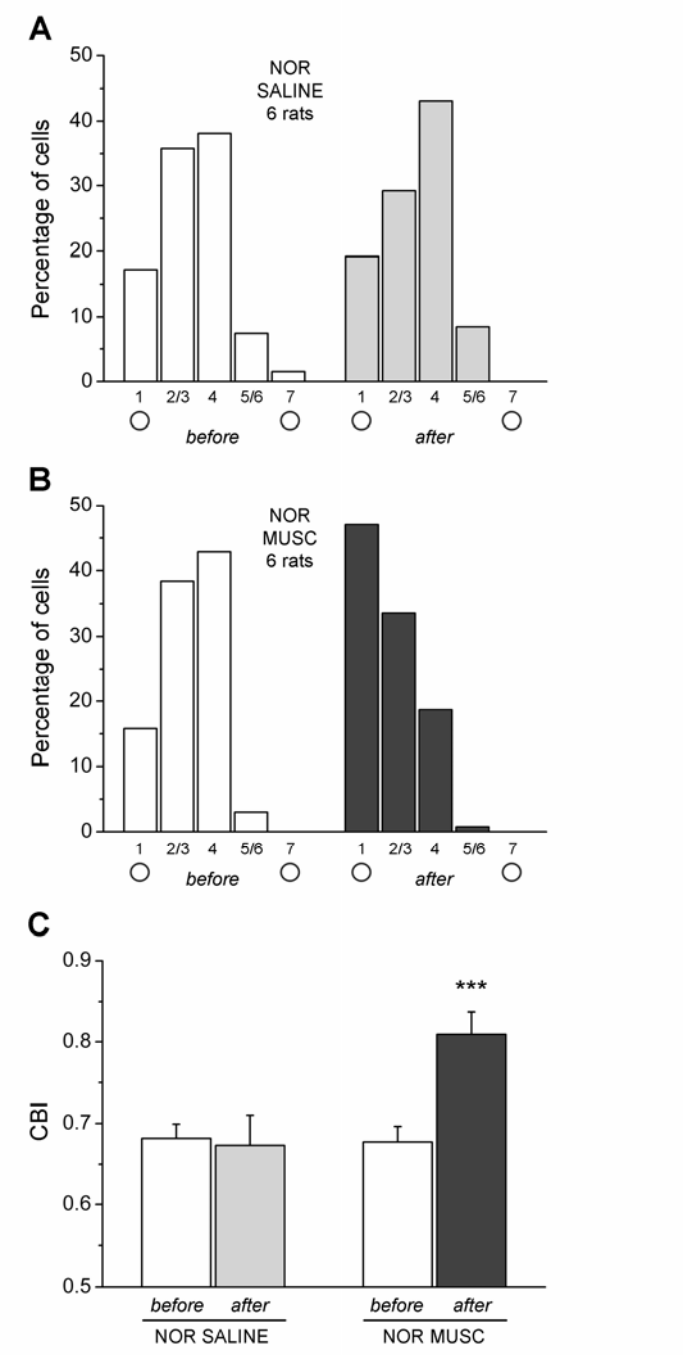


Figure 4.8 Contribution of callosal inputs to cortical binocularity. (A and B) OD distributions of normal (NOR) young rats (age P26–P30) before and after injection of saline (A) or muscimol (MUSC; B) into the opposite hemisphere. Saline has no effect on binocularity ((χ^2 test, $p = 0.48$), whereas rats injected with muscimol show a clear increase in the number of units driven exclusively by the contralateral eye (χ^2 test, $p < 0.001$). Number of animals as indicated. Before saline, n = 135 cells;

after saline, n = 130 cells; before muscimol, n = 133 cells; after muscimol, n = 134 cells. (C) CBIs of all treated animals. Data are mean ± standard error. There is a significant enhancement of contralateral bias after muscimol (one-way ANOVA followed by Holm-Sidak test, CBI before muscimol versus after muscimol, $p < 0.001$). Saline, n = 6 rats; muscimol, n = 6 rats. ***$p < 0.001$.

Peak discharge rates of cortical units before and after muscimol injection

The change in binocularity could be due to an increase in contralateral eye strength or to a decreased visual drive through the ipsilateral eye. To define the mechanism, I analyzed the peak discharge rates of cortical units following stimulation of each eye, before and after muscimol (or saline) administration. Injection of saline impacted neither contralateral nor ipsilateral eye responses (one-way ANOVA on ranks followed by Dunn's test, $p > 0.05$ for both comparisons; data not shown). Following muscimol infusion, I found a consistent reduction of responses through the ipsilateral eye (one-way ANOVA on ranks, $p < 0.001$; post hoc Dunn's test, IPSI before versus IPSI after, $p < 0.01$; Figure 4.9). Thus, callosal connections contribute ipsilateral eye inputs to cortical neurons, and when interhemispheric communication is silenced, OD shifts toward the contralateral eye (Figures 4.8B and 4.8C).

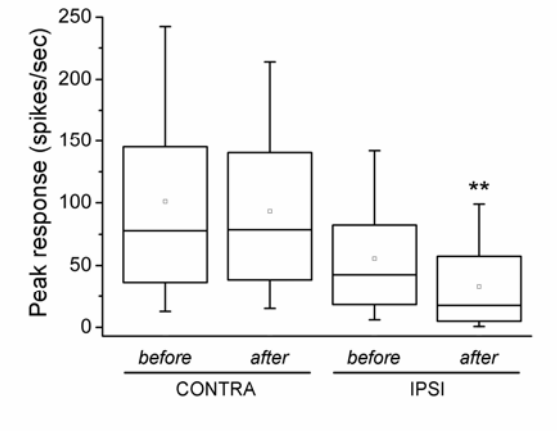


Figure 4.9 Reduction of ipsilateral eye inputs following muscimol infusion. Box chart showing peak firing rates of visual cortical neurons in naive rats injected with muscimol. There is a highly significant reduction of the responses evoked by the ipsilateral (IPSI) eye following muscimol administration into the opposite side (post ANOVA Dunn's test, ipsilateral eye, before versus after muscimol, $p < 0.01$). There is no significant effect on contralateral (CONTRA) eye response.

The horizontal lines in each box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbols denote the mean of the column of data. Before muscimol, n = 133 cells; after muscimol, n = 134 cells. **$p < 0.01$.

ROLE OF CALLOSAL CONNECTIONS IN OCULAR DOMINANCE PLASTICITY

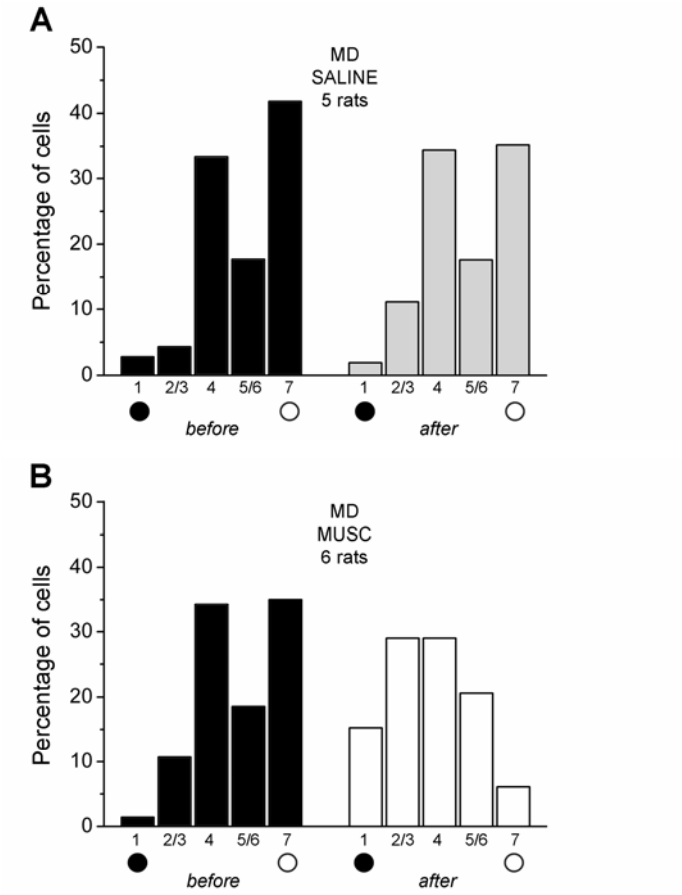
The experiments described above indicated that callosal connections contribute to normal OD by mainly carrying visual input from the ipsilateral eye. Since this important role in binocularity, I next studied the involvement of interhemispheric connections in the plastic shift of OD after MD.

Acute silencing of callosal inputs restores binocularity in monocularly deprived animals

Rats at the peak of the critical period (age P20-P23) were monocularly deprived for 7 days, and single units were recorded from primary visual cortex contralateral to the occluded eye before and after muscimol (or saline as control) injection into the opposite hemisphere (see Fig. 4.10A). All recordings were made in correspondence with the vertical meridian. Seven days of MD produced the expected change in eye preference of cortical neurons so that most units responded preferentially to the open, ipsilateral eye (Fig. 4.10A and 4.10B). Injection of saline into the opposite side produced no changes in eye preference, as indicated by statistical analysis of OD distributions (χ^2 test, MD before saline versus after saline, $P = 0.29$; Fig. 4.10A) and CBIs (one-way ANOVA followed by Holm-Sidak test, before saline versus after saline, $p = 0.86$;).

By contrast, muscimol infusion had a dramatic impact on the OD histogram. There was a complete disappearance of class 7 cells and a corresponding increase in the proportion of closed eye-driven units (Fig. 4.10B). Statistical analysis of OD histograms indicated that the OD shift was robustly attenuated after muscimol (χ^2 test, MD before muscimol versus after muscimol, $p < 0.001$). Analysis of CBIs of single animals strengthened the conclusions obtained from the pooled OD distributions. Deprived animals recorded before muscimol showed a consistent drop in CBI values as compared to naive animals (one-way ANOVA, $p < 0.001$; post hoc Holm-Sidak test, MD before muscimol versus naive, $p < 0.001$). Binocularity was almost entirely recovered by blocking the opposite hemisphere (one-way ANOVA, $p < 0.001$; post hoc Holm-Sidak test, MD after muscimol versus MD before muscimol, $p < 0.001$; Fig. 4.10C). It is noteworthy that the change in eye preference induced by acute muscimol injection in MD animals was much greater than that obtained in

naïve rats (t-test, $p < 0.01$; Fig 4.10D). Thus, removing the callosal input after a period of MD substantially alleviates the OD shift. I conclude that callosal connections play a key role in circuit modifications underlying OD plasticity.



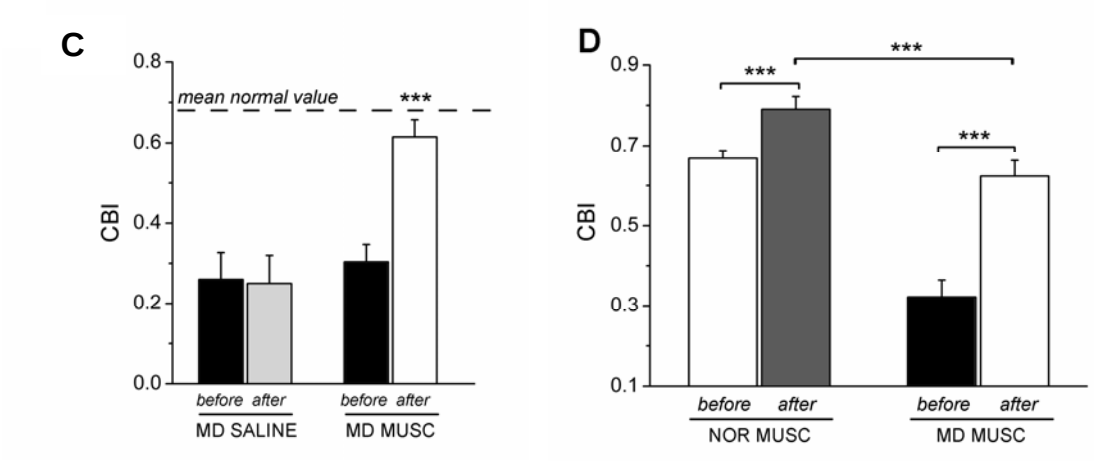


Figure 4.10 Recovery of binocularity in MD rats after acute blockade of callosal input. (A and B) OD distributions of rats monocularly deprived for 7 days, before and after injection of saline (A) or muscimol (MUSC; B) into the opposite side. Recordings were performed in the visual cortex contralateral to the closed eye (filled circle). Following injection of saline, OD remains shifted toward the undeprived eye (open circle; χ^2 test, $p = 0.29$; A). Muscimol causes a reduction of the cells driven exclusively by the open eye and a corresponding increase in the proportion of units controlled by the contralateral, deprived eye (χ^2 test, $p < 0.001$; B). Number of animals as indicated. Before saline, n = 141 cells; after saline, n = 108 cells; before muscimol, n = 140 cells; after muscimol, n = 131 cells. (C) CBIs of all treated animals. Data are mean ± standard error. There is a substantial change in OD following muscimol, but not saline, infusion in rats monocularly deprived for either 7 days (MD) (one-way ANOVA followed by Holm-Sidak test, CBI before muscimol versus after muscimol, $p < 0.001$). MD SALINE, n = 5 rats; MD MUSC, n = 6 rats. ***$p < 0.001$. (D) Comparison of the effects of acute callosal silencing in naive (NOR) versus monocularly deprived (MD) rats. Data are mean CBIs ± standard error.

Callosal inputs suppress deprived eye responses

The results reported above demonstrated a role for callosal connections in plasticity based on OD, that is a measure of the relative responsiveness of the visual cortex to stimulation of each eye. It was important to determine whether the recovery of binocularity observed after muscimol infusion in monocularly deprived animals depends on a potentiation of deprived eye inputs, on a depression of open eye responses, or both. I analyzed the peak firing rates of single units following stimulation of each eye, before and after muscimol or saline injection (Fig. 4.11). I found that acute saline infusion into the other hemisphere had no effect on visual responses (ANOVA on ranks followed by Dunn's test, $p > 0.05$; data not shown). I found a non significant decrease in the peak response of cells after stimulation of the open, ipsilateral eye (one-way ANOVA on ranks followed by Dunn's test, IPSI before muscimol versus after muscimol, $p > 0.05$). In contrast, there was a highly consistent enhancement of deprived, contralateral eye responses following muscimol (one-way ANOVA on ranks followed by Dunn's test, CONTRA before muscimol versus after muscimol, $p < 0.01$; Fig. 4.11). Thus, acute silencing of the callosal pathway alleviates the effect of MD by elevating the strength of inputs from the deprived eye. These data demonstrated that callosal afferents exert a functional inhibition of closed eye inputs during MD.

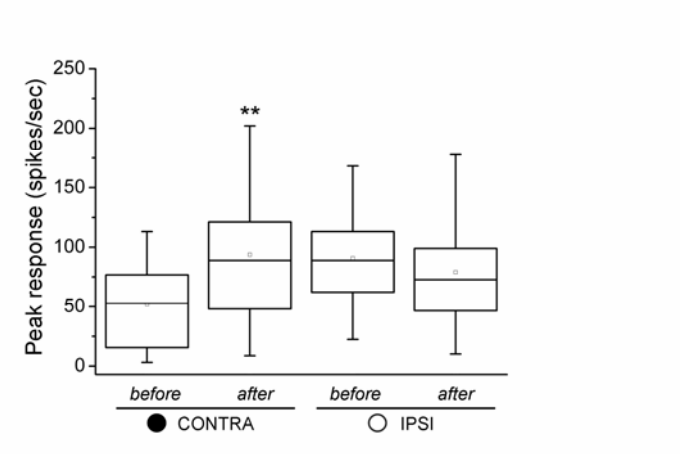


Figure 4.11 Acute silencing of callosal input rapidly unmasks deprived eye responses. Box chart showing peak firing rates of visual cortical neurons in monocularly deprived rats injected with muscimol. Contralateral (CONTRA), deprived eye responses are significantly increased after muscimol (post ANOVA Dunn's test, contralateral eye, before versus after muscimol, $p < 0.01$), while ipsilateral (IPSI), open eye responses are unaffected (post ANOVA Dunn's test, ipsilateral eye, before versus after muscimol, $p > 0.05$). The horizontal lines in each box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbols denote the mean of the column of data. Before muscimol, n = 140 cells; after muscimol, n = 131 cells. **$p < 0.01$.

Role of the callosum in adult MD

I next asked whether the enhancement of binocular responses by acute removal of transcallosal influences is restricted to the critical period. Rats older than P80 were monocularly deprived for 7 days and recordings were performed contralateral to the deprived eye. I found that adult MD produced no significant changes of the normal OD histogram (χ^2 test, normal adult versus MD adult, $p > 0.05$; Fig. 4.12A), consistent with previous reports (Pizzorusso et al., 2002; Caleo et al., 2007). Acute muscimol infusion slightly increased the contralateral eye bias in these animals (χ^2 test, before muscimol versus after muscimol, $p = 0.007$; Fig. 4.12B; t test, $p < 0.05$; Fig. 4.12C), an effect that is similar to that observed in young undeprived rats (see Fig. 4.9B). Thus, callosal inputs change their effect on OD as a specific result of critical period plasticity.

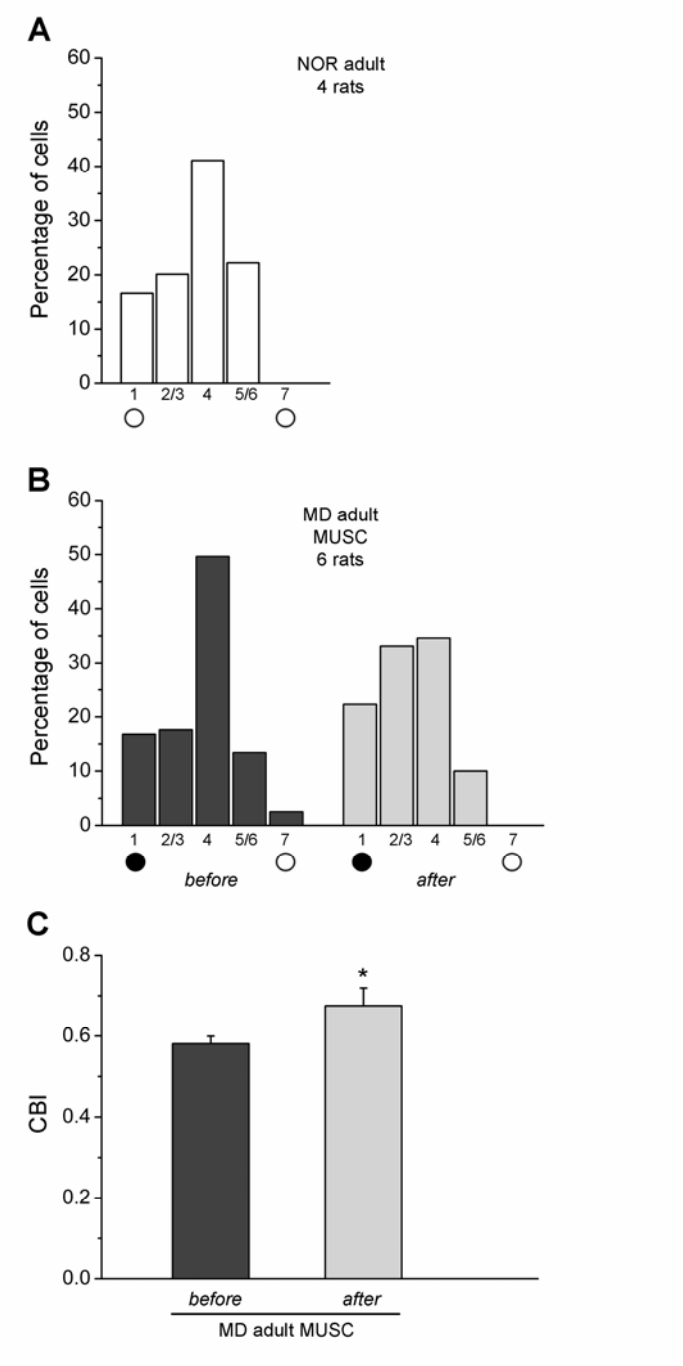


Figure 4.12 Acute silencing of callosal input slightly increases contralateral bias following adult MD. (A) OD distribution of normal (NOR) adult rats (n = 90 cells). Number of animals as indicated. (B) OD distributions of monocularly deprived (MD) adult rats before and after inactivation of the opposite hemisphere with muscimol (MUSC). Recordings were performed contralateral to the occluded eye. Note the small enhancement of contralateral bias following muscimol infusion (c2 test, p = 0.007). Number of animals as indicated. Before muscimol, n = 120 cells; after muscimol, n = 130 cells. (C) CBIs of monocularly deprived adult rats (n = 6). Data are mean ± standard error. There is a small increase of contralateral bias following muscimol (t test, $p < 0.05$). *$p < 0.05$.

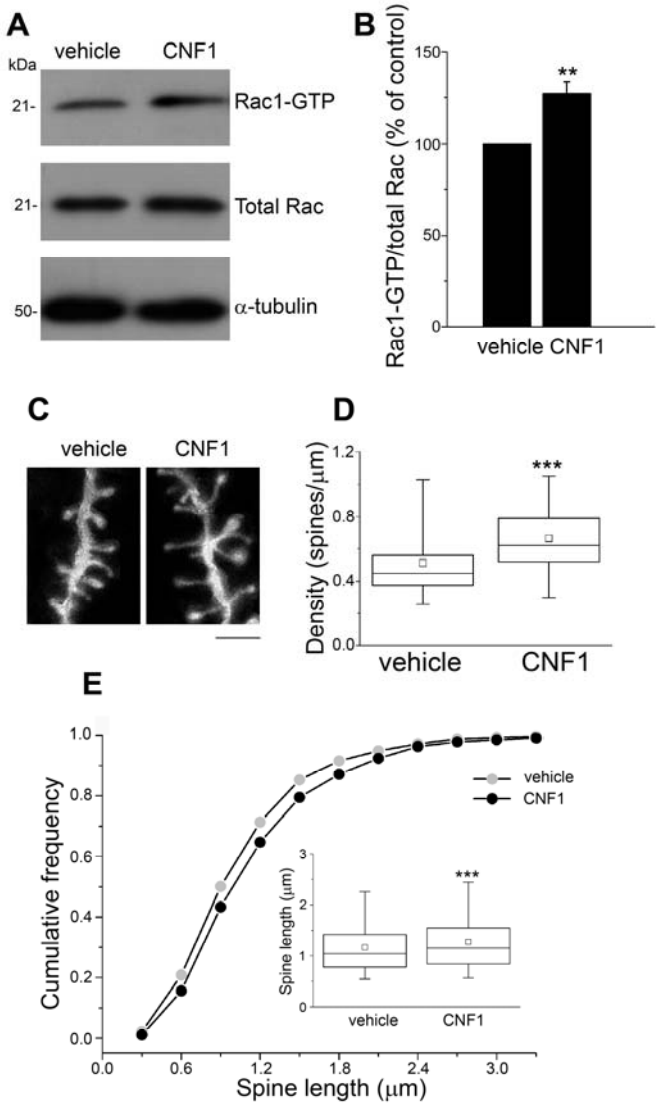
ACTIVATION OF RHOGTPASES TRIGGERS DENDRITIC SPINE REMODELING AND FUNCTIONAL PLASTICITY IN THE ADULT VISUAL CORTEX

The last section of this thesis is focused on the adult visual cortex and in particular on the analysis of the role of small RhoGTPases in visual cortex plasticity.
As shown above, adult MD is not effective in shifting the OD distribution of cortical neurons. Here I exploited Cytotoxic necrotizing factor 1 (CNF1), a protein toxin from *Escherichia coli* that activates constitutively Rho GTPases, to induce structural remodelling of dendritic spines in the adult cortex and test the hypothesis that such structural plasticity can reinstate a functional OD shift following MD in adulthood.

Effects of CNF1 injection into the visual cortex

Injection of CNF1 into the brain has been previously shown to trigger a sustained (28 days) activation of Rac1 (Diana et al., 2007; Pavone et al., 2009). I stereotaxically injected CNF1 (15 pM) or vehicle into the primary visual cortex of naïve adult rats (> P90). The activation state of Rac1 was assessed by a pull-down assay. A significant activation of Rac1 was observed 4 hr after the injection (not shown) and persisted at 10 days (paired t-test, $p < 0.01$; Fig. 4.13 A, B).
To evaluate the impact of CNF1 injection on dendritic spine structure, rats were intracortically injected with CNF1 or vehicle and perfused at 10 days. "DiOlistic" labelling of pyramidal neurons in layers II-III was performed on vibratome slices of primary visual cortex (Fig. 4.13C). I measured the length and number of protrusions on primary and secondary dendrites. The vast majority of the protrusions had a well-defined neck and head structure, characteristic of mature spines; filopodia accounted for only a minority of the protrusions, with no difference between CNF1-treated and control neurons (10.2 ± 0.57% and 10.8 ± 0.8%, respectively; t-test, $p = 0.99$). I found a highly consistent increase in spine density in basal dendrites of CNF1-treated animals with respect to controls (Mann-Whitney rank sum test, $p < 0.001$; Fig. 4.13D). Median spine length in basal dendrites from CNF1-treated cortices was also significantly longer than in control cortices (Mann-Whitney rank sum test, $p < 0.001$; Fig. 4.13E). Spine density and length in apical dendrites were unaffected by CNF1 (data not shown).

To examine whether enhanced spine density in CNF1 animals was accompanied by a corresponding increase in presynaptic excitatory markers, I measured by Western blot the expression of the vesicular glutamate transporter 1 (vGlut-1), the major excitatory vesicular transporter in the cortex. Quantitative immunoblotting analysis indicated a significant (40%) up-regulation of vGlut-1 in CNF1-treated cortices 10 days after injection (t-test, $p < 0.01$; Fig. 4.13 F, G). Interestingly, there was no significant change in the levels of GABA biosynthetic enzymes GAD65/67 in CNF1-treated animals (t-test, $p > 0.15$; Fig. 4.14). Altogether, these data demonstrate that injection of CNF1 into the adult rat visual cortex induces a long-lasting activation of Rac1, associated with an increase in dendritic spine density and a corresponding increase in the levels of the presynaptic excitatory marker vGlut-1.



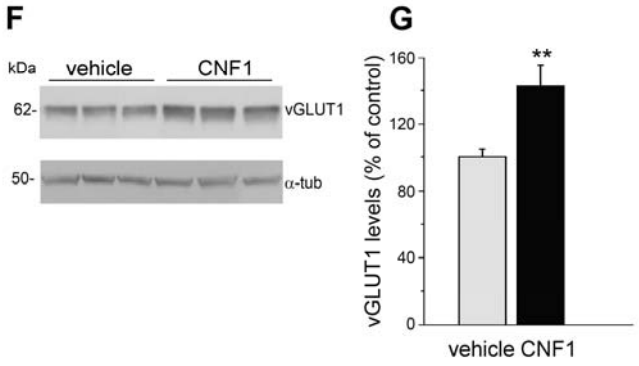


Figure 4.13 Enhanced Rac1 activation and increased spine density in the primary visual cortex following CNF1 treatment. (A) Representative immunoblot showing the amount of activated Rac1 (Rac1-GTP) in protein extracts from visual cortex 10 days after CNF1 (or vehicle) injection. □-tubulin, loading control. (B) Quantification of the ratio between Rac1-GTP and total Rac in CNF1- and vehicle-treated rats (three independent experiments). CNF1 significantly enhances Rac1 activation (paired t-test, $p < 0.01$). (C) Spine phenotype of visual cortex pyramidal neurons in animals treated with vehicle (left) or CNF1 (right). Scale bar = 1 μm. (D) Analysis of spine density in basal dendrites from layer II-III pyramidal neurons in visual cortex. Neurons of animals treated with CNF1 show a consistent and highly significant increase in the density of protrusions (Mann-Whitney rank sum test, $p < 0.001$). The horizontal lines in the box chart denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values, while the square indicates the mean of the data. (E) Cumulative frequency distribution of spine lengths for vehicle- (grey circles) and CNF1-treated animals (black circles). Inset shows the median spine length for the two groups. Spine length is significantly longer in CNF1-treated samples with respect to controls (K-S test and Mann-Whitney rank sum test, $p < 0.001$; 2,356 spines from 31 neurons and 1,761 spines from 21 neurons in CNF1- and vehicle-treated rats, respectively). (F) Representative immunoblotting for vGlut-1 in the visual cortex of vehicle- and CNF1-injected rats. Each lane represents the visual cortex of one animal. α-tubulin, loading control. (G) Quantification of immunoblots for vGlut-1 (8 samples per group examined in triplicate). CNF1 significantly enhances the expression of vGlut-1 (t-test, $p < 0.01$). Data are mean ± SEM.

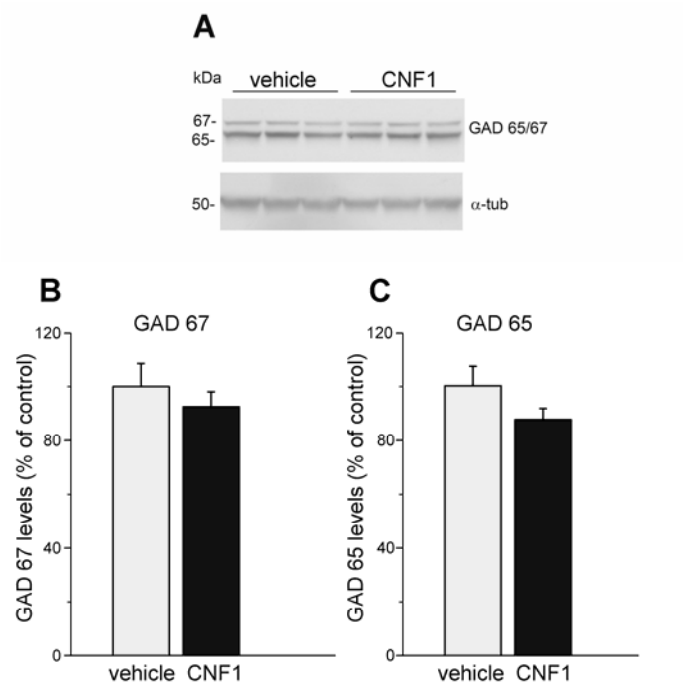


Figure 4.14 CNF1 injection does not alter GAD65/67 expression in visual cortex. (A) Representative immunoblotting for GAD65/67 on protein extracts from the visual cortex of vehicle- and CNF1-injected rats. Each lane represents the visual cortex of one animal. α-tubulin, loading control. (B, C) Quantification of immunoblots for GAD67 (B) and GAD65 (C; 8 samples per group examined in triplicate). There is no significant variation of GAD65/67 levels between vehicle and CNF1 samples (t-test, GAD67, $p = 0.47$; GAD65, $p = 0.15$). Data are mean ± SEM.

Histological controls were performed 10 days after CNF1 to demonstrate that the sustained activation of Rho GTPases had no deleterious effects on neuronal survival in the cortex. Cortical thickness and neuronal density (measured in NeuN-stained coronal sections) were superimposable in naïve, CNF1- and vehicle-injected rats (one way ANOVA, $p > 0.57$; Suppl. Fig. 4.15A, B). We also controlled inflammatory responses using staining for OX-42, a marker for microglia and neutrophils. We found that CNF1 had no impact on the number of OX-42-positive cells in primary visual cortex (one way ANOVA, $p = 0.63$; Suppl. Fig. 4.15C).

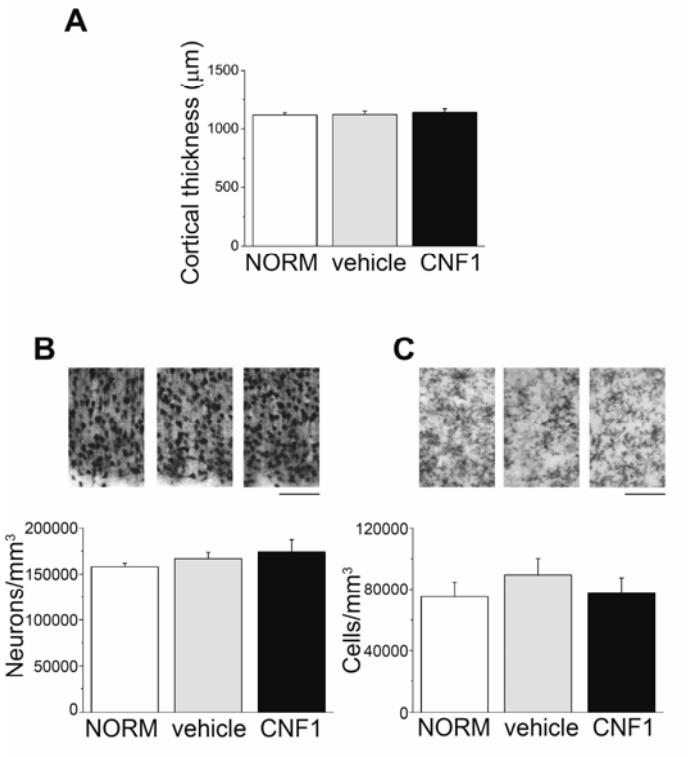


Figure 4.15 CNF1 has no adverse effects on neuronal survival in the visual cortex. (A) Mean cortical thickness in the various experimental groups. NORM, cortex of normal adult animals (n = 4); vehicle, cortex injected with vehicle solution (n = 4); CNF1, cortex treated with CNF1 (n = 8). There are no significant differences among the groups (one way ANOVA, p = 0.8). (B) (Top) Representative immunostaining for the neuronal marker NeuN in layer II-III of the visual cortex of animals in the three experimental groups. CNF1 produces no significant alterations in neuronal density (bottom; one way ANOVA, p = 0.57). (C) (Top) Representative immunostaining for the microglial marker OX-42. Density of microglial cells is not impacted by CNF1 administration (bottom; one way ANOVA, p = 0.63). Scale bar = 100 µm for panels B and C. All data are mean ± SEM.

CNF1 reactivates ocular dominance plasticity in the adult visual cortex

I used the classical paradigm of MD to examine whether CNF1-induced spine remodelling reinstates functional plasticity. Adult (> P90) rats were unilaterally injected into the visual cortex with CNF1 or vehicle, and 7 days later the eye contralateral to the injection was sutured shut. OD was evaluated by visual evoked potentials (VEPs) and extracellular recordings of single unit activity after 7 days of MD (Fig. 4.16A).

I found that the contralateral-to-ipsilateral (C/I) VEP ratio (i.e. the ratio of VEP amplitudes recorded by stimulating the contralateral and ipsilateral eye) was about 2 in naïve adult rats. Intracortical injection of vehicle solution followed by MD had no

effect on OD (Fig. 4.16B, MD+vehicle vs. naïve adult, post ANOVA Holm-Sidak test $p = 0.52$), while delivery of CNF1 produced a robust and consistent shift towards the ipsilateral open eye (one way ANOVA followed by Holm-Sidak test, MD+vehicle and naïve adult vs. MD+CNF1, $p < 0.001$; Fig. 4.16B). Thus, CNF1 triggers a plastic modification normally restricted to the critical period. Importantly, injection of a mutated form of CNF1 that lacks enzymatic activity (mut CNF1; Schmidt et al., 1998) was completely ineffective in shifting OD (MD+CNF1 vs. MD+mut CNF1, post ANOVA Holm-Sidak test, $p = 0.002$; Fig. 4.16B), demonstrating that activation of Rho GTPases by CNF1-induced deamidation is crucial for the reinstatement of plasticity.

The CNF1-induced OD shift following MD could be due to a depression of deprived eye inputs, or to a potentiation of the visual drive through the ipsilateral open eye. To define the mechanism, I analyzed absolute VEP amplitudes following stimulation of each eye in MD rats treated with CNF1 or control solution. Responses evoked by the contralateral, deprived eye were comparable in CNF1-treated and control rats (Mann-Whitney rank sum test, $p = 0.75$; Fig. 4.16C, D). Conversely, there was a highly significant increase in the strength of inputs from the ipsilateral, open eye in CNF1-injected animals (Mann-Whitney rank sum test, $p < 0.001$; Fig. 4.16C, D). Thus, the reinstatement of plasticity observed in CNF1 animals is selectively due to a potentiation of open eye responses.

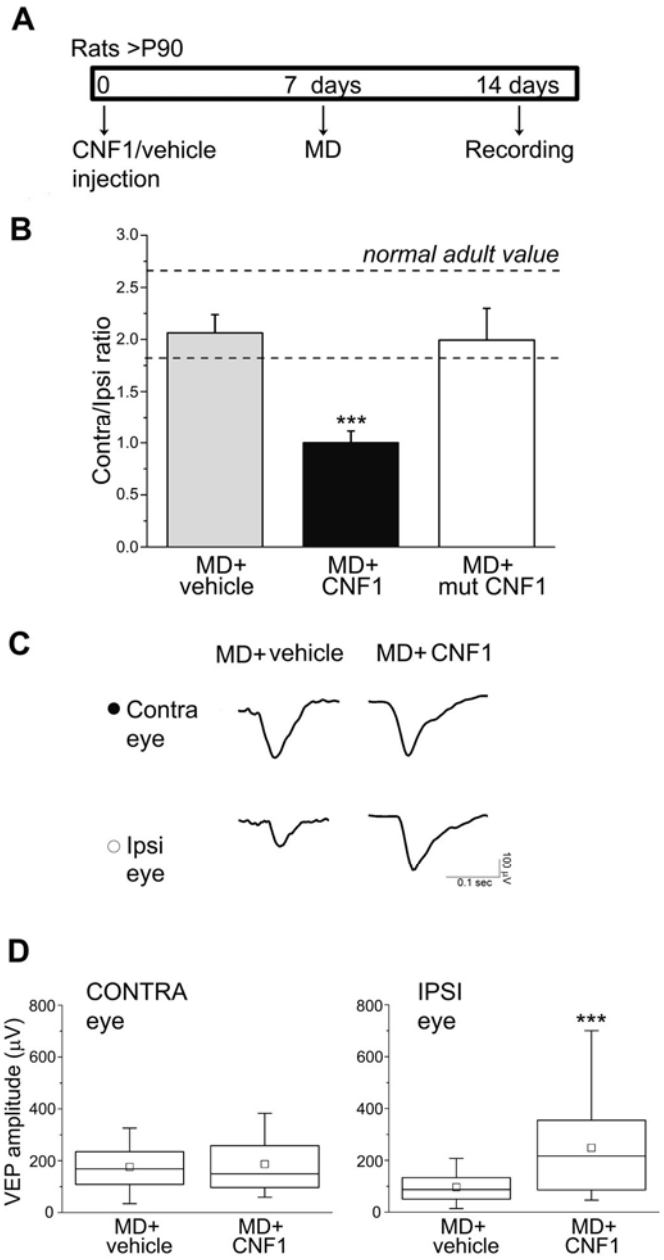


Figure 4.16. Activation of Rho GTPases reinstates OD plasticity in the adult cortex via potentiation of inputs from the ipsilateral, open eye. (A) Experimental protocol. (B) Contra/Ipsi VEP ratios in MD animals treated with vehicle (MD+vehicle; n = 11), with CNF1 (MD+CNF1; n = 8) and with a mutated form of CNF1 (MD+ mut CNF1; n = 5). In MD+vehicle rats, the C/I ratio is unchanged (post ANOVA Holm-Sidak test, $p = 0.52$) as compared to the normal adult range (indicated by the dashed lines), while in MD+CNF1 animals there is a dramatic decrease of the C/I ratio ($p < 0.001$). Injection of a mutated form of CNF1 (mut CNF1) is completely ineffective in shifting OD (MD+mut CNF1 vs. MD+vehicle, $p = 0.8$; MD+mut CNF1 vs. MD+CNF1, $p = 0.002$). Data are mean ± SEM. Normal adult range: mean value ± SD. (C) Representative examples of VEP responses for both eyes in MD+vehicle (left column) and MD+CNF1 rats (right column). Visual

stimulus: square-wave grating alternating at 1 Hz, spatial frequency 0.07 c/deg, contrast 90%. CONTRA, contralateral deprived eye; IPSI, ipsilateral open eye. (D) Quantitative analysis of absolute VEP amplitudes in MD+vehicle (n = 11) and MD+CNF1 rats (n = 8). There is no difference in contralateral eye VEP amplitude between the two groups (Mann-Whitney rank sum test, p = 0.75, left), while ipsilateral eye VEPs are significantly enhanced in CNF1-injected rats (Mann-Whitney rank sum test, p <0.001).

I next examined whether Rho GTPase activation alters the efficacy of each eye to evoke cortical spiking activity. Extracellular recordings of single unit discharges were performed contralateral to the deprived eye and OD was quantitatively assigned to each unit according to a five-point scale (Restani et al., 2009). I found that seven days of MD were totally ineffective in shifting the OD distribution in rats treated with vehicle. Indeed, the OD distribution of MD+vehicle animals was biased towards the contralateral eye and superimposable to that of naïve undeprived animals (χ^2 test, p = 0.6; Fig. 4.17A). By contrast, a significant shift of OD toward the ipsilateral, open eye was induced in MD rats injected with CNF1 (χ^2 test, p< 0.001; Fig. 4.17A). It is worth noting that this shift was mainly due to a loss of cells driven exclusively by the contralateral, deprived eye (class 1 cells, Fig. 4.17A). Computation of an OD score for each recorded neuron (Pizzorusso et al., 2002) confirmed that the OD distribution recorded in MD+CNF1 animals was significantly different from that of control rats (K-S test, p = 0.01; Fig. 4.17B). To examine how CNF1 application affects the response strength of each eye, I compared the peak discharge rates of single units in MD rats treated with CNF1 or with control solution. No difference was observed between the two groups for the responses evoked by the deprived, contralateral eye (Mann-Whitney rank sum test, p = 0.64; Fig. 4.17C, left). Remarkably, firing rates triggered by the ipsilateral open eye were significantly higher in CNF1-treated animals than in control animals (Mann-Whitney rank sum test, p = 0.01; Fig. 4.17C, right). These spiking data are in line with the selective potentiation of the ipsilateral eye observed by VEP recordings (see Fig. 4.16D). Moreover, the decrease of class 1 cells coupled to a globally stable strength of contralateral eye responses indicates that the very likely mechanism of CNF1-mediated plasticity is a takeover of originally monocular, contralaterally driven cells by inputs from the ipsilateral eye.

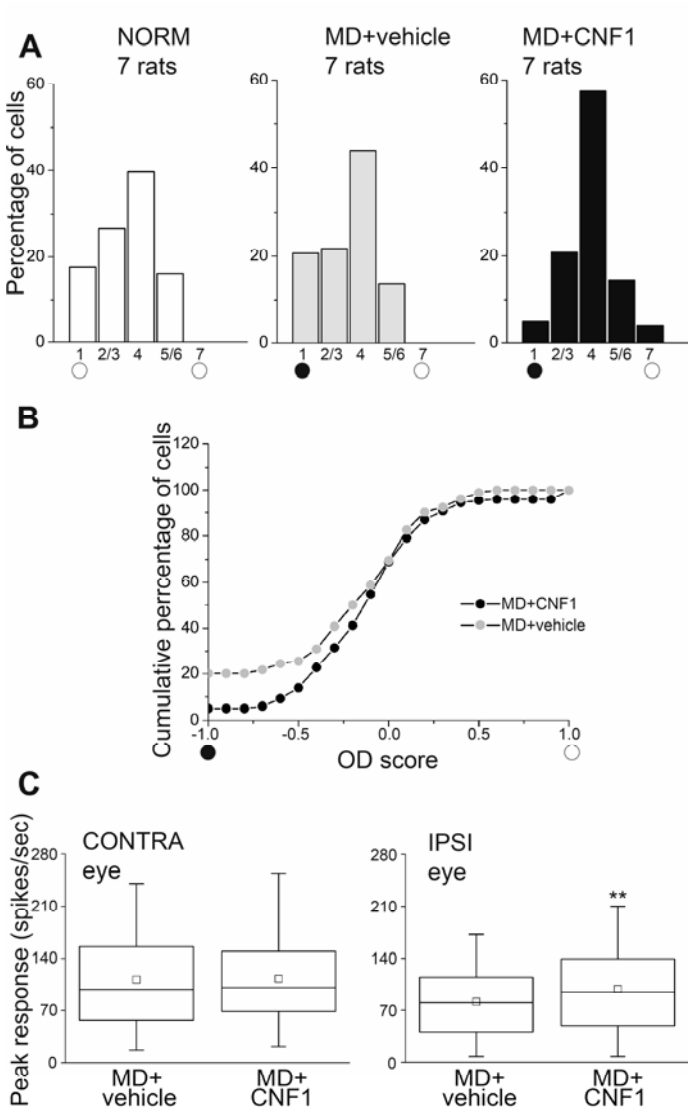


Figure 4.17 CNF1 injection in adult MD rats shifts the OD histogram towards the open eye. A) OD distributions of naïve adult rats (NORM) and rats monocularly deprived for 7 days and treated with either vehicle (MD+vehicle) or CNF1 (MD+CNF1). Recordings were performed in the visual cortex contralateral to the closed eye (filled circle). CNF1 triggers a significant OD shift (χ^2 test, $p < 0.001$) and a reduction in the proportion of neurons driven exclusively by the closed eye (class 1 cells). Number of animals as indicated. NORM, n = 131 cells; MD+vehicle, n = 184 cells; MD+CNF1, n = 216 cells. (B) Cumulative distribution of the OD score of MD+vehicle (grey circles) and MD+CNF1 rats (black circles). The two groups are significantly different from each other (K-S test, $p = 0.01$). (C) Box chart showing peak firing rates of visual cortical neurons in MD+vehicle and MD+CNF1 rats. Ipsilateral (IPSI), open eye responses are significantly increased by CNF1 treatment (Mann-Whitney rank sum test, $p = 0.01$), while contralateral (CONTRA), deprived eye responses are unaffected (Mann-Whitney rank sum test, $p = 0.64$).

I also noted an increase in the spontaneous discharge of cortical neurons in MD+CNF1 rats (Mann-Whitney rank sum test, p = 0.019; Fig. 4.18A), however, due to the enhanced evoked activity, overall there were no net changes in cell responsiveness (i.e. the peak-to-baseline ratio; Mann-Whitney rank sum test, p = 0.8; Fig. 4.18B).

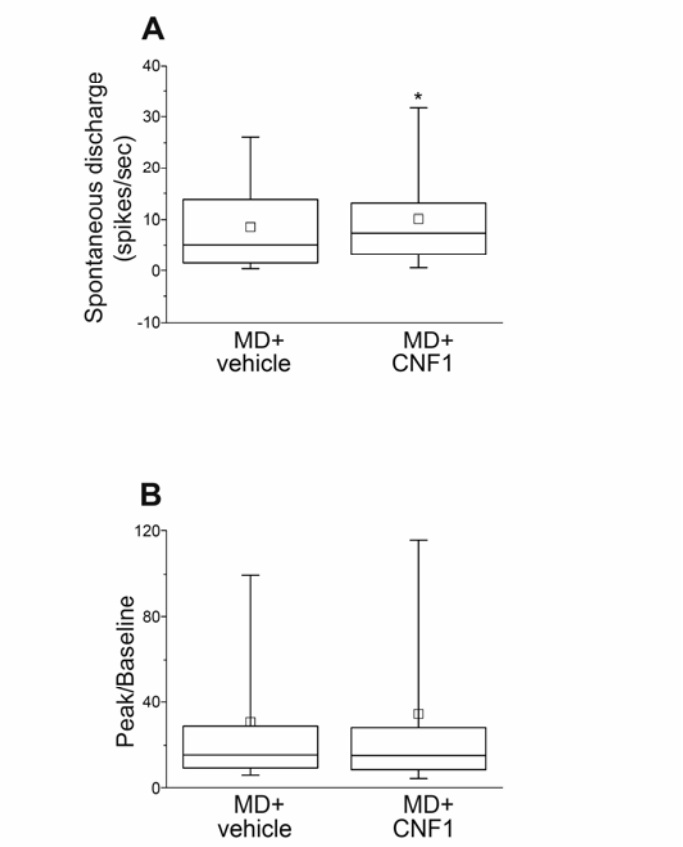


Figure 4.18 Spontaneous discharge and cell responsiveness in CNF1 and vehicle- treated MD rats. (A-B) Box chart showing spontaneous discharge (A) and cell responsiveness (B) of visual cortical neurons in MD+vehicle and MD+CNF1 rats. (A) In MD+ CNF1 treated rats spontaneous discharge is significantly increased (Mann-Whitney rank sum test, p = 0.019). (B) No changes were observed in peak-to-baseline ratio between the two groups (Mann-Whitney rank sum test, p = 0.8).

To exclude the possibility that activation of Rho GTPases per se causes an OD shift towards the ipsilateral eye, I measured OD in a subset of adult, CNF1-treated animals with normal vision. I found that the C/I ratio of VEP responses was unaltered by CNF1 treatment in naïve, undeprived adult rats (t-test, CNF1 vs. normal, p = 0.83; Fig. 4.19A). Receptive field size of cortical neurons was also comparable between naïve rats and naïve rats treated with CNF1 (Mann-Whitney rank sum test, p = 0.73; Fig. 4.19B).

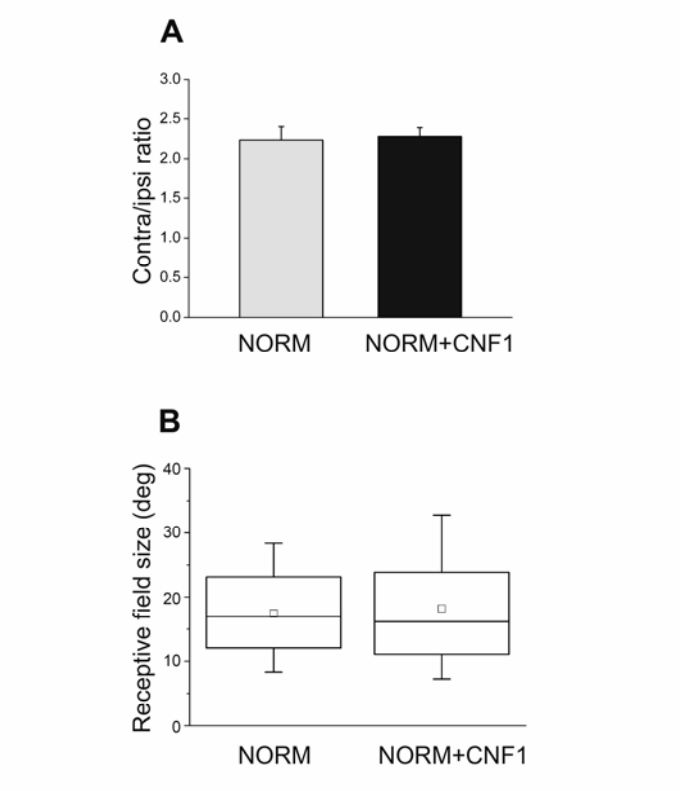


Figure 4.19 CNF1 injection per se does not alter visual cortical functional properties. (A) C/I VEP ratio is identical in naïve, undeprived adult rats (n = 4) and naïve rats treated with CNF1 14 days before (n = 4; t-test, p = 0.83). Data are mean ± SEM. (B) Receptive field size is not altered by CNF1 infusion in naïve rats (Mann-Whitney rank sum test, p = 0.73). Normal, n = 131 cells; CNF1, n = 55 cells

DISCUSSION

CALLOSAL CONTRBUTION TO OCULAR DOMINANCE IN RAT PRIMARY VISUAL CORTEX

The experiments in the first part of the thesis were designed to examine the role of callosal connections in constructing eye preference in the rat visual cortex. These data indicate that the callosum plays an important role in binocularity by providing input from the ipsilateral eye.

Callosal connections and binocularity: a review of the literature

The importance of callosal connections for binocularity in primary visual cortex has remained a matter of controversy. For example, lesions of the corpus callosum in kittens and cats have been shown to reduce binocularity (Yinon et al., 1992) and similar findings have been reported after an acute lesion (or inactivation by cooling) of the contralateral hemisphere (Blakemore et al., 1983). However, Minciacchi and Antonini (1984) reported no effect of callosotomy on OD in primary and secondary cat visual cortex (Minciacchi and Antonini, 1984). Another cat study found alterations in binocularity only when section of the callosum was performed during an early phase of development (Payne et al., 1980). The discrepancies between these different reports likely arise as a consequence of technical aspects, including age at which the callosal section is performed, and time elapsed between surgery and recording. This applies particularly to studies that used long intervals (months) between the callosal lesion and measurement of binocularity, thus allowing for plastic rearrangements that could affect the results. In rodents, the importance of callosal connections for OD is also controversial (Drager, 1975; Diao et al., 1983; Coleman et al., 2009). Using morphometric measures, Coleman et al. (2009) suggested that OD in the primary visual cortex of the mouse can be solely accounted for by the relative density of feed-forward geniculocortical inputs from the two eyes. Indeed, the contralateral eye pathway exhibits a higher degree of convergence on geniculate neurons than the ipsilateral eye pathway (Coleman et al., 2009).

A role for interhemispheric connections in determining cortical OD in rodents was suggested by previous experiments based on inactivation of callosal input activity. Diao et al. (1983) recorded single units in primary visual cortex of adult albino rats

before and after inactivation by cooling of the contralateral hemisphere. In most cells, cooling caused a reduction in the responses through the ipsilateral eye, so that the cumulative OD distribution shifted towards the contralateral eye during cooling (Diao et al., 1983).

Callosal pathways contribute to binocularity by providing ipsilateral eye input to visual cortex

The results obtained here showed clearly that callosal connections contribute to normal OD by mainly carrying visual input from the ipsilateral eye.

In the first set of experiments I found that the silencing of the genicolo-striate pathway via TTX injection into the geniculate causes an OD shift towards the ipsilateral eye. This effect has to be ascribed to the callosal connections because after a further silencing of the contralateral hemisphere with muscimol I found a complete disappearance of visually-driven activity.

Muscimol, a $GABA_A$ receptor agonist, was used to silence activity in the visual cortex based on previous reports (Reiter and Stryker, 1988; Caleo et al., 2007). TTX was employed for blocking activity in the geniculate because thalamic cells possess an hyperpolarization-activated cation current (I_h) and a low threshold calcium current (IT) that can generate spike bursts under conditions of hyperpolarization (Bal and McCormick, 1996; Sherman, 2005). Therefore, I chose to prevent directly the firing of geniculate cells by a sodium channel blocker.

I first confirmed that TTX and muscimol are effective in silencing visual responses within the geniculate and the cortex. In particular, one single injection of TTX into dLGN was found to silence the entire area mapping the central part of the visual field, and one single injection of muscimol blocked the binocular portion of the cortex around the site of delivery.

In TTX experiment, analysis of eye preference in the cortex was accomplished by both VEP and single unit recordings. VEP recordings have proven very valuable in previous studies of cortical function and plasticity (Hanover et al., 1999; Huang et al., 1999; Porciatti et al., 1999; Bartoletti et al., 2004; Frenkel and Bear, 2004; Maya Vetencourt et al., 2008). Indeed, they allow to measure the absolute strength of the response of each eye, in addition to ratios (Frenkel and Bear, 2004). To determine the callosal contribution to spiking responses in the cortex, the OD analysis was also made via extracellular recordings of action potentials. Both VEP and single unit

analysis demonstrated a clear shift of cortical OD towards the ipsilateral eye following intrageniculate TTX delivery.

When OD was measured in naïve rats before and after muscimol delivery into the opposite hemisphere (without a previous TTX intragenicolate injection), I found that the OD distribution of cortical neurons significantly shifted towards the contralateral eye following muscimol injection.

This OD shift towards the contralateral eye following removal of callosal input is opposite to the OD shift towards the ipsilateral eye that I have observed after geniculate silencing. The analysis of absolute strength of each eye indicated a dramatic decrease of contralateral eye responses and a slight reduction of ipsilateral eye input following geniculate silencing, while after silencing of callosal connections an opposite effect was observed. Indeed, following removal of callosal input, I found a consistent reduction of responses through the ipsilateral eye, while contralateral eye inputs were unaffected.

These complementary data sets indicate two sources of OD in primary visual cortex: the retinogeniculate pathway carrying primarily information from the contralateral eye, and the callosal pathway supplying mainly ipsilateral eye inputs. This interpretation is supported by the analysis of VEP latency before/after TTX injection. Indeed, in naïve animals before TTX, VEP latency for the contralateral eye was consistently shorter than for the ipsilateral eye, consistent with the direct retinogeniculate input to the cortex being mainly contralateral eye-driven, and with a significant transcallosal contribution for the ipsilateral eye. After TTX delivery, VEP latencies for both eyes increased (due to the physiological time window of interhemispheric communication) and the difference in latency was abolished, indicating that both contralateral and ipsilateral eye inputs reach the cortex through the same transcallosal route.

The present data provide a direct demonstration that, in addition to the geniculocortical pathway, callosal projections are a significant source of ipsilateral eye input to cortical neurons. In rodents, direct contralateral eye input (via the retinogeniculate pathway) is much stronger than ipsilateral eye input (due to massive decussation of optic fibers at the chiasm), however the proportion of binocular units

in the binocular segment of primary visual cortex is extremely high (Caleo et al., 1999a; Caleo et al., 1999b; Di Cristo et al., 2001; Caleo et al., 2007). My data demonstrate that some of the influence of the ipsilateral eye on cortical responses is provided via callosal afferents from the opposite hemisphere, where the ipsilateral eye is dominant. In this context, it is worth noting that contralateral bias is graded across the medio-lateral extent of the visual cortex in rodents (Drager, 1975; Gordon and Stryker, 1996; Caleo et al., 1999b). In particular, cells with receptive fields within the central 20° (located in the lateral aspect of primary visual cortex) are highly binocular, while dominance of the contralateral eye increases sharply at more peripheral locations in the visual field. Callosal connections are also particularly concentrated in the region mapping the central part of the visual field, i.e. at the border between area 17 and 18 (Cusick and Lund, 1981; Mizuno et al., 2007). Thus, it is tempting to speculate that the higher proportion of binocular units in the lateral aspect of primary visual cortex is due to the contribution of callosal connections providing ipsilateral eye input.

The finding that callosal afferents contribute to eye preference in the rat visual cortex is important for the interpretation of studies on OD plasticity. Recently, rodents have become increasingly popular for mechanistic studies of plasticity, since they allow coordinated molecular, physiological and behavioural analyses (Hensch et al., 1998; Huang et al., 1999; Frenkel and Bear, 2004; Tropea et al., 2006; Mrsic-Flogel et al., 2007; Maya Vetencourt et al., 2008). Classically, it has been assumed that cortical OD is only determined by thalamocortical afferents serving the two eyes. Similarly, competition between geniculocortical inputs has been proposed as the dominant mechanism driving OD plasticity following sensory deprivation. The present findings indicate that callosal connections are important players in the construction of normal OD in the visual cortex. The next section discusses the importance of interhemispheric afferents in the processes of OD plasticity.

ROLE OF CALLOSAL CONNECTIONS IN OCULAR DOMINANCE PLASTICITY

Having established a role for callosal inputs in controlling cortical binocularity, I next examined the involvement of corpus callosum in OD plasticity. My experiments showed that after MD, acute silencing of callosal afferents causes an OD recovery due to an unexpected increase in the strength of the deprived eye. Thus, acute removal of callosal influence following MD unmasks deprived eye inputs. These data indicate that callosal afferents act primarily to inhibit closed eye inputs under visual deprivation.

For these experiments I reasoned that the shift in eye preference following monocular occlusion might potentially derive either from changes in the direct thalamocortical pathway or from modifications in the transcallosal route. To assess the involvement of the callosal pathway in OD plasticity, I measured eye preference in MD rats before and after acute blockade of interhemispheric communication. This experimental protocol allows plasticity to proceed normally and probes the results of acute removal of callosal input, thus dissecting the OD shift that would be measured via the sole geniculocortical pathway. I found that silencing transcallosal input in animals that had undergone MD strongly alleviated the OD shift and produced a change in eye preference consistently greater than that obtained in normal animals. This is particularly clear from the data reported in Fig. 4.10D, which summarize the OD changes triggered by acute muscimol injection in both normal and monocularly deprived rats. It is important to note that acute muscimol resulted in a remarkable recovery of binocularity after MD, but did not fully recovered OD. More specifically, the quota of MD effects that is due to rearrangements in the geniculocortical pathway can be computed by comparing the OD post muscimol in naive and MD rats (mean CBI post muscimol: 0.81 ± 0.03 in naive versus 0.61 ± 0.04 in MD animals; t test, $p < 0.001$; Fig. 4.10D). Indeed, the post muscimol condition indicates the eye preference that is determined by the sole thalamocortical route, and the difference observed in naive versus deprived animals measures MD-induced alterations in this pathway. Thus, changes in both transcallosal and thalamocortical input contribute to MD effects. I found that the recovery of binocularity after callosal silencing in MD rats was selectively due to an enhancement of deprived eye responses. This differs

from the naïve situation in which callosal afferents supply ipsilateral eye inputs (Fig. 4.9). Deprived eye inputs were rapidly unmasked by muscimol injection into the opposite hemisphere, indicating that a mechanism of transcallosal inhibition is used to drive down the efficacy of afferents from the closed eye.

There is much debate in the literature on the mechanisms that lead to weakening of deprived eye strength after a period of MD. On one side, work by Bear and colleagues suggests that depression of the efficacy of thalamo-cortical afferents serving the closed eye is the major mechanism responsible for the OD shift following brief MD. Indeed, in young mice monocularly deprived for 3 days, the OD shift appears to be fully manifest by a change in thalamo-cortical excitatory synaptic transmission (Khibnik et al., 2010). On the other side, there is abundant evidence for a role for inhibition in modulating the induction of plasticity. Indeed, studies have indicated that inhibition is crucially involved in the effects of MD (for a review, see Hensch, 2005). For example, an adequate level of intracortical inhibition is required for OD plasticity to occur (Hensch et al., 1998). Visual deprivation potentiates inhibitory feedback between fast-spiking basket cells and star pyramidal neurons (Maffei et al., 2006). Microiontophoretic delivery of the GABAA antagonist bicuculline restores inputs from the deprived eye in the visual cortex of monocularly deprived cats (Duffy et al., 1976; Sillito et al., 1981; Mower et al., 2002). Thus, functional inhibition is one important factor determining abnormal eye preference following MD, but the source of this inhibition has remained unclear. One previous study of CRE-mediated gene transcription following MD suggested the idea of inhibitory influences from outside the primary visual cortex (Pham et al., 1999).

The present findings clearly demonstrate that the callosal input is a major source of inhibition and a key determinant of the OD shift. These experiments uncover a novel role for callosal inputs in OD plasticity. Specifically, I have shown that after monocular visual deprivation the transcallosal pathway changes from a mainly excitatory action (supplying ipsilateral eye input) to a predominantly inhibitory function (providing selective suppression of deprived eye afferents). But how could this inhibition be achieved? Callosal cells are mostly (95%) glutamatergic, and they retain this excitatory phenotype also after a period of MD (Restani et al., 2009). Thus, one possible hypothesis is that callosal axons recruit inhibitory neurons in the opposite hemisphere (Fig. 5.1). It has been reported that callosal fibers mainly evoke

a direct excitation of principal neurons in the opposite hemisphere (supplying ipsilateral eye input; present results), but can also produce a disynaptic inhibitory postsynaptic potential via a local GABAergic cell (Toyama et al., 1974; Innocenti, 1980). I suggest that connections between callosal fibers and inhibitory neurons may be strengthened during MD (Fig. 5.1B). Under these conditions, responses from the contralateral deprived eye could be effectively suppressed by callosal afferents; no changes would be apparent for the ipsilateral eye, since enhanced GABAergic inhibition via the callosum would compensate the normal transcallosal direct excitation (Fig. 5.1B). This scenario could explain how selective suppression of deprived eye inputs can be achieved during MD. Further studies with intracellular recordings are needed to corroborate this hypothesis. Another possible mechanism of disinhibition following muscimol treatment might be a modification of the strength of thalamic inputs from the deprived eye via alterations of geniculocortical axon arborization. I consider this interpretation unlikely, based on the following lines of evidence. First, geniculocortical projections are only modestly affected by MD in rodents, and these effects require at least 20 days of deprivation (Antonini et al., 1999). Second, the fast (within 40 minutes) and persistent elevation of deprived eye strength by acute muscimol application supports the removal of intracortical functional inhibition. Indeed, the rapid unmasking strongly favors functional versus anatomical rearrangements in the mechanisms of the OD shift.

These data prompt a reconsideration of the mechanisms involved in OD plasticity. I demonstrated that transcallosal inhibition plays a key role in the loss of deprived eye inputs. The importance of this mechanism for OD plasticity in higher species such as monkeys and humans remains to be investigated. It is worth pointing out, however, that transcallosal inhibition has been demonstrated to participate in plastic events occurring during several pathological conditions of the human brain. For example, it has been shown in neglect patients that some of the behavioral symptoms are attributable to a pathological state of increased inhibition exerted onto the damaged parietal cortex by the contralateral, intact hemisphere (Fecteau et al., 2006; Fierro et al., 2006). Indeed, inactivation of the unaffected hemisphere by transcranial magnetic stimulation ameliorates visuospatial neglect (Fecteau et al., 2006; Fierro et al., 2006). It has also been reported that changes in transcallosal inhibition contribute to the

occurrence of mirror movements in Parkinson's disease and ischemic patients (Cincotta et al., 2006; Li et al., 2007; Nair et al., 2007). Recently, a mechanism of transcallosal inhibition was found to be operant in normal human visual cortex to dampen neuronal responses at high contrasts (Bocci, Caleo and Sartucci, unpublished data).

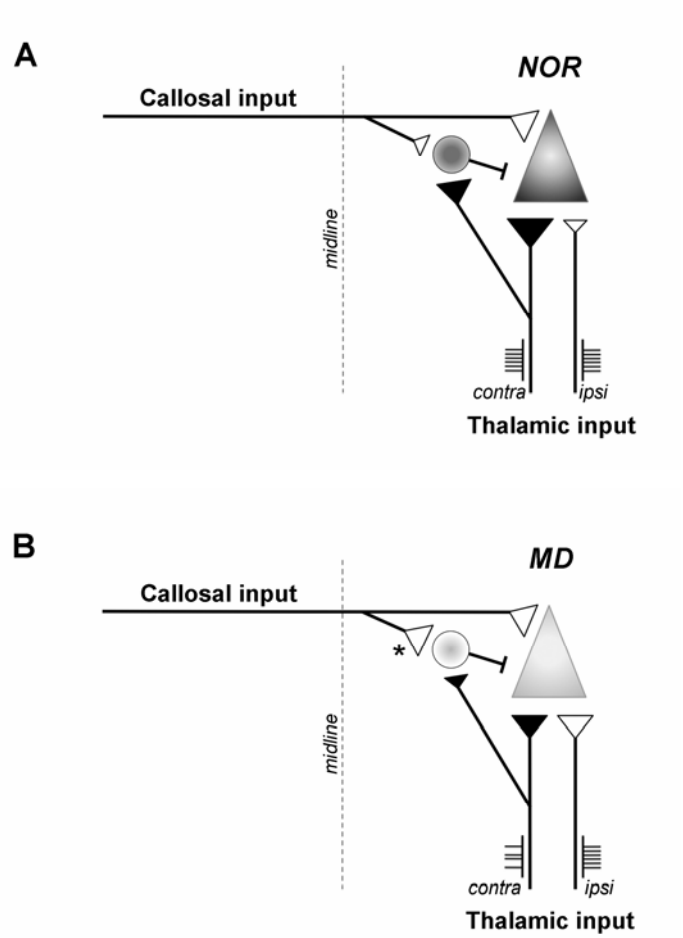


Figure 5.1 Simplified model of visual cortical circuitry in naive and monocularly deprived rats. Thalamic and callosal inputs to a principal neuron (triangle) and to an inhibitory cell (circle) are shown. Contralateral eye- and ipsilateral eye-driven synaptic terminals are in black and white, respectively. Size of the terminals indicates relative synaptic strength. (A) In normal animals, callosal afferents have a net excitatory action, contributing to ipsilateral eye responses. (B) MD might result in strengthening of the synaptic connections (asterisk) between callosal afferents and inhibitory cells in the opposite hemisphere, thus masking weak inputs from the contralateral, deprived eye. Remodeling of callosal connections during MD would have no net effect on ipsilateral eye responses, since the increased inhibition via the callosum (asterisk) would be balanced by the normal transcallosal excitatory drive. Thus, acute silencing of interhemispheric communication might selectively unmask contralateral, deprived eye inputs with no impact on ipsilateral, open eye responses.

ACTIVATION OF RHOGTPASES TRIGGERS DENDRITIC SPINE REMODELING AND FUNCTIONAL PLASTICITY IN THE ADULT VISUAL CORTEX

My experiments show that CNF1 infusion reinstates structural and functional plasticity in the adult visual cortex, demonstrating a key role for GTPase activation in experience-dependent adult plasticity. The Rho family of GTPases comprises RhoA, Rac1 and Cdc42, which have different actions on neurite elongation. Specifically, Rac1 and Cdc42 activation stimulates new spine formation and neuronal branching, while RhoA exerts an inhibitory action. (Tashiro et al., 2000; Sin et al., 2002; Govek et al., 2004; Van Aelst and Cline, 2004). For example transfecting neurons with a constitutively active form of RhoA results in a decrease in spine density, while activation of Rac1 increases the number of protrusions (Tashiro et al., 2000). In neurons of the *Xenopus* optic tectum, visual stimulation induces growth of the dendritic arbour, and this effect requires increased Rac1 and Cdc42 activity, and decreased RhoA activity (Sin et al., 2002). CNF1 can potentially increase the activity of all three Rho GTPases, however previous in vivo studies in mice have shown that the CNF1-mediated activation of Rac1 is long-lasting (28 days) while that of Rho is short-lasting (< 10 days; Diana et al., 2007). This might be due to faster degradation of activated Rac1 by the proteasome pathway (Diana et al., 2007). In line with this idea, the structural phenotype of CNF1-treated neurons at 10 days in the present study (increased spine density and length) correlated with an enhanced Rac1 activation (see Fig. 4.13). I have concentrated my analysis of CNF1 effects on neurons, as most of the previous studies investigating the role of Rho GTPases in brain development and plasticity have dealt with neuronal rather than glial cells (Govek et al., 2004; Van Aelst and Cline, 2004). It remains to be seen whether the activation of RhoGTPases by CNF1 also occurs in glial cells, and whether such glial changes contribute to the physiological effects observed in this study.

How can Rho GTPase activation reinstate plasticity in adulthood? Rho GTPases play critical roles in activity-dependent plasticity of excitatory synapses by controlling their structural and functional stability (Bonhoeffer and Yuste, 2002; Van Aelst and Cline, 2004). The increase in spine density and length following CNF1 suggests that CNF1 reinstates a high level of structural plasticity that is normally absent in the

adult neocortex (Grutzendler et al., 2002; Majewska et al., 2006; Holtmaat et al., 2009; Holtmaat and Svoboda, 2009; Chen and Nedivi, 2010), thus triggering the wiring up of new synapses. The increased levels of the excitatory marker vGlut-1 in CNF1-treated rats is consistent with this possibility.

Interestingly, on a functional level, CNF1-mediated OD shift following MD appeared to be specifically due to a potentiation of open eye responses. These data suggest that CNF1 sets in motion a mechanism of activity-dependent takeover by which newly formed dendritic sites are contacted preferentially by more active afferents from the open eye. In this scenario CNF1 would produce an increase in excitatory synaptic inputs on cortical neurons, as indicated by the enhanced spontaneous firing of cells in CNF1 treated cortices (Fig. 4.18A). These excitatory terminals would carry mainly inputs from the ipsilateral open eye. In keeping with this interpretation, the increase in spontaneous activity is accompanied by an enhancement of responses evoked through the ipsilateral eye, so that cell responsiveness (i.e the peak to baseline ratio) remains constant (Fig. 4.18B).

My data establish a clear relationship between spine growth and experience-dependent response potentiation. This relationship has been recently demonstrated in the adult somatosensory cortex (Wilbrecht et al., 2010). A link between response potentiation and new spine formation is also confirmed by the evidence that in adult mouse visual cortex the functional consequences of MD (strengthening of open eye responses) is accompanied by a net gain of spines (Hofer et al., 2009). On the other hand, a loss of spines has been associated with response depression in the visual cortex. For example, brief MD in young mice (triggering depression of deprived eye inputs) results in a net loss of dentritic spines in layer II-III pyramidal neurons (Mataga et al., 2004). It is worth noting that there is no alteration in spine density in the monocular cortex, where response depression does not occur (Mataga et al., 2004). Similarly, reduction in dendritic spine and fillopodia densities by Notch-1 signaling in cortical neurons enhances the effect of monocular deprivation, with a more marked weakening of closed eye responses (Dahlhaus et al., 2008).

Previous strategies to restore plasticity in adulthood have largely relied on the manipulation of intracortical GABAergic inhibition (He et al., 2007; Sale et al., 2007; Maya Vetencourt et al., 2008; Harauzov et al., 2010). For example,

pharmacological reduction of GABAergic inhibition (via either interference with GABA release or blockade of $GABA_A$ receptors) renders the adult rat visual cortex susceptible to MD (Harauzov et al., 2010). A period of dark rearing in adulthood increases the excitation/inhibition ratio and restores OD plasticity (He et al., 2006; He et al., 2007). Environmental enrichment and fluoxetine treatment also enhance adult plasticity via a reduction of intracortical inhibition, as their effects are completely blocked by coinfusion of the $GABA_A$ receptor agonist diazepam (Sale et al., 2007; Maya Vetencourt et al., 2008). Another strategy for the reinstatement of plasticity in adulthood is removal of extracellular inhibitory molecules (Pizzorusso et al., 2002). In this experiment, degradation of sugar chains of chondroitin-sulphate proteoglycans by the bacterial enzyme chondroitinase ABC reactivates OD plasticity (Pizzorusso et al., 2002). The data reported here represent the first demonstration that a reinstatement of plasticity can be based on manipulation of the structure of dendritic spines, the main site of excitatory synapse input.

In summary, these results indicate a novel role for Rho GTPases structural and physiological plasticity of the adult brain. Spine pathology is consistently observed in synaptopathies and mental retardation syndromes (Govek et al., 2004; Holtmaat and Svoboda, 2009), and a net loss of spines is found in neurodegenerative disorders such as Alzheimer's disease (Tsai et al., 2004). The plasticizing effects of Rho GTPase activation may be beneficial in the treatment of these pathologies, and as a strategy to promote brain repair after injury.

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