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ROLE OF THE CALLOSUM IN VISUAL CORTEX DEVELOPMENT AND PLASTICITY

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INDEX

INTRODUCTION

INTRODUCTION

THE VISUAL SYSTEM: ANATOMICAL FEATURES

Understanding how the brain changes as a function of experience is an issue of great interest for neuroscientists. The relative contribution of genes and environment in brain development has long been debated. Many studies have addressed the role of experience in development of visual system, taking advantage of the relatively easy manipulation of visual inputs. 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. There are some differences in the anatomical organization of visual pathways in the different species, even if the basic aspects are conserved in all 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 team of different types of neurons whose role it 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. In the rodent visual system the vast majority of the fibers cross at the chiasm (only 3-5% of the optic axons remain ipsilateral), while the percentage of decussating fibers is lower in carnivores and primates.

Past the chiasm, retinal ganglion cell axons run within the two optic tracts. Each optic tract carries a representation of the contralateral visual field. Retinal inputs terminate within two major subcortical visual structures, the superior colliculus (SC) and the dorsal geniculate nucleus (dLGN), a portion of the

thalamus. In rodents, all ganglion cells project to the SC, and 40% of the retinal fibers send a collateral to the dLGN.

The dLGN is the structure that relays input to visual cortex. In each dLGN there is a retinotopic representation of the contralateral visual field. In primates and humans, the dLGN contains six layers, each of whose 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). In rodent the dLGN contains two patches, each receiving eye-specific input (Godement et al., 1984). The inner core is ipsilateral, surrounded by a contralateral patch.

Projections of neuron in dLGN reach the primary visual cortex, or V1, in the occipital portion of the brain. The V1 is a layered structure (layer I-VI). The major layer of inputs from dLGN is 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). In rodents, there are no ocular dominance columns, because inputs coming from two eyes converge on the same postsynaptic target cell at the level of layer IV (Antonini et al., 1999). Although V1 received input from the two eyes, information concerns always the contralateral half of the visual field. 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). 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).

The V1 cortex is responsible for creating the basis of a three-dimensional map of visual space, and extracting features about the form and orientation of objects. Once basic processing has occurred in V1, the visual signal goes to secondary visual cortex, V2, which surrounds V1. Secondary visual cortex (V2) is principally responsible for perceiving colours and forms.

Primary visual cortex contains two main types of neurons: pyramidal cells are projection neuron, while nonpyramidal cells represent local interneurons. There are several different classes of pyramidal cells and interneurons, and their

physiological, anatomical and molecular diversity is the subject of ongoing research.

THE CORPUS CALLOSUM: ANATOMY AND PHYSIOLOGY

The corpus callosum (CC) is the largest white matter structure in the brain, and comprises in humans more than 200 millions of fibers. The CC connects homologous cortical areas of the two cerebral hemispheres (Houzel et al., 2002; Bloom and Hynd, 2005).

Nature and function of the corpus callosum have long been of interest, because alterations in this structure are noted in psychiatric and developmental disorders. Abnormalities in the size of callosum have been found in patients with schizophrenia, autism, mental retardation, Down's syndrome, developmental languages disorders (Bloom and Hynd, 2005). Despite the amount of work devoted to the CC, it is striking to realize how little we know about the nature and physiology of interhemispheric integration. The visual cortex is a good model to address these questions, due to the excellent knowledge of the physiological properties of V1 cells.

Visual callosal connections mature late in humans, around one month in cats and at postnatal day fiftteen (P15) in rodents. First it enlarges caudally and then develops rostrally, similarly myelinization occurs slowly and with a caudal-rostral development (Bloom and Hynd, 2005).

In primary sensory areas, intherhemispheric projections link essentially homotopic zones. In all mammals, each hemisphere receives information from the opposite visual hemifield. In cats and primates, with a large binocular visual field, only axons arising from the nasal half of the retina cross, while in rodents, with more lateral eye and limited binocular vision, a higher percentage of fibers crosses (Houzel and Milleret, 1999). Thus, the visual world is represented discontinuously as seen in cortical maps, split along the central vertical meridian. Despite this, we have perception of continuity, because one of the accepted essential function of CC is to guarantee the continuity of sensory maps across the hemispheres. This fusion is achieved by precise,

reciprocal, point-to-point callosal connections between cortical neurons, whose receptive fields are located along the vertical meridian.

The basic layout of the callosal connections linking primary visual cortex has been investigated mostly in cats, by anatomical and electrophysiological techniques. As emerging from literature, callosal connections form a dense stripe along the border of areas 17 and 18 (Payne, 1994). This zone is a transition zone, that shares cytoarchitectonic and physiologic features with cells of area 17 and 18. Notably, neurons in this boundary have receptive fields mapping in the vertical midline, together with a smaller portion of the ipsilateral hemifield (Blakemore et al., 1983; Payne, 1990; Payne and Siwek, 1991; Payne, 1994). In addition, split-chiasm experiments revealed that trans-callosal and ipsilateral, geniculocortical inputs converging onto a given target neuron are precisely matched in their selectivity for stimulus orientation, direction and velocity, and that receptive fields plotted through both pathways are virtually superimposed (Berlucchi and Rizzolatti, 1968; Milleret et al., 1994). It is noteworthy that analysis of single callosal axons in cats has demonstrated that some branches terminate within the core of area 17 (in addition to the dense terminations at the 17/18 border). These branches might provide mostly subthreshold activation of the cortical neurons (Houzel et al., 2002).

In rats, the entire mediolateral extent of striate cortex contains callosal cells and terminals (Olavarria and Van Sluyters, 1983). In particular, experiments with retrograde tracers have revealed a dual connectivity scheme: (i) at the 17/18 border, callosal connections would link cortical loci sharing the same receptive fields along the vertical meridian, as in cats; (ii) within area 17, callosal afferents would link mirror-symmetric cortical regions, representing mirror-symmetric positions in the periphery of the visual field (Lewis and Olavarria, 1995). These mirror-symmetric projections might be involved in processes requiring large-scale integration of features across the entire visual field (e.g. computaion of opticflow, detection of symmetry).

The vast majority of callosal neurons are large pyramidal cells; however, callosal neurons do not constitute a homogenous population, since they have different morpho-chemical phenotypes. Among those are spiny stellate, but also smooth stellate and fusiform cells, which suggests that at least some

callosal neurons could use inhibitory transmitters. This is compatible with the occasional observation of symmetric callosal synapses as well as with the electrophysiological disclosure of short-latency transcallosal inhibition (Payne and Siwek, 1991).

As I have discussed above, one of the main functions of visual callosal connections is to grant perceptual continuity across the vertical meridian. 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).

SHAPING THE BRAIN

To date it is well accepted that, after an initial period during which nervous system development is strictly controlled by genetic programmes, successive aspects of brain development require neuronal activity to reach complete maturation (Zhang and Poo, 2001; Sengpiel and Kind, 2002).

Many years have been spent by neuroscientists to address the question “nature versus nurture”, that is the debate on whether brain development what is genetically determined and what could be changed by environment and experience. One way by which environment could change the brain is via patterns of electrical activity generated by experience.

The visual system is an optimal model to address the issue about the permissive versus the instructive role of electrical activity. Permissive means that a threshold of activity must be reached for normal development to occur. Instructive means not only that electrical activity is necessary, but also that the levels or patterns of activity dictate specific aspects of morphological and functional development (Sengpiel and Kind, 2002).

There are two categories of electrical activity that have been studied by neuroscientists: spontaneous versus experience-dependent activity. Each one is predominant during a particular stage of development.

Role of spontaneous electrical activity in development of the visual system

Spontaneous activity plays a fundamental role in the early stage, when there is the initial anatomical and physiological development and when vision is not present yet. Action potentials are present in the retina, lateral geniculate nucleus and visual cortex very early in development. They could play a key role in the structuring of projections during development. *In vivo* recordings from animals before eye opening found action potentials in spontaneously active cells (Galli and Maffei, 1988; Weliky and Katz, 1999; Chiu and Weliky, 2001). Notably, recordings in anesthetized prenatal rats between embryonic days 18 and 21 demonstrated that the firings of neighboring retinal ganglion cells are strongly correlated, resulting in highly correlated bursts of action potentials (Maffei and Galli-Resta, 1990). Correlation in the activities of neighboring neurons in the retina could be the basis of developmental processes such as refinement of retinotopic maps in the brain and segregation of the inputs from the two eyes in the dorsal lateral geniculate nucleus (dLGN). Segregation of retinogeniculate axons was originally believed to be achieved by axon guidance and/or target recognition molecules, occurring independently of activity, similarly to retinotectacl projections (Sperry, 1963). However, twenty years ago, experiments of Stryker's lab had demonstrated that early spontaneous activity is necessary for segregation of ganglion cells axons in the dLGN (Shatz and Stryker, 1988; Sretavan et al., 1988). Sodium channel blocker tetrodotoxin (TTX) was intracranial infused in fetuses by minipumps to prevent action potentials. TTX was administered continuously into the fetal brain of the cat beginning from embryonic day 42, a time when axons from the two eyes are intermixed within the dLGN. They found that after two weeks ganglion cells axons were not segregated in the dLGN (Shatz and Stryker, 1988). Though there are no functional photoreceptors at these age, spontaneous waves of depolarization, named retinal waves, with a specific correlation are generated among neighboring ganglion cells and propagated across the developing ganglion cell layer, probably by means of cholinergic cells (Wong et al., 1993; Wong, 1999; Torborg and Feller, 2005). The hypothesis has been tested that retinal waves drive segregation of axons in the dLGN. In ferret pups, monocular intraocular blockade of spontaneous retinal waves of action

potentials by cholinergic agents altered the eye-specific lamination pattern of the lateral geniculate nucleus (Penn et al., 1998). The projection from the active retina was greatly expanded into territory normally occupied by the other eye, and the projection from the inactive retina was substantially reduced. In addition, increased retinal waves activity by elevated cyclic adenosine monophosphate (AMP) in one eye resulted in an expansion of the territories occupied by ipsilateral projection of that eye, suggesting an instructive role for retinal waves (Stellwagen and Shatz, 2002). Furthermore, in a mouse model (nob mouse) in which early in development spontaneous retinal activity is indistinguishable from that of wild-type mice, eye-specific segregation proceeds normally until eye-opening, when spontaneous and visually evoked activity in nob retinas become abnormal. In coincidence, retinogeniculate axons fail to preserve precise eye-specific layers (Demas et al., 2006). Transgenic rescue of the mutated protein restores spontaneous retinal activity patterns and prevents desegregation. Thus, normally structured spontaneous retinal activity stabilizes newly refined retinogeniculate circuitry (Demas et al., 2006).

Other studies have questioned the notion of an instructive role of spontaneous retinal activity in driving geniculate segregation.

Disrupting the correlated activity of neighboring ganglion cells in the developing ferret retina through immunotoxin depletion of starburst amacrine cells did not avoid normal eye segregation in the dLGN (Huberman et al., 2003). On the other hand when all spontaneous activity was blocked, segregation of projections from the two eyes fail to occur (Huberman et al., 2003). Thus, some features of spontaneous neural activity are not required for the formation of eye-specific projections to the dLGN, but a threshold is necessary to reach a normal development. The debate is still open (Cook et al., 1999; Huberman et al., 2003).

Spontaneous activity before visual experience drives formation of layers in the dLGN, but may also influence the formation of OD columns in the primary visual cortex (V1). OD columns are formed in cat, ferret or primate by segregation of geniculocortical terminals representing the two eyes. Initially they are overlapped, later segregated into distinct bands under the influence of

early electrical activity (LeVay et al., 1978; Katz and Shatz, 1996). Subsequent experiments in ferrets by the Katz's laboratory indicated that retinal activity is not required for the initial formation of OD columns (Crowley and Katz, 1999, , 2000; Feller and Scanziani, 2005; Huberman, 2007). Results supporting this theory became from experiments in which both eyes were removed very early in development, P0 in the ferret, when dLGN afferents have not reached yet the layer IV of the visual cortex. In these animals, tracer injections into individual LGN layers reveal the existence of ocular dominance columns in the visual cortex, thus ruling out the role of retinal activity in their formation (Crowley and Katz, 1999). In addition, inducing experimentally an imbalance in retinal activity by monocular enucleation between P7 and P14 unaffected segregation of OD columns, suggesting a role for molecular cues (Crowley and Katz, 2000). It is noteworthy that highly correlated activity within dLGN layers may be present in the absence of retinal input and could be sufficient to drive normal column segregation (Weliky and Katz, 1999).

However, spontaneous activity plays a role in the refinement and maintenance of the initial structure of OD columns. At two weeks from birth in cat, binocular deprivation by lid suture did not prevent geniculocortical segregation (Stryker and Harris, 1986). In contrast, complete blockade of retinal activity by making repeated intravitreal injections of tetrodotoxin (TTX) in both eyes showed a uniform labelling of cortical layer IV. Furthermore, electrophysiological recordings demonstrated that nearly all cortical cells were driven well by both eyes (Stryker and Harris, 1986). Moreover, disruption of retinal waves by epibatidine (a nicotinic acetylcholine receptor agonist) during the period in which geniculocortical afferent would normally segregate, prevents formation of OD columns (Penn et al., 1998; Cang et al., 2005; Huberman et al., 2006). Early blockade of spontaneous retinal activity also leads to a dramatic increase in receptive field size for binocular cells in primary visual cortex (Huberman et al., 2006). The reasons for such contradictory results in the literature still need to be clarified (Stryker and Harris, 1986; Penn et al., 1998; Crowley and Katz, 1999, , 2000; Cang et al., 2005).

After eye opening, spontaneous and visually driven activity coexist, but experience-dependent processes become predominant. Sensory experience is the major determinant of subsequent development and refinement necessary to

produce a mature visual system. This belief is clear since Hubel and Wiesel's experiments, in cat and monkey, showing that initial neuronal circuits can be modified by manipulating visual experience (Wiesel and Hubel, 1963). The studies of visual system plasticity take advantage of the possibility of easily manipulating visual experience for each eye. The classical paradigm to study the role of afferent visually driven activity is monocular deprivation (MD), which induces in cortical neurons a change in ocular dominance (OD) towards the open eye. Others well-studied paradigms of visual deprivation effects include dark rearing or the use of activity blockers.

EXPERIENCE-DEPENDENT PLASTICITY

Critical period and monocular deprivation

Wiesel and Hubel in the early 1960s pointed up that cortical neurons had an eye preference, and cells driven by the same eye were grouped together, originating the columns of ocular dominance (Hubel and Wiesel, 1963). Then, they started to manipulating early in life afferent visual input, by closing one eye, and they obtained an ocular dominance shift: the loss of visually driven activity in V1 through the closed eye, and a dramatic increase in the number of neurons responding preferentially to stimuli presented to the open eye (Wiesel and Hubel, 1963). This susceptibility to MD changes with age: it begins 5-10 days after onset of vision, it is most robust during a specific time window, then it declines and it is absent or minimal in the adult age (Wiesel and Hubel, 1963; Fagiolini et al., 1994; Gordon and Stryker, 1996). Later, OD shift has been reported in all mammals studied (Berardi et al., 2000). The brief time window during which MD produce maximal shift, i.e. experience is crucial for shaping the brain, is called critical period (Hubel and Wiesel, 1970; Berardi et al., 2000; Hensch, 2005). The critical period can be defined as "*a strict time window during which experience provides information that is essential for normal development and permanently alters already present neuronal connections*", by mechanisms that are activity-dependent (Hensch, 2005).

MD leads to anatomical and functional effects. In addition to the shift in OD, MD renders the closed eye amblyopic, i.e. with a lower visual acuity, even if

retina is physically healthy. Moreover stereoscopic vision is affected and contrast sensitivity drops down (Hensch, 2004, , 2005; Medini and Pizzorusso, 2008). 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 show that anatomical changes occurred days after detection of functional effects. Indeed, a OD shift is already detectable after a short period of MD (1-3 days) (Antonini and Stryker, 1993, , 1996; Antonini et al., 1998).

Wiesel and Hubel proposed a mechanism in which OD plasticity results from competitive interactions between the two eyes for the control of cortical units. This idea was supported by the fact that binocular lid suture was not effective to shift OD columns in mammals (Wiesel and Hubel, 1965; Sherman and Spear, 1982; Gordon and Stryker, 1996). In favour of a competitive view, an experiment performed by Stryker's laboratory 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). Competition-based model is related to heterosynaptic mechanisms, involving interactions between two sets of inputs (i.e. from the two eye) where open eye inputs drive down the synaptic efficacy of the deprived inputs (Miller et al., 1989; Harris et al., 1997). Active geniculate neuron corresponding to the open eye compete better than less active neurons, driven by closed eye, so they become functionally and structurally strengthened. Competition between thalamic inputs might be mediated via the acquisition of a neurotrophic factor from target structures (Maffei et al., 1992).

Data published since 1990s proposed an alternative view of the processes underlying MD, suggesting the idea that OD plasticity is due to different, homosynaptic mechanisms, related to specific forms of synaptic plasticity. These mechanisms engage separately each eye's pathway (Blais et al., 1999; Heynen et al., 2003; Frenkel and Bear, 2004). First, there is a depression of responsiveness of deprived eye, following by a potentiation of open eye responses. This model is known as Bienenstock-Cooper-Munro model (BCM theory) and postulates a bidirectional change of synapses: they can undergo

homosynaptic long term potentiation (LTP) but also homosynaptic long term depression (LTD) (Kirkwood et al., 1996; Bear and Rittenhouse, 1999; Sengpiel and Kind, 2002; Heynen et al., 2003). Homosynaptic depression occurs only at active synapses. Loss of responsiveness of deprived eye was proposed to be the result of homosynaptic depression, where spontaneous, residual activity coming from closed eye contribute to synaptic depression. To test this hypothesis, the effect of very brief MD by lid suture has been compared with that of monocular silencing by intra-ocular injections of TTX (Rittenhouse et al., 1999). Results showed that lid suture was more efficient in causing depression of deprived eye responses than blockade of all retinal activity by TTX.

Other evidence indicates the involvement of a phenomenon of homosynaptic depression in the effects of MD. In particular, brief MD sets in motion the same molecular and functional changes as the experimental model of homosynaptic LTD. Prior synaptic depression by MD also occludes subsequent induction of LTD (Heynen et al., 2003). These data support the view that homosynaptic LTD accounts for the loss of responsiveness of deprived eye during MD.

A delayed modification induced by MD is an experience-dependent potentiation of open eye responses. There is evidence supporting the idea that long-term potentiation of the synapses driven by the open eye is important for ocular dominance plasticity. First, alphaCAMKII activity appears to be required for both LTP in vitro and MD plasticity in vivo (Kirkwood et al., 1997; Taha et al., 2002). Second, a form of in vitro LTP (white matter - layer II-III LTP) is developmentally regulated with a decline over time that mirrors that of the critical period for ocular dominance plasticity (Kirkwood et al., 1996). Monocular inactivation by TTX is also able to enhance potentiation of uninjected eye, as shown by Visual Evoked Potentials (VEPs) (Frenkel and Bear, 2004).

Other data are at odds with the view that MD effects during the critical period are entirely ascribable to LTP- and LTD-like mechanisms. For example, in GAD65 knockout mice, that are not sensitive to brief MD, there is no impairment of LTD *in vitro* (Hensch et al., 1998). Strictly, 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). MD plasticity in vivo is blocked by overexpression of the protein, but LTD appears normal in these animals (Yang et al., 2005). Thus, it is still unclear whether MD effects are completely modelled by homosynaptic mechanisms. Other mechanisms likely come into play (see below).

Homeostatic synaptic plasticity

In the previous section I have discussed the view that critical period plasticity depends on Hebbian forms of plasticity, involving mechanisms similar to LTP and LTD (Rittenhouse et al., 1999; Heynen et al., 2003; Frenkel and Bear, 2004; Mataga et al., 2004). In the last years, another form of synaptic plasticity has emerged as a complement of Hebbian mechanisms, based on the concept of homeostasis. Indeed, neurons are able to maintain their responsiveness and synaptic strength within a certain range, despite perturbations of the levels of neuronal activity (Burrone and Murthy, 2003; Turrigiano and Nelson, 2004; Davis, 2006). Neurons in the visual cortex could use two strategies to counteract the reduced visually-driven activity during MD. First, deprived neurons could reduce the threshold for LTP induction (Kirkwood et al., 1996; Bear, 2003); second, visual responsiveness could be enhanced directly by increasing synaptic strength or intrinsic excitability (Desai, 2003; Maffei et al., 2004; Turrigiano and Nelson, 2004), a mechanism known as homeostatic response compensation.

A homeostatic response could include multiple mechanisms, acting by activity-dependent regulation of intrinsic firing properties (Marder and Prinz, 2003; Zhang and Linden, 2003), or through presynaptic and postsynaptic forms of excitatory synaptic plasticity (Turrigiano and Nelson, 2004; Davis, 2006), balancing of excitation and inhibition through neuronal networks (Maffei et al., 2004), or also using compensatory change in synapses number (Wierenga et al., 2006). All these changes seem to act in order to restore the neuronal firing rates to normal levels after perturbation. The most described example occurs at the neuromuscular junction (NMJ). Here, perturbation in presynaptic function leads to compensatory changes in postsynaptic excitability, in order to maintain

neuromuscular transmission (Davis and Bezprozvanny, 2001; Davis, 2006). Similarly, pharmacological blockade of postsynaptic receptors in NMJ rapidly increases presynaptic neurotransmission release (Frank et al., 2006). Synapses in the CNS require several hours to show a homeostatic response (Davis, 2006; Sutton et al., 2006). It is interesting to note that presynaptic changes in neurotransmission release could affect dynamics of short term plasticity, whereas postsynaptic modification in receptor number could scale synapse responsiveness without affecting short term kinetics of presynaptic output (Abbott and Nelson, 2000; Turrigiano, 2007). In the CNS, synapses seem to rescale their responsiveness by changes in the postsynaptic terminal, specifically by accumulating receptors for glutamate. Indeed, after relieving a TTX activity blockade, there is an increase of amplitude but not of frequency of miniature excitatory postsynaptic currents (mEPSC) (Lissin et al., 1998; O'Brien et al., 1998; Turrigiano et al., 1998; Wierenga et al., 2005). However, other *in vitro* studies reported that activity deprivation could induce changes in presynaptic release probability and number of release sites (Murthy et al., 2001; Burrone et al., 2002; Thiagarajan et al., 2005). A recent work has shed light on this issue (Wierenga et al., 2006). Authors showed that within 2.5 weeks after TTX treatment there is only a postsynaptic change, while after this period, a presynaptic alteration begins to develop. It is important to note that changes in quantal amplitude could be due not only to postsynaptic receptor accumulation, but also to an upregulation of the expression of the vescicular glutamate transporter VGLUT. Indeed, VGLUT expression is increased after activity blockade (De Gois et al., 2005; Erickson et al., 2006).

Pharmacological manipulations of activity induce bidirectional compensatory changes in mEPSC at glutamatergic synapses and this synaptic scaling is developmentally regulated, suggesting that it could be important in regulating cortical excitability during activity-dependent development (Desai et al., 2002; Maffei et al., 2004; Goel et al., 2006). As described above, one point that remains to be clarified is if synaptic scaling is induced by postsynaptic changes in firing, presynaptic changes in neurotransmitter release or local dendritic changes in receptors activation or calcium influx.

Another crucial issue in homeostasis concerns how changes in activity are signalled to synapses. Among the molecules that have been proposed there are

BDNF, cytokine tumor-necrosis factor α (TNF α) and the effector immediate-early gene product Arc. Effects of BDNF depend on brain region and developmental stage. TNF α is produced by glia, suggesting that not only pre and postsynaptic elements are involved in synaptic scaling, but a more complicate network (Turrigiano, 2007). Concerning Arc, it has been shown that its 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 has found 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 nondeprived eye responses in individual neurons. At the level of the entire population, OD shifts are explained by a reduction of deprived eye responses and a delayed increase of closed eye inputs, as I have already discussed. However, Mrsic-Flogel and colleagues found that in neurons devoid of open eye-input, the responses of deprived eye were unexpectedly stronger after MD. These findings demonstrate that the weak input of deprived eye is not able *per se* to induce response depression, which instead seems to be dependent on the input of the other eye. Second, increased visual drive in cells responding predominantly to the deprived eye is difficult to reconcile with Hebbian rules of synaptic plasticity. These results are best explained by homeostatic mechanisms. In favour of this homeostatic interpretation, authors found that the proportion of monocular, closed eye-driven cells remained constant after MD. In addition, most neurons in monocular cortex increased their responsiveness. Thus, in the deprived visual cortex compensatory mechanisms seem to exist that maintain firing rates within a certain range during MD.

In summary, it is likely that homeostatic and Hebbian rules concur to produce the OD shift after MD. Decorrelated input through the closed eye causes weakening of deprived-eye synapses, accordingly to Hebbian rules, during the first few days of MD (Heynen et al., 2003), but later a compensatory upscaling of responses occurs. This is consistent with the finding that the level of deprived-eye response depression stabilizes or even reverses (Frenkel and Bear, 2004) after 3 days of MD. Further studies are required to clearly understand the

mechanisms underlying homeostatic compensation of neuronal responsiveness. These mechanisms may include rearrangements in intracortical inhibitory circuitry or changes in synaptic strength/intrinsic conductances of individual cells (Mrsic-Flogel et al., 2007).

Critical period and inhibitory circuits

It is extremely simplistic to consider thalamocortical afferents as the only players in the process of OD plasticity. Visual cortex is an intricate circuit in which many neuron networks crosstalk, leading to a delicate balance between excitation and inhibition. Attempts to disrupt excitation/inhibition balance during MD have been performed. For example, hyperexcitation by glutamate or bicuculline as well as cortical silencing by 2-amino-5-phosphonovaleric acid (APV) prevent OD plasticity (Shaw and Cynader, 1984; Ramoa et al., 1988; Bear et al., 1990). However, these manipulations do not provide mechanistic information as any change in cortical activity is bound to interfere with plasticity. Hensch's group was the first to shed light on the role of local, inhibitory cortical circuits in OD plasticity (Hensch et al., 1998). He took advantage of a knockout (KO) mouse, lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65), the GABA biosynthetic enzyme. The other isoform, GAD67, localizes to cell somata and dendrites, and accounts for most of GABA synthesis in interneurons. Indeed, GAD67 KO die at birth, with GABA concentration less than 10% of wild-type value. In contrast, GAD65 is found primarily in the synaptic terminal, where it serves as a reservoir of GABA, because it can be recruited when needed to provide additional GABA synthesis, for example following intense activity. GAD65 KO mice survive and develop normal morphology and normal adult concentration of GABA, because GAD67 expression is normal. Only after an intense neuronal activation, GABA release in GAD65 KO mice is reduced with respect to wild-type. Interestingly, extracellular recordings revealed prolonged discharge in GAD65 mice, i.e. a tendency to continue to fire even after stimuli have passed the cell's receptive field. Hensch and coworkers found that in these KO mice MD was ineffective: cells continued to respond preferentially to the contralateral, closed eye following MD (Hensch et al., 1998). Remarkably, mechanisms of synapse modification in vitro, as LTP and LTD, were not

impaired in these mice, demonstrating no general deficit in activity-dependent plasticity. Enhancement of intracortical inhibitory transmission by benzodiazepines (diazepam) restored OD plasticity in GAD65 KO mice (Hensch et al., 1998). Notably, rescue of plasticity is possible at any age, indicating that critical period onset is dependent on a certain level of inhibitory transmission (Fagiolini and Hensch, 2000).

Consistent with this view, the onset of the critical period can be accelerated by premature enhancement of GABA-mediated transmission (Fagiolini and Hensch, 2000; Fagiolini et al., 2004). In transgenic mice overexpressing BDNF, inhibitory circuits mature precociously, and the critical period for plasticity begins and ends earlier than normal (Huang et al., 1999).

One class of interneurons that are particularly important for critical period plasticity are the parvalbumin positive cells. Specific blockade of fast-spiking fidelity in these cells decreases the OD shift after MD (Hensch, 2005). An interesting experiment of Hensch's group was to analyze if a particular GABAergic receptor was involved in OD plasticity. They used a mouse "knockin" mutation to alpha subunits that renders individual $GABA_A$ receptors insensitive to diazepam. Using this approach, they found that only $GABA_A$ receptors containing α1 subunit are responsible for critical period plasticity (Fagiolini et al., 2004). These receptors are preferentially localized at somatic synapses opposite to parvalbumin-positive large basket cell terminals. With age, these large parvalbumin-positive cells are enveloped in perineuronal nets of extracellular matrix, which provide more efficient inhibitory neurotransmission. When perineuronal nets are disrupted, perisomatic inhibition of the target is reduced (Saghatelyan et al., 2001) and OD shift can be induced by MD (Pizzorusso et al., 2002). The key role of GABAergic transmission in the regulation of OD plasticity is not restricted to mouse, as it has been demonstrated that OD columns are disrupted in cat by manipulating inhibitory transmission (Hensch and Stryker, 2004). Thus, a certain threshold of inhibition is necessary to trigger plasticity, but a higher level of inhibition, reflecting maturation of circuits, is responsible for critical period closure (Huang et al., 1999).

Effects of dark rearing

A widely used paradigm to study the role of vision in cortical development is to rear animals in complete darkness. Animals kept in darkness from birth show abnormal functional and anatomical maturation of the visual cortex, that appears immature far beyond the end of the critical period (Benevento et al., 1992; Fagiolini et al., 1994; Gianfranceschi et al., 2003). Visual deficits of dark reared (DR) rats include an extremely low spatial resolution (visual acuity), as measured electrophysiologically and behaviourally (Fagiolini et al., 1994; Gianfranceschi et al., 2003). Cortical neurons of DR animals exhibit rapid habituation, i.e. the visual response tends to disappear after repeated stimulus presentations (Sherman and Spear, 1982; Fagiolini et al., 1994). Receptive fields of cortical cells are larger than normal, and neurons have a reduced orientation selectivity. By Visual Evoked Potential (VEP) technique it has been found that also other parameters of visual responses are changed: the latency of visual response appears increased while temporal resolution is significantly reduced (Pizzorusso et al., 1997a). In addition, changes have been reported in the spontaneous discharge of cortical units of DR animals, and they are linked to alterations in intracortical inhibitory mechanisms (Benevento et al., 1992). Indeed, the mean rate of spontaneous activity was increased in DR animals (Gianfranceschi et al., 2003).

The increased spontaneous activity, the loss of orientation selectivity and the larger receptive fields indicate that intracortical inhibitory mechanisms could be seriously compromised. Indeed, dark-rearing resulted in a significant decrease in the density of GABA-immunoreactive neurons in all cell layers of primary rat visual cortex. Inhibitory currents are also reduced (Benevento et al., 1995; Morales et al., 2002). Deficits in inhibition could also explain the relatively prolonged duration of responses to moving stimuli, an effect resembling the prolonged discharge found in GAD65 KO animals (Hensch et al., 1998).

As discussed before, inhibitory circuit maturation is a key factor in determining critical period plasticity. It is well established that DR animals retain a great potential for plasticity, i.e. the critical period is extended. In DR animals, MD performed beyond the end of the critical period is effective in producing a shift in OD distribution (Cynader and Mitchell, 1980; Mower, 1991; Gianfranceschi

et al., 2003). This potential for plasticity decrease, however, if normal visual experience is allowed. Similarly, most of the cortical cells become orientation selective when dark rearing is followed by light experience (Cynader and Mitchell, 1980). Given that activity could switches on various molecules (Nedivi et al., 1993), visual-driven input may switch on a molecular cascade that finally leads to maturation of the visual cortex. More than ten years ago, various groups have demonstrated that among the molecules involved a key role is played by neurotrophic factors (Maffei et al., 1992; Berardi et al., 1994; Pizzorusso et al., 1997b; Huang et al., 1999). Experiments showed reduced expression and activity of neurotrophic factors in DR animals (Castren et al., 1992; Cotrufo et al., 2003). Two important experiments demonstrated physiological rescue of DR effects by neurotrophins. First, transgenic mice overexpressing BDNF were kept in darkness from birth, and visual cortex maturation and susceptibility to MD were analyzed. The authors found that visual acuity, receptive field size, critical period for OD plasticity and inhibitory transmission in DR BDNF mice were identical to those of normal, light-reared mice (Gianfranceschi et al., 2003). Second, enriched environment promotes the same rescue effects from DR as BDNF-overexpressing mice (Bartoletti et al., 2004). As reported in literature, animals kept in an enriched environment shown a precocious expression of BNDF (Cancedda et al., 2004). Therefore, BDNF overexpression rescues effects of DR, and it is sufficient to promote visual cortex maturation also under conditions of visual deprivation, likely acting at the level of GABAergic circuits.

Role of intrinsic cortical activity

Since Hubel and Wiesel, the role of afferent inputs on maturation and plasticity of the visual cortex has been amply investigated. However, to date only few reports have addressed the role of intrinsic cortical activity.

Synaptic model of cortical plasticity are based on Hebbian mechanisms: postsynaptic activity is important for potentiation because coincidence of postsynaptic activity and recently active presynaptic terminals enhances strength of synapses. Taking advantage of the robust OD shift induced by MD in visual cortex, Stryker's laboratory studied the role of postsynaptic cells during OD plasticity. His group inhibited pharmacologically postsynaptic

activity in V1 by means of the $GABA_A$ agonist muscimol (Reiter and Stryker, 1988). Muscimol was infused continuously in kitten visual cortex by osmotic minipumps and blocked cortical activity with no direct effects on geniculocortical terminals. MD was performed during the infusion period. As expected, in control animals MD normally shifted OD toward the open, more active eye. However, in muscimol infused cortex (after recovery from drug action), they found a completely different result: OD shift occurred in the direction of the less active, closed eye (paradoxical effect). One possible explanation is that the activity of deprived thalamocortical terminals is now better correlated with that of the inhibited postsynaptic cell, thus leading to synapse strengthening. Notably, recordings in areas bordering the muscimol infused region showed normal shift of OD in favour of the open eye. These data are very interesting, because they demonstrate a crucial role for postsynaptic neurons in OD plasticity. Indeed, postsynaptic target activity is a major determinant of the direction of OD plasticity.

A subsequent experiment from the same group provided evidence for the effect of MD on geniculocortical afferents when visual cortex was blocked by muscimol infusion (Hata and Stryker, 1994). After MD and delivery of muscimol for four weeks, they physiologically mapped activity of cortical cells to delimit the region of action of muscimol, and then labelled and analysed thalamocortical terminals. Anatomically, they found in the region around the cannula an expansion of territories controlled by the closed eye, similar to that found for the open eye far from the cannula, where muscimol did not diffuse.

The role of cortical activity in visual cortex development has been poorly investigated. An experiment addressed the role of intrinsic activity in the development of orientation selectivity in the ferret (Chapman and Stryker, 1993). The ferret visual system is similar to that of cat, but it is a better model to study precocious processes of cortical maturation because ferrets are born at an earlier stage of development. This study investigated the role of cortical activity in development of orientation selectivity by intracortical injection of TTX. Results of this manipulation were compared to those obtained in normally reared or binocularly deprived animals. The results showed that blockade of cortical activity prevents maturation of orientation properties, freezing orientation selectivity in an immature state.

Finally, the development of horizontal projections in primary visual cortex of cat it has been studied. These type of connections link cells with similar orientation selectivity by a patchy network of fibers, that may extend for several millimiters. Experiments provided evidence for a role of cortical activity in development of horizontal connections (Ruthazer and Stryker, 1996). Visual cortex was continuously silenced by TTX infusion in ferret at the third postnatal week, and horizontal connections were identified by retrograde labelling. In contrast to the normal, patchy distribution of normal ferrets, the data demonstrated a spatially random distribution of retrogradely labelled cells after TTX. Remarkably, bilateral enucleation allowed an initial development of clusters of horizontal connections. Thus, spontaneous cortical activity is sufficient for the initial organization of horizontal connections, but to reach mature anatomical features, horizontal connections need visually driven activity.

MOLECULAR MECHANISMS IN VISUAL CORTEX PLASTICITY

The molecular network involved in visual cortex plasticity remains only partly understood. Starting from the pioneering experiments on neurotrophins, several studies have been performed to gain knowledge of the molecules mediating the action of experience in plasticity (Berardi et al., 2003; Medini and Pizzorusso, 2008).

In 1990s, Maffei's group and other laboratories put forward the idea that competition in OD plasticity might depend on the uptake of limiting growth factors, such as neurotrophins (NT), secreted by cortical neurons and retrogradely transported (Maffei et al., 1992; Bonhoeffer, 1996; Harris et al., 1997; McAllister et al., 1999). Accordingly, exogenous delivery of NT in the visual cortex during MD prevented competition, rescuing cortex from MD effects (McAllister et al., 1999; Berardi et al., 2000). It has also been clarified that, with the exception of neurotrophin 3 (NT-3), all neurotrophins influence MD, but not all factors play the identical role on visual neuron properties (Lodovichi et al., 2000). In particular, neurotrophin 4 (NT-4) and NGF prevent the shift induced by MD, and they have no effects on spontaneous or visually-

driven activity (Gillespie et al., 2000; Lodovichi et al., 2000). In contrast, BDNF is less effective in preventing OD shift, and it changes both spontaneous and visually-evoked activity of cortical neurons.

A complementary strategy consisted in antagonizing the action of endogenous neurotrophins. Blockade of NGF led to alterations in visual acuity and binocularity of cortical neurons. In addition it induced a shrinkage of geniculate neurons, demonstrating a key role for neurotrophins in normal visual cortex development and plasticity (Berardi et al., 1994). Blockade of the TrkB ligands BDNF and NT-4 desegregated OD columns (Berardi et al., 1994; Cabelli et al., 1997). Other key studies were conducted in BDNF-overexpressing mice. These animals maintain normal cellular pattern and expression of BDNF in visual cortex, but have an accelerated postnatal rise of this neurotrophin (Huang et al., 1999). BDNF-overexpressing mice showed an acceleration of development of visual function, such as visual acuity, and of CP time course. Notably, a strong link was established between BDNF and intracortical inhibition, because GABAergic circuit maturation is accelerated by overexpressing BDNF (Huang et al., 1999).

One link between experience-dependent plasticity and NT action is represented by experience-dependent production and release of NT (Bozzi et al., 1995; McAllister et al., 1999). NT can modulate electrical activity, at pre and post-synaptic level, increasing neurotransmitter release, depolarizing neurons but they also act on gene expression (Sala et al., 1998; Kafitz et al., 1999; Lodovichi et al., 2000; Poo, 2001). This reciprocal regulation between activity and neurotrophins might be the reason because only active synapse in MD are reinforced (Caleo et al., 1999a; Kovalchuk et al., 2002).

It is well known that N-methyl-D-aspartic acid (NMDA) receptors are particularly implicated in mediating changes in synaptic efficacy. These receptors are not only transmitter and voltage-dependent, but they also permit Ca^{2+} influx, which is an intracellular signal. NMDA receptors involvement in visual cortical plasticity started with experiments showing that blockade of NMDA receptors resulted in no effects after MD (Bear et al., 1990; Roberts et al., 1998; Sawtell et al., 2003). Interestingly, NMDA receptors are developmentally regulated: in visual cortex, NMDA subunit composition

varies from prevalent presence of NR2B to a dominant present of NR2A, paralleling to visual cortex development and critical period plasticity. NR2B is responsible of long-lasting current, while NR2A originates currents with faster kinetics. Dark rearing, which freezes visual cortex development and plasticity, delays expression of NR2A subunit, suggesting that NR2B/NR2A switch has a role in visual cortex maturation and CP regulation. However, in mice with deletion of NR2A subunit, OD plasticity is weaker but restricted to normal CP, while orientation selectivity fails to mature (Fagiolini et al., 2003). Thus, NR2A might be not necessary for CP regulation, but might be involved in other properties of cortical maturation.

Electrical activity, neurotrophins, and NMDA receptors can set in motion three kinases important per OD plasticity: cAMP-dependent protein kinase (PKA), extracellular-signal-regulated kinase (ERK), α Ca^{2+}/calmodulin dependent protein kinase II (α CAMKII) (Beaver et al., 2001; Di Cristo et al., 2001; Taha et al., 2002; Cancedda et al., 2003). Each kinase has its own pathway that however influences also that of the others, for example via phosphorylation of the same downstream target (e.g., CREB). The overlap and crosstalk among these pathways explain why blockade of only one of these molecules affects OD plasticity. However, level of regulation could be of two types: cytoplasmatic, local and rapid, targeting molecules involved in synaptic transmission or cytoskeleton motility; or nuclear, delayed, engaging gene regulation.

Long-lasting modifications require changes in gene expression, also in visual cortex (Mower et al., 2002; Taha and Stryker, 2002). It has been found that visually-driven activity activate transcriptional factors, such as zif268 or CREB (Caleo et al., 1999b; Pham et al., 1999; Mower et al., 2002). Many other sets of genes are modulated by visual experience or deprivation, and one important molecular cascade regulated by activity is the IGF-1 receptor pathway (Nedivi et al., 1993; Tropea et al., 2006). An experience-regulated factor is not always necessary for OD plasticity, in fact mice knock out for zif268 respond with normal shift after MD (Mataga et al., 2001). To date the action of only a few factors has been found to be necessary for MD (Pham et al., 1999; Mower et

al., 2002). ERK inhibition prevents synaptic plasticity and MD effects in visual cortex, as exogenous application of IGF-1 (Di Cristo et al., 2001; Cancedda et al., 2003; Tropea et al., 2006). A challenge is represented by addressing the gene expression profile in single, identified types of neurons, following experience.

In the latter years, it became clear that neurons modify gene expression patterns in response to experience-dependent activity. These mechanisms could explain the way by which the brain produces long term changes in its circuits. Histone phosphoacetylation, seems to be important for synaptic plasticity in hippocampus and for activity-dependent gene transcription (Alarcon et al., 2004; Korzus et al., 2004). Recently, the regulation of chromatin structure has emerged as one mechanisms regulating visual cortex plasticity. The experiments have demonstrated the involvement of histone phosphoacetylation in OD plasticity (Putignano et al., 2007). The authors found that these modifications occurred within minutes, following visual experience. A mediator seems to be ERK, because ERK inhibition prevents visually-driven phosphoacetylation. Remarkably, this effect is developmentally regulated: in adult mice visual experience is able to activate ERK and other kinases, but it is unable to promote histone phosphoacetylation at a level comparable to that found in juvenile animals. Accordingly, restoring histone acetylation by pharmacological drugs is able to promote OD plasticity in adult mice (Putignano et al., 2007).

Plasticity has not only *intracellular* effectors, because it is known that the extracellular environment also contributes to regulate plasticity. One of the factors involved in experience-dependent plasticity is represented by tissue plasminogen activator (tPA). Biochemically, it is a serine protease and it is an immediate early gene induced by electrical activity (Qian et al., 1993). Proteolysis by tPA increased in V1 after two days of MD during the CP, but not in adulthood or in GAD65 knock out mice (Mataga et al., 2002). Accordingly, tPA inhibition impaired OD shift induced by MD (Mataga et al., 1996) and prevented recovery from MD following reverse occlusion (Muller and Griesinger, 1998). Targets of tPA include extracellular-matrix proteins, growth factors, membrane receptors, cell-adhesion molecules (Endo et al.,

1999; Wu et al., 2000; Nicole et al., 2001), that could all be involved in cortical plasticity. A further evidence for a key role of tPA in plasticity is represented by data showing an increase of spine motility in young animals after tPA delivery (Oray et al., 2004).

Another component of extracellular matrix (ECM) which has been investigated is represented by chondroitin-sulfate proteoglycans (CSPGs). These molecules are organized in typical structures, named perineuronal nets (PNNs), around soma and dendrites of parvalbumin-positive neurons. PNNs increase during development and their organization in the visual system is complete at the end of the critical period (Hockfield et al., 1990; Koppe et al., 1997; Pizzorusso et al., 2002). Dark rearing prevents PNNs formation (Pizzorusso et al., 2002), while axonal sprouting and regeneration are inhibited by CSPGs (Bradbury et al., 2002). CSPGs exert an inhibitory action in cortical plasticity, indeed degradation of CSPGs in adulthood by chondroitinase ABC is able to restore OD plasticity and to promote recovery from amblyopia (Pizzorusso et al., 2002; Pizzorusso et al., 2006). In addition, treatment with chondroitinase ABC is also able to increase spine density (Pizzorusso et al., 2006). It is interesting to note that ECM proteolysis could be regulated not only exogenously, but also autonomously by particular rearing conditions. Enriched environment promotes amblyopia recovery and decreases PNNs number (Sale et al., 2007).

To date it has been accepted that the adult brain has a decreased ability to repair and that myelin exerts an active inhibitory role in these processes (Schwab, 2004). Few years ago it has been demonstrated that certain factors preventing brain repair, such as Nogo, a growth inhibitor associated to myelin, are involved in the closure of the critical period. Mice with a mutation in Nogo receptors have an altered course of the CP, showing a prolonged OD plasticity induced by MD (McGee et al., 2005). The Nogo pathway does not seem to affect GABAergic inhibition or tPA activity, that are indeed normal in mutant mice. Rather, its signalling involves the low-affinity neurotrophin receptor p75 and Rho pathway. Thus, myelinization is able to inhibit not only recovery from injury, but could also promote the decrease of plasticity observed at the end of the CP (Sengpiel, 2005).

PLASTICITY IN ADULTHOOD

Since the classical experiments of Hubel and Wiesel in 1960s, plasticity is thought to occur only during a brief time window early in life, the critical period, as discussed above. However, recent evidence suggests some residual plasticity also in adult animals. This seems to be true not only for the visual system, but also for other models, such as the somatosensory cortex that shows persistent plasticity in some cortical layers also in mature animals (Diamond et al., 1994).

Several laboratories have reported OD plasticity in the visual cortex of adult mice, using intrinsic signal imaging, activity reporter gene Arc, and Visual Evoked Potentials (VEPs). Some but not all, authors reported OD plasticity in adult mice using extracellular unit recordings (Gordon and Stryker, 1996; Hensch et al., 1998; Sawtell et al., 2003; Lickey et al., 2004; McGee et al., 2005; Tagawa et al., 2005; Hofer et al., 2006b; Tohmi et al., 2006; Heimel et al., 2007). Interestingly, OD plasticity in adult mice has been reported in urethane- but not barbiturate-anesthetized animals (Fagiolini and Hensch, 2000; Pham et al., 2004). Using VEPs, plasticity has been measured in awake, head-restrained mice (Fagiolini and Hensch, 2000; Sawtell et al., 2003; Pham et al., 2004).

Important data were reported a few years ago by Sawtell et al. (2003) demonstrating not only that OD plasticity occurred in adult mice but also that the mechanism is different from that found during the critical period. First, they measured in normal mice the ratio of VEPs elicited by 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, a slightly longer MD (5 days) was able to induce a shift in OD. Hence, they studied daily VEPs amplitude in chronically implanted animals, to identify the mechanism: the adult OD plasticity was almost completely due to a potentiation of absolute amplitude of VEP of ipsilateral, open eye, that developed gradually after the third day of MD. This stands in contrast with the effects of MD in juveniles. Indeed, in young animals 3 days of MD were effective in shifting OD mainly by depression of contralateral, deprived eye

inputs, while there was only a delayed component of ipsilateral, open eye response potentiation. Thus, the mechanism by which OD plasticity occurs in adults seem to be different from juveniles. However, the same group has also demonstrated that functional NMDA receptors are necessary for adult plasticity, suggesting common molecular effectors. It is noteworthy that effects induced by adult MD seem to be different depending on whether the contralateral or ipsilateral projection is analyzed. Ipsilateral eye inputs undergo weakening after MD of ipsilateral eye, demonstrating that input depression is still possible in adult mice, despite the developmental downregulation of LTD (Tagawa et al., 2005; Hofer et al., 2006b).

An important point to investigate is the structural rearrangement consequent to MD in adulthood. In juvenile animals, functional changes come first, and thalamocortical axon remodelling takes some days to develop(Antonini and Stryker, 1993; Antonini et al., 1999). Progress in neuroscience techniques, such as the introduction of 2-photon microscopy, has allowed to study changes at the level of dendritic spines. It was found that spine motility increased after 2 days of MD, while after 4 days there was a significant spine loss, consistent with the initial strong reduction of responses elicited by the deprived eye (Gordon and Stryker, 1996; Mataga et al., 2004; Oray et al., 2004; Hofer et al., 2006b). 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). In adulthood, spine motility is still present in normal mice (Holtmaat et al., 2005). However, spine loss after MD is not observed, consistent with the absence of depression of deprived eye inputs (Sawtell et al., 2003; Hofer et al., 2006b). Rather, there is a slight spine gain, consistent with potentiation of the open eye (Mataga et al., 2004). Specifically, spine gain is detectable in apical dendrites of layer V pyramidal neurons (Hofer et al., abstract 346.23/D25; 2007 Neuroscience Meeting).

Promoting adult plasticity

I have already discussed about the possibility to enhance visual cortex plasticity by removing inhibiting factors, such as components of ECM or myelin-associated molecules (Pizzorusso et al., 2002; McGee et al., 2005; Pizzorusso et al., 2006). Enhancing plasticity in adulthood is of high interest

because is strictly connected to functional recovery. The same factors that promote OD plasticity could for example be exploited to allow recovery from amblyopia.

It has been demonstrated that plasticity and recovery can be obtained not only using pharmacological drugs, but also by manipulating environment and visual experience, i.e. by non-invasive protocols. In adult rats, enriched environment is sufficient to lead to complete recovery from early MD (Sale et al., 2007). Similarly, ten days of visual deprivation (dark rearing) in adult rats are effective in restoring susceptibility to MD, as measured by evaluating the ratio of VEPs elicited by the two eyes (He et al., 2006). A period of dark rearing followed by reverse suture is effective in promoting recovery from amblyopia consequent to early MD (He et al., 2007). However, this recovery is not complete, at least at the behavioural level (Pizzorusso et al., 2006; Sale et al., 2007).

Recently, it has been demonstrated by an interesting experiment that previous experience can influence the potential for plasticity in adult circuits (Hofer et al., 2006b). Inducing an OD shift by brief MD in juvenile or in adult mice leads to a stronger and more persistent change consequent to a second MD several weeks later. Remarkably, this induction is eye-specific because it occurs only if it is the same eye to be deprived. (He et al., 2006).

In conclusion, plasticity in adult mice was found by many laboratories and with different techniques. Plasticity in adulthood seems to be qualitatively and quantitatively different with respect to juvenile animals. The potential for plasticity remains maximal during the critical period (Hofer et al., 2006a; 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). In the adult rat visual cortex, plasticity can be reinstated either by manipulating the rearing environment or by degrading CSPGs (Pizzorusso et al., 2006; He et al., 2007; Sale et al., 2007).

CORPUS CALLOSUM AND VISUAL EXPERIENCE

So far I have focused attention on the effects of manipulating afferent and intrinsic activity in visual cortex development and plasticity. An important input to visual cortex is represented by afferents from the opposite hemisphere running within the corpus callosum. In this section I discuss how visual experience shapes callosal connections.

As I have already pointed out, callosal afferents terminate in both supragranular and the infragranular layers. In cats, terminals are particularly concentrated in a narrow transition zone between area 17 and 18 (Blakemore et al., 1983; Payne, 1990; Payne and Siwek, 1991; Mizuno et al., 2007). However, there is also evidence for callosal axon collaterals innervating the core of area 17 (Houzel et al., 2002). In rats, 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). Recently, axon labelling in mice has demonstrated a narrow strip of callosal terminals at the area 17/area 18 border (Mizuno et al., 2007).

The development of visual callosal connections is strongly activity-dependent (Innocenti, 1986; Mizuno et al., 2007). Indeed callosal axons are initially exuberant, but during development there is a partial elimination of callosal axon terminals (Innocenti and Caminiti, 1980). 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 partial elimination of immature callosal projection (Frost and Moy, 1989): there is a reduction of the total number of callosal neurons, but also a slightly narrower distribution of their terminals at the area 17/18 border. In addition, both bilateral eyelid suture or bilateral enucleation (Innocenti and Frost, 1980; Innocenti et al., 1985) decrease the number of callosal projecting neurons, with an important difference: enucleation produces also an abnormally wide distribution of callosal cells at the 17/18 border. This latter effect is similar to that described in cats reared with convergent or divergent strabismus, monocular enucleation, or monocular eyelid suture. All these manipulations produce a widespread distribution of callosal terminals (Innocenti and Frost, 1979; Berman and Payne, 1983; Frost et al., 1990).

These results demonstrating a role for activity and visual experience in callosal projections development suggest a mechanisms similar to that taking place in segregation of OD columns. Thalamocortical axons are overlapped early in life, then segregated during development. Similarly, vision seems to act in callosal projections rearrangement by modulating developmental elimination of callosal neurons and fibers (Innocenti, 1986; Mizuno et al., 2007).

There are also data showing that visual experience can influence the functional properties of callosal neurons. For example, a study demonstrated that MD in adulthood is able to induce functional changes in visual callosal map, leading to an increase of receptive field size and to a loss of orientation selectivity (Watroba et al., 2001).

The data described above indicate that sensory experience alters callosal afferents. In literature there are also some indications that manipulating the callosum may impact cortex development. Indeed, 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). These studies are important because they indicate a role for the callosum in development but have some shortcomings. In particular, section of the corpus callosum is a quite invasive protocol, and its effects on cortical maturation could be due to absence of callosal projections, but also to lesion-induced plasticity.

BOTULINUM NEUROTOXINS

Many living species produce toxins that are able to modify the physiology of other organisms in order to increase their chance of survival. Chemically toxins can be very simple molecules or heavy proteins, depending on organism. Most of the toxins are specific for a selected target, and during evolution toxins have been often shaped around their targets. Moreover, most known toxins have as target specific molecules of the nervous tissue, such as the most poisonous neurotoxins, tetanus and botulinum. In general, neurotoxins act by blocking neurotransmitter release.

Botulinum neurotoxins are produced by a bacterium, genus *Clostridium*, that produces spores widely present in the environment and resistant to various physical and chemical agents. These spores can contaminate food and under particular anaerobic conditions they could germinate and yield the vegetative bacteria, which produce toxins. Ingestion of food contamined by botulinum neurotoxins causes an intoxication, the botulism, first described at the beginning of the 17^{th} century. Recently, it has been described also an *infant botulism*, caused by colonization of the intestinal tract by *C. botulinum* (Schiavo et al., 2000; Simpson, 2004). Botulism could also be developed by intoxication of wounds, because anaerobic environment of wounds is a good growing substrate for *C. botulinum* (Schiavo et al., 2000; Simpson, 2004)

To date seven type of botulinum neurotoxins have been characterized, from BoNT/A to /G. Their common effect consists in the blockade of neurotransmitter release. In botulism the sustained blockade of acetylcholine at somatic and autonomic nerve terminals leads to a progressive weakness and paralysis of skeletal muscle, reduced salivation, lacrimation, nausea. The paralysis is descending, affects muscles of the trunk, including respiratory ones. Respiratory or cardiac failures are responsible of death in botulism: if respiratory failure is rescued by mechanical ventilation, patients survive botulism, with slow recovery. Indeed, the potent action of botulinum on neurotransmitter release is completely reversible. It is not surprising that botulinum neurotoxins have elicited so great interest, not only because of botulism, but also to exploit their specific and reversible action to better understand neuroexocytosis. The coevolution of botulinum neurotoxins and

their targets made toxins good tools to understand nerve physiology. Recently, BoNTs have been used as therapeutic agents for many human syndromes caused by hyperactivity of cholinergic nerve terminals and in pharmaco-cosmetics.

Structure of botulinum neurotoxins

BoNTs are synthesized in the bacterial cytosol without a leader sequence and they are released to the culture medium after bacterial lysis as a progenitor toxins, composed of inactive single polypeptide chain of 150 kDa and other non-toxic accessory proteins. These accessory proteins protect BoNTs during the passage in the stomach, then dissociate. The inactive form of the protein can be cleaved by different bacterial and tissue proteinases within a surface-exposed loop (Krieglstein et al., 1994; Turton et al., 2002) to form the active di-chain neurotoxin. The heavy chain (HC, 100 kDa) and the light chain (LC, 50 kDa) remain associated via both non-covalent protein-protein interactions and the conserved interchain S-S, the integrity of which is essential for neurotoxicity (Schiavo et al., 1990). The 3D crystal structure of BoNT/A has been determined (Lacy et al., 1998). The active form consists of three different domain of 50 kDa, endowed with distinct biological properties (Lacy and Stevens, 1999; Swaminathan and Eswaramoorthy, 2000):

- the zinc-dependent endopeptidase domain of the LC;
- the translocation domain in the N-terminal half of the HC;
- the binding domain in the C-terminal half of the HC;

These three functional domains are structurally distinct and arranged in a linear fashion, such that there is no contact between them.

Historically, BoNTs mechanism action have been studied at the neuromuscular junction, leading to the identification of four sequential step of action: rapid and specific binding to cholinergic nerve terminal membrane, internalization inside a vesicle, membrane translocation in the cytosol, expression of the L-chain proteolytic activity (Schiavo et al., 2000; Lalli et al., 2003).

Function

Binding

BoNTs diffuse in the body fluids from the site of adsorption to the pre-synaptic membrane of cholinergic terminals where they bind. The binding domain is cointained in the C-terminal of the HC, although additional region seem to play a role, because immunization with the C-terminal fragment of HC shows only a partial protection from intoxication with the intact BoNT molecule (Poulain et al., 1991). In the last years many efforts have been focused to identify BoNTs receptors, leading to the common idea that polysialogangliosides are involved in the binding (Kitamura et al., 1980; Montecucco et al., 1988). Indeed, preincubation of BoNTs with polygangliosides partially prevents the poisoning of the nerve terminal, and treatment of cultured cells with neuroamidase, that remove sialic acid residues, decreases the binding of clostridial toxins (Schiavo et al., 2000). However it is unlikely that polysialogangliosides are the sole receptors of these neurotoxins and there are many evidence that proteins of the nerve cell surface have a part in the process. Montecucco in 1980s hypothesized that BoNTs bind strongly and specifically to the presynaptic membrane because they display multiple interaction with sugar and protein binding sites. Indeed subsequent experiments demonstrated that both BoNT/B and BoNT/F bind strongly to the synaptic vesicle protein synaptotagmin II in the presence of polysialogangliosides (Nishiki et al., 1994; Li and Singh, 1998). Only a couple of years ago it has been identified the receptor for BoNT/A, SV2, the synaptic vesicle protein 2 (Dong et al., 2006).

Internalization

All available evidence indicates that BoNTs do not enter the cell directly from the plasma membrane but they are endocytosed inside the lumen of vesicular structures, as demonstrated by electron microscopic studies (Black and Dolly, 1986). The internalization is a process temperature and energy-dependent. The HC plays a fundamental role in the internalization step, at least for BoNT/A, /B, /E. The rate of nerve terminal inhibition by BoNTs seem to be dependent on synaptic activity: more active nerves are intoxicated faster than the less active, probably because the most active have higher level of vesicle recycling, and then higher level of internalization (Dong et al., 2006).

Translocation

After being internalized in the vesicles, the L chain needs to cross the hydrophobic barrier of the vesicle membrane to reach the cytosol where it can display its proteolytic activity. BoNTs need to be exposed to a low pH step before nerve intoxication can occur (Simpson, 1983). Low pH induces a conformational change from a water soluble neutral structure to an acid structure characterized by the surface exposure of hydrophobic patches, in order to allowed transmembrane translocation of both chains (Hoch et al., 1985; Schmid et al., 1993). Studies with model membrane systems have shown that BoNTs, following this low pH-induced membrane insertion, form ion channels in the planar lipid bilayer that regulate the transmembrane translocation of the L domain from the vesicle membrane to the nerve terminal cytosol. Interestingly, different serotypes show different speeds of translocation, and BoNT/E translocates rapidly relative to BoNT/A (Keller et al., 2004).

Zinc-endopeptidase activity

All BoNTs are specific proteases. The catalytic activity of BoNTs was discovered following the sequencing of the corresponding genes (Minton, 1995). There is a highly conserved 20-residues-long segment, located in the middle of the L chain, containing a zinc-binding motif typical of zinc-endopeptidases. Subsequently, the substrates of BoNTs enzymatic activity were identified through assays of proteolysis performed on synaptic proteins (Sollner et al., 1993).

BoNTs act through specific cleavage of a group of proteins integral to the exocytotic process, the SNARE proteins.

For synaptic vesicle exocutosis, it is necessary the interaction between specific integral proteins of the synaptic vesicles membrane (the v-SNARE) and receptor proteins of the target membrane (the t-SNARE). In the brain two t-SNARE have been identified: syntaxin, a nerve terminal integral membrane protein, and SNAP-25, a peripheral membrane protein of 25 kDa mass. In synaptic vesicles the integral membrane protein VAMP (or synaptobrevin) has been identified as the v-SNARE. The ternary complex of VAMP, syntaxin, and

SNAP-25 is extremely stable. When SNARE disassembly, the energy released upon hydrolysis of ATP is used for vesicle fusion and neurotransmitter release.
The seven BoNTs are very specific proteases. Indeed BoNT/B, /D, /F and /G cleave VAMP, each at a single site (Schiavo et al., 1992; Schiavo et al., 1993; Schiavo et al., 1994); BoNT/A and /E cleave SNAP25, each at a single site (Schiavo et al., 1993) while BoNT/C cleaves both syntaxin and SNAP-25 (Schiavo et al., 1993). Recombinant VAMP, SNAP-25 and syntaxin are cleaved at the same peptide bonds and at the same rate as the corresponding cellular proteins, indicating that there is no additional endogenous factors involved in the proteolytic activity of BoNTs. Primary and secondary structure of the neurotoxins are very similar, in addition variable cleavage sites and flanking regions do not account for the specificity of the three SNARE proteins. Hence, it is possible that SNARE targets could have a common structural element that would serve as a recognition motifs for the neurotoxins. Comparison of their sequence has revealed the presence of a nine-long residue motif, characterized by three carboxylate residues alternated with hydrophobic and hydrophilic residues, termed thereafter the SNARE motif (Rossetto et al., 1994). Experiments have supported the idea that the SNARE motif is the major determinant of the of BoNTs specificity for the three SNARE proteins (McMahon et al., 1992; Shone et al., 1993). Interaction with other regions of each SNARE could contribute to the selectivity of different BoNTs.
The regions of BoNTs involved in substrate binding are still unknown. It is tempting to suggest that the strongly conserved 100-residue-long NH2-terminal region is involved, as the removal of more than eight residues from the NH2 terminus leads to complete loss of activity.

The neuromuscular junction

The target of BoNTs action is the neuromuscular junction (NMJ), where they cause a selective blockade of the exocytosis of acetylcholine (Ach), thereby triggering a profound, albeit transitory, muscular paralysis.
The first electrophysiological investigation of the effect of BoNTs on NMJ was conducted by Burgen (Burgen et al., 1949) on the rat hemidiaphragm preparation. The results of this seminal study can be summarized as follows:

- large and persistent blockade of the end-plate potential (EPP), as a result of the impaired synaptic transmission at intoxicated synaptic terminals;
- reduction of the frequency, but not of the amplitude, of miniature end-plate potential (mEPP);
- no impairment of the processes of neurotransmitter synthesis, uptake, and storage or of the propagation of nerve impulse and Ca2+ homeostasis.

These data can be well explained by the activity of BoNTs. Indeed each BoNT cleaves a specific SNARE protein that is essential for the neuro-exocytosis process. The neurotoxin-impaired exocytosis apparatus is therefore able to mediate some spontaneous residual synaptic activity, but with reduced efficacy with respect to the amount of neurotransmitter released and to the rate of the overall process. Moreover nerve-evoked Ach release is strongly impaired, thereby the EPP can not reach the appropriate membrane potential level to trigger an action potential in the muscle fiber.

The muscle atrophy induced by BoNTs in animal models and in humans is largely reversible, even after repeated BoNTs injections, and this is important for therapeutic and research application.

BoNTs in the central nervous system

The action of BoNTs at the level of peripheral nervous system (PNS) has been characterized in detail (Meunier et al., 2002; Turton et al., 2002). More recently, it has been shown that BoNTs are also effective ate the central synapses (Verderio et al., 2004; Bozzi et al., 2006; Dong et al., 2006).

The first demonstration that BoNTs can block neurotransmitter release in central neurons came from Bigalke and coworkers, who demonstrated that BoNT/A inhibits acetylcholine release from synaptosomes prepared from the rat brain (Bigalke et al., 1981). BoNT/A also inhibits release of noradrenaline, and glycine (Bigalke et al., 1981), glutamate, serotonin, dopamine (Dolly et al., 1982; Sanchez-Prieto et al., 1987; Ashton and Dolly, 1988; Nakov et al., 1989). Interestingly, GABA synapses show less sensitivity to BoNT/A action (Bigalke et al., 1981; Ashton and Dolly, 1988). BoNT/E is found to inhibit GABA release much less than glutamate release (40% decrease of GABA versus 90% decrease of glutamate). The resistance to BoNTs action of GABAergic neurons is attributed to the fact that SNAP-25, the specific target of BoNTs, is

expressed at low levels in the synaptic terminals of inhibitory neurons, at least in hipocamopus (Verderio et al., 2004). Different targets result in different action of BoNTs: BoNT/B depresses glutamate and noradrenaline release (Ashton and Dolly, 1991; McMahon et al., 1992), while BoNT/C in cultured neurons blocks both inhibitory and excitatory synapses (Williamson et al., 1996), because it cleaves also syntaxin. BoNT/E blocks almost completely noradrenaline, dopamine and glutamate release (Stigliani et al., 2003; Costantin et al., 2005) BoNT/F affects acetylcholine, noradrenaline, dopamine (Fassio et al., 1999). It is noteworthy that BoNT/A ed /E behave differently despite sharing the same substrate, indeed inhibition by BoNT/A can be partially rescued by raising extracellular calcium concentrations (Capogna et al., 1997; Keller and Neale, 2001).

At the electrophysiological level, treatment with BoNT/A or BoNT/E prevents the occurrence of both spontaneous and evoked postsynaptic potential (EPSPs) in hippocampal slices (Capogna et al., 1997). The neurotoxins have no effect on post-synaptic glutamate sensitivity, the main action of BoNTs is pre-synaptic and consists of a strong reduction of neurotransmitter release probability.

In vitro studies have determined the effects of BoNTs on the differentiation and survival of central neurons. Morphological examination of intoxicated synapses does not reveal major alteration of structure. The only consistent chemage is an increase in the number of the synaptic vesicles close to the cytosolic face of the presynaptic membrane (Schiavo et al., 2000). Only BoNT/C has been documented to induced neuronal death (Berliocchi et al., 2005), revealing a key role for syntaxin in controlling axonal maintenance and cell viability.

An important difference among BoNT serotypes consists in their duration of action. A recent study by Foran and coworkers has employed cultured rat cerebellar neurons to systematically compare the half-life of the effect of BoNT/A, /B, /C, /E and /F. It was found that intoxication by serotypes E and F is short-lived, while the blockade caused by BoNT/A and C is much more persistent (Foran et al., 2003). Previous works have demonstrated that little cleaved SNAP-25 is evident 18 days after BoNT/E intoxication in cultured spinal cord, whereas BoNT/A-cleaved SNAP-25 remains relatively unchanged

for up to 80 days following toxin exposure (Keller et al., 1999). One explanation could be found in the different cleaved fragment. BoNT/E cleaves a 26-amino acid fragment from the C-terminal of SNAP-25 while BoNT/E removes only nine residues. The long-lasting effects induced by BoNT/A could due to persistent catalytic activity of the toxin inside nerve terminals, but also to persistence of BoNT/A cleaved SNAP-25, that competes with the remaining intact SNAP-25 and prolongs blockade (Keller et al., 1999; Keller and Neale, 2001; Foran et al., 2003). On the contrary BoNT/E protease is less stable and its product is speedly replenished by newly synthesized SNAP-25 (Keller et al., 1999; Keller and Neale, 2001; Foran et al., 2003).

Few studies have described the effects of BoNTs after direct intracerebral administration. *In vivo* microdialysis experiments in freely moving rats showed that pre-administration of BoNT/A, B and C reduced basal and K+-evoked serotonin and dopamine release in hippocampus (Murakami et al., 2001; Okada et al., 2001). Injections in striatum also showed a reduction in dopamine release, while delivery in entorhinal cortex produced substantial impairments in several memory tasks, during the blocking period (Ando et al., 2002; Bergquist et al., 2002). Later, Luvisetto and coworkers performed intracerebroventricular injections of BoNT/A and B in mice in order to evaluate toxicity (Luvisetto et al., 2003). The injected mice displayed clear signs of cholinergic dysfunction. At sub-lethal doses, the toxins induced behavioural impairments (Luvisetto et al., 2004).

A recent report has analyzed the effects of BoNT/E following unilateral injection into the rat hippocampus (Costantin et al., 2005). BoNT/E treatment resulted in a dramatic reduction of glutamate release. Since BoNT/E action could affect multiple neurotransmitter systems, it was important to determine the net effect on BoNTs by electrophysiological recordings. Data showed that BoNT/E potently inhibited spontaneous discharges of pyramidal neurons (Costantin et al., 2005). Thus, the net effect of BoNT/E is a silencing of spontaneous spike activity of hippocampal neurons. The action of BoNT/E persisted for at least three weeks in the injected hippocampus. Clear deficits in acquisition of spatial learning were evident in rats tested during the time window of action of the toxin (3-7 days after administration). Remarkably, BoNT/E-injected animals at time at which the effects of BoNT/E are

extinguished exhibited a normal performance in the Morris water maze (Costantin et al., 2005). Hence, BoNT/E is a novel tool to transiently silence neural activity in the CNS, and could be exploit to interfere with pathological conditions characterized by brain hyperactivity, such as epilepsy.

Therapeutic uses

The reversibile blockade of synapses caused by BoNTs, in particular BoNT/A, have become a multi-million dollar affair. Toxins are used in the treatment of many human syndromes caused by hyperactivity of nerve terminal, such as cervical distonya, blepharospasm, strabismus and hyperhydrosis but recently BoNT/A is also used for pharmaco-cosmetic treatment, for example to treat facial wrinkles (Harrison, 2003).

Recently, BoNTs have been used for the treatment of migraine headache and other types of pain, also if the effects could be only indirect, blocking neuronal release of molecules associated with pain (Binder et al., 2000; Aoki, 2003; Cui et al., 2004). In addition, BoNTs seem to be a good tool also for the treatment of epilepsy (Costantin et al., 2005; Antonucci et al., 2007).

Injections of minute amounts of BoNTs into the muscle led to a depression of the symptoms lasting few months. So far, BoNT/A is the most used serotype. However, if immunization problems arised against BoNT/A, other BoNT serotypes could be used. BoNT/B, BoNT/F and BoNT/E are very effective in their action but their effect is short lasting and hence they are not a valid alternative to BoNT/A, while encouraging results have been obtained with BoNT/C and BoNT/B (Eleopra et al., 1997; Eleopra et al., 1998).

The increasing use and success of BoNT/A is due to their high specificity and to the common view that, unlike tetanus neurotoxin, BoNTs remains locally in intoxicated cells. Recently, Antonucci and coworkers confirmed local action of BoNT/E but provided the first evidence for *in vivo* long-distance retrograde transport of BoNT/A (Antonucci et al., 2008a). Experiments demonstrated that BoNT/A-cleaved SNAP-25 appears not only at the injection site but also in distant regions that project to the infusion area. Experiments demonstrated retrograde transport and transcytosis of BoNT/A and not of the cleaved substrate. In addition, electrophysiological recordings showed functional

consequences of the spread toxin. No SNAP-25 fragments were detectable in nonconnected areas, providing clear evidence that BoNT/A does not spread in a passive way from the site of injection in the CNS. Previous data in the literature indicated the possibility that BoNT/A might undergo a limited extent of retrograde axonal transport from site of injection to the spinal cord or to the sciatic nerve, but it remained unclear whether the radioactivity corresponded to intact, catalytically active BoNT/A (Habermann, 1974; Wiegand et al., 1976).
The precise molecular mechanisms as well as the cellular compartments involved in this process remain to be determined. However, given the large use of BoNT/A in clinical treatment, the evidence that BoNT/A applied in the periphery can affect central circuits via retrograde transport and transcytosis is an important finding to be held into account.

AIM OF THE THESIS

The aim of this thesis is to investigate the role of the callosum in visual cortex development and plasticity. Surprisingly, there is very little information in the literature on this topic. Experiments have shown that the organization of callosal fibers is shaped by experience during the critical period, however the functional role of the callosum in cortical plasticity has not been investigated so far.

My work is divided into two part. In a first study, I took advantage of the bacterial enzyme BoNT/E to produce a unilateral silencing of cortical activity during the critical period. This experimental protocol allows one to investigate the role of intrinsic cortical activity in the maturation of the visual cortex in the blocked hemisphere. The experimental setting also allows to address the role of interhemispheric connections in cortical development, as the side contralateral to the blockade receives normal visual input through the direct retinogeniculate pathway, and selectively lacks afferent activity through the callosal pathway. The results of these experiments demonstrate that the corpus callosum plays a fundamental role in maturation of visual cortex.

In a second study, I investigated the role of the callosum in cortical plasticity during the critical period. I performed acute microinjection of the activity blocker muscimol into the visual cortex contralateral to the recording site in order to inactivate callosal neurons. Electrophysiological recordings performed before and after injection demonstrate the involvement of callosum in determining cortical binocularity. Thus, I investigated using the same protocol whether callosal neurons have a role in mechanisms underlying plasticity in MD. I reporte here for the first time that callosal inputs play a key role in functional weakening of less active connections during brain plasticity.

MATERIALS AND METHODS

MATERIALS AND METHODS

BoNT/E EXPERIMENTS

Animal treatment

Long-Evans hooded 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.

BoNT/E was obtained by Wako (Osaka, Japan), trypsin activated, purified and tested as previously described (Schiavo and Montecucco, 1995; Costantin et al., 2005). Unilateral stereotaxic injections of BoNT/E (30 nM) or vehicle (2% rat serum albumin in PBS) were made into left primary visual cortex of P14 rat pups under avertin anaesthesia (tri-bromo-ethanol, Sigma). Injections were performed at three locations by means of a glass pipette (40 μm tip diameter) mounted on a motorized three-axis micromanipulator connected to an injector. The three locations were 2.5 mm lateral to the midline and 1 mm anterior to lambda; 2.5 mm lateral and in correspondence with lambda; 2.5 mm lateral and 1 mm posterior to lambda. At each site, 0.5 μl were slowly delivered at a depth of 1 mm below dura.

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.

Histology

P14 rat pups received unilateral injections of BoNT/E (n = 9) or vehicle (n = 5). One-four days later, they were deeply anesthetized and perfused through the heart with 4% paraformaldehyde. Coronal sections (40 μm thick, cut on a freezing microtome) were reacted with antibodies recognizing either BoNT/E-cleaved or intact SNAP-25. Cleaved SNAP-25 was detected using a peptide-affinity purified polyclonal antibody raised against the BoNT/E truncated C-terminal peptide of SNAP-25 (CDMGNEIDTQNRQIDR). This antibody specifically recognizes cleaved SNAP-25 but not the whole protein (Costantin

et al., 2005). Intact SNAP-25 was revealed with a polyclonal antibody raised against a synthetic peptide corresponding to residues 195-206 of SNAP-25 protein.

For immunostaining, sections were blocked with 10% normal goat serum in PBS containing 0.3% Triton X-100 and then incubated overnight at 4°C with the primary antibodies. For detection of cleaved SNAP-25, 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. For detection of intact SNAP-25, sections were incubated with AlexaFluor568-conjugated secondary antibody (Molecular Probes).

A group of normal rats (n = 4) and rats unilaterally injected at P14 with vehicle (n = 4) or BoNT/E (n = 5) were perfused at P35 for the anatomical analysis. Serial cortical sections (one out of six) were Nissl-stained for the evaluation of cortical thickness. Additional serial sections were incubated overnight at 4°C with monoclonal antibodies specific either for the neuronal marker NeuN (Chemicon, 1:500) or the GABA biosynthetic enzyme GAD65 (Chemicon, 1:500), or in a solution of biotin-conjugated Wisteria Floribunda Agglutinin (WFA; 10 μg/ml). Bound primary antibodies were detected with biotinylated anti-mouse (1:200), followed by Cy3-conjugated ExtrAvidin (1:500, Sigma). WFA was revealed with a 1hr incubation in Cy3-conjugated ExtrAvidin (1:500, Sigma).

Additional rats (vehicle, n = 4; BoNT/E, n = 4) were injected unilaterally at P14 and perfused when they were older than P100. Serial cortical sections were stained with anti-GAD65 antibodies as described above.

Morphometry and confocal analysis

All data were collected blind to the experimental treatment.

Cortical thickness: The analysis was performed on coronal sections of primary visual cortex using Stereo Investigator software (Microbrightfield, Colchester, VT, USA) and a Zeiss microscope. In each section, I measured the distance from the pial surface to white matter. Three measures were taken from each

section and at least 6-7 sections were analyzed per animal. These values were averaged to obtain cortical thickness in individual animals.

Density of neurons: NeuN-stained sections were examined with a confocal microscope (Leica) using a 40X oil immersion objective. Neurons were counted in three-dimensional counting boxes (250 μm x 250 μm x 12 μm) positioned either in layer II/III or layer V/VI of primary visual cortex. An average of 1,600 neurons were counted for each experimental animal. Neuronal density was calculated by averaging values obtained from at least 6 sections per animal.

Quantification of GAD65 staining: All confocal images were collected with an Olympus confocal microscope using a 60X oil immersion lens. An initial analysis on stained sections from the different groups was performed to establish settings for laser intensity, gain, offset and pinhole size. Care was taken to avoid saturation at either end of the pixel intensity range (0-255). Confocal settings were then held constant through the study. Examination of animals from the various treatment groups was interdigitated to avoid bias caused by slow shifts in laser power. Five-eight sections per animal were analyzed. For each section, I imaged three adjacent fields (105 x 105 μm) located in layer II/III of primary visual cortex. In each field, a stack of ten GAD65 optical sections separated by 1 μm was collected at the top face of the tissue section. The image within each stack with the highest average pixel intensity was selected for the quantitative analysis of GAD65 immunoreactivity (Silver and Stryker, 2000; Tropea et al., 2003).

Perisomatic GAD65 signals ("puncta-ring") from 4-5 target neurons were outlined for each image and GAD65 signal intensity was calculated (Metamorph; Molecular Devices, Sunnyvale, CA, USA). For each neuron, signal intensity values were divided by the background labeling in the cell soma (which is devoid of GAD65 staining). A total sample of 90-180 neurons was analyzed for each animal.

The area occupied by GAD65-positive pixels in the neuropil was calculated for each image by applying a threshold (average background signal in the cell

somas multiplied by four) and masking all blood vessels and cells bodies. Calculation of the cross-sectional area of GAD65 staining was used here and in previous studies (Smith et al., 2000; Tropea et al., 2003) to estimate the density of presynaptic boutons.

Density of WFA-positive cells: Labelled cells were counted (Pizzorusso et al., 2002) blind to the treatment on a fluorescence microscope using a 20X objective (0.1 mm^2 fields). Six-eight sections were analyzed for each experimental animal.

Immunoblotting

Rats received unilateral BoNT/E injections at P14 and visual cortices were dissected 1, 7, 14 and 21 days post treatment. Proteins were extracted (Viegi et al., 2002) with lysis buffer (1% Triton X-100, 10% glycerol, 20mM Tris-HCl, pH 7.5, 150 mM NaCl, 10mM EDTA, 0.1 mM Na_3VO_4, 1μg/ml leupeptin, 1μg/ml aprotinin, 1mM PMSF). Protein extracts (10 μg) were separated by electrophoresis and blotted, and filters were incubated with an antibody specific for the BoNT/E-cleaved form of SNAP-25 (1:50 dilution) or with an antibody recognizing both BoNT/E-cleaved and intact SNAP-25 (1:1,000, Sternberger monoclonals). Blots were then reacted with HRP-conjugated secondaries (Bio-Rad) and developed by ECL (Amersham, UK). Filters were also probed with anti-β-tubulin monoclonal antibody (1:500 dilution; Sigma, St Louis, MO), which serves as an internal standard for protein quantification.

***In vivo* Electrophysiology**

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 surrouding 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. A portion of the skull

overlying the binocular visual cortex was carefully drilled and the dura was removed. A glass micropipette (2 MΩ) filled with NaCl (3M) was inserted into the binocular portion of primary visual cortex.

Single-unit recordings: The position of receptive fields of single units were mapped using a hand-held stimulator. Only cells with receptive fields within 20° of the vertical meridian were included in the analysis. Most (> 80%) of the cells in our sample were recorded from supragranular layers (i.e. at a depth less than 800 μm from the cortical surface). Spontaneous activity and peak response were determined from peristimulus time histograms (PSTHs) recorded in response to computer-generated bars, averaged over 10-20 stimulus presentations as described (Lodovichi et al., 2000).

Ocular dominance (OD) was evaluated according to the methods of Hubel & Wiesel (1962) (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 (CBI, contralateral bias index), 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.

VEPs recordings: the electrode was positioned at a depth of 400-500 μm into the cortex, where VEPs had their maximal amplitude. Transient VEPs were recorded in response to abrupt reversal of a horizontal square wave grating, generated by computer on a display (Sony) by a VSG card (Cambridge Research System) which was positioned in front of the rat's eyes to include the binocular visual field. 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. Transient VEPs were evaluated in the time domain by measuring the peak to trough amplitude and the latency of the major negative component (Pizzorusso et al., 2002). VEPs in response to a blank stimulus were also frequently recorded to estimate noise. Visual acuity was measured as the highest spatial frequency that still evoked a response above noise level at maximum contrast. Analysis of all electrophysiological data was performed blind to the experimental treatment of the animals.

Behavioural assessment of visual acuity

Behavioural assessment of visual acuity was performed starting from P60 in animals that received BoNT/E (n = 10) or vehicle (n = 7) at P14 and in naïve subjects (n = 6). Rats were tested blind to treatment in the visual water task as described (Prusky et al., 2000; Pizzorusso et al., 2006). Briefly, the device consisted of a trapezoidal-shaped pool, filled with water, partially divided at one end in two arms by a divider. Visual stimuli consisted of gratings of various spatial frequencies or grey fields, which were generated with two computer monitors and placed at the end of each arm. An hidden platform was placed below the grating. Animals were released from the nondivided end of the box and trained to associate the stimulus grating with the submerged platform (i.e. escape from water). The position of the grating and the platform was alternated in a pseudorandom sequence. Visual water task trains animals to distinguish initially a low (0.1 cycles/deg) spatial frequency vertical grating from grey, and then tests the limit of this ability at higher spatial frequencies. A trial was recorded as incorrect if an animal entered the arm without the platform. Animals were removed from the pool when they found the platform. The limit of discrimination was estimated by increasing the spatial frequency of the grating until performance fell below 70% accuracy. The highest spatial frequency at which 70% accuracy was achieved was recorded as the visual acuity (Prusky et al., 2000; Pizzorusso et al., 2006).

MUSCIMOL EXPERIMENTS

Animal treatment and surgical procedures

Long-Evans hooded 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.

Fifteen naïve animals during the critical period (age range: P24 – P29; saline, n = 7; muscimol, n = 8) were use for extracellular recording, while twelve animals were used for VEPs (saline, n = 5; muscimol n = 7). For monocular deprivation experiments, nineteen rats at the peak of the critical period (P20-P22) were deprived for seven days. Seven rats were used for single units recordings, while the other seven animals were used for VEP recordings.

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.

Cortical microinjection of muscimol (1 μl; 30 mM, Sigma) or saline was performed with a glass pipette (tip diameter, 40 μm) mounted on a micromanipulator. The solution was slowly delivered at a depth of 0.6 – 1 mm from the pial surface. All the experiments were performed blind to the experimental treatment.

In vivo electrophysiology

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. In all of the animals, a portion of the skull overlying the binocular visual cortex was carefully drilled on both sides of the skull. In the right hemisphere the dura was removed. A glass micropipette (2 MΩ) filled with NaCl (3M) was inserted into the

binocular portion of primary visual cortex in correspondence with the cortical representation of the vertical meridian (4.6 – 4.9 mm lateral from lambda). Single units or VEPs were recorded from a single track in the right hemisphere. The left hemisphere was then injected with saline or muscimol. After a delay of 30 min, I started to record single units or VEPs again during withdrawal along the same track.

Single-unit recordings: A sample of 10–12 neurons were recorded from a single pipette track in the right hemisphere. All of the cells were recorded 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, 28°/sec) presented on a monitor (Sony, 40 x 30 cm; mean luminance 15 cd/m^2). Signals were amplified 25,000-fold, bandpass filtered (500-5000Hz), 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 (Antonucci et al., 2007; Caleo et al., 2007; Resta 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; Antonucci et al., 2007). Peak response was evaluated as the peak firing rate (spikes per second) in the cell response to the stimulus.

Ocular dominance (OD) was evaluated according to the methods of Hubel & Wiesel (1962) (Hubel and Wiesel, 1962). Neurons in ocular dominance class 1 were driven exclusively 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 (CBI, contralateral bias index), 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 addition, I computed for each neuron the normalized OD score and plotted the cumulative probability for each experimental group. The OD score was defined as follows:

ODS= [(Pk(ipsi) – M(ipsi)) - (Pk(contra) - M(contra))] / [(Pk(ipsi) – M(ipsi)) + (Pk(contra) -M(contra))]; where Pk is the peak response and M is the mean spontaneous activity (Rittenhouse et al., 1999). This score is -1 for cells in class 1, +1 for cells in class 7, and 0 for cells in class 4.

VEPs recordings: the electrode was positioned at a depth of 100 μm within the cortex. Transient VEPs were recorded in response to abrupt reversal (0.5 Hz) of a horizontal square wave grating, generated by computer on a display (Sony, 40 x 30 cm; mean luminance 15 cd/m^2) by a VSG card (Cambridge Research System) which was positioned in front of the rat's eyes to include the binocular visual field. During recording through one eye, the other was covered by a black adhesive tape. 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. Transient VEPs were evaluated in the time domain by measuring the peak to trough amplitude and the latency of the major negative component (Porciatti et al., 1999; Pizzorusso et al., 2002). VEPs in response to a blank stimulus were also recorded to estimate noise.

Statistical analysis

Statistical analysis was performed with SigmaStat (version 3.1). Differences between two groups were assessed with a two-tailed t-test. A paired t-test was used in muscimol experiments to compare data obtained in the same animal, before and after treatment. Differences between three or more groups were evaluated with one way analysis of variance (ANOVA) followed by Tukey or Holm-Sidak test for data normally distributed, and with Kruskal-Wallis one-

way ANOVA with Dunn's post hoc test for data non normally distributed. Normality of distributions was assessed with Kolmogorov-Smirnov test. Differences between OD histograms and OD score distributions were assessed using a $\chi 2$ test (four degrees of freedom) or Kolmogorov-Smirnov test, respectively. Level of significance $p < 0.05$.

RESULTS

RESULTS

ROLE OF CALLOSAL CONNECTIONS IN CORTICAL DEVELOPMENT

Transient silencing of cortical activity by BoNT/E

I unilaterally injected BoNT/E (30 nM solution) or vehicle (2% rat serum albumin in PBS) into the visual cortex of rat pups at the time of eye opening (postnatal day 14, P14). Immunostaining for cleaved SNAP-25, performed 1-4 days after BoNT/E injection, demonstrated that BoNT/E effects were restricted to the injected side and that diffusion of the toxin was adequate to cover the whole extent of primary visual cortex in the infused hemisphere (Fig. 1A). Staining for intact SNAP-25 revealed a strong loss of immunoreactivity in the injected area and the normal, punctuate labelling typical of synaptic markers in the contralateral hemisphere (Fig. 1B). There was no detectable spread of BoNT/E effects to more ventral structures such as the superior colliculus (Fig. 1A) and hippocampus (data not shown). To further confirm the regional specificity of BoNT/E effects, I performed immunoblot analysis on protein extracts from the treated and untreated visual cortex at P15 (i.e., 1 day after injection), using an antibody that recognizes both intact and BoNT/E-truncated SNAP-25. The results were clear in indicating that SNAP-25 cleavage only occurred on the infused side (Fig. 1C) and that levels of intact SNAP-25 in the contralateral hemisphere were comparable to those of normal animals (post ANOVA Tukey test, $p > 0.05$).

Extracellular recordings of spiking activity and visual evoked potentials (VEPs) were used to demonstrate functional consequences of SNAP-25 cleavage. Recordings at P16-P17 showed that infusion of vehicle had no effect on visual responses (data not shown). Conversely, BoNT/E produced a blockade of neuronal activity in the injected hemisphere (Fig. 2A, B). No visually responsive cells and no measurable VEP responses could be found in the cortical area surrounding the injection sites (Fig. 2B). The silencing effects

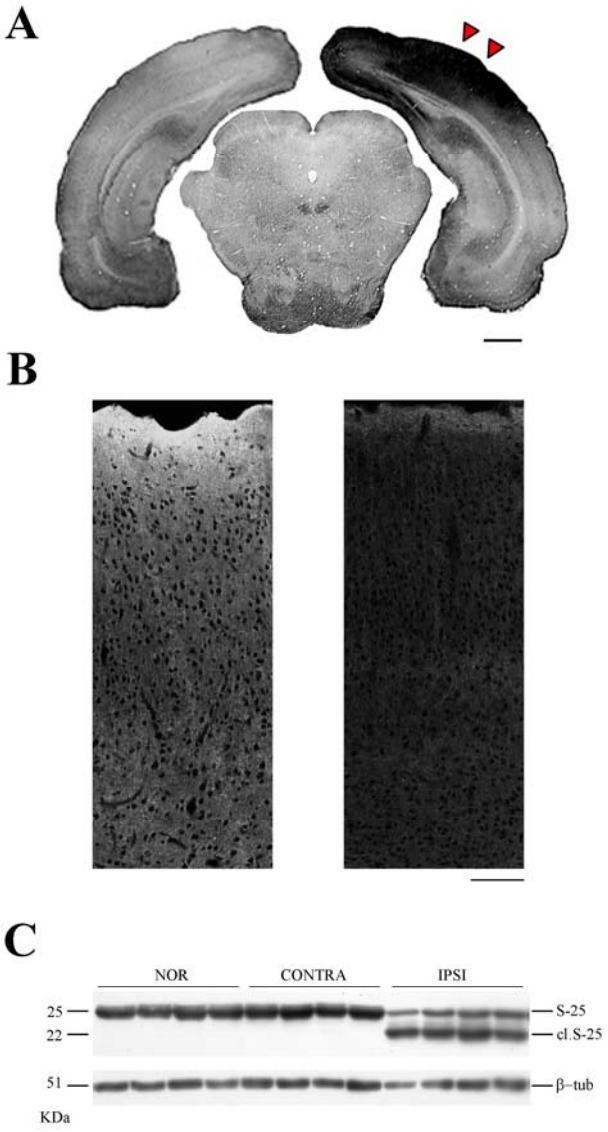

Figure 1

Characterization of BoNT/E effects in the visual cortex. (A) Immunostaining for cleaved SNAP-25 (dark labelling) in a coronal section through the occipital cortex and midbrain of a P16 rat, two days after unilateral BoNT/E injection. Cleaved SNAP-25 is evident in the binocular visual cortex (delimited by arrows) of the infused side (right), while no signal is detectable in the contralateral hemisphere (left). The superior colliculus is also devoid of staining. Scale bar = 1 mm. (B) Confocal images of immunostaining for intact SNAP-25 in a P16 rat that received a unilateral BoNT/E infusion two days earlier. While the uninjected hemisphere (left) displays the normal punctate staining characteristic of SNAP-25, immunoreactivity is almost completely abolished on the treated side (right). Scale bar=100 um. (C) (A) Representative immunoblotting for intact (S-25) and cleaved SNAP-25 (cl. S-25) on cortical protein extracts from P15 rats. Tissues were harvested from normal animals (NOR) and from the side contralateral (CONTRA) and ipsilateral (IPSI) to BoNT/E, 1 day after infusion. Each lane represents one animal. β-tub, β-tubulin (internal standard).

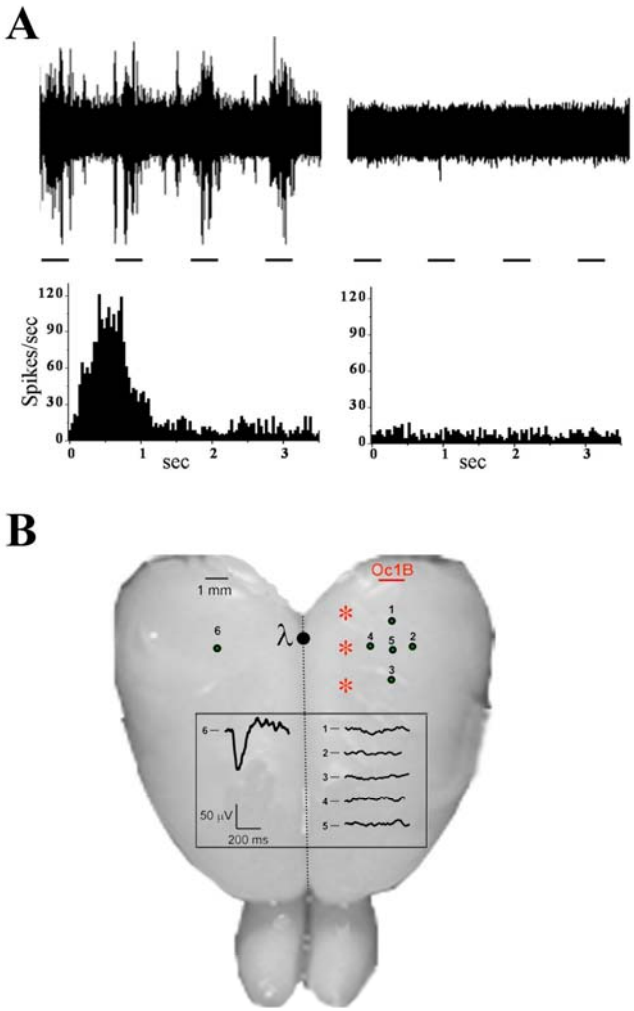

Figure 2

Functional consequences of BoNT/E infusion. (A) (top) Representative extracellular recordings of spiking activity from the binocular cortex ipsilateral (right) and contralateral (left) to BoNT/E infusion, 2 days after toxin injection. Passage of a light-bar stimulus across the visual field is indicated by the horizontal bars under the trace (each horizontal bar = 1.90 sec). (bottom) Examples of peristimulus time histograms recorded in the infused (right) and contralateral cortex (left). Visual response is abolished in the BoNT/E-treated hemisphere. (B) Representative physiological mapping of the effects of BoNT/E in a P17 rat (i.e., 3 days after injection). The BoNT/E injection sites (asterisks) and the location of six microelectrode penetrations are shown on a dorsal view of the rat brain. Five penetrations (numbered 1-5) were made in the binocular visual cortex (Oc1B) of the infused hemisphere and showed no VEP response (see traces in the box). A robust field potential response (track number six) could be evoked from the uninjected side. λ, lambda suture.

of BoNT/E were consistently limited to the injected side and indeed vigorous responses could be recorded in the contralateral hemisphere (Fig. 2A, B).

Duration of BoNT/E action was assessed by recordings from visual cortex at different times after delivery of the toxin. At P21-P23 (i.e., 7-9 days post BoNT/E) cortical cell discharges were still blocked in the infused hemisphere, and no VEP responses could be elicited (Fig. 3A). Visually responsive cells began to reappear at P28 in 4 out of 5 recorded animals. Accordingly, VEP recordings showed sluggish field potential responses (with significantly increased latency, $p < 0.05$ t-test vs. normal animals; see Fig. 3A) in these rats and ongoing blockade in the remaining animal. Visual activity was completely recovered by P35, i.e. 21 days after BoNT/E injection (Fig. 3A). Primary visual cortex was dissected from the recorded animals to analyze the time course of SNAP-25 cleavage. In keeping with the electrophysiological results, Western blotting demonstrated very high levels of cleaved SNAP-25 at P15 and P21, i.e. 1-7 days post BoNT/E (Fig. 3B-D). A decrease in BoNT/E-truncated SNAP-25 and a corresponding rise in intact SNAP-25 was observed in P28 animals (Fig. 3B-D). Cleaved SNAP-25 was no longer detectable at P35 (Fig. 3B-D). Thus, BoNT/E effects are completely extinguished 21 days after injection. To exclude that the long-term blockade of activity might have deleterious effects on neuronal survival in the cortex, I determined cortical thickness and neuron density at P35. This analysis revealed no changes in the BoNT/E-injected and contralateral cortex with respect to either the normal or vehicle-injected P35 cortex (one way ANOVA, $p > 0.19$ for all comparisons; Fig. 4A-C).

Silencing of one side results in bilateral impairments in visual cortical development

Functional development of binocular striate cortex was assessed at P34-P36, i.e. around the end of the critical period for rat visual cortex (Fagiolini et al., 1994). Data were collected from four groups: (i) naïve cortex, (ii) cortex infused with vehicle, and cortex (iii) ipsilateral and (iv) contralateral to BoNT/E-injection.

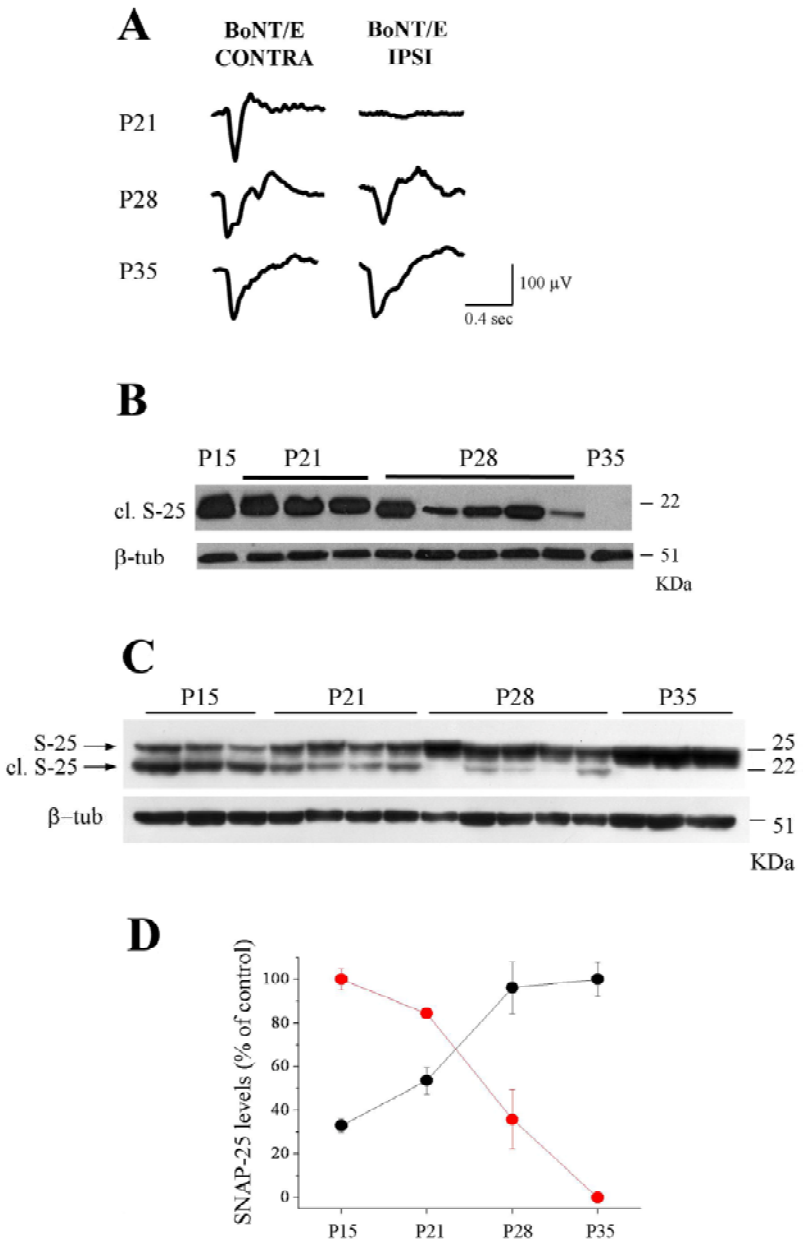

Figure 3

Reversibility of BoNT/E effects. (A) Representative VEP responses from both hemispheres of animals injected with BoNT/E at P14. Age of recording is indicated. Note reduced VEP amplitude and increased latency in the treated side at P28. Responses in the injected side are completely recovered by P35. (B) Immunoblotting for cleaved SNAP-25 on protein extracts from the visual cortex at different times after BoNT/E injection. Cleaved SNAP-25 is no longer detectable 21 days post BoNT/E. cl. S-25, cleaved SNAP-25; b-tub, b-tubulin (internal standard). (C) Intact and BoNT/E-truncated SNAP-25 at different times after one single injection of BoNT/E at P14. Filters were probed with an antibody raised against the amino-terminal domain of SNAP-25 that allows simultaneous visualization of intact (S-25) and BoNT/E-cleaved SNAP-25 (cl. S-25). b-tub, b-tubulin (internal standard). (D) Densitometric analysis of intact (black circles) and BoNT/E-cleaved SNAP-25 (red circles). BoNT/E effects are stable for about a week and decline thereafter, being completely off by P35. Error bars indicate S.E., and when not seen, are within the symbol.

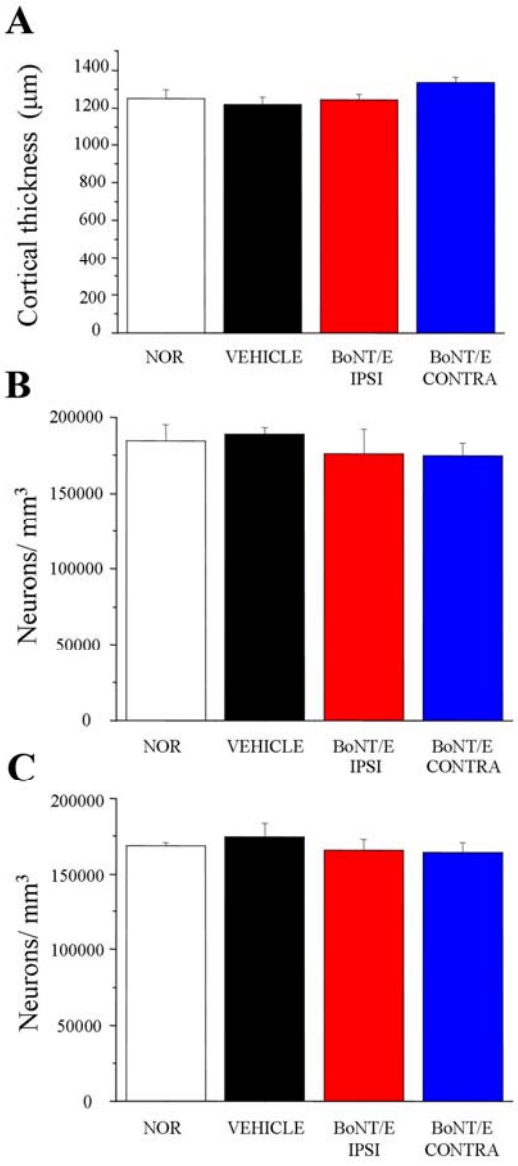

Figure 4

BoNT/E has no adverse effects on neuronal survival in the visual cortex. (A) Mean cortical thickness (± S.E.) in the various experimental groups. NOR, cortex of normal animals; VEHICLE, cortex injected with vehicle solution; BoNT/E IPSI and CONTRA, hemisphere ipsilateral and contralateral to BoNT/E infusion. (B, C) Mean neuronal density (± S.E.) in layers II-III (B) and layers V-VI (C) of primary visual cortex in the various groups.

I first measured the maturation of spatial resolution (acuity) in the cortex using VEPs (Fagiolini et al., 1994; Huang et al., 1999; Gianfranceschi et al., 2003). I found that visual acuity was about half of the normal value on both the injected and uninjected side of P35 animals treated with BoNT/E (one-way ANOVA, $p < 0.001$; post hoc Tukey test, $p < 0.05$; Fig. 5), while vehicle injections had no effect ($p > 0.05$). Acuity values were indistinguishable in the two hemispheres of BoNT/E-infused rats (post ANOVA Tukey test, $p > 0.05$). Thus, a unilateral silencing of striate cortex results in bilateral impairments in the development of visual acuity.

Single-unit recordings were performed to evaluate cortical binocularity in BoNT/E-treated and control rats. This analysis indicated that the ocular dominance (OD) of cortical neurons was not affected by the activity blockade. OD histograms and contralateral bias indexes (CBIs) were normal in both the BoNT/E-injected and contralateral hemisphere ($\chi 2$ test, $p > 0.05$ and one way ANOVA, $p > 0.3$; Fig. 6A, B).

Visual cortex maturation in rats is accompanied by a decline in the potential for experience-dependent plasticity (Fagiolini et al., 1994; Pizzorusso et al., 2002). I assessed sensitivity to MD in a group of BoNT/E-infused and control rats. I found that 4-5 days of MD starting from P34-P36 were ineffective in inducing a change in eye preference in normal and vehicle-infused rats (Fig. 7A, B). OD histograms and CBIs of these animals were superimposable to those measured in undeprived rats ($p > 0.05$; $\chi 2$ test and two-tailed t-test, respectively). In contrast, a robust OD shift to the open eye could be observed in both the ipsilateral and contralateral hemisphere of BoNT/E-injected rats (Fig. 7A, B). For this analysis, BoNT/E-infused animals were split into two groups and monocularly deprived either contralateral or ipsilateral to the injection, to assess plasticity of the treated and untreated hemisphere, respectively. Statistical testing demonstrated that CBI values were lower in the BoNT/E-injected and contralateral cortex as compared to normal and vehicle-infused controls (one way ANOVA, $p < 0.001$; post hoc Tukey test, $p < 0.05$). CBIs in the former groups did not differ (post ANOVA Tukey test, $p > 0.05$), indicating that the magnitude of the OD shift was similar in the two

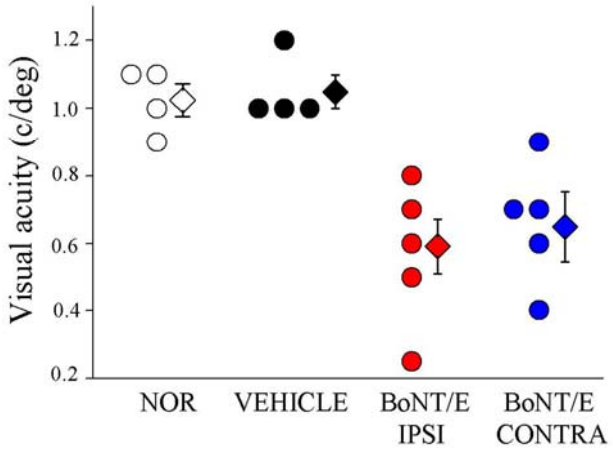

Figure 5

Bilateral impairments in spatial resolution in P35 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 in comparison with that in normal or vehicle-injected animals. Error bars indicate S.E. Abbreviations as in Fig. 3.

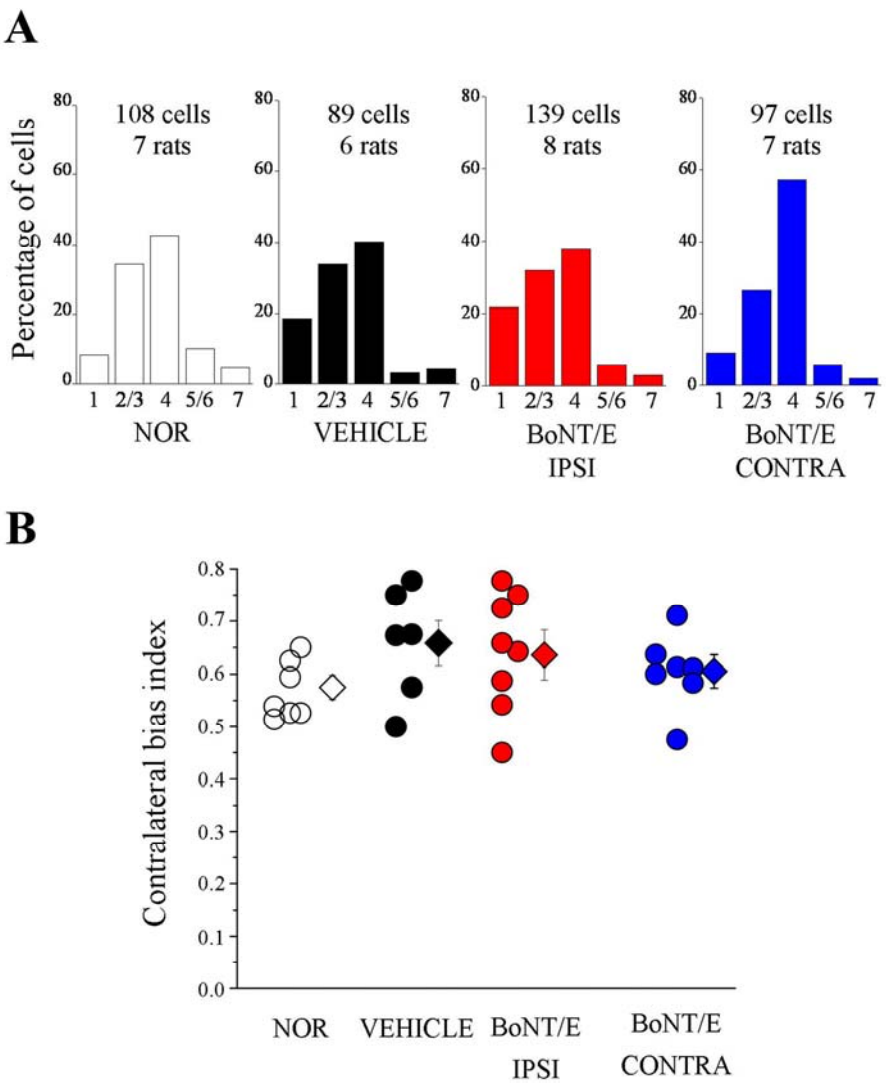

Figure 6

Activity blockade has no effects on cortical binocularity. (A) OD distributions of control and BoNT/E rats at P35. Control rats were either naïve (NOR) or injected with vehicle at P14 (VEHICLE). BoNT/E rats were injected at P14 and recorded both ipsilateral and contralateral to the infusion side (BoNT/E IPSI and BoNT/E CONTRA). Number of animals and cells as indicated. (B) CBI of control and BoNT/E-treated rats at P35. Circles, values of individual animals; diamonds, mean of the group. Error bars indicate S.E. and, when not seen, are within the symbol. No differences in OD can be detected among the groups (one way ANOVA, $p > 0.3$).

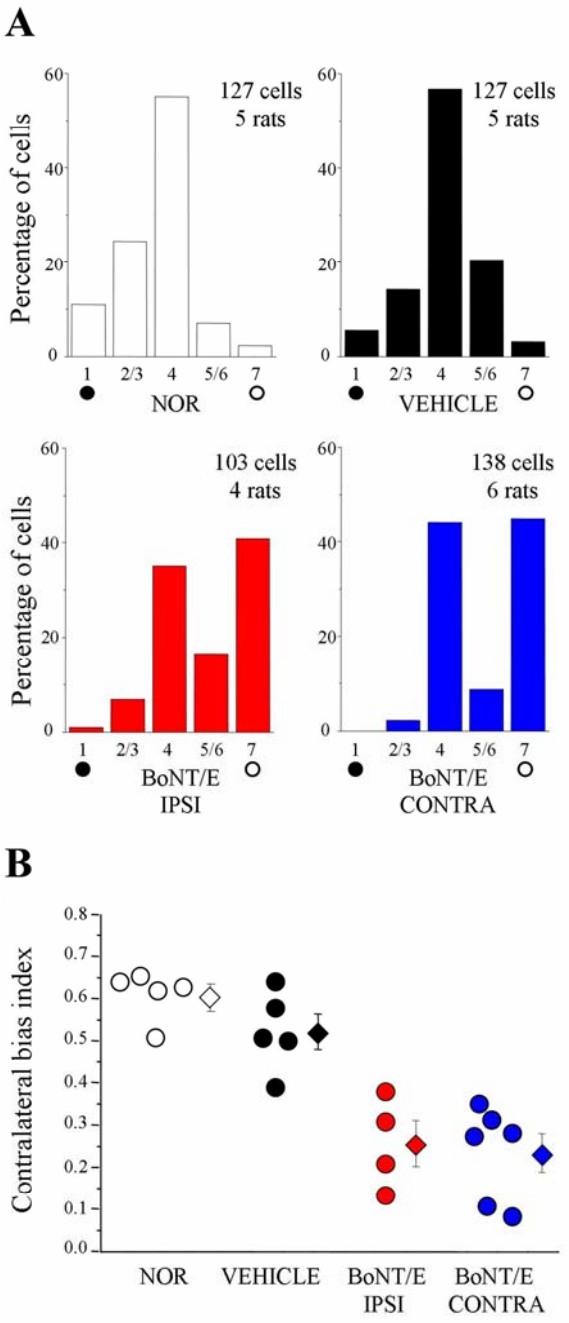

Figure 7

Bilateral prolongation of the critical period in P35 BoNT/E rats. (A) OD distributions in normal, vehicle- and BoNT/E-injected rats subjected to MD at P35. OD was assessed after 4-5 days of monocular occlusion. The deprived eye was always the eye contralateral to the cortical hemisphere being examined. Both hemispheres of BoNT/E rats display a significant shift towards the nondeprived eye (open circle). Number of animals and cells as indicated. Note that OD histograms for BoNT/E animals look bimodal, suggesting that two subsets of cortical cells are differentially sensitive to the activity blockade. (B) CBI for all animals recorded in each experimental group (circles, values of single animals; diamonds, mean ± S.E.). MD effects are consistently detectable in both hemispheres of BoNT/E-injected rats.

hemispheres of BoNT/E rats. I conclude that unilateral blockade of cortical activity prolongs the critical period for plasticity in both hemispheres.

Silencing of one side results in bilateral downregulation of inhibitory markers

Substantial evidence indicates that intracortical GABA-mediated inhibition is an important determinant of the critical period (Hensch et al., 1998; Huang et al., 1999; Hensch, 2005). I analyzed the expression of GAD65, an isoform of glutamic acid decarboxylase that is concentrated in presynaptic terminals, in layers II-III of striate cortex by quantitative confocal microscopy (Huang et al., 1999). Measurement of the area occupied by the immunoreactive staining indicated that both the cortex ipsilateral and contralateral to BoNT/E injection had a reduced density of GAD65 terminals in the neuropil (one way ANOVA, $p < 0.001$; post hoc Holm-Sidak test, $p < 0.05$ with respect to naïve and vehicle-infused cortex; Fig. 8A). Analysis of GAD65 fluorescence in "puncta-rings" surrounding the soma of cortical neurons also indicated a lower GAD65 signal in both hemispheres of the BoNT/E-treated rats (one way ANOVA on ranks, $p < 0.001$; post hoc Dunn's test, $p < 0.05$; Fig. 8B).

Cessation of OD plasticity in rats correlates with the developmental organization into perineuronal nets of chondroitin sulphate proteoglycans (Pizzorusso et al., 2002). I assessed the number of perineuronal nets by staining cortical sections with Wisteria floribunda agglutinin (WFA). Countings of WFA-positive cells in layers II-III at P35 revealed a decrease in the BoNT/E-injected hemisphere with respect to both the naïve and vehicle-infused cortex (one way ANOVA, $p < 0.01$; post hoc Holm-Sidak test, $p < 0.05$; Fig. 7C). The number of perineuronal nets was similarly reduced in the cortex contralateral to BoNT/E infusion (post ANOVA Holm-Sidak test, $p < 0.05$; Fig. 8C).

Transient blockade of activity leaves a lasting change in visual cortex

The impaired spatial resolution and the sensitivity to MD found in P35 BoNT/E rats might either represent a delay in developmental maturation of the visual cortex or persist into adulthood. To address this issue, a group of control rats and rats injected with BoNT/E at P14 were allowed to survive until P60-P100 (i.e., 30-70 days after the completion of BoNT/E effects) and tested

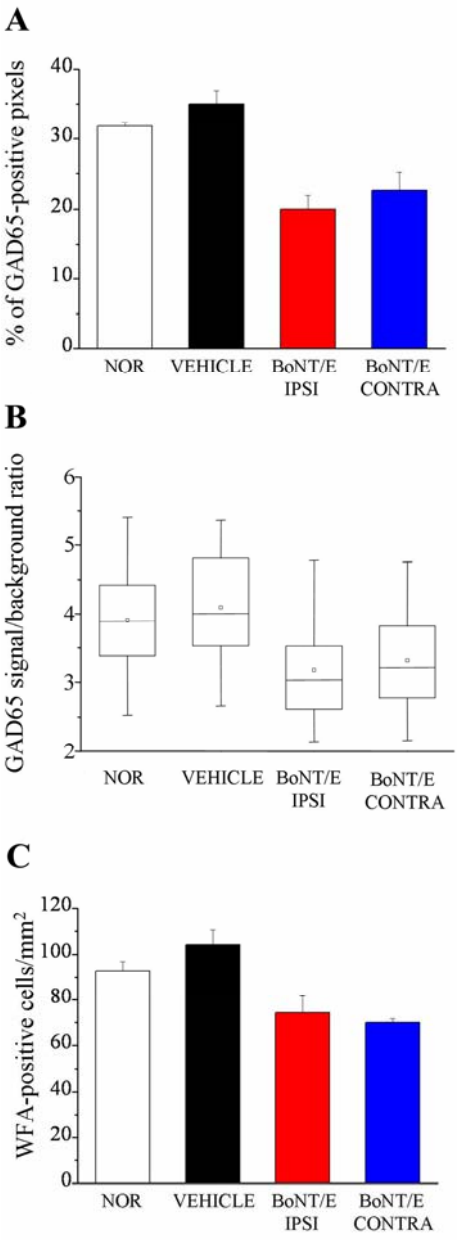

Figure 8

Bilateral reductions in markers of intracortical inhibition in P35 BoNT/E rats. (A) Mean density of GAD65-positive pixels in the neuropil (layers II-III) is significantly reduced in the cortex ipsilateral and contralateral to BoNT/E infusion with respect to both naïve and vehicle-injected cortex. Error bars indicate S.E. (B) Quantification of GAD65 immunofluorescence in GABAergic terminals around the soma of target neurons. The box chart summarizes the distribution of the GAD65 signal-to-background ratio (intensity of GAD65 label in "puncta-rings" divided by the background staining in the soma) for the cells in each experimental group. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbol in the box denotes the mean of the column of data. GAD65 staining is less intense in both hemispheres of BoNT/E-injected rats as compared to normal and vehicle-injected cortex (one way ANOVA followed by Dunn's test, $p < 0.05$). (C) Mean density (± S.E.) of perineuronal nets surrounded neurons in layer II/III. Density of WFA-positive cells is reduced ipsilateral and contralateral to BoNT/E infusion.

behaviourally for the determination of visual acuity. Using a two-alternative forced-choice discrimination task (visual water box) (Prusky et al., 2000), I found significantly lower visual acuities in the animals treated with BoNT/E with respect to normal and vehicle-injected rats (post ANOVA Holm-Sidak test, $p < 0.01$; Fig. 9A, B). Thus, BoNT/E rats exhibit a persistent deficit in visual acuity at the behavioural level. To determine whether spatial resolution was equally impaired in both hemispheres of adult BoNT/E rats, the animals were recorded for the determination of VEP acuity. The results were clear in indicating that visual acuity was low both ipsilateral and contralateral to BoNT/E injection (one way ANOVA, $p < 0.001$; post hoc Tukey test, $p < 0.01$ vs. normal and vehicle rats; Fig. 10A). Acuity values did not differ between the two hemispheres of BoNT/E rats ($p > 0.05$). I conclude that a unilateral blockade of cortical activity during the sensitive period produces bilateral impairments in visual function that extend into adulthood.

Plasticity was assessed with 4-5 days of MD in a second group of P60-P80 rats. Monocular occlusion had no effect in control rats but significantly skewed the OD distribution towards the open eye in both the BoNT/E-injected and contralateral cortex ($\chi 2$ test, $p < 0.05$; Fig. 10B). Analysis of CBI values of individual rats indicated that the OD shift was comparable in the two hemispheres of BoNT/E rats (one way ANOVA, $p = 0.01$; post hoc Holm-Sidak test, $p > 0.05$; Fig. 10C). Thus, the two hemispheres retained a similar potential for plasticity in adult age.

To assess whether a persistent downregulation of intracortical inhibition might explain this enduring plasticity, coronal cortical sections from vehicle- and BoNT/E-injected rats (age > P100) were reacted with anti-GAD65 antibodies. A quantitative confocal analysis revealed that both hemispheres of BoNT/E-treated rats had a reduced density of presynaptic inhibitory boutons in the neuropil with respect to vehicle-injected rats (layers II-III; one way ANOVA, $p = 0.011$; post hoc Holm-Sidak test, $p < 0.05$; Fig. 11). Analysis of GAD65 fluorescence in “puncta-rings” surrounding the cell body of target neurons also revealed similar bilateral reductions of GAD65 staining in BoNT/E rats (data not shown).

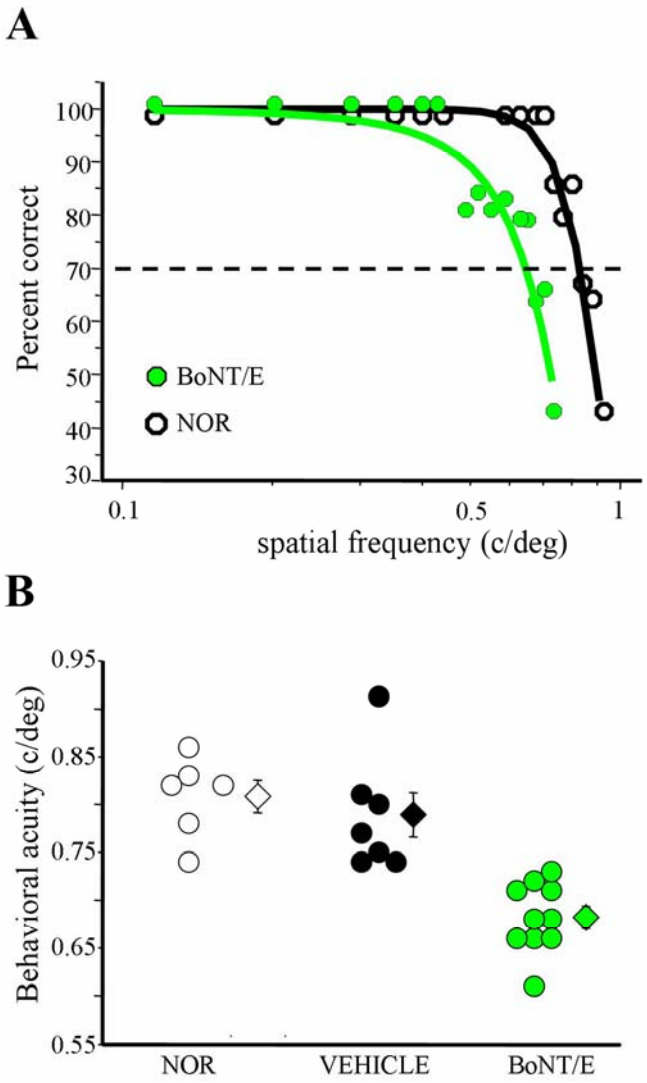

Figure 9

Persistent impairments in behavioral acuity in adult BoNT/E rats. (A) Representative frequency-of-seeing curves for a normal (open symbols) and a BoNT/E rat (green symbols). Each point is the average performance at a spatial frequency. A sigmoid curve is fit to the data and the point at which the curve intersects 70% accuracy is taken as the grating threshold. (B) Summary of data on behavioral visual acuity. Each circle represents one animal. Mean visual acuity (diamonds, ± S.E.) is significantly reduced in BoNT/E animals with respect to normal or vehicle-injected rats (ANOVA; post hoc Holm-Sidak test, $p < 0.01$).

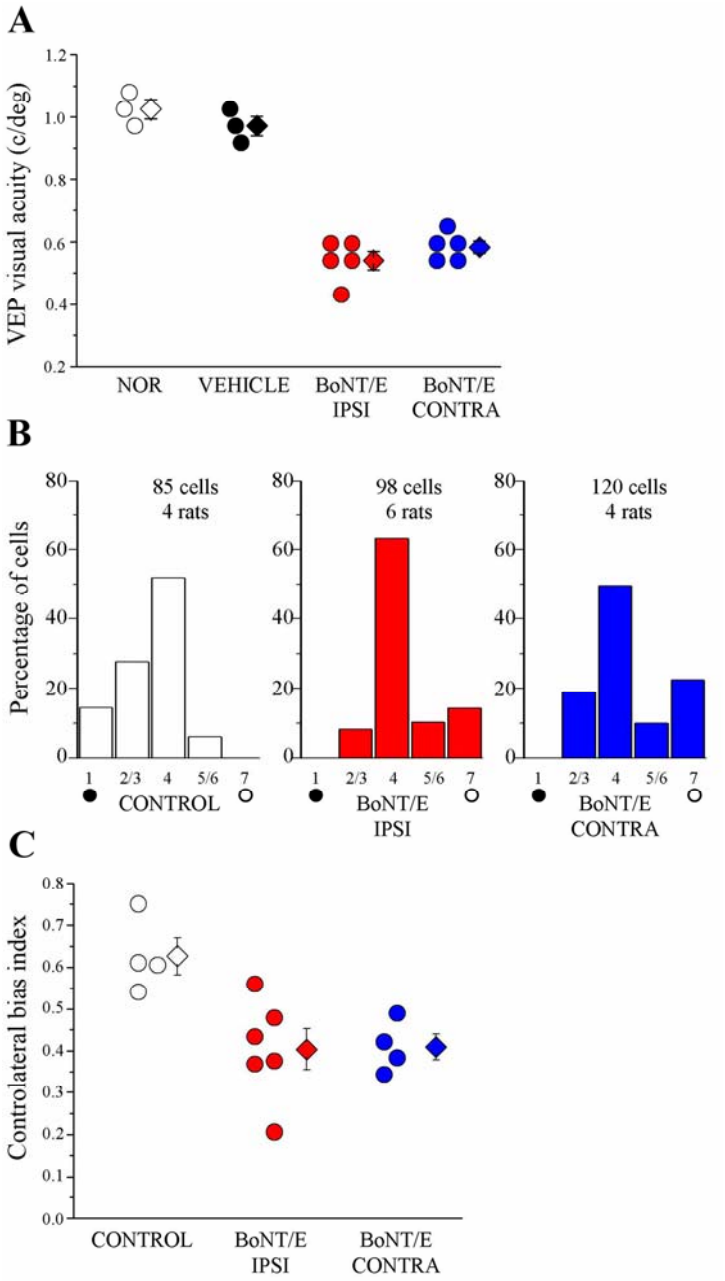

Figure 10

Persistent bilateral impairments in visual acuity and enduring plasticity in adult BoNT/E rats. (A) VEP visual acuity of P60-P100 animals. Acuity is poor in both hemispheres of BoNT/E-treated rats (circles, single animal values; diamonds, mean). Error bars indicate S.E. and, when not seen, are within the symbol. (B) OD distributions in animal subjected to MD at age > P60. The control group includes one vehicle-injected rat and three normal animals. In each hemisphere, OD was assessed after 4-5 days of occlusion of the contralateral eye. An OD shift in favour of the open eye (open circle) is observed in both hemispheres of BoNT/E-treated rats, but not in the control group. Number of animals and cells as indicated. (C) CBI for all animals recorded in each experimental group (circles, values of single animals; diamonds, mean ± S.E.). CBI values are lower than normal in both hemispheres of BoNT/E-injected rats (post ANOVA Holm-Sidak test, $p < 0.05$).

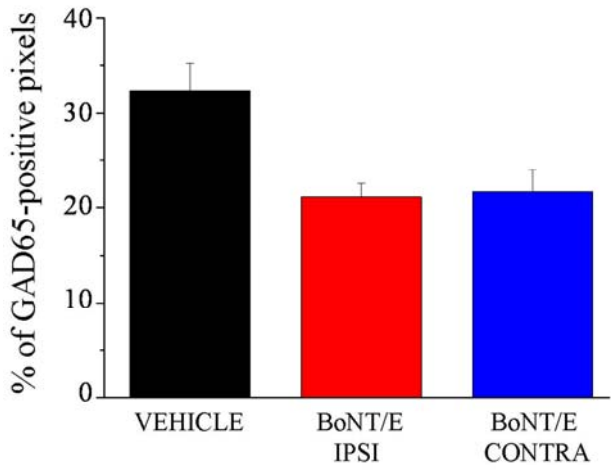

Figure 11

Mean density of GAD65-positive pixels in the neuropil (layers II-III) of rats older than P100. Control rats received vehicle at P14. The density of presynaptic inhibitory terminals is significantly reduced in the cortex ipsilateral and contralateral to BoNT/E infusion with respect to the vehicle-injected cortex. Error bars indicate S.E.

Reduction of spontaneous activity levels after silencing of the contralateral hemisphere

The dramatic long-term consequences of abolishing transcallosal input point to a crucial role for interhemispheric interactions in shaping cortical development. To investigate the mechanisms responsible for these effects, I performed single unit recordings in the untreated hemisphere of BoNT/E-treated rats, 7 days after toxin infusion (i.e., during the period of blockade). The analysis revealed that while responses to light bar stimuli remained extremely brisk (see also Fig. 3A), there was a remarkable decrease in the spontaneous discharge of cortical cells. Spontaneous firing rate had a median value of 3.37 spikes/sec (interquartile ranges: 2.16-6.57) in the untreated hemisphere of BoNT/E rats. These values were substantially lower than those found in normal age-matched animals (median: 13.03 spikes/sec; interquartile ranges: 7.03-18.92). The difference was highly significant (Mann-Whitney rank sum test, $p < 0.001$). To determine whether this reduction in spontaneous activity reflects a developmental plastic event, or it is rather an acute effect of callosal deprivation, I carried out an experiment in which single cortical units were recorded in the same animal before and after silencing of the contralateral hemisphere. BoNT/E was not suited for this kind of experiment, as the toxin requires several hours to become fully active (Antonucci et al., 2008b). Therefore, I selected the $GABA_A$ agonist muscimol as the blocking agent. About 10-12 cortical cells were recorded along a micropipette penetration and muscimol (30 mM) or saline as control was injected into contralateral striate cortex. I found that muscimol, but not saline, blocked cortical activity within 30 minutes (Fig. 20A). After this period, I started to record from single units again during withdrawal along the same track. I found a clear reduction of spontaneous activity levels following muscimol, but not saline, infusion (Fig. 12A). Quantification of spontaneous discharge indicated that cells recorded after muscimol had a significantly reduced firing frequency (one way ANOVA on ranks, $p < 0.001$; post hoc Dunn's test, $p < 0.05$ vs. all the other groups; Fig. 12B). These data demonstrate reductions of spontaneous discharge following blockade of the contralateral hemisphere.

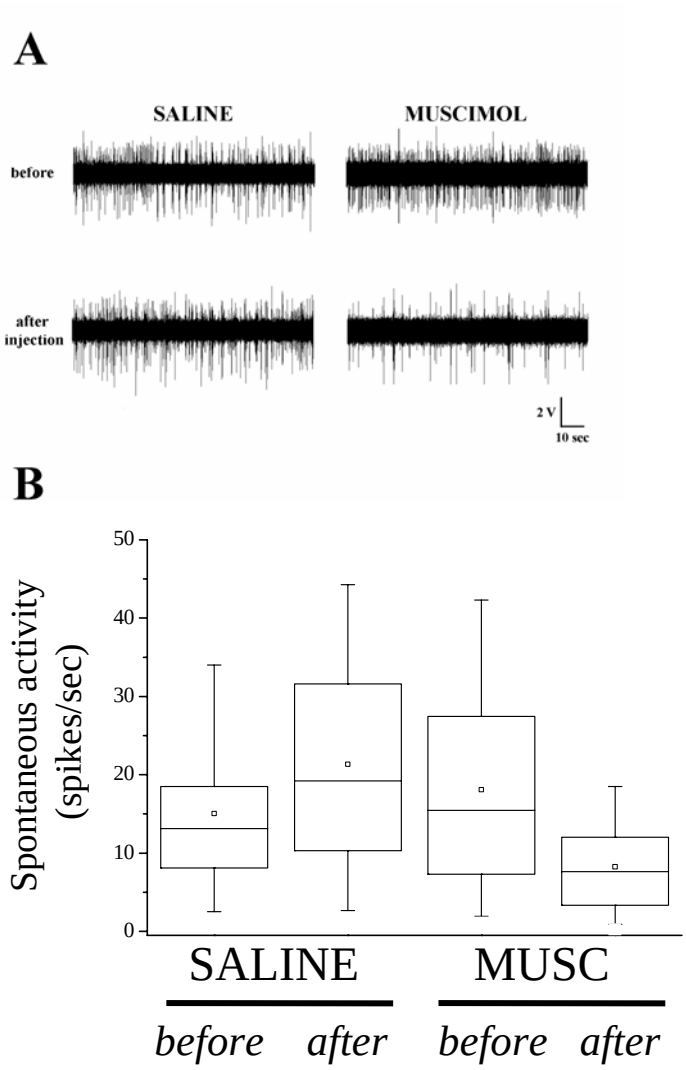

Figure 12

(A) Representative recordings of spontaneous spiking activity from visual cortical neurons, before and after muscimol (or saline) injection into the contralateral hemisphere. (B) Quantification of spontaneous firing rates before and after muscimol infusion into the contralateral hemisphere. The box chart summarizes the distribution of spontaneous activity for the cells in each experimental group. The horizontal lines in the box denote the 25th, 50th, and 75th percentile values. The error bars denote the 5th and 95th percentile values. The square symbol in the box denotes the mean of the column of data. Spontaneous discharge is significantly reduced after muscimol delivery to the opposite side (one way ANOVA, $p < 0.001$ followed by Dunn's test, $p < 0.05$).

ROLE OF CALLOSAL CONNECTIONS IN CORTICAL PLASTICITY

Control of cortical binocularity by the corpus callosum

The experiments described in the previous section indicated that blockade of interhemispheric communication reduced spontaneous activity and leads to altered development of the visual cortex during the sensitive period. I next asked whether callosal connections are involved in shaping other aspects of cortical function, such as binocularity. In the previous experiments binocularity was not altered in the hemisphere contralateral to BoNT/E (see Fig.6). However, the recordings were performed at P35 (i.e. when BoNT/E action is off), leaving open the possibility that OD was indeed affected during the period of blockade, and the return of activity was sufficient to restore normal binocularity by P35.

Experiments were performed in naïve rats during the developmental critical period (age P24-P29). I compared binocularity of cortical cells before and after acute silencing of the striate cortex contralateral to the recording site. The spiking activity of cortical neurons from the binocular portion of the primary visual cortex was recorded extracellularly along a micropipette penetration. Then I injected either the $GABA_A$ agonist muscimol (30 mM; 1 μl) or saline as control into the contralateral binocular cortex. After 30 minutes, I started to record from single units again along the same track.

I found that the ocular dominance (OD) distribution of cortical neurons significantly shifted towards the contralateral eye following muscimol, but not saline, injection. 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 (Pizzorusso et al., 2002; Pizzorusso et al., 2006). Saline infusion had no effect on binocularity, as shown by analysis of both OD distributions (χ^2 test, naïve before saline vs. after saline, $p = 0.15$; Fig. 13A). Analysis of contralateral bias index (CBI), that represents an evaluation of interindividual variability of ocular dominance, yielded the same result (paired t-test, $p = 0.36$; Fig. 14A).

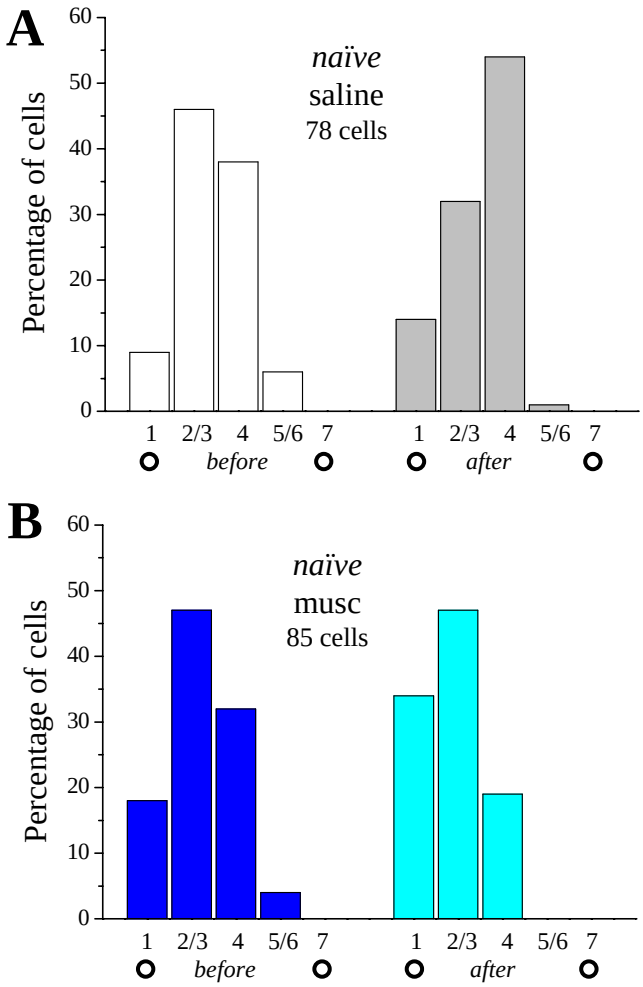

Figure 13

Callosal connections contribuite to binocularity. (A,B) OD distributions of naïve young rats (age P24-P29) before and after injection of either saline or muscimol into the opposite hemisphere. Saline has no effect on the OD histogram, while rats injected with muscimol show a decrease in the number of units driven by the ipsilateral eye.

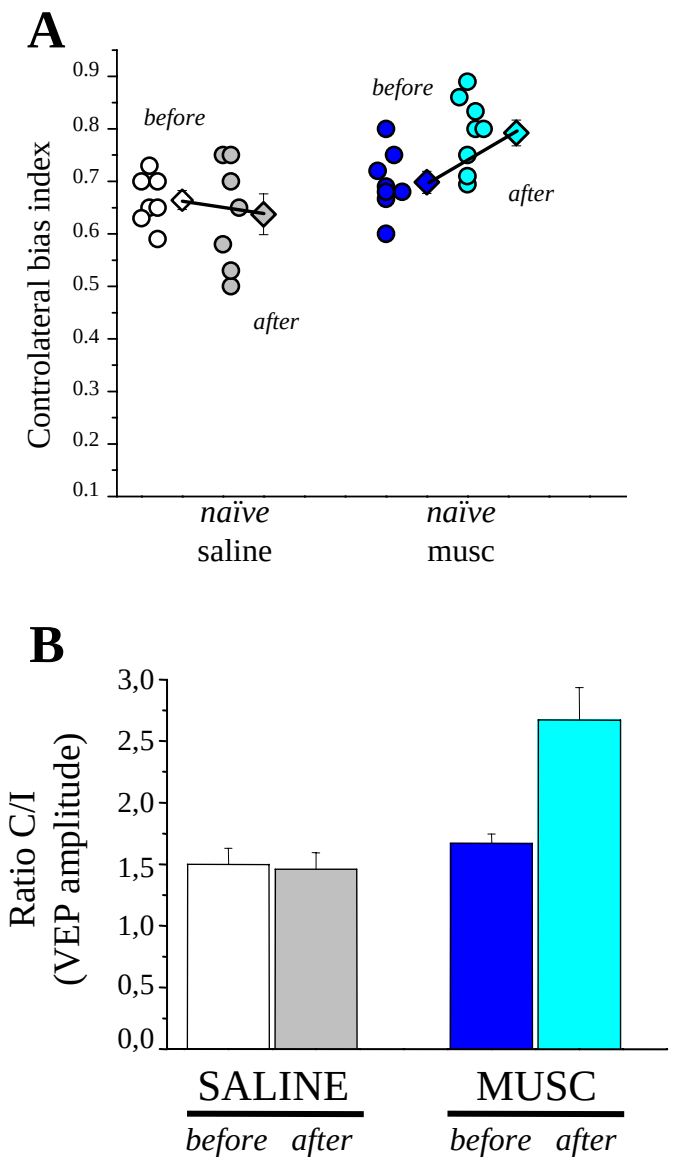

Figure 14

(A) CBIs recorded before and after saline or muscimol injection into the opposite hemisphere. Circles represent values of single animals and diamonds are the mean of the group. Error bars indicate S.E., and when not seen, are within the symbol. Muscimol injection shift CBIs values significantly (paired t-test, $p = 0.013$)
(B) C/I VEP ratio of rats injected with saline or muscimol. After muscimol, the C/I ratio increased significantly ($p = 0.002$)

Conversely, muscimol injection decreased the percentage of units driven by the ipsilateral eye (χ^2 test, naïve before muscimol vs. after muscimol, $p = 0.016$; Fig. 13B). The shift of OD towards the contralateral eye was confirmed by analyzing the cumulative distribution of the ocular dominance score, which allows a finer and more robust statistical comparison (Kolmogorov–Smirnov test, before muscimol vs. after muscimol, $p = 0.002$; data not shown). Accordingly, CBIs were significantly higher (paired t-test, $p = 0.013$; Fig. 14A). These results suggest that acute silencing of callosal input affects cortical OD as measured by recording extracellular spiking activity.

To further validate this change in binocularity, I recorded VEPs elicited by each eye before and after delivery of saline or muscimol into the opposite hemisphere. VEPs represent the integrated responses of a population of neurons, and are commonly used to evaluate alterations in binocularity (Huang et al., 1999; Porciatti et al., 1999; Sawtell et al., 2003; He et al., 2006). I assessed OD calculating the contralateral-to-ipsilateral (C/I) ratio, that is the ratio of VEP amplitude recorded by stimulating each eye separately. I found that C/I ratio in saline injected animals did not change after injection (paired t-test, $p = 0.79$; $n = 5$ rats; Fig. 14B, *left*), while in animals injected with muscimol in the opposite cortex, C/I ratio increased significantly (paired t-test, $p=0.002$; $n = 7$ rats, Fig. 14B, *right*). Thus, a shift in OD towards the contralateral eye is apparent following acute silencing of the opposite hemisphere.

This OD shift could be due either to an increased in contralateral eye response, or to a decreased visual drive through the ipsilateral eye. To define the mechanism, I analysed peak firing rates of single units following stimulation of each eye, before and after muscimol delivery (Fig.15). I found a clearly significant depression of ipsilateral eye responses after muscimol (one way ANOVA on ranks, $p < 0.001$; post hoc Dunn's test, $p < 0.05$ Fig. 15, *right*). In contrast, there was only a slight change in the peak response of cells after stimulation of the contralateral eye (one way ANOVA on ranks, $p < 0.001$; post hoc Dunn's test, $p < 0.05$; Fig. 15, *left*).

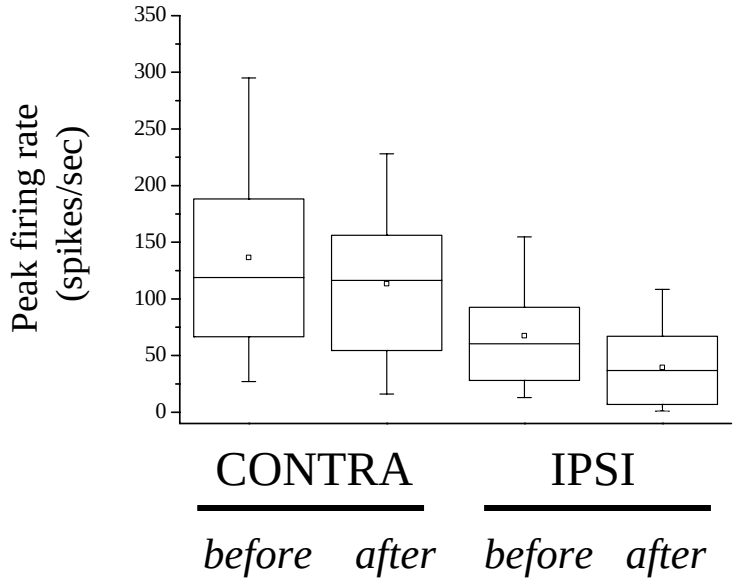

Figure 15

Peak firing rates of visual cortical neurons in animal injected with muscimol. Note that peak firing rates of contralateral eye decreased slightly, while the peak responses of ipsilateral eye were much more reduced (by about 50%).

As a control, I also examined peak firing rates in animal infused with saline. I found no significant changes following saline in either the contralateral eye- or the ipsilateral eye–driven responses (data not shown).

Involvement of the callosum in cortical plasticity

Since interhemispheric connections contribute to cortical binocularity, I studied their involvement in the plastic shift of OD after monocular deprivation (MD). Rats at the peak of the critical period (age P20-P22) were monocularly deprived for 7 days, and single units were recorded from primary visual cortex contralateral to the occluded eye before and after muscimol injection into the opposite hemisphere (see Fig. 20B). MD produced the expected change in eye preference of cortical neurons so that most units responded preferentially to the open, ipsilateral eye (Fig. 16A, left). Muscimol infusion had a dramatic impact on the OD histogram. There was a complete disappearance of class 7 cells and a corresponding rise in the proportion of closed eye-driven units (Fig. 16A, right). Deprived animals infused with saline solution shown no change in OD (data not shown).

Statistical analysis of OD histograms indicated that a nearly normal eye preference was restored after muscimol (χ^2 test, MD before muscimol vs. after muscimol, $p < 0.001$; MD after muscimol vs. naïve before muscimol, $p = 0.17$; compare Figs. 13B, 16A). Analysis of CBIs of single animals (Fig. 16B) and computation of the OD score (Fig. 20C) strengthened the conclusions obtained from the pooled OD distributions. Deprived animals recorded before muscimol exhibited a robust drop in CBI values as compared to naïve animals (one way ANOVA, $p < 0.001$; post hoc Holm-Sidak test, $p < 0.01$; Fig. 16B). This fall was almost entirely recovered by blocking the opposite hemisphere (post ANOVA Holm-Sidak test, MD after muscimol vs. naïve before muscimol, $p > 0.05$; Fig. 16B). 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, $p < 0.01$; Figs. 16B). 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.

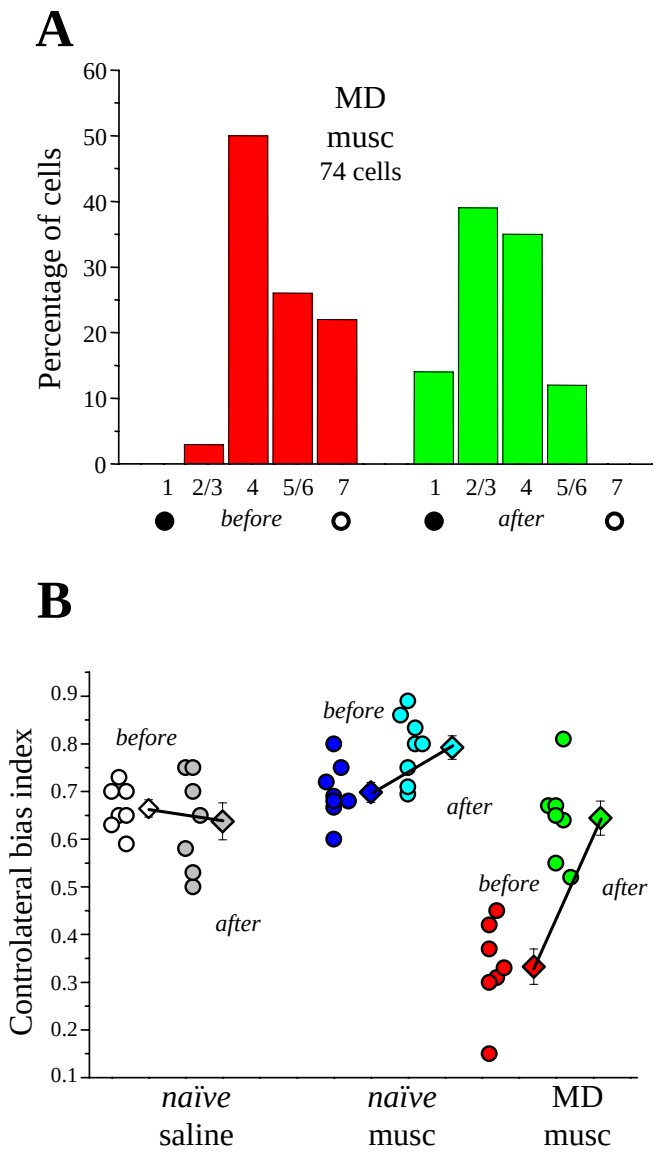

Figure 16

(C) OD distribution of rats subjected to MD for 7 days. Recordings were performed in the right hemisphere (contralateral to the deprived eye) before and after muscimol injection into left visual cortex. Muscimol causes a disappearance of cells driven exclusively by the open eye and a corresponding increase in the proportion of units controlled by the contralateral, deprived eye. (D) CBIs for all animals recorded in each experimental group before and after muscimol injection. Circles represent values of single animals and diamonds are the mean of the group. Error bars indicate S.E., and when not seen, are within the symbol. Note the substantial change in OD following muscimol infusion in monocularly deprived rats.

I next analyzed whether there is any correlation between the initial OD class (before muscimol injection) and the entity of OD change produced by acute callosal silencing (measured as the difference between the OD score of each unit before and after muscimol delivery). Such plot is shown in Fig 17. It is clear that the major shift is restricted to cells that have initial OD class 7 (one way ANOVA on ranks, $p < 0.001$; post hoc Dunn's test, $p < 0.05$; Fig. 17)

Functional masking of deprived eye responses by callosal input

So far, I have inferred the role of interhemispheric connections in OD plasticity from measures of relative responsiveness of the visual cortex to stimulation of each eye. It remains to be seen whether the recovery in OD observed after muscimol infusion in MD animals depends on a potentiation of deprived eye inputs, on a depression of open eye responses, or both. I analysed the peak firing rates of single units following stimulation of each eye, before and after muscimol injection (Fig. 18A,B). I found a highly significant potentiation of deprived eye responses after muscimol (paired t-test, $p < 0.001$; Fig. 18B). In contrast, there was no change in the peak response of cells after stimulation of the open, ipsilateral eye (paired t-test, $p = 0.40$; Fig. 18A,B).

To further address the mechanisms of recovery of binocularity, I performed visual evoked potential (VEP) recordings in animal deprived from P20-P22 for 7 days, before and after muscimol injection. VEPs evoked by each eye were recorded from superficial layers contralateral to the deprivation. As expected (Frenkel and Bear, 2004), I found that after monocular occlusion open eye inputs were dominant and responses from the contralateral, deprived eye were weak (Fig. 18C). The electrode was left in place and muscimol was delivered to the opposite hemisphere. Notably, I found that muscimol delivery selectively elevated deprived eye responses (Fig. 18D, left; paired t-test, $p < 0.001$). There was no effect on the amplitude of the field potential evoked by stimulating the ipsilateral open eye (Fig. 18D, right; paired t-test, $p = 0.61$). The rapid unmasking of deprived eye inputs following blockade of transcallosal connections indicates that functional inhibition (rather than anatomical retraction) of less active inputs is the major determinant of the OD shift.

I finally investigated whether the recovery of contralateral, deprived eye input following muscimol could trigger recovery of visual acuity. I found, as

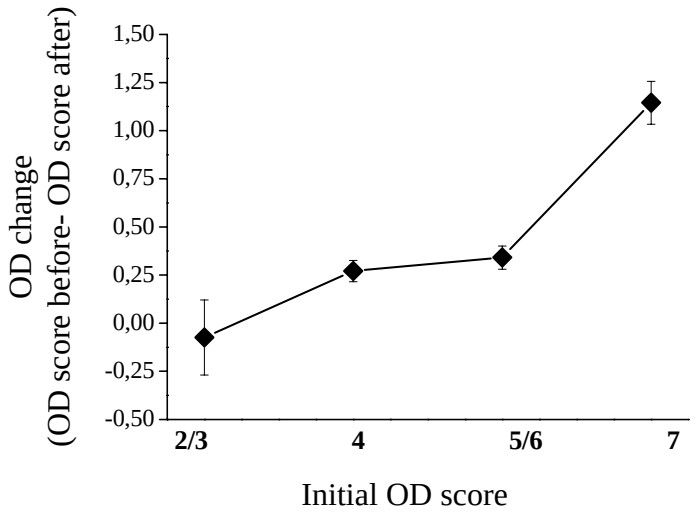

Figure 17

Class 7 cells exhibit the greater shift after muscimol. Correlation between the initial OD class (before muscimol injection) and the entity of OD change produced by acute callosal silencing (measured as the difference between the OD score of each unit before and after muscimol delivery). It is clear that the major shift is restricted to cells that have initial OD class 7 (one way ANOVA on ranks, $p < 0.001$; post hoc Dunn's test, $p < 0.05$)

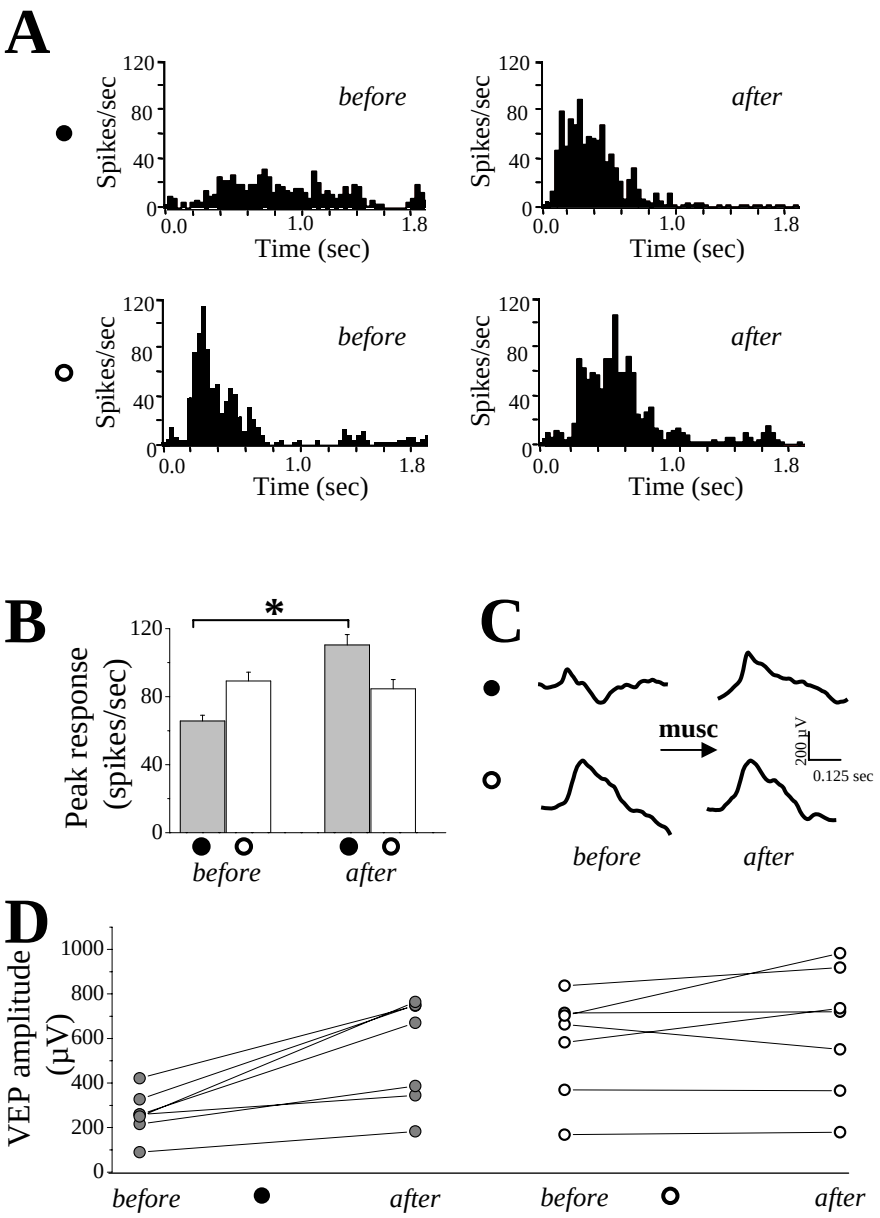

Figure 18

Silencing callosal input rapidly unmasks deprived eye responses. (A) Examples of peristimulus time histograms recorded in response to stimulation of the deprived (top, filled circle) and open eye (bottom, open circle), before and after muscimol injection into the opposite hemisphere. Visual stimulus, light bar drifting at 28°/sec; period of the stimulation, 1.9 sec; for each record, the cell discharge has been averaged over 20 stimulus periods. Deprived eye responses are unmasked after muscimol (top right). (B) Histograms showing peak firing rates of both eyes, before and after muscimol injection into the opposite side. Peak responses of the deprived eye are significantly increased (asterisk), while open eye responses are unaffected. Error bars indicate S.E. (C) Representative examples of VEPs responses for both eyes. (D) VEP amplitudes for deprived (left, filled circle) and open eye (right, open circle), before and after muscimol delivery. Deprived eye responses increase consistently after inactivation of callosal input.

expected, that visual acuity of the deprived eye before injection was lower than normal (mean, 0.4 c/deg; $n = 4$). After muscimol injection, despite of an increased amplitude of VEPs at low spatial frequencies, the spatial resolution did not change (see fig. 19), Acuity values were indistinguishable before and after callosal silencing (paired t-test, $p > 0.05$, Fig 19B). Thus, acute blockade of callosal projections results in a substantial recovery of deprived eye input, but is not sufficient to recover the amblyopic effect of MD.

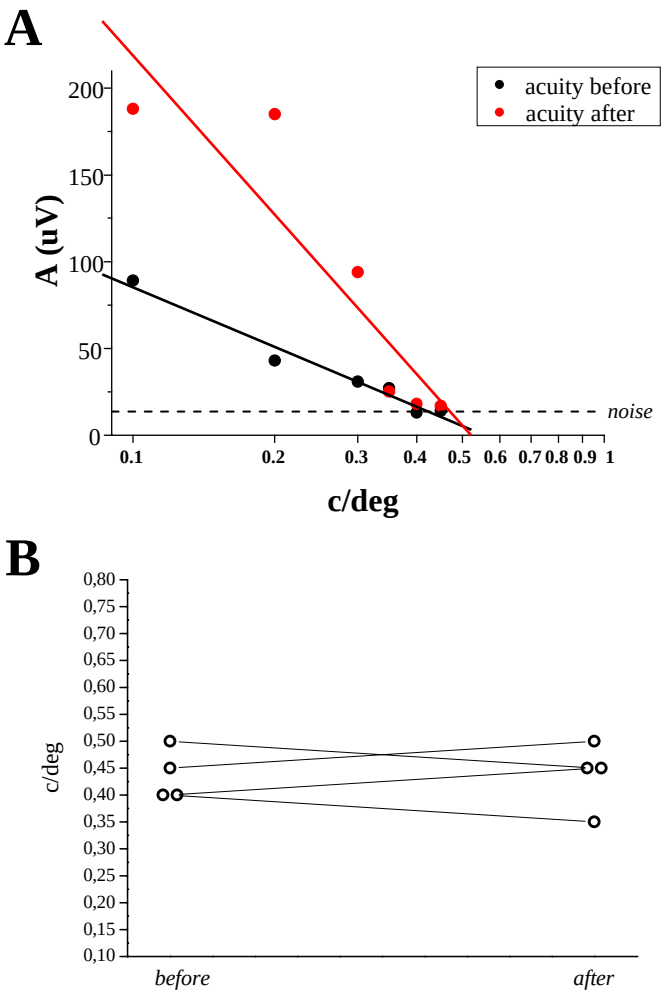

Figure 19

Visual acuity of the deprived eye in muscimol injected animals. Visual acuity measured electrophysiologically before and after muscimol delivery. (A) Representative example of acuity determination in a rat. Note increase of VEP amplitudes at low spatial frequencies after callosal silencing, but no change of spatial resolution.
(B) Acuity values for single animals. No statistical difference can be detected (paired t-test, $p>0.05$)

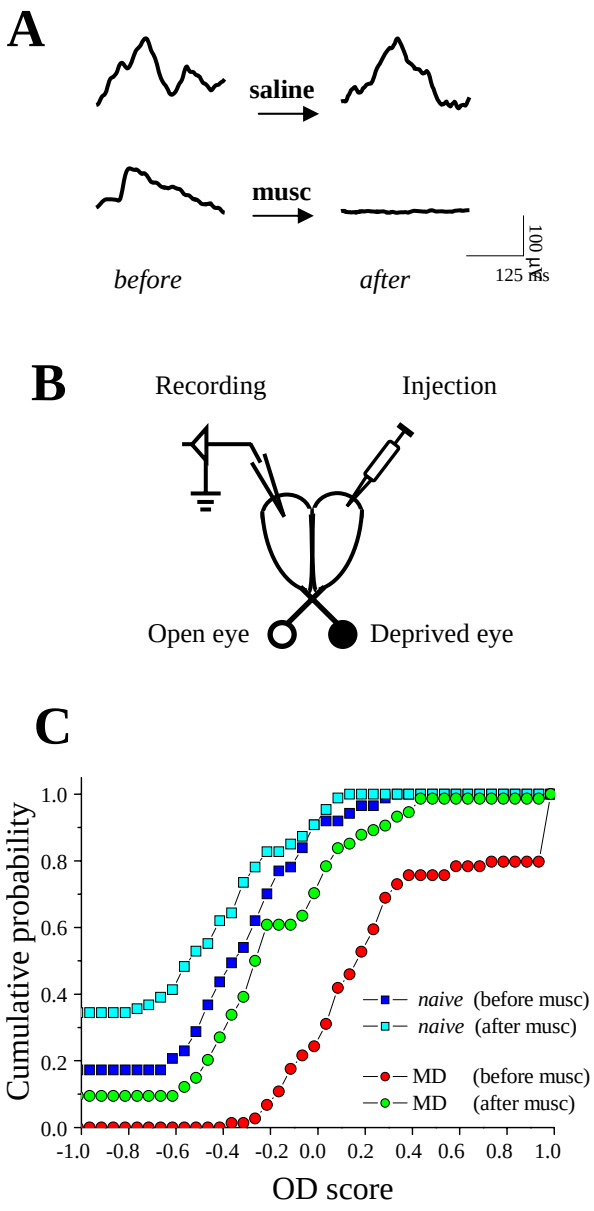

Figure 20

(A) Muscimol silences visual responses. Example of VEP recordings demonstrating blockade of injected visual cortex after muscimol. (B) Experimental design. Single units and/or VEPs were recorded along a single track in the right hemisphere (contralateral to the deprived eye). Muscimol was then injected into left striate cortex. After 30 min, we started to record again from the same track in the right hemisphere. (C) Cumulative distribution of the OD score in *naïve* and MD rats, before and after muscimol injection. Muscimol produces a large shift in OD score in monocularly deprived rats (Kolmogorov–Smirnov test, MD before musc vs. MD after musc, $p < 0.001$). OD score distribution of MD animals after muscimol is almost superimposable to that of *naïve* rats before injection (Kolmogorov–Smirnov test, $p > 0.05$).

DISCUSSION

DISCUSSION

In this study, I have provided evidence for a novel role of callosal connections in controlling maturation and plasticity of the visual cortex. First, I have examined the effects of a prolonged blockade of callosal input during development. Second, I have investigated the involvement of the corpus callosum in plasticity mechanisms induced by monocular deprivation. The results from these experiments will be discussed in turn.

ROLE OF THE CALLOSUM IN VISUAL CORTEX DEVELOPMENT

BoNT/E as a novel tool to transiently blocks cortical activity

In this study I have exploited the clostridial neurotoxin BoNT/E to investigate the effects of a unilateral, transient blockade of cortical activity during the sensitive period. BoNT/E is a highly selective metalloprotease that elicits persistent but reversible inhibition of transmitter release via cleavage of the synaptic protein SNAP-25 (Schiavo et al., 2000; Costantin et al., 2005; Davletov et al., 2005). Proteolysis of SNAP-25 by BoNT/E prevents the formation of a functional SNARE complex, resulting in a highly selective blockade of synaptic activity (Schiavo et al., 2000; Montecucco and Molgo, 2005). The action of BoNT/E is completely reversible, as the protease is inactivated with time and intact SNAP-25 is wholly replenished (Keller et al., 1999; Costantin et al., 2005). Thus, BoNT/E combines a potent and long-lasting action with full reversibility at the cellular level (Bozzi et al., 2006). These features make BoNT/E an ideal tool to address the role of synaptic activity in the brain. Indeed, a single administration of the toxin is sufficient to switch off activity for a period of two weeks, thus avoiding the need for more invasive techniques such as long-term minipump infusion of activity blockers (tetrodotoxin, muscimol), that is often impractical in small developing rodents. Importantly, I found no adverse effects of BoNT/E on neuronal survival. Indeed, neuronal cell density was perfectly normal in the cortex of BoNT/E-infused rats (see Fig. 4). This is consistent with previous reports in vitro (Osen-Sand et al., 1996).

Differently from tetanus toxin, BoNT/E is not axonally transported (Lalli et al., 2003; Antonucci et al., 2008a). I made several controls to make sure that there was no unintended diffusion or transport of the toxin from the injection site. These controls rely on the detection of intact and cleaved SNAP-25 by immunohistochemistry and immunoblotting (see Fig. 1). Following the fate of BoNT/E in vivo is inherently difficult due to the very tiny amounts of injected toxin. In this context, detection of cleaved substrate molecules provides the most sensitive assay for tracking movement of BoNT/E. Indeed, single toxin molecules can cleave a large number of SNAP-25 target molecules, providing a dramatic amplifying effect.

I have shown that intracortical delivery of BoNT/E at the time of eye opening (P14) results in strictly unilateral silencing of activity until near the peak of the critical period for visual cortex, i.e. P25-P28 in rats (Fagiolini et al., 1994; Lodovichi et al., 2000). Considerable evidence indicates that eye opening represents a crucial event for refinement of cortical circuitry (Maffei et al., 2004; Gandhi et al., 2005). Thus, injections of BoNT/E at P14 allow to target the process of cortical maturation since its initial phases.

Functional properties of the visual cortex were assessed at P35, i.e. the time of complete recovery from BoNT/E effects. A second group of animals were recorded in adulthood (P60-P100).

There are three main findings from this BoNT/E experiments:

(i) a transient unilateral silencing of intrinsic cortical activity produces deficits in visual acuity and an extension of the critical period for OD plasticity in the blocked hemisphere;

(ii) these same effects are detectable in the striate cortex of the opposite side, pointing to a crucial role for interhemispheric connections in postnatal development; (iii) impairments in cortical maturation persist into adulthood, indicating that a transient blockade of synaptic activity leaves a permanent trace in cortical circuitry.

Impaired development of the visual cortex in the blocked hemispere

My data demonstrate the requirement for intrinsic activity in several aspects of visual cortex development. It was previously shown that silencing of action

potentials completely prevents maturation of orientation selectivity and clustering of horizontal connections in ferret visual cortex (Chapman and Stryker, 1993; Ruthazer and Stryker, 1996). I show here that a transient blockade of cortical activity causes a reduction of visual acuity and an extension of the period of susceptibility to MD. These effects are accompanied by a decrease in GAD65 immunoreactivity. A reduced inhibitory tone may explain the deficits in spatial resolution and prolongation of the plastic state. Indeed, substantial evidence indicates that intracortical inhibitory connections control maturation of visual function and critical period plasticity (Huang et al., 1999; Berardi et al., 2003; Hensch, 2005). For example, a reduction of GABA-mediated transmission in visual cortex accounts for some of the classic effects of dark rearing (Gianfranceschi et al., 2003; Iwai et al., 2003). Changes at the level of excitatory circuitry, such as alterations in AMPA or NMDA receptors (He et al., 2006), could also underlie the effects of the activity blockade. This possibility remains to be evaluated.

I found no measurable effects on the OD of cortical neurons after BoNT/E blockade. It is likely that BoNT/E affects OD, but that the return of intrinsic activity is sufficient to restore normal binocularity by the time the recordings were performed (P35). This interpretation is supported by my data showing that acute silencing of interhemispheric communication affects binocularity (see below).

Impaired development of the contralateral, uninjected hemisphere

A second major conclusion from this study is that interhemispheric communication plays a fundamental role in functional development of the cortex. The callosal pathway matures quite early in development (by P15 in rodents; (Mizuno et al., 2007) and links retinotopically corresponding loci in the two hemispheres (Lewis and Olavarria, 1995; Olavarria, 1996). Interhemispheric linkages serve important functions, including binding together the separate representations of the two halves of the visual field (Berlucchi and Rizzolatti, 1968; Engel et al., 1991). I have shown here a significant reduction in spontaneous cortical activity after silencing of the

contralateral side. This is consistent with previous studies in cats indicating that callosal projections provide a kind of general sustaining input to the contralateral hemisphere (Blakemore et al., 1983; Yinon et al., 1992). This activity deprivation might explain the impaired cortical development on the side contralateral to BoNT/E injection. Indeed, the striate cortex contralateral to the blockade exhibits poor spatial resolution, prolongation of the critical period for OD plasticity, and reductions in markers of intracortical inhibition. It is worth noting that the magnitude of these effects is comparable in the blocked and untreated hemisphere. Thus, maturation of the blocked cortex is superimposable to that of the opposite side, that only lacks callosal input and maintains normal afferent activity through the direct retinogeniculate pathway.

The persistent plasticity found in both hemispheres was accompanied by a bilateral reduction in the density of WFA-positive profiles in the visual cortex. This is consistent with previous reports indicating that the density of perineuronal nets in the cortex is tightly linked to the potential for plasticity. Indeed, delaying maturation of perineuronal nets by dark rearing prolongs plasticity, and removal of perineuronal nets in adulthood reinstates sensitivity to MD (Pizzorusso et al., 2002).

The very similar developmental deficits observed ipsilateral and contralateral to the activity blockade indicate a fundamental role for callosal linkages in coordinating the process of cortical maturation. In teleological terms, parallel development of the two sides of the brain is needed to ensure a match in information processing between the cerebral hemispheres. Indeed, this coordinated maturation grants uniform sampling of visual information and the ability to combine the two partial cortical maps into a single, coherent representation.

In rats, callosal cells occupy lateral as well as medial portions of striate cortex (Olavarria and Van Sluyters, 1985; Lewis and Olavarria, 1995). In the region of the border between areas 17 and 18, which contains a representation of the vertical meridian of the visual field, cells projecting through the corpus callosum are concentrated throughout the depth of the cortex. In contrast, in

medial portion of striate cortex, where peripheral portions of the visual field are represented, callosal cells are preferentially found in infragranular layers (Olavarria and Van Sluyters, 1985; Lewis and Olavarria, 1995). This organization is different from that observed in higher mammals such as cats and primates, where callosally projecting cells are mainly concentrated at the border between area 17 and 18 (Olavarria, 1996; Houzel and Milleret, 1999). The anatomical difference raise the issue whether callosal influences are as important for cortical development in higher mammals as reported here for the rat. Reports in the literature suggest that this may indeed be the case. Early section of the callosum in cats produces a reduction in behaviourally measured visual acuity, supporting a role for interhemispheric communication in cortical maturation (Elberger, 1984). Monkeys that received unilateral lesions of primary visual cortex in infancy display impairments of stimulus detection in the intact visual hemifield (see Fig. 3 of (Moore et al., 1996). Thus, development of visual performance depends to a great degree on interhemisperic communication in several species.

Development of other functional properties might rely more on retinothalamic input and/or local intracortical plasticity rather than on callosal influences. For example, development of orientation selectivity in ferret visual cortex is not affected by activity blockade in the contralateral hemisphere (Chapman and Stryker, 1993).

Long-lasting consequences of activity blockade

A third important finding of these experiment is that a transient activity blockade leaves a lasting change in visual cortex. Indeed, I observed basically no recovery of visual acuity in either hemisphere of animals injected with BoNT/E at P14 and allowed to survive till P60-P100. In these adult animals, both the previously blocked and the uninjected hemisphere maintained a similar susceptibility to monocular suture. The data indicate that silencing neural activity produces persistent effects on visual function and the cortical plasticity machinery. It has been recently shown that adult plasticity is shaped by prior experience (Hofer et al., 2006b). My data demonstrate that malleability of adult circuits can be conditioned by activity deprivation during

early life. The persistent sensitivity to MD found on the site opposite to the blockade indicates that lack of callosal input during development is sufficient to maintain the adult cortex in a plastic state.

The long-lasting effects of BoNT/E on cortical development and plasticity likely involve changes in gene expression. It has been demonstrated that long-term functional modifications in the hippocampus require changes in structure and functionality of genes (Bliss and Collingridge, 1993; Nguyen et al., 1994). It would be interesting to know the specific gene pathways that are altered by BoNT/E treatment, and compare the injected vs. contralateral hemisphere.

I found that the expression of the GABA biosynthetic enzyme GAD65 exhibited a long lasting reduction in both visual cortices of the BoNT/E treated rats. It is possible that restoration of an appropriate level of GABAergic transmission (for example by infusion of the GABAergic agonist diazepam; (Fagiolini and Hensch, 2000; Iwai et al., 2003) rescues the developmental deficits (i.e. the reduced visual acuity and the enduring plasticity). This hypothesis remains to be investigated.

The results also indicate that the transient activity blockade of one hemisphere is able to maintain plasticity in both visual cortex. Several recent findings in mouse visual cortex suggest that at least some measure of OD plasticity persists into adulthood (Sawtell et al., 2003; Tagawa et al., 2005; Hofer et al., 2006b), making one wonder whether the susceptibility to MD reported here for the adult BoNT/E rats reflects at least some of this normal, extended plasticity. I consider this interpretation unlikely, however, as I and others have consistently failed to reveal MD effects in normal adult rats by both single unit and VEP recordings (Fagiolini et al., 1994; Guire et al., 1999); present results).

In summary, I have also revealed a previously unknown functional matching in the developmental maturation of the two cerebral hemispheres. Such coordinated development allows homogeneous processing of sensory information coming from the two visual hemifields, thus ensuring consistency of the visual percept. The importance of the callosal pathway in integrating

cerebral processing is still apparent in adults. Patients with unilateral occipital cortex injury show reduced spatial and temporal sensitivities in the sighted hemifield (Hess and Pointer, 1989; Rizzo and Robin, 1996).

ROLE OF CALLOSUM IN PLASTICITY OF THE VISUAL CORTEX

Role of the callosum in cortical binocularity

To investigate the role of the callosum in cortical binocularity, we recorded visual responses before and after acute blockade of callosal input. This was achieved via delivery of muscimol into the striate cortex contralateral to the recording. Extracellular recordings of spiking activity demonstrated an enhancement of the contralateral bias of single units following injection of muscimol into the opposite hemisphere.

To further characterize this effect, I performed VEP recordings. I found a significant increase of the contra/ipsi (C/I) VEP ratio after silencing of the callosal input. In order to distinguish if the effect was due to an increase of contralateral eye responses, or to a decrease of ipsilateral eye input, I analysed the peak firing rates of single units. After silencing of callosal linkages, there was a small reduction of contralateral eye responses, and a much more dramatic decrease of ipsilateral eye responses. Indeed, peak firing rates following stimulation of the ipsilateral eye were reduced by about 50%. Thus, the enhancement of contralateral bias produced by muscimol is mainly due to a decreased input from the ipsilateral eye.

These data suggest that in the normal rat visual cortex, binocularity depends to a great degree on the function of callosal fibers. The idea is that cortical afferent input from the contralateral eye is much stronger than that from the ipsilateral eye, and that some of the influence of the ipsilateral eye on cortical responses arrives via callosal connections from the opposite hemisphere, where it is the dominant eye (see Fig. 21A).

This view is supported by recent experiments in our laboratory, in which cortical binocularity was measured before and after silencing of the retinogeniculate pathway (via TTX infusion into the geniculate). This protocol allows one to isolate visual responses driven exclusively by callosal afferents. We found that C/I VEP ratios drop dramatically after TTX injection into the geniculate. Specifically, there is a small reduction of ipsilateral eye responses and a much greater, dramatic decrease of contralateral eye-driven activity. Thus, in normal rats, the callosal pathway mainly carries visual input from the ipsilateral eye (see Fig. 21A).

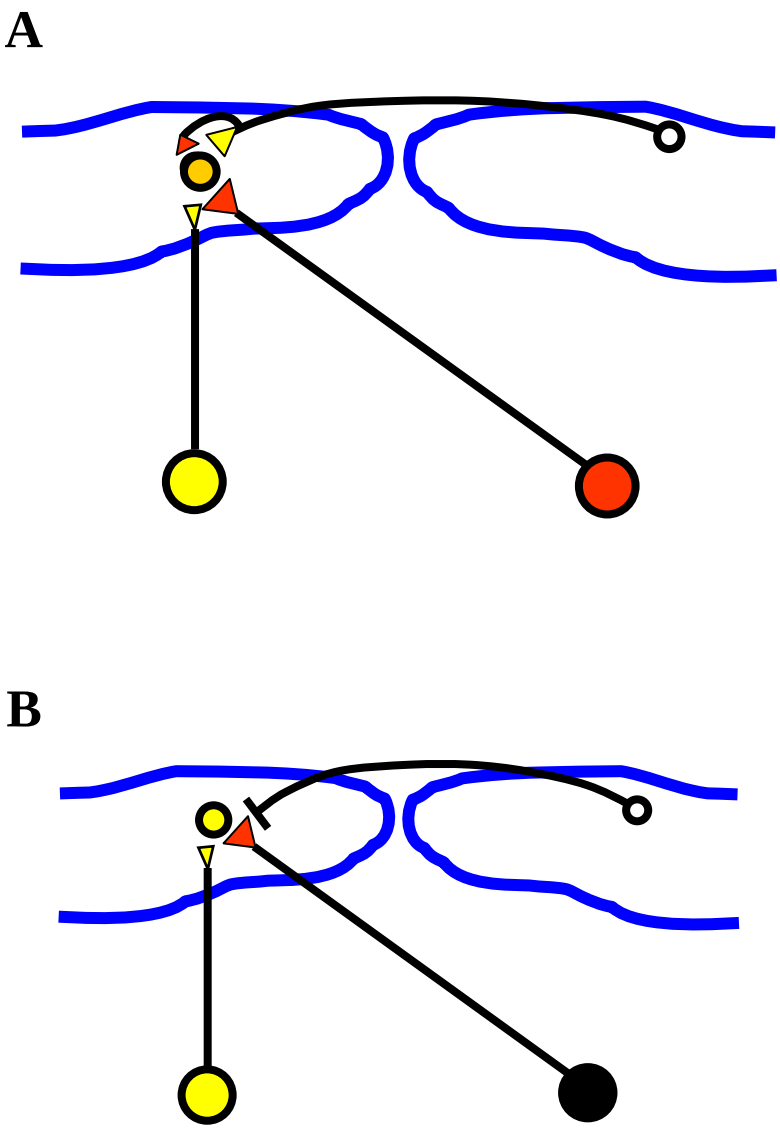

Figure 21

(A) Role of the callosum in naïve animals. Callosum is involved in determining binocularity. It contributes greatly to ipsilateral responses and in a minor way to contralateral ones.
(B) Role of the callosum in plasticity. In deprived animals, callosal inputs exert a selective functional inhibition onto deprived eye afferents.

Previous studies in cats have yielded contradictory results concerning the role of the callosum in binocularity. Most of these studies were conducted in animals with section of the corpus callosum. Some authors reported changes in the OD distribution of lesioned animals (Elberger and Smith, 1985; Payne, 1990; Yinon et al., 1992), while others did not (Minciacchi and Antonini, 1984). One study found alterations in binocularity 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 the surgery and the recording. In particular, previous studies used long intervals (months) between the callosal lesion and measurement of binocularity, thus allowing for plastic rearrangements that could affect the results. Furthermore, section of the callosum is an invasive procedure leading to a cascade of secondary events (i.e. inflammatory reactions), that could further impact visual responses.
In contrast to these previous studies, our experiments are based on acute functional blockade of callosal input by muscimol injection into the contralateral visual cortex. This allows to compare binocularity in the same animal, with and without the contribution of callosal afferents.

Previous functional and anatomical studies in cats have shown that the vast majority of callosally projecting cells are excitatory (Payne and Siwek, 1991; Sun et al., 1994). Indeed, transcallosal cells are pyramidal neurons which form asymmetric synapses in the opposite hemisphere (Shatz, 1977; Sun et al., 1994). A minor proportion of callosal fibers form symmetric (presumably inhibitory) synapses (Peters et al., 1990). I am currently investigating the neurochemical phenotype of callosal cells in rats, by combining retrograde tracing with either GABAergic or glutamatergic markers. The notion of a primarily excitatory function of callosal fibers is supported by my data indicating (i) reduced spontaneous discharge and (ii) reduced visual drive in cortical cells (mainly from the ipsilateral eye) following acute blockade of callosal input.

Role of the callosum in plasticity of the visual cortex

Given the role of callosal input in controlling cortical binocularity, it is possible an involvement of corpus callosum in OD plasticity. My experiments, indeed, uncover a novel role for callosal inputs in OD plasticity. 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 dissect the precise involvement of each pathway in OD plasticity, I compared responses of the open and deprived eye before and after acute silencing of interhemispheric communication. I found that silencing transcallosal input in animals that had undergone MD reversed almost completely the OD shift. More interesting, the recovery of binocularity was selectively due to potentiation of deprived eye responses. Deprived eye inputs were rapidly unmasked by muscimol injection into the opposite hemisphere, while there were no effects on open eye responses. This indicates that the callosal pathway exerts a selective functional inhibition onto deprived eye afferents, that is relieved after muscimol infusion. The rapid unmasking strongly favours functional vs. anatomical rearrangements in the mechanisms of the OD shift (see Fig. 21B).

An important issue is whether the degree of recovery of deprived eye responses is the same for cells of all OD classes. To address this point, I performed an analysis of the correlation between initial OD class (before muscimol) and the degree of OD shift following blockade of callosal input (see Fig. 17). I found that the recovery of binocularity was particularly striking and robust for cells with initial class 7. It follows that functional inhibition by callosal afferents is crucial for yielding a population of monocular, open eye-driven cells after MD.

We found that acuity of the deprived eye was not rescued by muscimol infusion, despite the enhancement of closed eye responses at low spatial frequencies (Fig 19). Thus, amblyopia cannot be relieved by removing transcallosal inhibition. The amblyopic effect of MD might derive mainly from alterations in the direct thalamocortical pathway.

An enhanced intracortical inhibition has been previously shown to contribute to the reduced ability of deprived afferents to activate cortical neurons (Maffei et al., 2006). Indeed, microiontophoretic delivery of the $GABA_A$ antagonist bicuculline restores inputs from the deprived eye in the visual cortex of MD cats (Burchfiel and Duffy, 1981; Sillito et al., 1981). The present findings demonstrate that the callosal input is the major source of inhibition. The present findings demonstrate that the callosal input is the major source of inhibition. There are at least two possible ways by which callosal input activity can result in a reduction of deprived eye responses. One possibility is that this functional inhibition is exerted directly by callosal neurons. To verify this hypothesis I am performing an experiment aimed at determining the neurochemical phenotype (i.e., glutamatergic vs. GABAergic) of callosal neurons, in normal and MD rats. If inhibition is exerted directly by callosal fibers, one would expect a switch from excitatory to inhibitory of at least some callosal cells.

A second possibility is that callosal inputs mediate functional inhibition via the recruitment of intracortical GABAergic circuits in the other hemisphere. It would be interesting to know whether transcallosal fibers in MD animals are preferentially innervating inhibitory cells.

It is noteworthy that an excitatory function of at least some callosal fibers is maintained also in MD animals. Indeed, muscimol delivery to the opposite hemisphere reduced spontaneous activity in MD rats, as it does in normal animals (my unpublished data).

My data prompt a reconsideration of the mechanisms involved in OD plasticity, that were originally thought to result solely from activity-dependent competition between the thalamic afferents serving the two eyes (Sherman and Spear, 1982). I demonstrate that transcallosal inhibition is a key player in the shift of eye preference following MD. 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 that transcallosal inhibition has been demonstrated to participate in plastic events occurring during several brain pathological conditions. For example, it has been shown in neglect patients that some of the behavioural 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). In these patients, silencing the intact side with transcranial magnetic stimulation results in substantial, long-lasting amelioration of the behavioural performances. 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).

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