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EXPERIENCE-DEPENDENT REACTIVATION OF OCULAR DOMINANCE PLASTICITY IN THE ADULT VISUAL CORTEX

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INTRODUCTION

CHAPTER 1

EXPERIENCE-DEPENDENT PLASTICITY: CRITICAL PERIOD OF OCULAR DOMINANCE PLASTICITY

The word 'plasticity' refers to the ability of the nervous system to reorganize its connections functionally and structurally in response to changes in environmental experience, underlying the adaptive development of neuronal circuitry, but also learning and memory.

It has been widely demonstrated the existence of time windows in early postnatal life, named 'sensitive periods', during which neural circuits display a heightened sensitivity to external stimuli. 'Critical periods' are a special class of sensitive periods wherein proper experience is required at fixed developmental times and results in irreversible changes in brain function (Knudsen, 2004). The question of what mechanisms underlie the activation and regulation of central nervous system (CNS) critical periods is of great interest in the field of neuroscience. Manipulation of such mechanisms may potentially allow reactivation of neural circuit plasticity during times when the adult brain is normally less plastic. Furthermore, understanding the mechanisms involved in the development and plasticity of neural connections is also important for specifying possible deviations from the proper developmental plan, and hence the aetiology of developmental brain disorders.

It is now understood that many regions of the brain have critical periods that occur at different times and are activated and regulated by distinct mechanisms (for a review, see Hensch, 2004), but the visual cortex has long been a proving ground for studying

experience-dependent plasticity because visual experience can be easily manipulated and the consequences of manipulations can be readily measured at the anatomical, physiological and molecular levels. Classic experiments have been performed in cats and primates, but recent findings regarding the cellular and molecular mechanisms underlying the experience-induced changes in visual cortical function have been derived predominantly from rodents, owing to the simplicity of the rodent visual system and the relative ease of genetic manipulation.

1.1 Survey of the mammalian visual system with reference to rodents

This paragraph addresses the basic aspects of visual system organization with particular reference to similarities and differences between the visual system of rodents and that of other mammalian species.

The retina is a sensory structure responsible for converting light into neural signals that are relayed to the brain through the optic nerve. The basic functional organization of the mouse retina is largely similar to that of other mammalian species (Adams, 1964; Rodieck, 1971). In all mammals the optic nerves from each eye join at the optic chiasm, where fibers destined for one or the other side of the brain are sorted out. The majority of retinal ganglion cell (RGC) axons carrying information from a given retina cross at the chiasm and project to the contralateral retinorecipient nuclei.

The proportion of RGC axons that do not cross and thus project to the ipsilateral retinorecipient nuclei, however, varies considerably among species and is closely related to the position of the eyes in the skull. In species with laterally positioned eyes and therefore a relatively small binocular field, the proportion of ipsilaterally projecting RGCs tends to be small. In particular, in rodents the proportion of RGCs projecting ipsilaterally is 2-3%

(Dräger and Olsen, 1980) against the 25-40% observed in cats and primates (Perry et al., 1984; Tassinari et al., 1997). Retinal fibers then enter each optic tract, which projects mainly to three subcortical targets, i.e. the superior colliculus, several nuclei in the pretectal complex of the epithalamus and the lateral geniculate nucleus (LGN) of the thalamus (Dräger, 1974; Hofbauer and Dräger, 1985; Provencio et al., 1998). The LGN is the thalamic nucleus that relays visual information from the retina to the visual cortex. RGC project in an orderly manner to points in the LGN, so that in each LGN there is a retinotopic representation of the contralateral half of the visual field (Wagner et al., 2000). In all studied species of carnivores and primates the LGN cells that receive contralateral retinal inputs and those receiving ipsilateral retinal inputs are clustered into cellular layers (Kaas et al., 1972; Hickey and Guillery, 1974). By contrast, in rodents there is not evidence of a layered organization of LGN; contralateral and ipsilateral retinal fibers are segregated in a patchy fashion (Reese and Jeffery, 1983; Godement et al., 1984; Reese, 1988).

The projections of LGN neurons reach the primary visual cortex (V1), in the occipital portion of the brain (Caviness and Frost, 1980; Simmons et al., 1982). The visual cortex consists of six layers of cells (layers I-VI) between the pial surface and the underlying white matter and contains a complete representation of the contralateral visual hemifield. The principal layer of inputs from the LGN is layer IV; then information flows systematically from one cortical layer to another, to higher visual cortical regions, to visual regions of the other hemisphere, to midbrain regions and also back to the LGN (Ruiz-Marcos and Valverde, 1970; Caviness, 1975; Yorke and Caviness, 1975; Frost and Caviness, 1980; Wagor et al., 1980; Cusick and Lund, 1981; Simmons et al., 1982; Olavarria and Montero, 1989). In carnivores and primates V1 is clustered into narrow columns, running from the pial surface to the white matter, devoted to the analysis of orientation (orientation columns) and binocular interactions (ocular dominance columns)

(Hubel and Wiesel, 1963; 1972; LeVay et al., 1975; Hubel et al., 1978; Shatz and Stryker, 1978; Ts'o et al., 1990). These regularly spaced columnar systems communicate with each other by means of long-range horizontal connections that link cells within a layer, allowing the integration of sensory information over the cortex (Livingstone and Hubel, 1984; Kisvarday et al., 1989; Gilbert, 1992).

In rodents, the binocular segment of V1, in which the binocular part of the visual field (nasal 30-40° of the visual field) is represented, occupies the lateral third of area V1. Not surprisingly, the proportion of cells in the binocular region that can be activated preferentially by stimuli presented through the contralateral eye is much larger than the proportion of cells activated preferentially by stimuli presented through the ipsilateral eye (Dräger, 1975; 1978; Métin et al., 1988; Porciatti et al., 1999; Kalatsky and Stryker, 2003). There is no evidence of cell clustering into radial columns based on eye preference. Indeed, monocular cells responding only to the stimuli presented to one eye, or binocular cells dominated by one eye, are intermingled with cells that preferentially (or solely) respond to stimuli presented to the other eye (Dräger, 1975; 1978; Fagiolini et al., 1994; Antonini et al., 1999). Even though rodents do not have anatomically distinct ocular dominance columns, they do, nevertheless, have a critical period during early postnatal life in which the relative representations of the two eyes in the binocular region of the cortex are sensitive to visual experience (Dräger, 1978; Fagiolini et al., 1994; Gordon and Stryker, 1996).

1.2 Critical period for ocular dominance plasticity in the visual cortex

Although the maturation of visual system circuitry starts well before the time of eye opening, and the targeting of thalamocortical connections occurs at very early developmental stages controlled by genetic programmes and spontaneous activity

(Crowley and Katz, 1999; Sur and Leamey, 2001; Crowley and Katz, 2002; Sur and Rubenstein, 2005), a proper development of the visual system requires sensory experience.

There is evidence that the total absence of sensory input leads to a delay in the functional and anatomical maturation of the visual cortex, that appears still immature far beyond the end of the critical period. The visual cortex of adult animals reared in darkness from birth (dark rearing, DR) displays serious physiological deficits including reduced orientation and direction tuning, lower cell responsiveness and increased latency, larger receptive field sizes, altered spontaneous activity, rapid habituation to repeated stimulus presentation, immature ocular dominance distribution and lower visual acuity (Fregnac and Imbert, 1978; Timney et al., 1978; Benevento et al., 1992; Fagiolini et al., 1994; Pizzorusso et al., 1997). A total lack of visual experience also affects the fine structure of visual cortical neurons, measured as alterations in the size, morphology and density of dendritic spines (Valverde, 1971; Wallace and Bear, 2004). Regular developmental processes seem to be restored once the animals are exposed to light, thus allowing the recovery of both neuronal response properties (Buisseret et al., 1978; 1982) and normal anatomical features (Wallace and Bear, 2004).

A classic model used for understanding how experience-dependent activity shapes the maintenance and refinement of brain circuitry is ocular dominance (OD) plasticity, i.e. the rapid changes in visual cortex circuitry resulting from unbalanced inputs from the two eyes.

Hubel and Wiesel first reported that thalamocortical inputs from the two eyes segregate in V1 of cats to form OD columns and that these neural circuits are influenced by manipulations of the animal visual experience. Reducing input from one eye by lid suture (monocular deprivation, MD) during development dramatically affects the binocularity properties of cortical neurons, leading to a loss of cortical physiological responses to that

eye and an increase in the number of neurons responding preferentially to stimuli presented to the open eye. As a direct behavioural consequence, the deprived eye becomes amblyopic: its visual acuity is strongly reduced and its contrast sensitivity blunted (Wiesel and Hubel, 1963a; Hubel and Wiesel, 1970; Olson and Freeman 1975; Movshon and Dürsteler, 1977; Olson and Freeman, 1980). Remarkably, physiological responses in the deprived retina and thalamus remained completely unaffected (Wiesel and Hubel, 1963b; Sherman and Stone, 1973; Kratz et al., 1979; Baro et al., 1990). Hubel and Wiesel observed that in kittens the susceptibility to the effects of MD begins suddenly near the start of the fourth week of life, remains robust until some time between the sixth and eighth weeks, and then declines completely after the third month, thus defining a critical period for MD effectiveness. MD starting in adulthood produces no detectable outcome (Hubel and Wiesel, 1970; Olson and Freeman, 1980).

The effects of MD and the relative existence of a critical period have been subsequently described also in several species of mammals, including primates (Hubel et al., 1977; Blakemore et al., 1978; LeVay et al., 1980; Horton and Hocking, 1997), rabbits (Van Sluyters and Stewart, 1974), hamsters (Emerson et al., 1982) and ferrets (Issa et al., 1999). The effects of deprivation can be reversed to a limited extent during the critical period by reversing the visual deprivation, but they later become irreversible (Wiesel and Hubel, 1965a; Movshon, 1976; van Sluyters, 1978; Blakemore et al., 1981; Antonini et al., 1998).

Anatomical tracing studies revealed that the imbalance of activity between the two eyes results in the actual loss of synaptic inputs from the thalamic regions representing the closed eye, and in the expansion of those representing the open eye, both in cats and primates. While in normal animals monocular cells responding only or binocular cells responding preferentially to visual stimuli presented via a particular eye cluster into

alternating, equal-sized OD columns, closure of one eye reduces the cortical territory of the closed eye to shrunken broken stripes, with its former territory now invaded by inputs representing the open eye (Hubel et al., 1977; Shatz and Stryker, 1978; LeVay et al., 1980). A more detailed morphological analysis has been shown that the thalamo-cortical axonal arbors driven by the deprived eye are less branched, have reduced length and decreased maximal innervation density, and show a reduction in spine density and size, while non-deprived thalamic arbors display an increase in all these parameters (Tieman, 1991; Antonini and Stryker, 1993; 1996). Due to the excellent correlation between the functional changes induced by MD and the reorganization of the geniculocortical afferents, it was believed that the latter was the structural correlate of OD shift. The finding that a functional OD shift occurs first in the extragranular layers and not in layer IV, and that the reorganization of the geniculo-cortical projections follows several days later came as a surprise (Trachtenberg et al., 2000). In search of a better structural correlate, Trachtenberg and Stryker (2001) analyzed the reorganization of horizontal connections in the upper layers of the cortex after inducing strabismus in cats during the critical period. They found that rapid plasticity of binocular responses in the upper layers of the cortex is mirrored by similarly rapid anatomical changes in the long-range horizontal connections between OD columns in these layers. Thus, horizontal connections seem to be a better structural correlate for the functional changes than geniculo-cortical afferents. Several question remain, however, with respect to the involvement of the connections between neurons of layer IV and II/III in OD plasticity and to the mechanism by which thalamocortical remodelling is guided by higher cortical stages (see Bence and Levelt, 2005).

Similar to higher mammals, MD in rodents shifts the physiological responsiveness of neurons in the binocular zone of V1 towards the open eye, and this plasticity is confined to a well-defined critical period (Dräger, 1978; Fagiolini et al., 1994; Gordon and Stryker,

1996). At least in the mouse, this is at first due to a rapid weakening of deprived-eye response, and later to strengthening of open-eye response (Frenkel and Bear, 2004). Interestingly, the OD shift is found in all layers, but it is more pronounced in extragranular layers than in layer IV, with the greatest shift in infragranular cells (Gordon and Stryker, 1996), suggesting that in rodents, as in other species, intracortical as well as geniculo-cortical synapses undergo plasticity with MD. Anatomical changes accompany functional plasticity in the developing visual cortex of the mouse, as they do in higher mammals (Antonini et al., 1999). Moreover, the advent of two-photon microscopy allowed the in vivo study of visual cortex spine dynamics during development and after visual deprivation: Oray et al. (2004) showed that spine motility in the binocular region of V1, contralateral to the deprived eye, is 35% higher than motility in control, nondeprived animals, indicating that sensory deprivation in a plastic cortex is able to initiate a rapid sequence of events that lead to increased structural dynamics at the level of individual spines. Such an increase in spine dynamics may reflect structural destabilization of a population of spines whose function is affected by visual deprivation. This, in turn, could precede a robust pruning of spine protrusions, probably correlated to the rapid reduction in the deprived-eye drive (Mataga et al., 2004).

It is worth to stress that also in humans vision during a definite time period is required for normal development of spatial acuity, global motion detection, and other visual system characteristics. Lasting visual impairments can result from several conditions that degrade or unbalance vision prior to adolescence, including strabismus, uncorrected refractive errors and cataracts (Lewis and Maurer, 2005). In particular, amblyopia is clinically important because, aside from refractive errors, it is the most frequent cause of vision loss in infants and young children, occurring naturally in about 2-4% of the population (Levi, 2006).

1.3 Physiological mechanisms of plasticity expression

Once it has been defined the crucial role of experience and the existence of a critical period during the development of visual system, the next step is to understand what is changing in the nervous system following alterations in visual experience. There are several caveats, however, to consider in establishing that variations in cortical responsiveness are due to particular synaptic modifications, because changes in environmental experience can affect the sensory systems at multiple levels (deprivation may also affect orientation and direction selectivity) and the synaptic modifications underlying a shift in OD may occur at multiple synapses. Furthermore, there may be multiple forms of synaptic plasticity (Hooks and Chen, 2007). Wiesel and Hubel proposed a mechanism in which OD plasticity operates through a competitive interaction between inputs from the two eyes for the control of cortical neurons, depending on the activity state of the postsynaptic neurons. This hypothesis was supported by the fact that binocular lid suture is not effective to shift OD columns in mammals (Wiesel and Hubel, 1965b; Gordon and Stryker, 1996; Antonini and Stryker, 1998). An experiment performed by Stryker's laboratory showed that patterned vision is not necessary for visual cortical plasticity, and that an imbalance in spontaneous retinal activity alone can produce a significant OD shift, thus supporting the competitive view (Chapman et al., 1986). In addition, a reversible blockade of the discharge activities of cortical neurons by intracortical infusion of tetrodotoxin (TTX) or muscimol completely prevents the OD shift that would normally be seen after MD, or causes a paradoxical shift in favour of the deprived eye (Reiter et al., 1986; Reiter and Stryker, 1988; Hata and Stryker, 1994; Hata et al., 1999). However, the mechanism underlying binocular competition has remained elusive. The classic competition-based model is related to heterosynaptic mechanisms, where open eye inputs drive down the synaptic efficacy of the deprived inputs (Miller et al., 1989; Harris et al., 1997). Previous studies have implicated

activity-dependent uptake of neurotrophins, as the mediator of binocular competition (Maffei et al., 1992; Cabelli et al., 1995), but subsequent experiments have shown that neurotrophins actually have cell-specific effects, such as the regulation of inhibitory circuitry, which may provide an alternative explanation of their importance for OD plasticity (Berardi and Maffei, 1999; Huang et al., 1999). Chronic electrophysiological recordings in mice at the peak of the critical period indicate that binocular competition may actually be the consequence of separable processes with distinct time courses mediating depression of deprived-eye and potentiation of non deprived-eye responses (Frenkel and Bear, 2004; Mrsic-Flogel et al., 2007; Kaneko et al., 2008a,b).

The homosynaptic view It is tempting to speculate that the loss or gain of visual responsiveness of neurons in V1 during the critical period is simply the result of homosynaptic long-term depression (LTD) or potentiation (LTP) of excitatory connections somewhere in the visual circuit (Smith et al., 2008). However, the role of LTP and LTD in OD plasticity is hotly debated. The induction of LTP has been extensively demonstrated at multiple synapses of the visual cortex ex vivo, although the mechanism appear to vary across layers (Wang and Daw, 2003). Additionally, in rats, NMDA receptor (NMDAR)-dependent LTP can be induced in layers II/III and IV in vivo following tetanic stimulation of LGN, and this LTP is sufficient to increase the magnitude of visually evoked responses (Heynen and Bear, 2001), suggesting that homosynaptic LTP, possibly at thalamortical synapses, can mimic the effects of open-eye potentiation after MD. Many manipulations known to disrupt homosynaptic LTP have been applied during OD plasticity. One example of this is the finding that OD plasticity is disrupted in mice with either disrupted αCaMKII autophosphorylation or lacking the protein entirely, which suggests a role for LTP (Gordon et al., 1996; Taha et al., 2002). Similarly, open-eye potentiation is absent in mice with a postnatal deletion of NR1 targeted to cortical layers II-IV (Sawtell et al., 2003). Further

suggestion comes from the recently discovered phenomenon of stimulus-selective response potentiation: in juvenile mice, the magnitude of visually driven thalamo-cortical responses in layer IV increases following repeated presentation of an oriented stimulus and this potentiation is dependent on NMDAR activation. Moreover, it has been shown that GluR1 delivery to synapses, that is crucial for LTP, is required for visual experience-dependent plasticity (Frenkel et al., 2006). Stronger evidence exists that LTD-like mechanisms influence depression of deprived-eye responses. The biochemical signature of LTD (in terms of AMPA receptor phopshorylation and cell-surface expression) has been used as a 'molecular fingerprint' to ask whether similar changes occur in visual cortex following a period of MD. To date, this has been examined in the rat visual cortex and the results support the hypothesis that MD induces this type of LTD in visual cortex (Heynen et al., 2003; Yoon et al., 2009). A second approach to address whether LTD is induced by MD is to ask whether naturally occurring synaptic depression in vivo occludes LTD ex vivo. This issue has been recently examined in rodents: LTD measured in slices is reduced (occluded) by 3 days of MD in vivo in both layer IV and II/III (Heynen et al., 2003; Crozier et al., 2007). Furthermore, the reduction in deprived-eye responses after lid suture is likely due to hebbian processes, as monocular inactivation with TTX (which prevents decorrelated inputs) blocks this depression (Frenkel and Bear, 2004). However, the question of the relative contribution of this synaptic modification to the functional consequences of MD is still controversial. An approach to this question has been to correlate deficits in LTD and OD plasticity in genetically or pharmacologically modified mice. A mutation that disrupts LTD dependent on metabotropic glutamate receptor (mGluR) does not alter the normal OD shift in response to MD (Renger et al., 2002). GAD65 knockout mice, which lack normal OD plasticity, show no deficit in induction of LTP or LTD in layer II/III of mouse binocular visual cortex (Hensch et al., 1998a), while similar studies at younger ages show

an impairment of LTD (Choi et al., 2002). A dissociation of LTD and OD plasticity has been suggested also by the study of several protein kinase A (PKA) regulatory subunit mutants. For example, the RIβ knockout mouse has a deficit in layer II/III LTD but exhibits a normal OD shift after 4 days of MD (Hensch et al., 1998b). However, two additional studies deleting both of the two RII subunits of PKA further complicate this story. RIIα knockout mice display normal LTD in layer II/III, whereas both LTP and OD plasticity are reduced (Rao et al., 2004). By contrast, RIIβ knockout mice exhibit normal LTP at the same synapse, but lack both LTD and OD plasticity (Fischer et al., 2004). Also calcineurin, the only known Ca2+/calmodulin-activated protein phosphatase in the brain, has been identified as a molecular constraint involved in OD plasticity, but a transient increase in calcineurin activity, that prevents the shift of responsiveness in the visual cortex following MD, does not impair LTD induction (Yang et al., 2005).

Given that many different plasticity mechanisms exist in the visual cortex (Wang and Daw, 2003; Rao and Daw, 2004), it is likely that a large portion of these seemingly conflicting results may be attributable to laminar differences between the molecular pathways supporting LTD and LTP, but essentially the LTD/LTP mechanisms alone are unlikely to account for the OD plasticity. Indeed, several alternative hypotheses have also been advanced to account for the phenomenology of OD plasticity. For example, balanced levels of excitation and inhibition have shown to be critical for enabling plasticity (Hensch, 2005a; Hensch and Fagiolini, 2005).

Excitatory-inhibitory balance In all species tested so far anatomical and physiological evidences indicate that synaptic inhibition matures later than excitatory transmission in the neocortex (Blue and Parnavelas, 1983; Luhmann and Prince, 1991; Benevento et al., 1992; Guo et al., 1997; Micheva and Beaulieu, 1997; Gao et al., 2000; Mower and Guo, 2001; Murphy et al., 2005). By controlling excitation, GABAergic circuits are ideally posed to

control the engagement of activity-dependent synaptic modification. Thus, the mismatch in the maturation of excitation and inhibition may define a window of opportunity for activity-dependent plasticity to occur. Taking advantage of gene-targeting technology, this hypothesis has been directly tested by reducing GABA synthesis or by prolonging glutamatergic synaptic responses, both adjustements yielding a similar shift of balance in favour of excitation in vivo.

Mice carrying a targeted disruption of the GAD65 gene show and identical OD distribution to wild-type mice; however, the response to a brief period of MD during the critical period is strikingly different. These mice, indeed, show no shift in their responsiveness in favour of the open eye and the cortical neurons continue to respond better to the contralateral eye input. The enhancement of inhibition obtained by local delivering of diazepam produces a complete OD shift in the infused mutant visual cortex (Hensch et al., 1998a). Equally, in NR2A knockout mice, OD distribution is similar to control animals. Unlike GAD65 knockout mice, brief MD is able to induce a slight shift in favour of the open eye but, interestingly, the overall magnitude of this plasticity is significantly weakened. Long-term MD (> 2 weeks) produces no further shift, confirming that saturation is reached within 4 days. Also in this case, diazepam infusion concomitant with MD fully rescues OD plasticity (Fagiolini et al., 2003). A direct physiological consequence of excitatory-inhibitory unbalance in GAD65 and NR2A KO mice is enhanced activation in response to visual stimulation, as assessed by the observation that visual cortical neurons display a tendency for prolonged discharge outlasting the visual stimulus (Hensch et al., 1998a; Fagiolini et al., 2003). Whereas a robust prolonged discharge appears throughout life in both mutants, it is only evident early in the life of wild-type animals before the critical period, when intrinsic inhibition is weak and OD plasticity is absent. With the natural appearance of OD plasticity during the critical period

in wild-type mice, the prolonged discharge drops off sharply (Fagiolini and Hensch, 2000). Taken together, these results indicate that a delicate equilibrium between excitation and inhibition intrinsic to visual cortical circuits is necessary to detect the imbalanced activity between competing inputs from the deprived and non-deprived eyes. Consistent with this view, the onset of the critical period can be accelerated in wild-type animals by premature enhancement of GABA-mediated transmission (Huang et al., 1999; Fagiolini and Hensch, 2000). Moreover, feedforward inhibition can enhance the precise timing of postsynaptic firing (Pouille and Scanziani, 2001). Specific spike timing-dependent windows for synaptic plasticity have been elucidated in developing and neocortical structures (Bi and Poo, 2001). Spike-timing forms of plasticity rely upon physiologically realistic, millisecond-scale changes in the temporal order of pre- and post-synaptic action potentials. Prolonged discharge in both NR2A and GAD65 knockout mice would impair plasticity by altering the pattern of neural activity encoding visual input. The normal development of inhibitory circuitry, as well as diazepam infusion in transgenic mice, improve temporal processing of sensory input, allowing OD shift in response to MD to take place (Hensch and Fagiolini, 2005).

Among the vast diversity of GABAergic interneurons in neocortex, two major sub-classes of parvalbumin-containing cells target the axon initial segment and soma. Both are ideally situated to control either spike initiation (chandelier cells) or back-propagation (basket cells) required for synaptic plasticity in the dendritic arbour (DeFelipe, 1997; Somogyi et al., 1998). Because distinct $GABA_A$ receptor subunits are enriched at these two parvalbumin-cell synapses, their individual contributions to visual cortical processing and plasticity have been identified by point mutations that selectively remove diazepam sensitivity: systematic use of the mouse knock-in technique showed that only one of these subtypes, the α1-subunit-containing interneurons (i.e. basket cells), drives cortical

plasticity (Fagiolini et al., 2004). Two scenarios centred on the parvalbumin-positive basket cells have been proposed (Hensch, 2005a). One is an 'instructive' model, in which powerful, fast somatic inhibition edits one-by-one the action potentials that can pass into the dendritic arbour by back-propagation through the cell body. Sloppy gating by weak inhibition at the soma would prevent a competitive outcome by allowing excess back-propagation and spurious coincident activity with infrequent inputs from the deprived retina. Consistently with this model, large-basket cells extend a wide, horizontal axonal arbour that can span ocular dominance columns in cat visual cortex and, receiving input from one eye, inhibits targets of the other eye (Buzas et al., 2001). A second 'permissive' model is based on the observation that basket cells are organized in electrically-coupled networks, endowed with the ability to detect synchrony (Galarreta and Hestrin, 2001a,b; Meyer et al., 2002). Whereas simultaneous inputs (for example, from the same eye) rapidly co-excite cells through gap junctions, even a 2 ms input jitter (for example, between opposite eyes) is sufficient to dampen the coupling by reciprocal $GABA_A$ synapses, which are also enriched in α1 subunits (Gao and Fritschy, 1994). As a result, these neurons are maximally active on a columnar scale, time-locked to release growth or plasticity factors when strong synchronous activity arrives in the neocortex.

It is worth to point out that during development the inhibitory tone surpasses two functional thresholds in the visual cortex: the first one enables OD plasticity and the second one causes the end of the critical period. A recent study shows that pharmacological reduction of intracortical inhibition obtained through the infusion of either MPA (an inhibitor of GABA synthesis) or picrotoxin (a $GABA_A$ antagonist) directly into the visual cortex reactivates OD plasticity in response to MD in adult rats (Harauzov et al., in press). Moreover, also other manipulations resulting in reductions of cortical inhibition promote adult plasticity (He et al., 2006; Maya-Vetencourt et al., 2008).

Homeostatic synaptic plasticity Homeostatic synaptic plasticity mechanisms are emerging as important complements to Hebbian forms of plasticity in the activity-dependent refinement of synaptic connectivity (Turrigiano and Nelson, 2004; Davis, 2006; Turrigiano, 2008). Homeostatic plasticity acts to stabilize the activity of a neuron or neuronal circuit against perturbations that alter excitability, providing a robust mechanism for generating stability in network function in the face of experience-related changes in synaptic input. Plasticity phenomena that conform to this definition of homeostatic plasticity include the activity-dependent regulation on intrinsic neuronal firing properties (Desai, 2003; Marder and Prinz, 2003; Zhang and Linden, 2003); pre- and post-synaptic forms of excitatory synaptic plasticity, such as synaptic scaling, that adjust all of a neuron's excitatory synapses up or down in the right direction to stabilize firing (Turrigiano and Nelson, 2004; Davis, 2006); the balancing of excitation and inhibition within neuronal networks (Maffei et al., 2004); compensatory changes in synapse number (Kirov et al., 2004; Wierenga et al., 2006); and meta-plastic mechanisms that adjust the relative threshold of LTP and LTD induction (Bienenstock et al., 1982; Abraham and Bear, 1996). The best studied mechanism of homeostatic regulation is synaptic scaling of excitatory synapses, which was first described in dissociated rat cortical cultures, where blockade of activity with TTX increases and blocking GABA-mediated inhibition decreases the amplitude of miniature excitatory postsynaptic currents (mEPSCs) (Turrigiano et al., 1998). Interestingly, the rules for synaptic scaling depend on the synapse type: inhibitory synapses onto pyramidal neurons are scaled in the opposite direction from excitatory synapses, suggesting that the firing rate is regulated through reciprocal changes in excitation and inhibition (Kilman et al., 2002; Swanwick et al., 2006). Homeostatic adjustements in synaptic strength include post-synaptic and pre-synaptic modifications in synaptic function (Turrigiano et al., 1998; Murthy et al., 2001; Thiagarajan et al., 2005;

Wierenga et al., 2005; 2006) and require that neurons sense and translate changes in activity into compensatory changes in synaptic strength, but the nature of the activity signal that controls synaptic scaling is still debated. Neurons could sense changes in their own firing rate through intracellular calcium levels and the modification of one or more intracellular signalling pathways (e.g. the calcium/calmodulin-dependent protein kinase family, the immediate early gene Arc, the polo-like kinase 2 and the cyclin-dependent kinase 5) globally scales synaptic weights up or down (Thiagarajan et al., 2002; 2005; Rial Verde et al., 2006; Shepherd et al., 2006; Ibata et al., 2008; Seeburg et al., 2008). Recently, several molecules important for trans-synaptic signalling and cell adhesion have been implicated in synaptic scaling (Goddard et al., 2007; Cingolani et al., 2008). Finally, synaptic scaling could require widespread changes in network activity, perhaps through activity-dependent release of a soluble factor by many neurons or glia simultaneously, such as BDNF and TNFα (Rutherford et al., 1998; Stellwagen and Malenka, 2006; Kaneko et al., 2008a).

Homeostatic plasticity appears to stabilize circuit function in vivo in a number of organisms and brain areas (Turrigiano, 1999; Davis and Bezprozvanny, 2001; Marder and Goaillard, 2006). Synaptic scaling has been most thoroughly studied in vivo in the visual system, using standard visual deprivation paradigms to mimic in vivo the activity blockade in culture. There is now increasing evidence that synaptic scaling in excitation and inhibition plays important roles during various critical periods of visual system development (Desai et al., 2002; Maffei et al., 2004; Maffei and Turrigiano, 2008a). In particular, it has been suggested that the potentiation of non deprived-eye responses following MD might arise through homeostatic mechanisms that boost the excitability of cortical neurons in response to a drop of sensory input. A recent study using in vivo calcium imaging to monitor eye-specific activation of individual neurons within binocular

layer II/III of visual cortex reported that binocularly driven neurons maintain their overall level of responsiveness to the two eyes, so that the decrease in the responsiveness to the deprived-eye stimulation is compensated by an increase in responsiveness to non deprived-eye stimulation. Interestingly, in monocular visual cortex, the population of neurons driven only by the deprived eye has homeostatic-mediated stronger responses after deprivation, as do all neurons after binocular deprivation (Mrsic-Flogel et al., 2007). In support of the notion that synaptic scaling underlies gain of responsiveness to the non-deprived eye, blocking TNFα signalling in visual cortex either pharmacologically or genetically has no effect on the loss of responsiveness to the deprived eye but prevented the gain of responsiveness to the non-deprived eye (Kaneko et al., 2008a). Complicating the interpretation of these studies is the recent report that the mode of homeostatic plasticity within layer II/III of the visual cortex during the critical period depends strongly on the method of visual deprivation: lowering visual drive through TTX or dark rearing induces synaptic scaling, whereas eyelid suture causes an increase in the intrinsic excitability of monocular cortex pyramidal neurons (Desai et al., 2002; Maffei and Turrigiano, 2008b). This suggests that also the homeostatic response observed after MD is likely due to homeostatic intrinsic plasticity rather than synaptic scaling, but further studies will be necessary to elucidate this point.

In conclusion, these studies highlight the notion that experience-dependent plasticity is unlikely to be explained by a single form of synaptic plasticity, but rather arises through a complex interplay between multiple forms of change in synaptic strength, including modifications in inhibitory circuitry, homosynaptic depression and potentiation, and global changes in circuit gain.

1.4 Molecular substrates of ocular dominance plasticity

A complete understanding of critical period plasticity requires linking the change in circuit function with the molecular mechanisms that make circuit changes possible. The molecular mechanisms that control the developmental plasticity of visual cortical connections are not fully understood. This paragraph reviews evidences establishing some factors as determinant for visual cortex plasticity.

Glutamatergic receptors The properties of NMDA receptors (NMDARs) suggest that these molecules might play a central role in visual cortex plasticity, acting as ‘coincident detectors’ for Hebbian plasticity. The involvement of NMDARs in OD plasticity has been repeatedly proposed by pharmacological experiments (Kleinschmidt et al., 1987; Gu et al., 1989; Bear et al., 1990), but such manipulations have potent suppressive effects upon normal synaptic transmission. Recently, the use of different NMDAR antagonists or antisense oligonucleotides to reduce expression of NR1 subunit of the NMDA receptor has overcome this problem, showing that it is possible to block the effects of MD without affecting visual responses (Roberts et al., 1998; Daw et al., 1999a). The direct dependence of OD plasticity on NR1 subunits has been further demonstrated using conditional NR1-knockout mice (Sawtell et al., 2003). An interesting property of NMDARs is that their subunit expression, determining the calcium influx, is developmentally and activity regulated. In particular, subunit composition varies in the visual cortex, from low to high NR2A/NR2B ratio, with a time course paralleling that of functional visual cortical development and the critical period (Roberts and Ramoa, 1999). It has been shown that in dark-reared animals the NR2A/NR2B ratio is lower than in light-reared animals (Quinlan et al., 1999 a,b; Tongiorgi et al., 2003). However, recent results have demonstrated that NR2B over-expressing animals don’t show an increased susceptibility to plasticity (Philpot

et al., 2001) and in mice with the deletion of NR2A subunit the sensitivity to MD is weakened, even if restricted to the normal critical period (Fagiolini et al., 2003). Interestingly, a very recent study highlights a co-regulation of OD plasticity and NMDAR subunit expression in GAD65 knockout mice. In the visual cortex of these animals there are reduced NR2A levels and slower NMDA currents. In addition, application of benzodiazepines, which rescues OD plasticity, also increases NR2A levels, suggesting that changes in inhibition would engage mechanisms that converge to regulate NMDA receptors, thereby enabling plasticity (Kanold et al., 2009).

Further results establish a role for AMPA receptors (AMPARs) in the deprived-eye response depression following MD, reporting that a brief MD during the critical period alters AMPAR phosphorylation and reduces the expression of AMPARs on the surface of visual cortical neurons (Heynen et al., 2003). Finally, there is also direct evidence that metabotropic glutamate receptors (mGluRs) are involved in visual cortex plasticity, with distinct roles depending on the receptor subtype and cortical layer (Daw et al., 1999b; Wang and Daw, 2003; Rao and Daw, 2004). Recently, using the molecular genetic approach, it has been shown an important role for mGluRs in the regulation of OD plasticity during development, since a 50% reduction in mGluR5 expression prevents OD plasticity induced by 3 days of MD (Dolen et al., 2007; Dolen and Bear, 2008).

Neurotrophins There is a conspicuous number of observations suggesting that neurotrophins control visual cortical plasticity during the critical period. Early studies in the rat demonstrated that intraventricular as well as intracortical infusion of NGF prevents OD shift following MD (Domenici et al., 1991; 1992; Lodovichi et al., 2000). Moreover, infusion of antibodies that specifically activate the NGF receptor trkA equally blocks OD plasticity (Pizzorusso et al., 1999). With the exception of NT-3, exogenous supply of all neurotrophins affects the outcome of MD. However, the effects of neurotrophins on OD

plasticity are sometimes accompanied by alteration of other properties of visual cortical neurons, such as their pattern of discharge and orientation selectivity (Gillespie et al., 2000; Lodovichi et al., 2000). Other studies, which followed the opposite course of antagonizing the action of endogenous neurotrophins, have also shown that neurotrophins are important for normal visual cortical development and plasticity (Berardi et al., 1994; Domenici et al., 1994a; Cabelli et al., 1997). In addition, neurotrophin production and release is developmentally regulated and depend on electrical activity, in particular on visual activity (Castren et al., 1992; Bozzi et al., 1995; McAllister et al., 1999). In turn, neurotrophins can modulate electrical activity and synaptic transmission at both presynaptic and postsynaptic levels (Carmignoto et al., 1997; Berardi and Maffei, 1999; Poo, 2001). They can have both fast actions, for instance by increasing transmitter release (Sala et al., 1998; Jovanovic et al., 2000) or by directly depolarizing neurons (Kafitz et al., 1999), and slow actions, by modulating gene expression (Poo, 2001). This reciprocal regulation between neurotrophins and neural activity might provide a means by which active neuronal connections are selectively strengthened. Indeed, neurotrophins seem to require the presence of electrical activity to exert their actions (Sala et al., 1998; Caleo et al., 1999; McAllister et al., 1999). The classic hypothesis (‘neurotrophic hypothesis’) states that competition for limited amounts of neutrotrophins is the effector of activity-dependent plasticity in the cortex, and the conventional explanation for OD plasticity is that the deprived eye does not activate cortical cells as well as the open eye, thereby failing to stimulate them to release sufficient neurotrophins to sustain the deprived-eye pathway (Thoenen, 1995; Bonhoeffer, 1996; McAllister et al., 1999). The possibility of an anterograde action of neurotrophins as opposed to target-derived action has also emerged from literature (Caleo et al., 2000; Kohara et al., 2001; von Bartheld, 2004). This significantly changes the frame of thought: in addition to thinking that cortex-derived

factors guide stabilization of thalamic afferents on cortical neurons, we may have to consider that thalamic fibers themselves release factors which promote and guide the formation and maintenance of their synapses onto cortical neurons and that corticothalamic afferents may contribute to the development of the pattern of thalamocortical connectivity.

However, some recent experiments show that a possible mechanism of action of neurotrophins on OD plasticity is an orchestrated modulation of synaptic efficacy, rather than a direct effect on thalamocortical afferents alone. In visual cortex synaptosomes, both NGF and BDNF potentiate glutamate and acetylcholine release, while only BDNF does so for GABA release. Like BDNF, NT4 potentiates GABA and glutamate release but is much less effective in enhancing acetylcholine release (Sala et al., 1998). Putting this information together with data on the expression of trk receptors in the visual cortex and with data on retrograde transport of cortically injected NGF (Domenici et al., 1994b), it can be concluded that NGF is likely to act directly on cholinergic afferents from the basal forebrain and on a population of glutamatergic cortical neurons; BDNF targets are principally cortical glutamatergic pyramidal cells and inhibitory interneurons, whereas NT4 acts on glutamatergic thalamic afferents and probably pyramidal neurons and inhibitory interneurons (Berardi and Maffei, 1999). In line with this, it has been shown that infusion of exogenous NGF in the cat has little or no effect on MD outcome and this seems related to a different cholinergic arborisation in the visual cortex of the two species. Similarly, BDNF infused in the cat visual cortex paradoxically results in the expansion of connections subserving the deprived eye, as previously observed with the intracortical infusion of the GABA receptor agonist muscimol (Reiter et al., 1986; Carmignoto et al., 1993; Fiorentini et al., 1995; Galuske et al., 2000; Gillespie et al., 2000; Silver et al., 2001). The relationship between neurotrophins and the development of inhibitory processes has been investigated, using an elegant transgenic mouse with postnatal

overexpression of BDNF in the forebrain. In these animals, BDNF overexpression accelerates the maturation of intracortical GABA-mediated inhibition and this is paralleled by a precocious development of visual acuity with respect to wild type and an accelerated time course of the critical period, resulting in an early shift of the critical period of about one week (Huang et al., 1999).

It should be noted that recent studies of a mutant mouse heterozygous for the null allele of BNDF demonstrate that a 50% reduction in the BDNF levels has no effect upon OD plasticity (Bartoletti et al., 2002). Similarly, Stryker and colleagues, using a conditional transgenic mouse, show that TrkB inactivation does not affect the induction of OD plasticity following MD (Kaneko et al., 2008b). However, since the redundancy of neurotrophin action on the modulation of synaptic transmission, these data do not exclude that neurotrophic factors play a fundamental part in the plasticity of visual cortex: the compensating action of other neurotrophins could account for the absence of alterations in visual cortex plasticity in these mutant mice.

Neuromodulatory systems Several studies have aimed to uncover the contribution of neuromodulators to cortical plasticity (Gu, 2002). As with many other molecules involved in cortical plasticity, the distribution of different receptors and fibres is developmentally regulated and dependent on cortical input (Foote and Morrison, 1984; Stichel and Singer, 1987; Prusky et al., 1988; Mower, 1991; Nakazawa et al., 1992; Vu and Törk, 1992; Mechawar and Descarries, 2001; Latsari et al., 2002). The involvement of these transmitters in visual cortex plasticity was first investigated by Kasamatsu and Pettigrew who showed that depletion of noradrenaline in kitten visual cortex disrupts OD plasticity (Kasamatsu and Pettigrew, 1976; 1979; Kasamatsu et al., 1979; 1981) and infusion of noradrenaline in kitten visual cortex enhances plasticity (Kasamatsu et al., 1979; 1981; Kuppermann and Kasamatsu, 1984; Imamura and Kasamatsu, 1988; 1991). Further

experiments demonstrated that intracortical infusion of noradrenaline combined with MD reduces the proportion of binocular neurons in adult cat visual cortex, restoring neuronal plasticity to the normally aplastic visual cortex of adult animals (Kasamatsu et al., 1979). In addition, OD changes are inducible in adult cat visual cortex by electrical stimulation of the locus coeruleus (Kasamatsu et al., 1985) or peripheral administration of an exogenous precursor of noradrenaline (Mataga et al., 1992). Experiments using osmotic minipumps to infuse β-adrenergic antagonists in kitten visual cortex indicated that activation of β_1-receptors seems to be mostly involved in regulation of OD plasticity (Kasamatsu and Shirokawa, 1985). Noradrenaline may support cortical plasticity through a NMDA receptor-gated mechanism. In vitro slice experiments have provided evidence that noradrenaline facilitates synaptic plasticity by enhancing NMDA receptor-mediated response component (Bröcher et al., 1992; Kirkwood et al., 1999). Noradrenaline could increase the probability of activation of NMDA receptors by its action on membrane K^+ conductance (Foehring et al., 1989) and second messengers, such as cAMP (Imamura et al., 1999).

Also the involvement of acetylcholine in OD plasticity has been examined in kittens through the lesion of basal forebrain. This alone results not sufficient to prevent OD shift in the visual cortex following MD, but combining this kind of lesion with depletion of cortical noradrenergic innervation reduces the physiological response to MD (Bear and Singer, 1986). Further experiments using pharmacological compounds to chronically block cholinergic receptors in visual cortex showed that blockade of muscarinic but not nicotinic receptors disrupts OD shifts in visual cortex of monocularly deprived kittens. In particular the chronic blockade of muscarinic M_1 but not M_2 receptor subtypes prevents OD shift (Gu and Singer, 1989; 1993). Similarly to that observed for noradrenaline, acetylcholine effects

could be attributed to a facilitation of NMDA receptor activation (McCormick and Prince, 1985; Markram and Segal, 1992).

The role of serotonin in OD plasticity has been investigated chronically infusing a specific neurotoxin into the visual cortex of kittens undergoing MD: the results showed that serotonin depletion prevents the susceptibility to experience-dependent modifications. Equally, the combined infusion of two broad serotoninergic receptor antagonists reduces OD plasticity (Gu and Singer, 1995). In addition, it has been demonstrated that the serotonin 5-HT_{2c} receptor subtype plays a key role in activity-dependent synaptic modifications in visual cortex (Wang et al., 1997). To explain the facilitatory action of serotonin in OD plasticity it has been proposed also in this case a mechanism associated with NMDA receptors (Nedergaard et al., 1987; Reynolds et al., 1988; Panicker et al., 1991; Hoyer and Martin, 1997). It is worth to point out, however, that administration of the selective serotonin reuptake inhibitor fluoxetine has been recently shown to restore OD plasticity in adult animals. The effects induced by fluoxetine are associated with a marked reduction of GABAergic inhibition, thus suggesting that serotonin could affect visual cortical plasticity also modulating intracortical inhibition (Maya Vetencourt et al., 2008).

Intracellular signalling of cortical plasticity Experiments using transgenic mice and/or pharmacological manipulations have identified three signalling kinases that can modulate synaptic strength and are critical for inducing OD plasticity: extracellular signal-regulated kinase 1,2 (ERK-1,2), cAMP-dependent protein kinase (PKA), and calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα; Beaver et al., 2001; Di Cristo et al., 2001; Taha et al., 2002; Taha and Stryker, 2005). These kinases may rapidly promote OD plasticity by directly phosphorylating plasticity-regulating molecules at the synapse (such as glutamate or GABA receptors) or cytoplasmatic substrates crucial for synaptic transmission, neuronal excitability and morphological stabilization (e.g. synapsin I,

potassium channels, MAP2), or they may signal to the nucleus to mediate changes in gene transcription (Berardi et al., 2003). The intracellular mechanisms mediated by kinase signalling can lead to the activation of cAMP-responsive element-binding protein (CREB), which in turn controls CRE-mediated gene expression of proteins essential for establishment and maintenance of plastic changes (Cancedda et al., 2003; Suzuki et al., 2004). Indeed, it has been recently demonstrated that CRE-mediated transcription is upregulated by MD during the critical period in the visual cortex contralateral to the deprived eye and that CREB is necessary for OD plasticity (Pham et al., 1999; Liao et al., 2002; Mower et al., 2002). As with many other molecules that mediate changes in plasticity, CREB levels also decreases with age (Pham et al., 1999).

Recently, the regulation of chromatin structure as emerged as one of mechanisms regulating visual cortex plasticity, since it has been demonstrated the involvement of histone phosphoacetylation in OD plasticity. In juvenile mice, visual stimulation that activates CREB-mediated gene transcription also induces ERK-dependent MSK and histone H3 phosphorylation and H3-H4 acetylation, an epigenetic mechanism of gene transcription activation. In adult animals, ERK and MSK are still inducible; however, visual stimulation induces weak CREB-mediated gene expression and H3-H4 posttranslational modifications. Finally, stimulation of histone acetylation in adult animals by means of trichostatin is able to promote OD plasticity (Putignano et al., 2007). The gene expression modifications deriving from the induction of histone acetylation could explain the way by which long-term changes of brain circuitry take place.

Additional classes of molecules are also likely to be important for calcium-dependent cellular processes that may mediate brain plasticity. For example, a link between calcium signalling and cytoskeletal dynamics comes from a recent microarray screen, which has found that the calcium sensor cardiac troponin C (part of a complex that

mediates calcium-dependent actin-myosin interaction) is elevated in visual cortex during the critical period, and is regulated by visual activity (Lyckman et al., 2008). Additionally, calcineurin, a calcium/calmodulin-activated phosphatase, has proven to be an effective negative regulator of OD plasticity: indeed, calcineurin overexpression reversibly prevents an OD shift during the critical period in mouse (Yang et al., 2005), suggesting that the balance between protein kinases and phosphatases is critical for visual cortex plasticity.

Extracellular environment Downstream effectors that implement the program initiated by the signalling mechanisms described in the preceding section are largely unknown. However, it is becoming clear that the extracellular environment, and in particular the extracellular matrix (ECM), plays an important part in controlling spine dynamics and visual cortical plasticity. Recent studies have shown a key role in OD plasticity for the major components of brain ECM, the chondroitin-sulfate proteoglycans (CSPGs). During development CSPGs condense at high concentration in lattice-like structures, called perineuronal nets (PNNs), which completely ensheath visual cortical neurons, in particular parvalbumin-positive neurons. The time course of PNN condensation in the visual cortex tightly matches the visual cortex critical period for the effects of MD (Hartig et al., 1992; Köppe et al., 1997; Brükner et al., 2000; Pizzorusso et al., 2002). In addition, the development of CSPGs is regulated by visual activity, since the process of PNN condensation is prolonged by dark rearing (Hockfield et al., 1990). The enzymatic degradation of CSPGs from the adult visual cortex reactivates OD plasticity in monocularly deprived adult animals, suggesting that adult ECM exerts a powerful inhibitory control on OD plasticity (Pizzorusso et al., 2002). The outcome of the study of ECM influence on OD plasticity led to analyse the role of endogenous extracellular proteases in the visual cortical plasticity during the critical period. It has been shown that pharmacological inhibition of tPA hampers visual cortical plasticity (Mataga et al., 1996;

Muller and Griesinger, 1998), and MD is ineffective in mice with deletion of the tPA gene both at the functional and structural level. Plasticity can be rescued in tPA knockout mice by the exogenous administration of tPA during the period of MD. Moreover, the link between tPA and experience-dependent plasticity is strengthened by the observation that in wild type animals MD elicits a fast and transient increase of tPA activity during the critical period but not in the adult (Mataga et al., 2002; 2004). The released tPA increases extracellular proteolysis directly or by the activation of plasmin. These proteases have a wide spectrum of targets and the available information is not sufficient to dissect which of these targets must be cleaved for plasticity to proceed. However, converging data point to an important role for tPA in 'freeing up' the extracellular matrix to promote the structural reorganization of connections during deprivation (Mataga et al., 2004; Oray et al., 2004).

Another candidate for plasticity regulation has been revealed by a recent study highlighting the critical role of myelin, particularly via its interaction with the Nogo receptor (NgR). The authors first characterized the density and laminar distribution of NgR and its ligands in mouse visual cortex: while total levels of myelin as well as of NgR increase only slightly during the critical period, layer IV shows the greatest increase in myelin. The main result is that the absence of either Nogo or NgR prevents the closure of the critical period and preserves plasticity: indeed, these transgenic mice exhibit an undiminished OD plasticity, even when MD is imposed in four month-old animals. Interestingly, the Nogo/NgR-dependent regulation of visual cortical plasticity does not seem to involve a change in GABAergic inhibition or tPA activity, as parvalbumin and tPA immunoreactivity are normal in NgR knockout mice. Therefore, Nogo/NgR must act either independently or further downstream in the signalling cascade, presumably converging to regulate cortical anatomical rearrangements (McGee et al., 2005).

In addition to the results described up to here, in the past few years several studies have investigated the molecular mechanisms of visual cortex plasticity using genetic screens, and have opened the door for examination of new families of molecules in plasticity (e.g proteins related to IGF-I pathway or immune/inflammation system signals). Expression of most of these molecules is developmentally regulated and differentially altered by sensory experience (Ossipow et al., 2004; Majdan and Shatz, 2006; Tropea et al., 2006; Lyckman et al., 2008). These studies further highlight that OD plasticity invokes a complex, interrelated set of mechanisms, involving a large number of molecules of different classes. An important goal for the field of cortical plasticity is to understand how the many molecular mechanisms guiding plasticity are recruited, how they interact and converge to permit and instruct plasticity, and over which time scale they act.

1.5 Experience-dependent plasticity in adult visual cortex

It is widely accepted that experience-dependent plasticity is a prominent feature of the developing visual cortex. However, adult cortical circuits can be still modified by a variety of manipulations, such as perceptual learning and visual deprivation (Karmarkar and Dan, 2006).

Perceptual learning refers to a robust gain in performance on basic perceptual tasks that are induced by sensory experience and are dependent on practice. Studies in both humans and animals have shown that in adults with normal vision practice can improve performance in various aspects of visual perception, including stimulus spatial frequency, orientation, luminance contrast, motion-speed and motion-direction discrimination (Fine and Jacobs, 2002; Fahle, 2005). The characteristics of such learning processes suggests that they involve early stages along sensory pathways, in some instances primary sensory cortices. Despite this recent progress in localizing the visual areas involved in perceptual

learning, elucidation of the underlying mechanisms at the cellular level remains a challenge (Karmarkar and Dan, 2006). Interestingly, a number of studies over the last years suggest that perceptual learning may provide an important new method for treating ambyopia (Levi, 2005; Levi and Li, 2009).

While the cortical modifications mediating perceptual learning appear to be induced by increased exposure to certain visual stimuli, significant changes can also be caused by deprivation of inputs in part or all of the visual field. Although it is induced by abnormal visual experience, the capacity of the adult cortex for such reorganization is functionally advantageous, since it allows the neuronal machinery rendered inactive by peripheral injury to be reused for processing other inputs. This could in turn facilitate functional recovery of perception. One form of visual deprivation is caused by lesioning a portion of the retina and thus causing a scotoma in the visual field. Retinal lesions initially silence the visual cortical region retinotopically mapped to the scotoma, but most cells recorded in the cortical projection zone of the retinal lesion exhibit 'ectopic' excitatory visual receptive fields which are displaced in the immediate vicinity of the lesion already after a few hours from the placement of lesions. The presence of ectopic receptive fields, combined with the presence of normal cortical representation of the retinal region surrounding the lesion, indicate a clear expansion of the cortical representation of the part of the retina near the lesion (Kaas et al., 1990; Heinen and Skavenski, 1991; Gilbert and Wiesel, 1992). To determine the loci along the visual pathway at which the reorganization takes place, the course of topographic alterations in the primary visual cortex and dorsal lateral geniculate nucleus (LGN) have been compared. At a time when the cortical reorganization is complete, the silent area of LGN persists, indicating that changes in cortical topography are due to alterations of long-range intrinsic horizontal connections (Darian-Smith and Gilbert, 1994; 1995; Calford et al., 2003).

Another form of deprivation-related plasticity is OD plasticity. Even if this issue is extremely controversial, recent studies showed that in adult mice a long period of MD can cause a shift in cortical OD. The 2-3 days of MD effective in juveniles must be extended to at least 5 days in order to induce OD shift in adult mouse V1 (Sawtell et al., 2003; Tagawa et al., 2005). However, adult plasticity involves different functional changes in cortical circuits: while developmental OD plasticity is due to a rapid reduction of responses to the deprived inputs followed by a later enhancement of non-deprived inputs (Frenkel and Bear, 2004), adult OD shifts in mice are primarily accounted for by increased responses to the non-deprived eye (Sawtell et al., 2003; Tagawa et al., 2005). Recent studies showed that prior experience can facilitate adult OD plasticity: indeed, inducing a saturating OD shift by brief eye closure in juvenile or adult mouse visual cortex enables faster and more persistent OD changes in response to a second MD several weeks later (Hofer et al., 2006). Similarly, housing adult animals in the dark for a brief period allows strong plasticity in the visual cortex, probably restoring a state similar to that in juveniles (He et al., 2006; 2007).

The advancement of our understanding of the mechanisms responsible for plasticity will offer the potential for novel strategies aimed to promote the reorganization of neuronal circuits in the mature nervous system.

CHAPTER 2

THE INFLUENCE OF ENVIRONMENT ON BRAIN AND BEHAVIOUR: NEURAL CONSEQUENCES OF ENVIRONMENTAL ENRICHMENT

Within the hoary 'nature versus nurture' debate a relevant progress in understanding the influence of environmental experience on the development, refinement and maintenance of appropriate nervous system connections was obtained by introducing environmental enrichment (EE) as experimental protocol. EE is an alteration of the standard laboratory conditions that modifies the quality and intensity of environmental stimulation, reaching an optimization of the rearing environment. A comprehensive definition of EE was provided for the first time by Rosenzweig et al. (1978) as 'a combination of complex inanimate and social stimulation'. Enriched animals are reared in large groups (6-10 individuals can be considered the most common used condition) and housed in wide stimulating environments where a variety of objects differently shaped (e.g. running wheels, platforms, boxes, toys, tunnels, shelters, stairs and nesting material) are present and changed frequently (specifically, the objects are completely replaced at least once a week). The goal of EE is to improve the animals' quality of life by providing them with high levels of multi-sensory stimulation, increasing physical activity and social interactions, stimulating natural behaviours and cognitive abilities (since the novelty due to frequent objects' replacement attracts the explorative curiosity of most laboratory animals). The significance of EE is based on the comparison of the enriched conditions with the standard environment condition (SC), that consists in housing 2-5 individuals in laboratory standard cages where no particular objects are present except for food, water and litter, and with the

impoverished environment condition (IC) that consists in housing the animals singly in cages identical to those used for SC or even smaller.

Although EE research has been mostly done upon rodents, similar effects occur in several species of mammals (gerbils, ground squirrels, rabbits, cats and primates), but also in some avian species (Rosenzweig and Bennett, 1969; Cornwell and Overman, 1981; Hansen and Berthelsen, 2000; Kozorovitskiy et al., 2005; Lazic et al., 2007; Jansen et al., 2009).

Since EE is a rather mild and non-invasive treatment, the results obtained are of great interest and applicability also for humans in many different fields, from psychology to medical clinic. EE is likely to affect the human brain, but direct research has been predominantly limited to the study of behavioural effects. All results are consistent with an increasing body of knowledge that implicates an enriched, stimulating environment in beneficial psychological and behavioral outcomes. The important work of Ramey and Ramey has shown that, starting at an early age, the use of a comprehensive enriched environment can increase IQ by a mean of 15 points in children from disadvantaged homes (Ramey and Ramey, 1998). Moreover, it has been demonstrated that subjects who participated in an enrichment program (nutritional, educational, and physical enrichment) at the age of 3-5 years have lower scores for schizotypal personality and antisocial behavior at the age of 17 years and for criminal behaviour at the age of 23 years, compared with control subjects (Raine et al., 2003).

2.1 Environmental enrichment affects animals' behaviour

In the 40s Hebb was the first to introduce the idea of the 'enriched environment' as experimental concept. He described that rats that he took home as family pets, and that he reared in an extremely more complex environment than a laboratory rodent's setting,

showed behavioural improvements in learning task. Subsequently, the finding that a more complex and stimulating rearing environment enhances performance on learning tasks was repeated by Hebb's students (Bingham and Griffiths, 1952; Forgays and Read, 1962). These pioneering observations have been followed by a large body of studies which highlighted that environmental enrichment is able to modify animals' behaviour leading to a sensitive improvement of complex cognitive functions, particularly learning and memory (for an exhaustive review, see Rampon and Tsien, 2000). Independently on the gender and age of tested animals, EE effects are especially evident in hippocampal-dependent tasks involving spatial memory, such as the Hebb-Williams maze (Kobayashi et al., 2002), the Lashley III maze (Greenough et al., 1972), the radial maze (Leggio et al., 2005) the Morris water maze (Tees et al., 1990; Falkenberg et al., 1992; Paylor et al., 1992; Moser et al., 1997; Kempermann et al., 1998a; Nilsson et a., 1999; Williams et al., 2001; Lee et al., 2003; Leggio et al., 2005; Fréchette et al., 2009) and the discrimination between different spatial contexts (Mitra and Sapolsky, 2008). EE animals have better performances also in nonspatial tasks, exhibiting enhanced learning and memory retention in the object recognition test, contextual and cued-fear conditioning (Rampon and Tsien, 2000; Duffy et al.,. 2001; Lee et al., 2003). However, Rampon and Tsien noticed that enrichment has no effect on the social transmission of food preference test, a task that is also dependent on the hippocampus and measures olfactory discrimination memory.

The consequences of EE upon emotional reactivity are less documented and remained controversial for a long time. While some authors reported no or inconsistent effects (Huck and Price, 1975; Fernandez-Teruel et al., 1997), others have observed modifications of emotional and stress reactions in EE animals (Chamove, 1989; Escorihuela et al., 1994). A more careful examination of the animals' behaviours in prenatally or postnatally stressed rats (Francis et al., 2002), aged mice (Thouvarecq et al.,

2001) and mice considered as pathologically anxious (Chapillon et al., 1999; Roy et al., 2001; Iwata et al., 2007) led to assume that animals reared in EE display a lower level of emotional reactivity than those reared in standard conditions (for a review, Chapillon et al., 2002). EE also modifies the behaviour of strains of mice not known to be pathologically anxious, such as C57BL/6 and B6CBA, and of Wistar and Long-Evans rats: indeed, it has been reported that environmental complexity decreases stress reactivity of these animals, as indexed by performance on an elevated plus maze (Fernandez-Teruel et al., 1997; Caston et al., 1999; Chapillon et al., 1999; Friske and Gammie, 2005; Zhu et al., 2006; Galani et al., 2007; Hoffman et al., 2009), defensive response to predators or intruders (Haemisch et al., 1994; Klein et al., 1994) and open field exploration (Widman and Rosellini, 1990; Chapillon et al., 1999; Nikolaev et al., 2002). The impact of environmental complexity on emotional behaviour is also evident in the differential expression of c-Fos in various amygdala subnuclei following exposure to aversive stimuli (Nikolaev et al., 2002). Recently, it has been demonstrated that EE is also protective for depressive disorders, leading to behavioural antidepressive-like effects in forced-swimming test both in normal rats and in animal models of depression (Brenes Sàenz et al., 2006; Llorens-Martin et al., 2007; Brenes et al., 2008).

Finally, it has been shown that EE conditions can improve sensory information processing, leading to a difference in behavioural measures of visual acuity (Prusky et al., 2000a) and auditory spatial discrimination (Cai et al., 2009).

2.2 Environmental enrichment affects brain anatomy

The behavioural improvements observed in EE animals are paralleled by deeply changes at the anatomical level in the brain. In the initial studies, it has been observed that 30 days of exposition to an enriched living condition result in different brain weights between

littermates housed in EE and SC or IC conditions, not imputable to differences in body weight (Rosenzweig et al., 1962 a,b; Bennett et al., 1969). These changes have been noticed in the entire dorsal cortex, but the largest difference was found with respect to the occipital cortex (9.4%).

This result prompted several groups to investigate in detail the anatomical outcome of living in EE, taking the visual cortex as preferred model. It has been widely reported that the cerebral cortex in EE animals develops significantly thicker compared to littermates housed in impoverished and standard environments (Bennett et al., 1964a,b, 1970; Diamond et al., 1964, 1966, 1972; Walsh et al., 1971). Subsequent studies have pointed out that exposure to EE leads in the different cortical layers to an increment in size of neurons' cell soma and nucleus (Altman and Das, 1964), dendritic branching and lenght (Holloway, 1966; Volkmar and Greenough, 1972; Globus et al., 1973; Greenough and Volkmar, 1973; Uylings et al., 1978; Green et al., 1983), number of dendritic spines (Globus et al., 1973), synaptic size and density (Mollgaard et al., 1971; West and Greenough, 1972; Diamond et al., 1975; Greenough et al., 1978; Bhide and Bedi, 1984; Turner and Greenough, 1985; Beaulieu and Colonnier, 1987), postsynaptic thickening (Diamond et al., 1964), gliogenesis (Diamond et al., 1966) and angiogenesis (Ekstrand et al., 2008). Further experiments revealed that significant cerebral effects of enriched versus impoverished (or standard) experience could be induced at any part of the life span and with relatively short periods of exposure (Bennett et al., 1964b; Riege, 1971; Ferchmin and Eterovic, 1986).

The anatomical changes are not limited to cortical regions: indeed, similar effects to that reported for cerebral cortex, have been found for pyramidal cells of CA1 and CA3 and for dentate granule neurons of hippocampus (Walsh et al., 1969; Diamond et al., 1976; Fiala et al., 1978; Altschuler, 1979; Walsh and Cummins, 1979; Rosenzweig and Bennet,

1996; Rampon et al., 2000a; Ekstrand et al., 2008), for the striatum (Comery et al., 1995), the amygdala (Ichikawa et al., 1993) and the cerebellum (Floeter and Greenough, 1979; Kleim et al., 1997).

Recent studies have shown that exposure to EE increases hippocampal neurogenesis and integration of the newly born cells into functional circuits. A short (3h) daily exposure to a complex environment for 14 days in adults is sufficient to induce a long-term increase in the rate of neurogenesis (Kempermann et al., 1997; Nillson et al., 1999; van Praag et al., 2000; Bruel-Jungerman et al., 2005). The definitive proof of increased neurogenesis in the hippocampus of EE animals has been provided by Kempermann et al. (1997; 1998) in mice and by Nillson et al. (1999) in rats using the proliferation marker bromodeoxyuridine (BrdU). EE induces an ≈70% increase in the number of newborn dentate gyrus cells, but this is not associated with any detectable changes in differentiation of the progenitor cells towards a neuronal or glial fate. Indeed, most (80-85%) of these cells expresses a neuronal phenotype, a proportion similar to that observed in naïve rats. This suggests that EE effects are expressed independently of the cell lineage, resulting in a net increase in both neuronal and glial cells in the dentate gyrus (Nilsson et al., 1999). EE does not seem to affect proliferation of progenitor cells, rather it appears to increase the number of surviving newly formed granule cells in the dentate gyrus (Kempermann et al., 1997; Nilsson et al., 1999). Increased cell survival reflects differences in apoptotic rates: indeed, apoptotic assessment using the TUNEL method revealed a decreased proportion of neurons undergoing cell death (Young et al., 1999). The functional significance and biological role of neurogenesis are not well understood. It is tempting to speculate that newborn granule cells in the dentate gyrus might contribute to the improved performance in the spatial learning tests observed in EE animals (Gould et al., 1999; Shors et al., 2001). To address this issue, it has been examined whether the

increase in the number of surviving adult-generated cells following EE contributed to improved memory function, reducing neurogenesis throughout the EE period by means of antimitotic agents. The antimitotic treatment during EE completely prevents both the increase in neurogenesis and EE-induced long-term memory improvement, thus establishing that newborn cells in the dentate gyrus contribute to the expression of the promnesic effects of EE (Bruel-Jungerman et al., 2005). However, this result is controversial (Meshi et al., 2006) and EE results in many different types of structural and functional changes that could supply to memory facilitation, including increased dendritic branching and spine and synapse number in the cortex and the hippocampus (reviewed in van Praag et al., 2000; Mohammed et al., 2002).

2.3 Electrophysiological responses to environmental enrichment

Few studies have addressed the possible relationship between enriched living and electrophysiologically measurable modifications of synaptic transmission. In hippocampal slices taken from enriched housed rats excitatory postsynaptic potential (EPSP) slopes and amplitudes are greater with respect to age-matched control (Green and Greenough, 1986; Foster et al., 1996; Foster and Dumas, 2001; Irvine and Abraham, 2005). Similarly, it has been demonstrated that EE selectively increases glutamatergic responses in the cerebral cortex of the rat: excitatory postsynaptic currents (EPSCs) display a large amplitude increase, accompanied by a rise-time decrease and reduced pair pulse ratio in layer II/III of the auditory cortex (Nichols et al., 2007). Exploration of enriched environments elicits pattern of electrical activity in hippocampal neurons of area CA1 that are similar to patterns of electrical stimulation used to induce LTP in hippocampal slices. In two recent studies, long-term potentiation (LTP) and long-term depression (LTD), two different

paradigmatic models of synaptic plasticity, have been compared in hippocampal slices from enriched and control rats. Their data indicate that significant enhancement of LTP and LTD occurr in hippocampal area CA1 following 5-8 weeks of EE (Duffy et al., 2001; Artola et al., 2006). Moreover, it is interesting to notice that enhancements in LTP and LTD, seen after a 5-week exposure to EE, are not reversed after 3-5 week exposure to standard housing (Artola et al., 2006). Two phenomena may contribute to this enhancement. One is a facilitation of the induction of synaptic plasticity. The finding that paired-pulse facilitation is decreased in enriched rats compared with control animals suggests that exposure to EE enhances transmitter release and, thus, decreases the demand for presynaptic activation to reach the postsynaptic thresholds for inducing LTP and LTD. Consistently, LTP induction requires a smaller number of high-frequency stimuli in enriched animals and it is very likely that enhanced LTD is also due, at least in part, to a facilitation of its induction. EE may also actually increase the dynamic range of synaptic modification: indeed, repeated LTP and LTD induction produces larger synaptic changes in enriched than in control rats. These data reveal that exposure to different environmental experiences can produce long-lasting effects on the susceptibility to synaptic plasticity, involving pre- and postsynaptic processes (Artola et al., 2006). Less is known about the changes induced by EE in other brain regions. However, in a recent work it has been demonstrated that EE significantly increases LTP and largely diminishes LTD in the anterior cingulated cortex (ACC). Sensory experience changes synaptic plasticity in the ACC via postsynaptic mechanisms, by altering the dynamic regulation of NMDA receptor subunits: indeed, the component of NR2B-containing NMDA receptors is enhanced in EE-exposed animals (Shum et al., 2007).

Surprisingly, little attention has been paid to changes in the response properties of cortical neurons after EE. Rich and stimulating environments seem to improve the sensory

information processing of cortical neurons. The latency of evoked potentials recorded in the visual cortex of rats was shown to be significantly shorter after their rearing in a complex environment (Edwards et al., 1969). A similar result was reported for evoked potentials recorded in the vibrissae representational zone (Seo, 1992). More recently, Coq and Xerri (1998) reported that an enriched environment promoting tactile stimulation through palpation and manipulation of objects induces a selective expansion of the forepaw map areas serving the glabrous skin. Moreover, the expanded cutaneous zones display a finer-grained topographic organization characterized by smaller receptive fields. In the somatosensory 'barrel' cortex EE reduces the functional representation of the facial whiskers and extracellular recordings demonstrated suppressed evoked neuronal responses and smaller receptive fields in the cortical layer II/III of enriched rats (Polley et al., 2004). Similarly, animals raised in enriched conditions have higher responsiveness to light stimuli, contrast sensitivity, as well as spatial and temporal frequency detection, and sharper orientation tuning in primary visual cortex with respect to impoverished animals (Beaulieu and Cynader, 1990 a,b). Neurophysiologic responses are sensitive to EE also in the auditory cortex: EE substantially increases response strength, selectivity and directional sensitivity, but decreases threshold and latency of auditory responses (Engineer et al., 2004; Percaccio et al., 2005; 2007; Cai et al., 2009).

2.4 Environmental enrichment modulates gene expression

Efforts dedicated to understanding potential molecular mechanisms underling the previously described changes of EE on brain and behaviour started several years ago, prompted by the promising goal to reveal molecules that can be manipulated to reproduce the beneficial effects of the enriched experience.

It has been early found that an enriched experience causes increased rates of protein synthesis and increased amounts of proteins in the cortex (Bennett et al., 1964a). Subsequent studies showed that EE lead to increased amounts and expression of RNA in rat brain (Ferchmin et al., 1970; Grouse et al., 1978).

New possibilities to further characterize brain molecular changes elicited by EE came from the recent development of gene chip analysis techniques and real-time PCR, allowing the simultaneous screening and comparison of differential gene activation in dependence on different environmental conditions. Although they produce only 'snapshots' of a highly dynamic process, such studies are instructive and suggest that a large number of genes change their expression levels in response to EE.

The two studies so far analyzing the effects of enrichment on gene expression in the mouse (Rampon et al., 2000b) and rat cerebral cortex and hippocampus (Keyvani et al., 2004) reported changes occurring even after only 3h of enriched environment exposure, but persisting until two weeks from the start of enriched housing procedure. The differential expression of genes after 3 and 6 h of exploration in EE reveals the early molecular events resulting from environmental stimulation. Almost half (46%) of the environmentally responsive genes codes for proteins involved in macromolecule synthesis and processing, including enzymes involved in DNA, RNA and protein processing, and transcription factors and translational regulatory enzymes, standing at the beginning of molecular changes with variable target pathways and essential for various structural and functional endpoint changes in the nervous system. A distinct group of genes found to be differentially expressed after brief EE encodes proteolytic proteins and chaperones involved in signalling and apoptosis (e.g. caspase-6 and the Bcl-2 associated protein Bax), indicating a molecular correlate of the antiapoptotic effect of enrichment training. EE animals also show alterations in the expression of structural proteins involved in the

establishment of new synapses and reorganization or strengthening of existing synapses: in particular, it has been shown that the expression of proteins belonging to cell-adhesion molecule (CAM) family (e.g. integrins) and to associated Rho family, involved in the induction of events in surface adhesion, synapse formation and neuronal plasticity, is increased 3-fold during the early phase of enrichment. A cluster of genes encoding proteins implicated in synaptic vescicle trafficking and modulation of neurotransmitter release, including synaptobrevin and clathrin-AP2, is up-regulated after 3 and 6 h of enriched training. Changes in the expression of these genes clearly suggest that presynaptic processes are being modified by enriched experiences. Finally, it has been described that exposure to EE dynamically regulates the expression of a number of genes, whose products are associated with neuronal excitability (e.g. the 78-kDa glucose-regulated protein and neurokinin A) (Rampon et al., 2000b) Moreover, Pinaud et al. (2001) demonstrated that animals exposed daily, for 1 h, to EE exhibit a marked up-regulation in the cerebral cortex, hippocampus and striatum of the immediate early gene Arc mRNA, an activity-dependent neuronal marker involved in multiple forms of neuronal plasticity.

By comparing the gene expression profiles following a short experience in EE with those from animals exposed to EE for two weeks, it results that some group of genes are equally regulated (Keyvani et al., 2004): i) transcription factors, such as different zinc finger transcription factors (e.g. JunB, ElF-4E, Krox20, NGF1-B); ii) synapse-related molecules (e.g. synapsin, synaptogyrin, clathrin, Rho proteins); iii) proteolytic proteins and molecules mediating apoptosis (e.g. proteins belonging to Bcl family, ubiquitin-specific protease, ClpP protease, aspartyl aminopeptidase and prolidase). However, most of the genes regulated by a longer housing in EE are different from those whose expression levels change at the early stages of the enriched experience. A number of genes associated with the regulation of neurotransmission and neuronal spiking activity are affected by EE. The

expression levels of different members of neurotransmitter (e.g. glutamate, GABA, dopamine and noradrenaline) receptors and of ion channels and transporters (e.g. Na,K-ATPase and Na-, K-channels) are dynamically modified following EE (Keyvani et al., 2004). Rampon et al. observed that the expression level of postsynaptic density 95 (PSD-95), important not only for anchoring the NMDA receptor at the postsynaptic membrane but also for coupling this receptor to pathways controlling synaptic plasticity, increases after 2 days and 14 days of enrichment. EE is also associated with changes in the expression of molecules downstream of the NMDA receptor, including up-regulation of calmodulin (that modulates clustering of neurotransmitter postsynaptic receptors) and down-regulation of neurogranin (that regulates calmodulin availability). In the same manner, long-term EE modulates the expression of a group of kinase/phosphatase network molecules (e.g. CaM kinase, PKC, calcineurin, protein phosphatase), playing a pivotal role in remodelling of neuronal circuits (Keyvani et al., 2004). Prolonged EE also alters the mRNA levels of many genes associated with structural changes occurring during neuronal growth and synaptogenesis (e.g. the cytoskeletal proteins dynactin and cortactin, N-cadherin, dynamin-like protein 1, myosin heavy chain) (Rampon et al., 2000b; Keyvani et al., 2004). The study by Keyvani et al. identified a differential regulation following EE also for the expression of growth factors/receptors (e.g. FGF-, IGF-receptors, BDNF, VEGF). Moreover, there are other molecules, whose expression is regulated by enriched experience, that might play an indirect role in the context of brain plasticity, i.e. metabolic enzymes (implicated in energy metabolism, oxidative stress and mithocondrial activity) and molecules involved in immune response (e.g. complement protein C1q, MHC class and T-receptor molecules). Noticeably similar functional groups of genes were regulated in different brain areas, particularly in the hippocampus and striatum. However, different expression patterns were found in distinct brain areas at the individual gene level and there

were only a few genes regulated in parallel in different brain regions: the higher responsiveness of the hippocampus to EE could be due to a more pronounced susceptibility of this structure for plasticity changes (Keyvani et al., 2004; McNair et al., 2007; Thiriet et al., 2008).

Neurotransmitter systems characterized by diffuse projections to the entire brain are particularly sensitive to environmental stimuli. First studies by Rosenzweig et al. (1962a; 1967) reported an increase in acetylcholinesterase activity, indicating an effect on the cholinergic system. Subsequent studies confirmed and extended this initial observation to other neurotransmitter systems. It has been shown that EE augments mRNA expression levels of serotonin 1A receptor and serotonin concentration in the cerebral cortex and hippocampus (Rasmuson et al., 1998; Galani et al., 2007; Brenes et al., 2008). Enriched experience increases also noradrenaline concentration and potentiates β-adrenoceptor signalling pathway in the cerebral cortex, cerebellum and brainstem (Escorihuela et al., 1995; Naka et al., 2002).

Alterations of neurotransmitter aminoacids systems in mice subjected to differential housing have initially been revealed by Cordoba et al., (1984): in particular, a significant increase for aspartate was found in spinal cord, whereas glutamate significantly decreased in colliculi and cerebral cortex; similarly, glycine increased in cerebral cortex and decreased in colliculi and pons-medulla, while GABA increased in spinal cord, pons-medulla and cerebellum and decreased in thalamus and hypothalamus. Subsequent studies failed in uncovering a clear influence of EE on GABArgic transmission, but showed that enrichment significantly affects excitatory glutamatergic system. In this context, Rampon et al. (2000c) investigated the influence of EE on knockout mice in which NMDA receptor was selectively deleted in the CA1 subregion of the hippocampus: they found that the learning deficits exhibited by these mice in three hippocampus dependent behavioural

tasks were rescued after two months of EE, thus establishing that CA1 NMDA receptor activity seems not essential for experience-induced behavioural and synaptic plasticity. An explanation proposed by Tsien group is that the compensation might be due to an enhancement in connectivity outside the functionally deleted hippocampus, for instance in the neocortex. This possibility is strenghtened by another study, aimed at investigating learning and memory function in transgenic mice in which the NMDA receptor function is enhanced in the forebrain via overexpression of the NR2B subunit. These mice show overall improvement in their performances in learning and memory tasks; however, EE do not further increase their already augmented abilities. The occlusion of the effects induced by environmental stimulation suggests the existence of overlapping mechanisms between EE and genetic enhancement of the NMDA receptor functions (Tang et al., 2001). As a step toward the detailed dissection of the molecular mechanism underlying EE, Tang et al. presented biochemical evidence that GluR1, NR2A and NR2B proteins in the forebrain begin to increase after 2 weeks of EE, indicating that NMDA and AMPA receptors functions might be directly modified by environmental experience. An alteration in the expression of AMPA and NMDA subunit receptor following EE has been observed also in the hippocampus (Naka et al., 2005; Andin et al., 2007). EE triggers another neurochemical modification in the brain: indeed, very recent studies reported that differential rearing affects also the opioid system, leading to sensitivity alterations in opioid receptor populations (Smith et al., 2003; 2005).

The group of molecules with potent functions that most likely respond to external stimuli are the neurotrophic factors (or neurotrophins), a class of secreted proteins promoting neuronal development, survival and plasticity NGF, BDNF, NT-3 and NT-4. EE plays a powerful role in the modulation of synthesis and secretion of neurotrophic factors throughout the brain, resulting in higher levels of mRNA for NT-3, NGF and BDNF in the

visual cortex and hippocampus (Falkenberg et al., 1992; Torasdotter et al., 1996; 1998) and for a candidate-plasticity gene, the nerve growth factor induced-A (NGFI-A or Zif/268), throughout the brain (Pinaud et al., 2002), and increased protein levels of NGF, BDNF and NT-3 in several brain regions, including cerebral cortex, hippocampus, cerebellum and basal forebrain (Ickes et al., 2000; Pham et al., 2002; Zhu et al., 2006). Neurotrophins act on neurons by binding to two distinct classes of membrane receptors: one class consists of a single receptor, p75, that binds NGF and other neurotrophins with relatively low affinity; the other class consists of multiple receptor tyrosine kinases, including trkA, trkB, trkC and their isoforms. It has been reported that immunohistochemical analysis of brain tissue from the medial septal area reveals higher staining intensity and fibre density with both the low-affinity and the high-affinity NGF receptors in EE animals (Pham et al., 1999). It has been also demonstrated that EE dynamically affects the protein levels of full-length and truncated TrkB in the different regions of visual system (Franklin et al., 2006). In addition, EE increases hippocampal phosphorylation of the transcription factor cyclic-AMP response element-binding protein (CREB; Williams et al., 2001), which is known to regulate BDNF expression.

A recent study by Fischer et al. (2007) suggests that EE-induced effects might be mediated, at least in part, by chromatin remodelling. They demonstrated for the first time that EE increases the acetylation of histone 3 and 4 (H3, H4) in the hippocampus and, to a lesser extent, in the cortex of wild-type mice. Histone post-translational modifications regulate chromatin susceptibility to transcription: high levels of histone acetylation on a specific DNA segment is generally correlated with increased transcription rates. This strongly suggests that epigenetic control of gene transcription through histone acetylation could be the final gate opened by EE to promote plasticity (Pizzorusso et al., 2007).

2.5 Environmental impact on developmental plasticity of the brain

The influence of EE on the development and plasticity of the nervous system has been scarcely investigated. In the past few years, new evidence has been provided showing that EE has a remarkable impact on the developmental plasticity of the visual system. The most striking effect on visual system development elicited by an EE paradigm starting at birth is a marked acceleration in the maturation of visual acuity (VA), a very sensitive and predictive index of visual system maturation. This has been initially assessed in the mouse both electrophysiologically by visual-evoked potential (VEP) recordings and behaviourally by a discrimination task (visual water box task) (Cancedda et al., 2004), and then replicated in the rat (Landi et al., 2007a). The acceleration effect is strong, as in enriched animals VA development is anticipated by 7 days with respect to control animals: in the timescale of human visual development, it would be as a child reached his final VA at around three years of age (i.e. approximately two years before the age at which children's acuity development normally ends). This precocious VA development induced by EE is accompanied by a precocious developmental decline of the possibility to induce LTP of layer II-III field potentials after theta-burst stimulation of the white matter in the visual cortex, a well-established in vitro model of developmental plasticity (Cancedda et al., 2004). The study of molecular mechanisms underlying the effects of EE revealed that one crucial factor is the neurotrophin BDNF: mice reared from birth in EE have increased levels of the BDNF protein in their visual cortex at P7 (Cancedda et al., 2004; Sale et al., 2004). The acceleration of visual cortical development in EE animals closely resembles that previously reported in transgenic mice overexpressing BDNF in the forebrain (Huang et al., 1999). A widely accepted model is that precocious higher BDNF levels triggers the development of the inhibitory GABAergic system, which, by affecting receptive field development and synaptic plasticity, could determine both the faster maturation of VA and

the accelerated decline of synaptic plasticity. In line with this hypothesis, an increased expression of the GABA biosynthetic enzymes GAD65 and GAD67 has been found in EE pups at both P7 and P15 (Cancedda et al., 2004; Sale et al., 2004). Another molecular factor crucially involved in EE effects on visual system development turned out to be IGF-I. IGF-I is increased postnatally in the visual cortex of enriched rats, and post-weaning administration of IGF-I in this structure mimics EE effects on VA acceleration. Furthermore, blocking endogenous IGF-I action in the visual cortex of developing EE subjects completely prevents EE effects on VA maturation (Ciucci et al., 2007). One of the targets of BDNF and IGF-I signalling is the activation of CREB. Cancedda et al. (2004) demonstrated that EE from birth accelerates the time course of CRE/CREB-induced gene expression and that treatment of non-EE mice with rolipram, a specific inhibitor of the high-affinity phosphodiesterase type IV that activate cAMP system, resulting in an increased phosphorylation of the transcription factor CREB, partially mimics EE effects on CREB pathway and on visual acuity development. Even if the work by Cancedda and colleagues focused on the visual system, it is very likely that the EE effect is not specific to the visual cortex, as suggested by the influence on CRE-mediated gene expression observed also in the somatosensory cortex.

The surprising finding that EE affects BDNF and GABAergic inhibition before eye opening indicates that some of the EE effects on visual system development could be totally independent of vision. This issue has been addressed by Bartoletti et al. (2004) in a study in which EE and dark rearing (DR) have been combined together. Lack of visual experience from birth prevents the VA development and prolongs the duration of the critical period in standard housed animals (Blakemore and Price, 1987; Fagiolini et al., 1994). These effects can be completely counteracted by providing DR animals with the opportunity to experience EE while in the dark: DR-EE rats show a normal closure of the

critical period for OD plasticity and a normal VA development. Also in this case the effect of EE is very similar to that found in BDNF overexpressing mice, in which a rescue of DR effects on visual system development is evident (Gianfranceschi et al., 2003), and the influence of EE on GABAergic inhibitory circuits has been confirmed (Bartoletti et al., 2004).

A more recent finding is the demonstration that also retina development is affected by experience provided by EE both at electrophysiological and molecular level. Landi et al. (2007) monitored the development of retinal responses in enriched and non-enriched rats using pattern electroretinogram, a sensitive measure of retinal ganglion cells (RGCs) function. Retinal acuity development is sensitive to EE on the same time scale as cortical acuity (Landi et al., 2007a). Furthermore, enriched mice displayed a pronounced acceleration in the process of RGC dendrite segregation into ON and OFF sublaminae (Landi et al., 2007b). BDNF turned out to be a key molecule in both processes, as demonstrated by the higher BDNF levels in the RGC layer of enriched animals and by the lack of EE effects in the retina of enriched pups in which BDNF was blocked by means of antisense oligonucleotides (Landi et al., 2007a, b). A clear influence of EE on retinal development has also been reported during prenatal life. Recent data by Sale et al. (2007) demonstrated that exposing pregnant females to EE (maternal enrichment) determines a marked acceleration of retinal anatomical development in the embryos, accelerating the migration of neural progenitors and anticipating the time-course of naturally occurring cell death. Interestingly, the effects found in the foetus are mediated by IGF-I. Anatomical modifications, indeed, are accompanied by a marked increase in IGF-I expression in the retinas of enriched pups and in the milk of mothers. Furthermore, the neutralization of IGF-I in enriched mothers by means of administration of antiIGF-I antiserum prevents the

action of maternal enrichment on retinal development, and chronic IGF-I injection to standard pregnant females mimics the effects of EE in the foetuses (Sale et al., 2007).

2.6 Environmental influences on aging progression

Over the last 30 years, environmental conditions have been identified as a significant contributor to cognitive aging. Human studies frequently demonstrated associations between environmental factors, particularly supportive social environments, and positive states of health (Winocur and Moscovitch, 1983; 1990; Winocour, 1998). Moreover, cognitive enrichment, which is manifested in education, job complexity, and/or leisure activities, has beneficial effects, which help to preserve several cognitive functions, and can delay the development of dementia. The beneficial effects of cognitive enrichment in humans can be explained by the cognitive reserve hypothesis, which postulates that cognitive enrichment promotes a more efficient use of existing neural networks and recruitment of alternative networks when required, rather than reducing rate of development of brain pathology (for a review, see Kramer et al., 2004; Milgram et al., 2006). The behavioural benefits of enrichment have structural correlates, which likely include increased synapses in specific brain regions: Jacobs et al. (1993) examined neuron structure derived from Golgi stained materials in subjects differing in amount of education, showing that dendritic measure increase in length with increased levels of education. Finally, a clear effect of fitness training as prophylactic agent for the functional integrity of the aged brain was found (Kramer et al., 2004).

Given the design limitations of human studies, application of animal models provide an alternative means for better understanding the role of EE on brain aging. While much of the work on animal models has been conducted on middle-aged animals, the aged brain appears to remain responsive to EE. Studies of environmental effects on age-related

changes in cognitive function widely indicated that living in complex, stimulating environments (both from youth and only during old age) rescues the cognitive deficits or, at least, ameliorates the performance on tests of learning and memory (particularly complex maze solving) in aged animals (Doty, 1972; Cummins et al., 1973; Warren et al., 1982; Berman et al., 1988; Winocur, 1998; Soffié et al., 1999; Kempermann et al., 2002; Kobayashi et al., 2002; Frick and Fernandez, 2003; Bennett et al., 2006; Leal-Galicia et al., 2008). The effect of long-term exposure to EE starting at weaning is quite obviously much greater than that of short term exposure in aged rats (Kobayashi et al., 2002). Moreover, it is important to point out that only continuous enrichment significantly reduces age-related memory decline (Bennett et al., 2006). Apart from environmental influences on cognitive functions, it is important to consider the impact of environment on other psychological processes (attentional, motivational and stress-related factors) that affect cognitive performance in an indirect way. There is growing evidence of an important link between environmental stress and cognitive function in old age (Lupien et al., 1998). Several studies have shown that handling and other forms of external stimulation protect old animals from potential stressors, decrease anxiety-like behaviours and, at the same time, preserve learning and memory function by reducing glucocorticoid toxicity in hippocampal cells (Meaney et al., 1991; Mohammed et al., 1993; van Waas and Soffié, 1996; Zambrana et al., 2007). Consistently, it has been observed that EE reduces the reactivity to stress of the cholinergic and dopaminergic systems in the prefrontal cortex of aged animals (Segovia et al., 2008 a,b).

Aging-related impairments of hippocampus-dependent memory may be caused, in part, by altered synaptic mechanisms including LTP. The magnitude of both the early and late phase of LTP in hippocampal CA1 region is significantly reduced in aged compared to young adult mice: Huang et al. (2007) showed that enriched aging mice regain a magnitude

of LTP comparable to that of young control adults. In the same work a significant increase of CREB, which could contribute to the positive effect on LTP, was also observed in EE animals (Huang et al., 2007). Previous research indicates that an age-related increase in the amplitude of the Ca^{2+}-dependent, K^{+}-mediated afterhyperpolarization (AHP) is related to cognitive decline. Following 8-10 weeks of differential rearing, the amplitude of AHP in hippocampal pyramidal cells is significantly reduced in aged animals (Kumar and Foster, 2006).

Aging also impairs sensory functions such as tactile sensitivity, sensorimotor coordination and locomotion: senescent rats exhibit a degraded topographic organization of the cutaneous map of the hindpaw and a severe deterioration of somatotopic features of the forepaw map in the primary somatosensory cortex (Spengler et al., 1995; Coq and Xerri, 2000). Sensorimotor experience retain a capacity to modulate the alteration of the somatotopic representations during the aging process: indeed, Coq and Xerri (2001) showed that prolonged enrichment could partially offset the age-related breakdown of the forepaw cutaneous map.

The enhanced cognitive performance associated with environmental stimulation may be linked to environmentally-induced changes in brain structure and function. Indeed, several experimental studies have shown that EE attenuates the age-related changes in cortical weight and thickness (Cummins et al., 1973; van Gool et al., 1987; Mohammed et al., 2002), dendritic branching (Connor et al., 1981; Kolb et al., 1998; 2003), spine density (Kolb et al., 1998; 2003), and gliogenesis (Soffié et al., 1999). Once again the EE effects are not limited to the cerebral cortex but are also detectable in other brain regions, in particular the hippocampus and the cerebellum, that are strongly involved in those cognitive functions most affected by aging. It has been reported that EE enhances hippocampal neurogenesis in aged animals (Kempermann et al., 1998; 2002; Segovia et al.,

2006; Leal-Galicia et al., 2008) and attenuates dendritic degeneration and the loss in dendritic spines in the hippocampus and cerebellum (Greenough et al., 1986; Mohammed et al., 2002).

At the synaptic level, these anatomical modifications seems to be reflected by increased synaptophysin immunoreactivtity, a synaptic vescicle-specific protein, in both the cortex and the hippocampus, suggesting that altered presynaptic plasticity may contribute to memory enhancement in aged animals (Saito et al.. 1994; Nakamura et al., 1999; Frick and Fernandez, 2003; Leal-Galicia et al., 2008). However, although increases in synaptophysin immunoreactivity have most often been interpreted as an increase in presynaptic terminals, an electron micrography study indicates that presynaptic terminals are not increased in response to EE. Instead, the number of vescicles per terminal is increased in aged animals in response to enrichment (Nakamura et al., 1999). Because synaptophysin is a constituent of neurotransmitter-containing presynaptic vescicle membranes, an increase in synaptophysin may reflect an increase in neurotransmission.

Actually, in addition to the EE-induced changes in stress-response of cholinergic and dopaminergic systems previously described, it has been demonstrated by using microdyalisis perfusion that EE increases concentration of glutamate and GABA in the CA3 region of hippocampus of aged rats (Segovia et al., 2006). Interestingly, there is also evidence that EE mitigates the senile impairment of the cholinergic system, increasing the choline acetyltransferase (ChAT) activity in several brain regions (Fernández et al., 2004). The aged brain is subjected to multiple cellular processes that result in damage to neurons and neural networks. Lipofuscin deposits in neurons are a prominent sign of aging in the brain and are thought to indicate chronic oxidative stress. EE significantly reduces the number of lipofuscin-loaded granule cells in the dentate gyrus of hippocampus (Kempermann et al., 2002). In addition, EE induces the production of proteins that

suppress the oxyradical production, such as gluthatione, stabilizes cellular calcium homeostasis and inhibits apoptotic biochemical cascades (Mattson et al., 2001; Fernández et al., 2004).

2.7 Enriched environment and disorders of the nervous system

An important line of research deals with the potential therapeutic effects of EE in experimental animals suffering from various nervous system dysfunctions (for a review, see Will et al., 2004; Nithianantharajah and Hannan, 2006).

Brain injury In the 1960s, it was shown for the first time that EE enhances functional recovery after brain damage: Schwartz (1964) reported that rats subjected to neonatal bilateral cortical lesions enhanced their performance in the Hebb-Williams maze when raised in enriched environments. Following this paper, a large number of reports indicated that EE promotes at least some degree of improvement after CNS injuries derived from both traumatic lesions and ischemic insults (reviewed in Will and Kelche, 1992; Mohammed et al., 2002; Will et al., 2004). Beneficial effects of enriched experience has been largely reported in animals that sustained either focal or global ischemic injury, which results from a sustained deficit in cerebral perfusion. Enriched rehabilitation after middle cerebral artery (MCA) occlusion or other kind of ischemia induction results in a long-lasting functional improvement both in memory and sensorimotor deficits, highly correlated with a reduction in infarct size, in the number of proliferating astrocytes and in the volume of the glial scar (Ohlsson and Johansson, 1995; Johansson, 1996; Briones et al., 2000; Biernaskie and Corbett, 2001; Farrell et al., 2001; Risedal et al., 2002; Dahlqvist et al., 2004; Gobbo and O'Mara, 2004; Wang et al., 2008; Xu et al., 2009). Recently, Buchhold et al. (2007) showed that EE significantly rescues the impaired behavioural

phenotype following stroke in aged animals. Ischemic rats exposed to EE have increased total dendritic length as well as enhanced number of dendritic segments and dendritic spine density in the apical region of the hippocampal area (Briones et al., 2000). EE promotes synaptic plasticity in injured animals, increasing synaptic density in parietal cortex and inducing structural changes in synaptic junctions, with a decreased width of synaptic clefts and increased thickness of postsynaptic densities in the parietal cortex and hippocampus (Xu et al., 2009). In addition, post-ischemic EE normalizes cell proliferation levels, increases the numbers of putative neural stem cells in the subventricular zone and of doublecortin-positive neuroblasts, which extend in migratory chains toward the infarct and could be important for tissue regeneration (Komitova et al., 2005). Enriched rehabilitation largely affects gene expression in brain regions close or remote with respect to injury (Keyvani et al., 2004), for example increasing the expression of trophic factors (Dahlqvist et al., 1999; 2003; Gobbo and O'Mara, 2004) and rescuing deficits in glucocorticoid and mineralocorticoid receptor expression (Dahlqvist et al., 1999; 2003). Functional recovery after experimental stroke could be enhanced by fostering plastic processes in brain regions outside the lesion. Indeed, animals exposed to enriched rehabilitation had significantly higher dendritic arborisation and spine density in the undamaged contralateral cortex (Biernaskie and Corbett, 2001; Johansson and Belichenko, 2002).

The most common animal model of traumatic brain injury (TBI) is lateral fluid percussion (FP) model, that produces persistent impairments predominantly in learning and memory, associated to bilateral hippocampal lesions. Exposure to EE following brain injury attenuates cognitive and neuromotor dysfunctions raised by FP, preserving tissue integrity and increasing neurogenesis in the ipsilesional granule layer of the dentate gyrus (Hamm et al., 1996; Passineau et al., 2001; Hicks et al., 2002; Wagner et al., 2002; Gaulke et al., 2005; Maegele et al., 2005). Other kind of brain damage whose symptoms are

alleviated by EE are surgical lesions of the anterior thalamic lesions (Loukavenko et al., 2007; Wolff et al., 2008), frontal cortex lesions (Kolb and Gibb, 1991), hippocampal formation lesions (Galani et al., 1997; Bindu et al., 2005; Dhanushkodi et al., 2007), section of the fimbria-fornix fibre bundles (Van Rijzingen et al., 1997) or lesions of the sensorimotor cortex (Held et al., 1985). Similarly to what observed in experimental models of ischaemic stroke, EE partially attenuates the post-lesional neurodegeneration (Dhanushkodi et al., 2007), enhances dendritic branching (Kolb and Gibb, 1991) and affects gene expression, increasing BDNF and decreasing DAT levels (Chen et al., 2005; Wagner et al., 2005). Importantly, EE also prevents much of the sensory and motor dysfunction that accompanies contusive spinal cord injury in the rat (Lankhorst et al., 2001; Berrocal et al., 2007; Fischer and Peduzzi, 2007). Stem cells provide a promising form of therapy to replace neuronal circuits lost following an injury. However, it is unlikely that replacement therapy alone will be sufficient to maximize recovery. A growing area of interest is the influence of environment facilitating recovery in transplanted animals (for a review, see Döbrössy and Dunnett, 2001). EE enhances migration of transplanted stem cells toward the region of injury and there is a trend toward increased survival of stem cells. EE also increases the number of endogenous progenitor cells in the subventricular zone of transplanted animals (Kelche et al., 1988; 1995; Grabowski et al., 1995).

EE studies using transgenic mouse models of neurodegenerative disorders have provided important new insights into gene-environment interactions and experience-dependent plasticity in brain disease. A range of striking effects have been observed in several models of brain disorders after exposure to EE. These findings have implications for developing novel clinical approaches to the prevention and treatment of brain disorders. These environmental manipulations can also provide a powerful tool to dissect cause and effect

among molecular and cellular correlates of pathogenesis, and so identify novel targets for future development of therapeutics (Nithianantharajah and Hannan, 2006; Laviola et al., 2008).

Huntigton's disease EE induces significant behavioural, cellular and molecular changes in transgenic mouse models of Huntington's disease (HD), an autosomal dominant brain disorder, characterized by degeneration of the cerebral cortex and striatum, producing progressive movement disorders, cognitive deficits and psychiatric symptoms. The pathogenic mechanism by which the trinucleotide CAG repeat expansion mutation, expressed as an extended polyglutamine tract, induces neuronal dysfunction and death is not yet fully understood. It has been shown that EE greatly delays the onset and progression of motor symptoms and the degenerative loss of cerebral volume in different transgenic models of HD (van Dellen et al., 2000; Hockly et al., 2002; Schilling et al., 2004; Spires et al., 2004). Recent evidence also suggests that EE can ameliorate cognitive deficits in R6/1 HD mice (Nithianantharajah et al., 2008). Investigations on the mechanisms mediating these experience-dependent effects have identified that EE increases neurogenesis in the dentate gyrus of the hippocampus and modifies synaptic composition (resulting, for example, in increased synaptophysin and PSD-95 levels) in the frontal cortex and hippocampus (Lazic et al., 2006; Nithianantharajah et al., 2008). At the molecular level EE prevents striatal and hippocampal deficits in BDNF, as well as the cortical reduction of the dopamine and cAMP-regulated phosphoprotein 32 kDa (DARPP-32) and the striatal depletion of CB1 receptors, possibly by rescuing transcription or protein transport problems (Glass et al., 2004; Spires et al., 2004). Thus, EE could overcome deficiencies in gene expression, synaptic function and experience-dependent plasticity, ameliorating the deficits in HD mice; however, it is possible that EE also affects the abnormal protein-protein interactions, resulting in intracellular inclusions of huntingtin

protein fragments, that occur in HD. A better understanding of how EE induces its beneficial effects might also provide direction for the development of therapeutic approaches. Interestingly, recent epidemiological study of human HD has shown a clear role for environmental factors in modulating the clinical onset of HD, although the nature of these factors remains unknown (Wexler et al., 2004). Moreover, it has been reported that a more stimulating environment improved physical, mental and social functioning in a small cohort of HD patients (Sullivan et al., 2001).

Alzheimer's disease Alzheimer's disease (AD) is a neurodegenerative pathology associated with progressive memory loss and severe cognitive decline, mainly affecting the neocortex and hippocampus. The disease is characterized by two pathological hallmarks, i.e. senile plaques (extracellular aggregates of amyloid, derived from proteolysis of the precursor protein APP) and neurofibrillary tangles (intraneuronal aggregations of hyperphosphorylated forms of the microtubule-associated protein tau). Most AD cases are sporadic and seem to result from an interaction of multiple genetic and environmental factors. However, there are also familial forms of AD that are inherited in an autosomal dominant fashion. Linkage and cloning studies have identified three genes (APP, presenilin 1 (PS1) and presenilin 2 (PS2)), which have been the focus for transgenic modelling studies. A genetic risk factor for the sporadic form of AD has also been found: polymorphisms in the apolipoprotein E (APOE) gene, particularly the ε4 allele are thought to increase the risk of AD onset. Levi and colleagues were the first to examine the effect of differential rearing in a mouse model of AD, using transgenic mice containing human APOE3 or APOE4 allele. Enriched mice transgenic for human APOE3 showed improved learning and memory associated with higher hippocampal levels of the presynaptic protein synaptophysin and of NGF, whereas mice transgenic for human APOE4 were unaffected by EE (Levi et al., 2003). The effect of EE on APP/PS1 transgenic mice was investigated

by Jankowsky et al. (2005) and Costa et al. (2007), both showing that differential housing significantly mitigates deficits observed in AD mice across multiple cognitive domains. In line with this, APP and AD11 transgenic mice raised in EE display enhanced cognitive activity, resulting indistinguishable from wild-type mice of the same age (Wolf et al., 2006; Berardi et al., 2007; Cracchiolo et al., 2007). Moreover, Arendash et al. (2004) observed that aged APP transgenic mice exposed to EE improve their performance in cognitive-based tasks. The effect of EE on Aβ levels and plaque deposition and their impact on cognitive improvement is controversial. Jankowsky and colleagues (2003; 2005) repeatedly reported that enriched APP/PS1 transgenic mice develop a higher amyloid burden with commensurate increases in aggregated and total Aβ levels, particularly in the hippocampus, as well as Arendash et al. (2004) failed to observe change in Aβ deposition in enriched APP transgenic mice. By contrast, Lazarov and colleagues (2005) found that enriched APP/PS1 transgenic animals have decreased hippocampal and cortical Aβ levels and amyloid deposits compared with standard-housed controls. In addition, the enzymatic activity of neprilysin, an Aβ-degrading endopeptidase, resulted to be elevated in the brains of EE mice. A reduction in brain β-amyloid deposition following EE exposure has been also shown in APP, TgCRND8 and AD11 transgenic mice (Ambrée et al., 2006; Berardi et al., 2007; Cracchiolo et al., 2007; Mirochnic et al., 2009). This discrepancy may reflect both the variety of experimental paradigms and the age analysed, but also the fact that the relationship between molecular and cognitive aspects of AD is highly complex. In AD11 transgenic mice EE significantly reduces the appearance of other AD neuropathological hallmarks, i.e. the aggregation of hyperphosphotylated tau and the cholinergic deficit (Berardi et al., 2007). Some studies have also investigated the effects of EE on neurogenesis in AD mouse models. While conditional PS1 knockout mice and mice overexpressing either wild-type human PS1 or the mutant form P117L show a deficiency

in EE-induced neurogenesis (Feng et al., 2001; Wen et al., 2004; Choi et al., 2008), it has been recently reported that EE promotes hippocampal neurogenesis in APP and TgCRND8 transgenic mice to wild-type levels (Wolf et al., 2006; Herring et al., 2009; Mirochnic et al., 2009). Moreover, it has been demonstrated that EE increases angiogenesis and facilitates blood Aβ clearance through a differential regulation of Aβ receptor/transporter molecules (Herring et al., 2008). Lazarov et al. (2005) carried out a microarray analysis in order to identify gene expression changes in APP/PS1 transgenic mice placed in EE compared with control mice, that could be predictive of the improvements in cognition. This study revealed a total of 41 genes differentially regulated in response to EE. The vast majority of genes showing elevated expression encodes polypeptides involved in a variety of processes associated with learning and memory, synaptic plasticity, neurogenesis, vasculogenesis, neuronal cell growth and cell survival pathways (e.g. NGF-1A, BDNF; CaMKIIα). Similar results have been obtained by other groups, performing gene expression and protein quantification analysis of hippocampal tissue in different AD transgenic mice (Costa et al., 2007; Cracchiolo et al., 2007; Herring et al., 2008). It is particularly interesting that transthyretin, a protein involved in Aβ clearance, is up-regulated in transgenic mice raised in EE (Costa et al., 2007). A quantitative RT-PCR study further confirmed that EE promotes the up-regulation of neurotrophic factor expression (NT-3, BDNF, IGF-I and VEGF) in the hippocampus of AD mice (Wolf et al., 2006). Finally, environmental stimulation attenuates pro-oxidative processes and triggers anti-oxidative defense mechanisms, as indicated by diminished biomarkers for reactive oxygen and nitrogen species, downregulation of pro-inflammatory and pro-oxidative mediators and upregulation of superoxide dismutase 1 (SOD1) and SOD2 (Herring et al., 2009). Although it remains to better elucidate the mechanisms underlying the effects of EE, these studies, together with epidemiological investigations (see the previous

paragraph, for a review Valenzuela and Sachdev, 2006), suggest that both mental and physical activity help to slow down or prevent the cognitive decline associated with AD.

Parkinson's disease Parkinson's disease (PD) is a neurodegenerative disorder, involving progressive motor symptoms and impairment in cognitive function, caused by degeneration of dopaminergic neurons in the substantia nigra and characterised by the formation of intracytoplasmic inclusions known as Lewy bodies. The genetics of PD is complex and gene mutations for various familial forms of PD continue to be identified, making it difficult until recently to establish animal models with appropriate construct validity. Nevertheless, established rodent models involving the use of toxins, such as 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP), to induce targeted degeneration of dopaminergic (DA) neurons have proved to be highly informative. In models of PD complex living environments have been applied before or after neurotoxic insult and was shown to provide behavioural and neurochemical beneficial effects. For instance, mice raised in EE are more resistant to MPTP compared to mice raised in a standard environment. Indeed, while mice raised in SC showed a 75% loss of DA neurons, mice raised in EE showed only 40% of such loss. This is achieved through the downregulation of the striatal expression of the dopamine transporter DAT. In addition, EE promotes an increase in the expression of BDNF and GDNF in the striatum (Bezard et al., 2003; Faherty et al., 2005). Similarly, rats housed in enriched conditions prior to receiving the infusion of 6-hydroxydopamine (6-OHDA) neurotoxin show improved motor function (Jadavji et al., 2006). The effects of life experience on the incidence of PD in humans is suggested only from epidemiological studies (Olanow and Tatton, 1999). These data directly suggest that positive early-life experiences, involving increased mental and physical activities, may have beneficial consequences, reducing the probability of developing PD.

Amyotrophic lateral sclerosis Amyotrophic lateral sclerosis (ALS) is the most common form of motoneuron disease, with muscle wasting and paralysis as main symptoms, and is characterized by the progressive degeneration of motoneurons in the cortex, brainstem and spinal cord. Although twin studies support a role for both genetic and environmental factors in ALS, the environmental contribution is still poorly understood. The predominantly used mouse model of ALS overexpresses the mutant human form of the Cu/Zn superoxide dismutase-1 (SOD1). The onset and progression of disease symptoms were recently studied in transgenic ALS mice housed in EE, showing that differential rearing significantly improves motor performance of these animals (Stam et al., 2008).

Epilepsy A growing number of studies describe the beneficial effects of EE in animal models of epilepsy, a neurological condition that disrupts the normal electrical activity and promotes neurodegeneration and abnormal wiring in the brain (for a review Dhanushkodi and Shetty, 2008). Although experience-related risk factors have been associated with epilepsy, susceptibility to epilepsy has been suggested to be partly genetic. However, the most used experimental animal models of epilepsy have been generated using proconvulsant drugs (such as kainic acid) and electrical stimulation (kindling). Animals reared in EE conditions exhibit decreased susceptibility to seizures and hippocampal neurodegeneration when challenged with kainic acid (Young et al., 1999). Consistent with this idea, Auvergne et al. (2002) demonstrated that prior housing of adult rats in EE increases their threshold for amygdala kindling induced seizures. Some studies suggest that housing animals in EE is beneficial for minimizing learning and memory deficits and the probability for developing behavioural abnormalities and depression that typically ensue at various time points after the induction of status epilepticus (Faverjon et al., 2002; Rutten et al., 2002; Koh et al., 2005; 2007). Nevertheless, it remains unknown whether exposure to EE after the induction of acute seizures or SE is efficacious for preventing or decreasing

the intensity of chronic epilepsy. The mechanisms that reduce seizure, behavioural impairment and neurodegeneration in enriched animals are unknown. However, it is believed that processes such activation of specific signal transduction pathways (pCREB, Arc, Homer1a and Egr1) and increases in multiple growth factor including BDNF and GDNF could be involved (Young et al., 1999; Faverjon et al., 2002; Koh et al., 2005).

Developmental disorders Recently, beneficial effects of EE have been reported also in animal models of developmental disorders of CNS, such as Down syndrome, Fragile X syndrome and Rett syndrome. Down syndrome is the most significant genetic cause of mental retardation and involves trisomy of chromosome 21. Currently, there are several murine models with segmental trisomy; however, the Ts65Dn mouse model is the most commonly used. Using this model, Martinez-Cue and others provided some suggestive evidence that EE increases exploratory behaviour in transgenic Down animals, but it improves learning and spatial memory only in females (Martinez-Cue et al., 2002; 2005). Fragile X syndrome is a pathology of development, due to a mutation of the fragile X mental retardation 1 (FMR1) gene, that leads to transcriptional silencing of the FMR1 gene. *Fmr1*-knockout mice, which lack the normal fragile X mental retardation protein (FMRP), show both cognitive and neuronal alterations. A recent study showed that EE rescues alterations in exploratory behaviour in these transgenic mice. Furthermore, EE increases dendritic branching, spine number, appearance of mature spines and expression of the AMPA receptor subunit GluR1 in the visual cortex (Restivo et al., 2005). Rett syndrome, commonly associated with mutations of the methyl CpG-binding protein 2 (MECP2) gene, is characterised by an apparently normal early postnatal development followed by deterioration of acquired cognitive and motor coordination skills in early childhood. EE significantly reduces the motor coordination deficit in heterozygous Mecp2$^{+/-}$ females, but not in Mecp2$^{-/y}$ males. Similarly, BDNF protein levels are enhanced

in the cerebellum of enriched Mecp2$^{+/-}$ females, whereas Mecp2$^{-/y}$ males show identical deficits of cerebellar BDNF, despite the enriched housing. These findings demonstrate a positive impact of EE in a Rett syndrome model, which could be dependent on the existence of one functional copy of Mecp2 (Kondo et al., 2008). In another study, it has been observed that EE provides subtle improvements to locomotor activity and contextual fear conditioning in Mecp2(1lox) mice. Additionally, EE reduces ventricular volumes, which correlates with improved locomotor activity, suggesting that neuroanatomical changes contribute to improved behaviour (Nag et al., 2009). EE also reverses almost all behavioral alterations observed in rats prenatally exposed to valproic acid (VPA), an animal model of autism. Enriched compared to standard VPA rats have higher sensitivity to pain and lower sensitivity to nonpainful stimuli, stronger acoustic prepulse inhibition, lower locomotor, repetitive/stereotypic-like activity and enhanced exploratory activity, decreased anxiety, increased number of social behaviors and shorter latency to social explorations (Schneider et al., 2006).

CHAPTER 3

AIM OF THE THESIS AND EXPERIMENTAL DESIGN

The functional impact of environmental enrichment in the adult healthy and pathological brain could be explained by the restoration of neural plasticity. Despite vast correlative evidence consistent with this hypothesis, thus far the possibility has not been directly tested.

In the present work, I explored environmental enrichment as a strategy for promoting plasticity in the adult brain and as a tool to investigate the molecular mechanisms restricting plasticity to the critical period of early postnatal life. My work consisted of two separate research lines.

On the one hand, I used the paradigmatic model of experience-dependent plasticity in the visual cortex, i.e. ocular dominance plasticity, assessing the effectiveness of monocular deprivation in the visual cortex of adult enriched rats by electrophysiological recordings of both visual evoked potentials and single-unity activity. In standard animals monocular deprivation causes a marked ocular dominance shift of cortical neurons only during the critical period. It has been reported that also the susceptibility of depth vision to the effects of visual deprivation declines with age in mammals (Timney, 1983; 1990). I evaluated whether the disruption of binocularity caused by monocular deprivation impaired depth perception abilities in adult enriched animals, by using a behavioural procedure based on two different versions of the visual cliff task.

I then examined the molecular mechanisms underlying the enhancement of plasticity induced by environmental enrichment. I first focused on the serotoninergic system. It has been recently demonstrated, indeed, that the pharmacological increase of serotonin levels through chronic administration of fluoxetine restores neural plasticity in

the visual cortex of adult animals (Maya-Vetencourt et al., 2008). Moreover, some evidence suggests that the serotoninergic system is sensitive to an increased environmental stimulation (Rasmuson et al., 1998; Galani et al., 2007; Brenes et al., 2008). I measured total levels of serotonin in the visual cortex of enriched animals by means of *ex vivo* HPLC and I evaluated the effects of environmental enrichment on visual cortical plasticity in adult animals monocularly deprived and intracortically infused, during the monocular deprivation week, with the serotonin synthesis inhibitor para-chlorophenylalanine (pCPA), in order to investigate whether the increase of serotonin levels was causally linked to the restoration of plasticity.

Afterwards, given the well-known role of cortical inhibitory circuits in the regulation of plasticity time-course (Hensch, 2005a), I used in vivo brain microdialysis and immunohistochemistry to assess whether the exposure to environmental enrichment affected GABA-mediated inhibition in the adult visual cortex. In addition, long-term potentiation and long-term depression of layer II-III field potentials were examined as an electrophysiological evaluation of the excitation-inhibition balance. I next investigated whether the reduction of intracortical inhibition in the visual cortex induced by environmental enrichment was related to the reinstatement of ocular dominance plasticity. To do this, I strengthened GABAergic transmission in a separate group of monocularly deprived enriched rats by intracortical chronic infusions (via osmotic minipumps) of the benzodiazepine agonist diazepam. Finally, I measured BDNF protein expression in the visual cortex of adult rats housed in environmental enrichment using semi-quantitative immunohistochemistry and I assessed whether the rise of cortical BDNF levels had a critical role in the plastic effects promoted by environmental enrichment, using intracortical infusion of BDNF antisense oligonucleotides during the monocular deprivation week. In order to clarify the interactions between the molecular factors studied,

I also analysed BDNF and GAD67 levels in the visual cortex of a group of rats bilaterally infused with pCPA. My results led to propose a model of the mechanisms underlying the reinstatement of plasticity induced by environmental enrichment in adult animals.

On the other hand, I investigated whether the reactivation of experience-dependent plasticity could be exploited as therapeutic approach to promote the recovery of visual functions (i.e. visual acuity and binocularity) in amblyopic animals. The traditional amblyopia therapy consists in patching the fellow preferred eye, thus forcing the brain to use the visual input carried by the weaker eye, but the success rate of this treatment strongly depends on the age of onset. For this reason the development of strategies that promote amblyopia recovery in the adult is of great interest. I evaluated (both electrophysiologically using visual evoked potentials and behaviourally with the visual water box) the recovery of visual acuity in adult enriched animals rendered amblyopic by long-term monocular deprivation starting during the critical period. The recovery of binocularity was assessed by recording the contralateral-to-ipsilateral VEP ratio in the visual cortex contralateral to the long-term occluded eye in the same animals in which visual acuity measurements was done.

CHAPTER 4

MATERIALS AND METHODS

4.1 Rearing environments

A total of 195 Long-Evans hooded rats were used in this study, which has been approved by the Italian Ministry of Public Health. At the postnatal day (P) 60-70 the animals were assigned to either enriched environment group (EE) or to standard condition (SC) group.

Figure 1: EE is a manipulation of the standard laboratory conditions that modifies the quality and intensity of environmental stimulation, by providing the animals with increased levels of multi-sensory stimulation, physical activity and social interactions, and by eliciting natural explorative behaviors.

Environmental enrichment consisted of a large cage (100 x 50 x 82 cm) with two or more floors linked by stairs, containing several food hoppers, running wheels and differently shaped objects (platforms, boxes, toys, tunnels, shelters, and nesting material), which were repositioned once per day and completely substituted with others once per week (Fig. 1).

Every cage housed at least six adult rats. Standard housing consisted of a standard cage (40 x 30 x 20 cm), housing a maximum of three adult rats. Litter and food were the same in both environmental conditions; food and water were provided *ad libitum*.

4.2 Animal Treatment

For the experiment of ocular dominance (OD) plasticity in response to monocular deprivation (MD), after 2 weeks of EE or SC rearing (~P75) rats were anesthetized with avertin (1 ml/hg of body weight administered intraperitoneally). MD was performed trimming lid margins and suturing with 7-0 silk. Rats were allowed to recover from anesthesia and were returned to their cages. Eyelid closure was inspected daily until complete cicatrisation; subjects with even minimal spontaneous re-opening were excluded from the study. For the experiment of amblyopia recovery MD was performed at P21; also in this case the effectiveness of lid closure was checked daily and animals showing occasional lid reopening were not included. Adult rats (P60-P70) were then subjected to reverse suture (RS), under the same anesthesia: the long-term deprived eye was reopened, while the other eye was sutured as previously described. Great care was taken during the first week after RS to prevent inflammation or infection in the previously deprived eye through topical application of antibiotic and cortisone. One day after RS, rats were divided in two groups, i.e. they were either reared under SC or transferred to EE conditions.

4.3 Drug administration

A different group of rats were subjected to MD as described before. During the same anesthesia, an osmotic minipump (model 2002; Alzet, Palo Alto, CA) connected via PE tubing to a stainless steel cannula (30 gauge) was implanted in the visual cortex

contralateral to the deprived eye (2 mm lateral to lambda). Osmotic minipumps (flow rate, 0.5 µl/hr) were filled with: diazepam (2 mg/ml in 50% propylene glycol), BDNF antisense oligonucleotide (1 mg/ml in 9 % saline; Eurogentec, San Diego, CA, USA), pCPA (5 mg/ml in 9% saline; Sigma, St. Louis, MO, USA) or vehicle solution.

4.4 In Vivo Electrophysiology

Visual evoked potentials (VEPs) and single-cell activity were recorded from the binocular portion of the visual cortex (Oc1B). Rats were anesthetized with an intraperitoneal injection of 20% urethane (Sigma, St.Louis, MO, USA; 0,7 ml/hg of body weight) and mounted in a stereotaxic apparatus allowing full viewing of the visual stimulus. Additional doses of urethane were used to keep the anesthesia level stable throughout the experiment. The closed eye was reopened using scissors and both eyes were restrained in a fixed position by means of adjustable metal rings surrounding the external portion of the eye bulb. The pupil was always clearly observable between eyelid margins and we checked the occurrence of eventual dilatation, that would be treated with cortisone-based collyrium. Body temperature was continuously monitored with a rectal probe and maintained at 37.0°C with a thermostated electric blanket during the experiment. An electrocardiogram was monitored and respiration was facilitated by means of an oxygen mask. A portion of the skull (2 x 2 mm) overlying the OcB1 was carefully drilled and the dura madre was removed. A resin-coated microlectrode (Harvard apparatus, Edenbridge, UK) with tip impedance of 2 MΩ filled with NaCl (3M) was inserted into the cortex perpendicularly to the stereotaxic plane 4.8-5.2 mm lateral to the lambda (intersection between sagittal- and lambdoid-sutures). In the same penetration either VEP and single-cell recordings were performed.

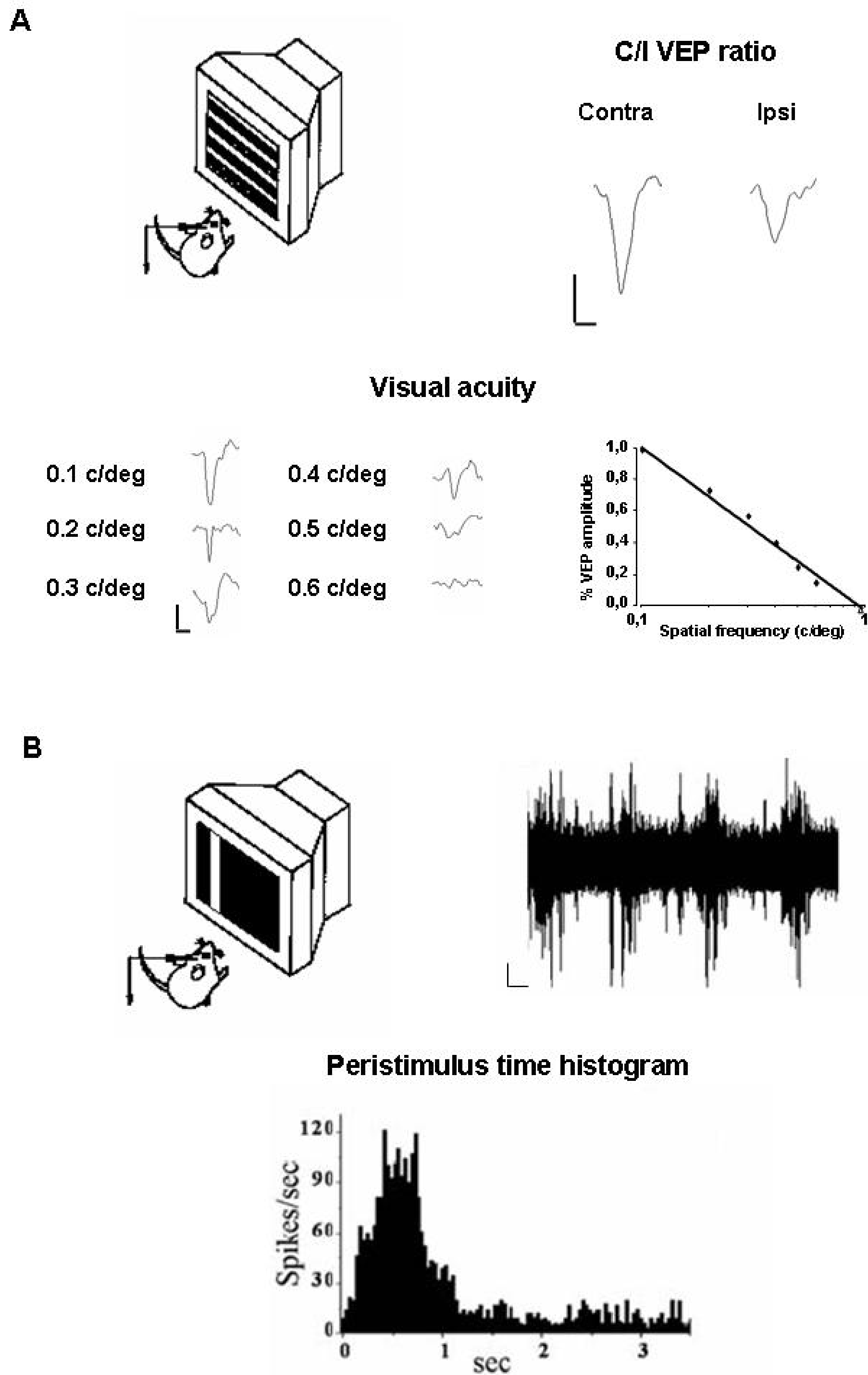


Figure 2: An illustrative diagram of in vivo electrophysiology. VEPs and single-cell activity were recorded from the binocular portion of the visual cortex. **(A)** For VEP recordings typical visual stimuli were horizontal sinusoidal gratings of different spatial frequency and contrast. C/I VEP ratio was calculated as the averaged ratio of VEP amplitudes recorded by stimulating the eye contralateral and ipsilateral, respectively, to the visual cortex where the recording is performed. Visual acuity was obtained by extrapolation to zero amplitude of the linear regression through the data points in a curve where VEP amplitude is plotted against log spatial frequency. Calibration bars: 50 μV, 100 ms. **(B)** For single-unit recordings visual stimuli were

light bars of different orientation moving on a computer display. A representative extracellular recording of spiking activity in response to the optimal oriented bar drifting is shown. Calibration bars: 1V, 1s. Spontaneous activity, peak response and receptive field size were determined from peristimulus time histograms (PSTHs).

VEP recordings Visual evoked potential (VEP) recordings were performed as previously described (Porciatti et al., 1999). Microelectrodes were advanced 100 or 400 μm within the cortex. At that depth VEPs had their maximal amplitude. Typical visual stimuli were horizontal sinusoidal gratings of different spatial frequency and contrast, generated by a VSG2/2 card (Cambridge Research System, Cheshire, UK) and presented on the face of a monitor suitably linearized by gamma correction. The display (mean luminance 25 cd/m2, area 24 x 26 cm) was placed 20 cm in front of the animal and centred on the previously determined receptive fields. Electrical signals were amplified (10000 fold), band-pass filtered (0.1–100 Hz), digitized (12 bit resolution) and averaged (at least 50 events in blocks of 10 events each) in synchrony with the stimulus contrast reversal. Transient VEPs in response to abrupt contrast reversal (0.5 Hz) were evaluated in the time domain by measuring the peak-to-trough amplitude and peak latency of the major component. We measured as binocularity index the VEP contralateral to ipsilateral ratio (C/I ratio), i.e. the ratio of VEP amplitudes recorded by stimulating the eye contralateral and ipsilateral, respectively, to the visual cortex where the recording is performed (Porciatti et al., 1999). During recording through one eye, the other was covered by a black adhesive tape. To prevent sampling bias for each animal at least three well-spaced penetrations were performed and at least ten series of responses from each eye alternatively were recorded. Care was taken to equally sample VEPs across the two cortical depths so that all layers contributed to the analysis. Visual acuity of each eye was obtained by extrapolation to zero

amplitude of the linear regression through the data points in a curve where VEP amplitude is plotted against log spatial frequency (Pizzorusso et al., 1996) (Fig. 2).

Single-unit recordings Single-unit recordings were performed as previously described (Lodovichi et al., 2000). Visual stimuli were light bars (contrast 90%; thickness, 3°; speed, 28°/sec) of different orientation computer-generated on the display. For each penetration site the position of receptive fields were mapped using a hand-held stimulator. Only cells with receptive fields within 20° of the vertical meridian were included in the analysis. Electrical signals were amplified (25000 fold), band-pass filtered (500–5000 Hz), digitized (12 bit resolution) and averaged (at least 10-20 stimulus presentations). During recording through one eye, the other was covered by a black adhesive tape. To prevent sampling bias for each animal at least three well-spaced penetrations were performed and at least 20-25 series of responses from each eye alternatively were recorded. Care was taken to equally sample cells across the whole cortical depth so that all layers contributed to the analysis. Cells were recorded at an interdistance of 60-70 µm from each other. Spontaneous activity and peak response were determined from peristimulus time histograms (PSTHs) recorded in response to the optimal oriented bar drifting (Fig. 2). OD classes were evaluated according to the method of Hubel and Wiesel. Neurons in ocular dominance class 1 were driven only by stimulation of the contralateral eye; neurons in ocular dominance classes 2 (ratio of contralateral to ipsilateral peak response ≥ 2) and 3 (ratio of contralateral to ipsilateral peak response between 1.2 and 2) were binocular and preferentially driven by the contralateral eye; neurons in ocular dominance class 4 were equally driven by the two eyes (ratio of contralateral to ipsilateral peak response between 0.83 and 1.2); neurons in ocular dominance classes 5 (ratio of contralateral to ipsilateral peak response between 0.5 and 0.83) and 6 (ratio of contralateral to ipsilateral peak response ≤ 0.5) were binocular and preferentially driven by the ipsilateral eye; neurons in ocular dominance class 7 were

driven only by the ipsilateral eye. For each animal, the bias of the OD distribution toward the contralateral eye [contralateral bias index (CBI)] was calculated as follows: CBI = [(N(1) – N(7)) + 2/3 (N(2) – N(6)) + 1/3 (N(3) – N(5)) + NTOT]/2NTOT, where N(i) is the number of cells in class i, and NTOT is the total number of recorded cells in a specific animal. OD score for each cell was calculated as follows: {[peak(ipsi) – baseline(ipsi)] – [peak(contra) – baseline(contra)]}/ {[peak(ipsi) – baseline(ipsi)] + [peak(contra) – baseline(contra]}, where peak is the maximal spike frequency evoked by visual stimulation (peak response), ipsi is the ipsilateral eye, baseline is the mean spiking frequency in the absence of stimulation (spontaneous activity), and contra is the contralateral eye. Cell responsiveness was assessed according to standard criteria in terms of the amplitude of modulation of cell discharge in response to an optimal visual stimulus (peak response divided spontaneous discharge). Receptive field size were determined from PSTHs, assuming as visual response the signal above a value equal to: mean spontaneous discharge + 2 standard deviation (Fagiolini et al., 1994).

4.5 In vitro electrophysiology: LTP and LTD

Each animals was decapitated and the brain was rapidly removed and immersed in ice-cold dissection buffer containing (in mM) 130 NaCl, 3.1 KCl, 1.0 K_2HPO_4, 4.0 $NaHCO_3$, 5.0 dextrose, 2.0 $MgCl_2$, 1.0 $CaCl_2$, 10 HEPES, 1.0 ascorbic acid, 0.5 myoinositol, 2 pyruvic acid, and 1 kynurenate, pH 7.3. Slices of visual cortex, 0.35 mm thick, were obtained using a vibratome (Leica, Nussloch, Germany) and gently transferred to an interface slice chamber. Here, the slices were maintained for 1h in an atmosphere of humidified 95% O_2 and 5% CO, and superfused with 35°C artificial cerebrospinal fluid (ACSF) at a rate of 1 ml/min. The ACSF was saturated with 95% O_2 and 5% CO, and had the same composition as the dissection buffer except that kynurenate was omitted. The

recording solution was identical to the dissection buffer, except for the following differences (in mM): 1.0 $MgCl_2$, 2.0 $CaCl_2$, 0.01 glycine, and no kynurenate. Chemicals were purchased from Sigma (St. Louis, MO, USA). Slices were perfused at a rate of 2 ml/min with 35°C oxygenated recording solution. Electrical stimulation (20-2000 µA, 100 µsec duration) was delivered with a bipolar concentric stimulating electrode (Frederick Haer Co., Bowdoinham, ME) placed at the border of white matter and layer VI. Field potentials (FPs) in layer III were recorded by means of a glass micropipette (1-3 MΩ) filled with NaCl (3 M). Synaptic responses were amplified (1000 fold), band-pass filtered (1 Hz–4 kHz), digitized stored on a computer. The amplitude of the maximum negative field potential in layer III was used as a measure of the evoked population excitatory synaptic current. In every experiment, a full input-output curve was generated. Baseline responses were obtained every 30 sec, with a stimulation intensity that yielded half-maximal responses. After achievement of at least a 15 min stable baseline (FP amplitude within 15% of change and with no evident increasing or decreasing trends), theta burst stimulation (TBS) or low frequency stimulation (LFS) were delivered respectively to induce either LTP or LTD. To induce LTP, two to five episodes of theta burst stimulation (TBS) were delivered at 0.1 Hz. TBS consists of 12 stimulus trains delivered at 5 Hz; each train consists of four pulses at 100 Hz. In LTD experiment, LFS consists of 15 min of pulses delivered at 1 Hz. After stimulus delivery FPs were recorded every 30 sec during 30 min.

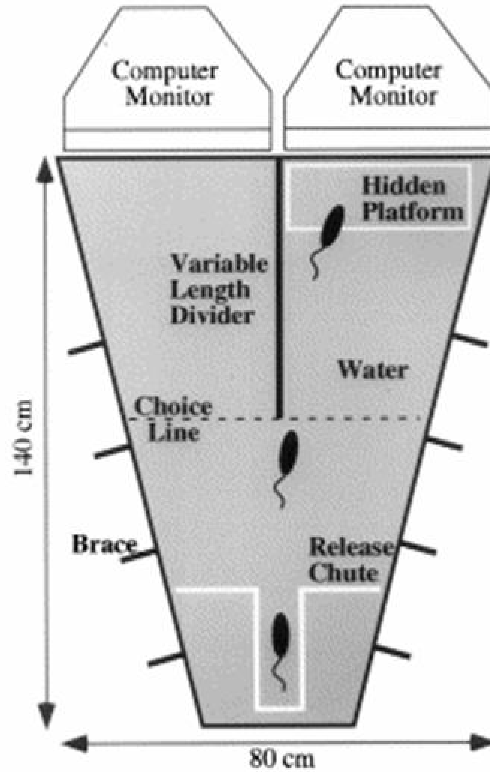
4.6 Behavioural assessment of visual acuity

Behavioural assessment of VA were performed as previously described (Prusky et al., 2000b). The basic apparatus consists of a trapezoidal-shaped pool with two computer-controlled monitors placed side-by-side at one end. The pool is made of 6 mm clear

Plexiglas and comprises a rectangular floor (140 cm long × 80 cm wide) and 55 cm (high) walls. The pool is wider at one end (80 cm) than the other (25 cm) and the two long walls and narrow end are finished on the inside with flat black paint to reduce reflections. Midline dividers (40 cm high) of different length sit in guides and extend from the end wall between the monitors into the pool, bisecting it along its long axis. The dividers are painted flat black on both sides to make them opaque and reduce reflections within the pool. The length of the divider sets the choice point and effective spatial frequency: it is the closest an animal can get to the monitors without entering one of the two arms. A portable escape platform (37 cm long × 13 cm wide × 14 cm high) is placed below one of the monitors. The pool is filled with tepid (22°C) water to a depth of 15 cm. White tempera dissolved in the water renders the platform invisible from water level. Visual stimuli are vertically-oriented square-wave gratings of different spatial frequency and contrast, displayed on two identical computer monitors suitably linearized by gamma correction (mean luminance 25 cd/m2, area 24 x 26 cm) that face into the wide end of the pool. The bottoms of the screens are at water level (Fig. 3). The rationale of this task is to use the animal's ability to associate a grating with escape from water, as a strategy to measure its VA. Animals must first be conditioned to distinguish a low spatial frequency from homogeneous grey with high reliability before the limit of this ability can be assessed at higher spatial frequencies. There are three phases to the task: pre-training shaping, task training and acuity testing. In a pre-training phase, animals are shaped gradually to locate a platform hidden below a screen displaying a low spatial frequency grating. A grating with a large period (~0.1 cycles per degree, c/deg) is displayed on one of the screens, and the platform is positioned directly below the grating. On the first trial, animals are removed from their holding cage and released, facing the screen, into the pool a few centimetres from the platform. Upon being released, most animals swim directly forward and touch the platform, then climb

upon it. They are allowed to remain on the platform for a few seconds and are subsequently removed and returned to their holding cage. On the next trial, the location of the grating and the platform are switched to the opposite side and another trial is run. After this routine is repeated a few times, the release distance from the platform is gradually increased until animals can reliably swim to the platform from the opposite end of the pool. In the training phase, animals are conditioned to distinguish between a low spatial frequency sine-wave grating (~0.1 c/deg) and homogeneous grey. The alternating pattern of the grating and platform position is replaced with a pseudorandom pattern where no more than three trials are allowed on one side. On all trials, animals are required to swim until they find the platform. If an animal breaks the plane perpendicular to the end of the divider on the side of the tank with the monitor displaying grey, the trial is recorded as an error, and after finding the platform, the animal is immediately required to run another trial. After animals have achieved near-perfect (80% or more) performance over 20–40 trials on a pseudorandom schedule in the training phase, the testing of VA can begin. For the testing phase small incremental changes in the spatial frequency of the stimulus are made between successive blocks of trials until the ability of animals to distinguish a grating from grey falls to chance. A pseudorandom display schedule is again used to determine on a trial-by-trial basis which monitor will display the test grating. If the animal makes a correct choice, the spatial frequency of the stimulus is increased by adding one cycle on the screen, and another trial is executed. This procedure continues for the low spatial frequencies until an error is made. Once an error occurs, additional trials are run until four correct responses are made in sequence, or seven correct choices are made in a block of ten trials. After trials covering approximately half of the animal's projected threshold are completed, the minimum number of trials is increased to three, and then again increased to four around three-quarters of the projected threshold. Animals are tested

A



B

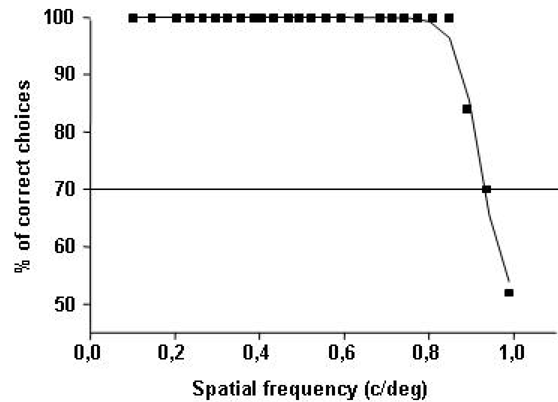


Figure 3: (A) Schematic diagram and components of the visual water box. View from above showing the major components including pool, midline divider, platform, starting chute and two monitors. The pool is filled with clean water. White tempera is dissolved in the water to render the platforms invisible from water level. Following release, animals choose to swim on the side of the pool displaying the grating in order to find the hidden platform and escape from the water. **(B)** For the testing phase small incremental changes in the spatial frequency of the stimulus are made between successive blocks of trials until the ability of animals to distinguish a grating from grey falls to chance. Visual acuity has been taken as the spatial frequency corresponding to 70% of correct choices on the sigmoidal function fitting the psychometric function.

in groups of five to eight in a session of 10-15 interleaved trials, with each session lasting 45–60 min and no more than three sessions are performed in a single day. Visual acuity has been taken as the spatial frequency corresponding to 70% of correct choices on the sigmoidal function fitting the psychometric function (Fig. 3). Since we measured VA of the open eye (not deprived) before performing RS, behavioural assessment of visual acuity in long-term monocularly deprived EE and SC rats started at P50. Then, after RS (at P60-P70) and the period of differential rearing, we measured VA of the formerly deprived eye. Therefore, VA measurement of the formerly deprived eye was completed when animals were about P100.

4.7 Visual cliff task

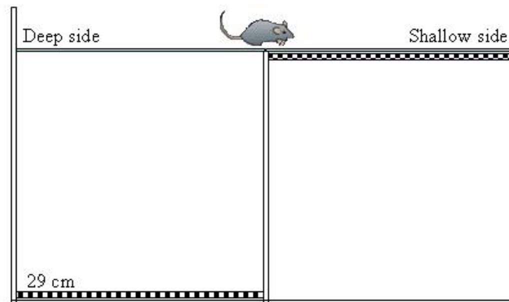
The apparatus consisted of a rectangular arena (100 × 40 × cm) constructed in poly(vinyl chloride) with black walls and bordered by black curtains to prevent the rat's escape. The arena consisted of two 38 by 50 plexiglass plates. On the shallow side a patterned floor was placed immediately below the glass plate; on the deep side the patterned floor was placed 29 cm below the glass plate. The patterns consisted in 2,5 cm black and white checked photographic paper covering the floor's surface. Incandescent lamps placed below the two patterned floors illuminate both deep and shallow surfaces to equate the brightness of the two sides. A telecamera was hanging up the apparatus and was connected to a computer by which the experimenter could observe and record the rat's behaviour. Each animal was placed on the shallow side and the experimenter measured the total time the rat spent exploring each of the two side of the arena. The trial ended when 5 minutes had elapsed. The box was cleaned between trials to stop the build-up of olfactory cues. The standard measure for the statistical analysis was the time spent exploring the two side of the apparatus. A discrimination index (DI) was calculated as the difference between the

time spent exploring the shallow and the deep side divided by the total time spent exploring the arena [(*s* - *d*)/(*s* + *d*), where *s* represents the shallow side and *d* represents the deep side] (Fig. 4). Since in the same apparatus the depth of both side could be varied, we tested the depth perception of the animals by a different procedure, taking advantage of a natural tendency of rats to jump from an elevated surface to surfaces below them.

Visual cliff task- exploration test:

Discrimination index =

(shallow side time – deep side time)/ total time



Visual cliff task- descent test:

Choice index (for each differential depth) =

(descents to shallow side – descents to deep side)/ total descents

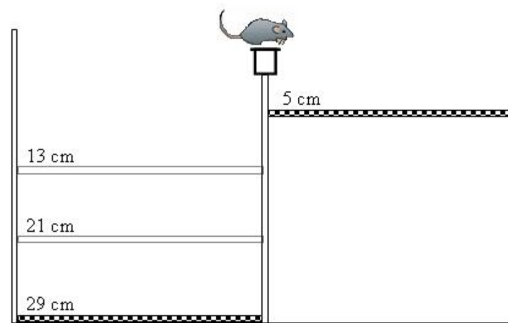


Figure 4: Schematic diagram and components of the visual cliff apparatus. **(A)** Exploration test. The apparatus consisted of a rectangular arena, consisting of two 38 by 50 plexiglass plates. On the shallow side a patterned floor was placed immediately below the glass plate; on the deep side the patterned floor was placed 29 cm below the glass plate. Each animal was placed on the shallow side and the experimenter measured the total time the rat spent exploring each of the two side of the arena. Discrimination index (DI) = $(s - d)/(s + d)$, where s represents the time spent on the shallow side and d the time spent on the deep side. **(B)** Descent test. In the apparatus described above the depth of the plates of both side could be varied. Rats were trained to descend from a central platform onto the nearer of the two plates and the differential depth between the two sides was made progressively smaller (24, 16, 8 cm). Each animal was placed on the center platform and waited for the choice. Choice index (CI) = $(s - d)/(t - f)$, where s represents the number of descents to the shallow side, d represents the number of descents to the deep side, t represents the total number of trials and f represents the number of falls.

Rats were mildly food deprived and trained to descend from a platform onto the nearer of two plates located on the two sides underneath. Jumps to the shallow side were rewarded. The differential depth between the two sides was made progressively smaller (24, 16, 8 cm) by means of mechanical scissor jack bolted under both the plates of the apparatus. Each animal was placed at one end of the center platform and was allowed 3 minutes to descend. The end of the platform on which the rat was placed was alternated. The position of the deep side with respect to the platform was changed randomly. The experimenter observed the rat behaviour trough the computer so as not to bias the animal's response. Each rat was tested four times for each depth. The surfaces of both sides and the platform were cleaned after each animal was tested. A preference for the shallow or the deep side was defined as placement of the four paws on the shallow or deep side within 3 min. Trials on which rats fell off the platform were recorded as falls in the data analysis. The actions of rats that failed to respond within the 3- min time were scored as "no-go" responses. A choice index (CI) for each differential depth was calculated as the difference between the number of correct choices and errors ("no-go" responses were scored as 0), divided by the total number of trials without falling [$(s - d)/(4 - f)$, where s represents the number of

descents to the shallow side, *d* represents the number of descents to the deep side and *f* represents the number of falls] (Fig. 4).

4.8 Sample preparation

Brain areas were dissected on ice, weighed to the mg sensitivity and added with an extraction buffer volume (Ascorbic Acid 25% solution 8.2%, $Na_2S_2O_5$ 5% solution 1.64%, HClO4 0.83M) in μl corresponding to three times the weight in mg of the specimen. This suspension was pottered and leaved on ice for thirty minutes and then centrifuged 30 minutes at 18000 rpm, 4°C. The supernatant was used as sample for the 5HT analysis.

4.9 *In vivo* Brain Microdialysis

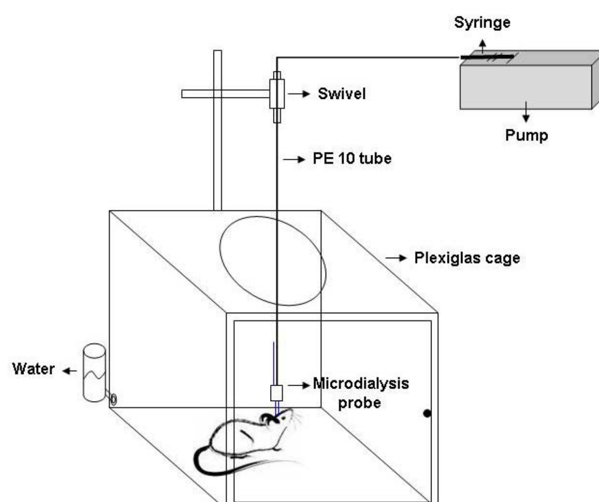


Figure 5: Schematic diagram of in vivo brain microdialysis. Sampling of dialysates was performed in freely moving animals. The microdialysis probe was inserted into the guide shaft and connected to a dialysis system pumping artificial CSF. After a stabilization period, six samples were collected every 20 min along 2 hours.

To perform brain microdialysis, rats were anesthetized with avertin and stereotaxically implanted with stainless steel guide shafts above the binocular visual cortex at coordinates: 7.3 mm posterior to bregma, 4.4 mm lateral to the mid-sagittal suture and 1 mm ventral to the skull. In vivo sampling of dialysates was performed inserting a microdialysis probe into the guide shaft. The probe was connected to a dialysis system pumping an artificial CSF (142 mM NaCl, 3.9 mM KCl, 1.2 mM CaCl2 , 1 mM MgCl2, 1.35 mM Na2HPO4 , pH 7.4) at a flow rate of 1 µl/min. The probe (Custom BR 1mm probe, BASi Instruments LTD, UK) protruded 1 mm from the tip of the guide shaft. Six hours after insertion of the probe (stabilization period), sampling was carried out. Six samples (20 µl/each) were collected every 20 min along 2 hours for each freely moving animal (Fig. 5). After brain microdialysis, animals were deeply anesthetized with chloral hydrate and perfused transcardially with PBS 1x followed by fixative (4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.4; PB). Brains were removed, post-fixed for 4-6h in the same fixative at 4°C, cryoprotected by immersion in 30% sucrose with 0,01% sodium azide solution in PB at 4°C and frozen by isopentane. Coronal sections (40 µm) were cut on a microtome and collected in PBS. Brain sections were then stained for cresyl violet to verify probes' location in Oc1B. Only those animals with a correct location of the probe were taken into account for further analysis.

4.10 High Performance Liquid Chromatography (HPLC)

For 5-HT analysis, standard solutions were prepared dissolving 5HT and Tryptophan (Sigma) with same proportion of extraction buffer solution components (Ascorbic Acid 25% solution 8.2%, $Na_2S_2O_5$ 5% solution 1.64%, HClO4 0.83M). Samples or standard solution 5HT content was measured by HPLC analysis according to Atkinson et al. (2006). Briefly, 20 µl of sample or standard solution were injected on to a Synergy 4 µm Hydro-RP

separation column (I.D.: 75.0mm x 3.00mm; Phenomenex srl, Bologna, Italy), fitted with a securityGuard C18 cartridge column (I.D. 4.0mm x 3.0 mm, Phenomenex srl, Bologna, Italy). The column was eluted isocratically at 0.8ml/min and 29°C with mobile phase (Ammonium Acetate 100mM pH 4.5: Methanol 12.5:1 v/v, filtered with 0.2 µm membrane) in a Waters Alliance 2690 HPLC apparatus. Detection was performed with a Waters 474 Scanning Florescence Detector with excitation and emission wavelengths set at 290nm and 337nm respectively and data analysis was performed with Waters Millenium software.

For the analysis of GABA and glutamate levels from microdialysates pre- column derivatization of samples and standards (Waters Alliance 2690 HPLC) with o-phtalaldehyde was performed. 20 µl of sample or standard solution were injected on to a Nova Pak C18 60 Å 4 µm separation column (I.D.: 3.9 mm x 150mm; Waters, Milford, MA, USA). The column was eluted at 0.9 ml/min and 30°C with a gradient program as follows: by definition, solvent A: 0.1M Sodium Acetate pH 5.8/methanol 20/80; solvent B: 0.1M Sodium Acetate pH 5.8/methanol 80/20; solvent C: 0.1M Sodium Acetate pH 6.0/methanol 80/20. Concerning the gradient program, initial isocratic step 5%A, 95%C from 0 to 5 min; 15%A, 85%B from 4 to 5 min and then isocratic until 9 min; 22%A, 66%B until 14.5 min and then 34%A, 66% B until 17 min; 5%A, 95%C until 19 min and then isocratic until 23 min. Homoserine was used as internal standard. Detection was performed with Waters 474 with excitation and emission wavelengths set at 350nm and 450nm respectively and data analysis was performed with Waters Millenium software.

4.11 Immunohistochemistry

Animals were deeply anesthetized with chloral hydrate and perfused transcardially with PBS 1x followed by fixative (4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.4;

PB). Brains were gently removed, post-fixed for 4-6 h in the same fixative at 4°C, cryoprotected by immersion in 30% sucrose with 0,01% sodium azide solution in PB at 4°C and frozen by isopentane. Coronal sections (40 µm) were cut on a microtome and collected in PBS. **BDNF:** Free-floating sections were incubated for 1-2 hours in a blocking solution (10% BSA, 0,3 % Triton X-100, in PBS, pH 7.4). Sections were then incubated overnight at 4°C in a solution of chicken polyclonal anti-BDNF antibody (1:400, Promega, Madison, WI, USA; 10% BSA, 0.1% Triton X-100 in PBS 1x). Primary antibody was revealed with biotinylated donkey anti-chicken (1:200, Promega, Madison, WI, USA; 10% BSA, 0.1% Triton X-100 in PBS 1x) followed by fluorescein-conjugated extravidin (1:300, Sigma, St.Louis, MO, USA, in PBS 1x). Sections of the two experimental groups were reacted together with the same immunohistochemical procedure. Images were acquired at 20x magnification (NA = 0.7; field 707 x 707 µm acquired at 1024 x 1024 pixels) using a confocal Olympus microscope and imported to the image analysis software MetaMorph to analyse the number of BDNF positive cells. To compare different specimens, the parameters of acquisition (laser intensity, gain, offset) were optimized at the start and then held constant throughout image acquisition. Counts were done on the entire thickness of Oc1B and normalized to the Oc1B area (mm^2). For each animal, at least five Oc1B sections were analyzed. **GAD67:** Free-floating sections were incubated for 1-2 hours in a blocking solution (10% BSA, 0,3 % Triton X-100, in PBS, pH 7.4). Sections were then incubated overnight at 4°C in a solution of mouse monoclonal anti-GAD67 antibody (1:1000, Chemicon MAB5406, Millipore, Billerica, MA, USA; 1% BSA, 0.3% Triton X-100 in PBS 1x). Primary antibody was revealed with Alexa 568 anti-mouse (1:400, Molecular Probes, Invitrogen, Carlsbad, CA, USA; 1% BSA, 0.1% Triton X-100 in PBS 1x). Images were acquired and analysed with the same method described for BDNF staining.

CHAPTER 5

RESULTS

5.1 Environmental enrichment reactivates OD plasticity in adult animals

VEP recordings

I initially examined the effects of a seven days (7 d) monocular deprivation (MD) on the ocular dominance (OD) plasticity of animals housed in environmental enrichment (EE) in adulthood. Long–Evans rats were raised in standard conditions (SC) until P60, then placed in EE for 21 d and subjected to MD during the last week of differential rearing. I recorded visual evoked potentials (VEPs) in the binocular region of the primary visual cortex (Oc1B) contralateral to the deprived eye to measure the response of cortical neurons to MD. VEPs are routinely used to assess both visual acuity (VA) and OD (binocularity) alterations in the visual cortex of rodents (Porciatti et al., 1999). In particular, OD distribution is evaluated calculating the contralateral-to-ipsilateral (C/I) VEP ratio, that is the ratio of VEP amplitudes recorded by stimulating the eye contralateral and ipsilateral, respectively, to the visual cortex where the recording is performed. The C/I VEP ratio is in the 2-3 range for adult normal (noMD-SC) rats, reflecting the predominance of crossed fibers in rodent retinal projections (Porciatti et al., 1999). As expected, 7 d of MD did not affect the C/I VEP ratio in the visual cortex contralateral to the occluded eye in control adult animals housed in standard conditions (MD-SC), confirming the absence of OD plasticity in adult rats (noMD-SC: $n = 4$, C/I VEP ratio = 2.39 ± 0.2; MD-SC: $n = 10$, C/I VEP ratio = 2.23 ± 0.24; One-way ANOVA, post-hoc Holm Sidak method, $p = 0.631$) (Fig. 6 a,b). However, when MD was performed in animals briefly housed in EE conditions (MD-EE), I observed a marked OD shift in favour of the non deprived eye,

reflected by a significant decrease of C/I VEP ratio (n = 11, VEP ratio = 1.02 ± 0.07; One way ANOVA, p < 0.001, post hoc Holm-Sidak method, see fig. 6 a,b), thus demonstrating the reactivation of OD plasticity in the visual cortex.

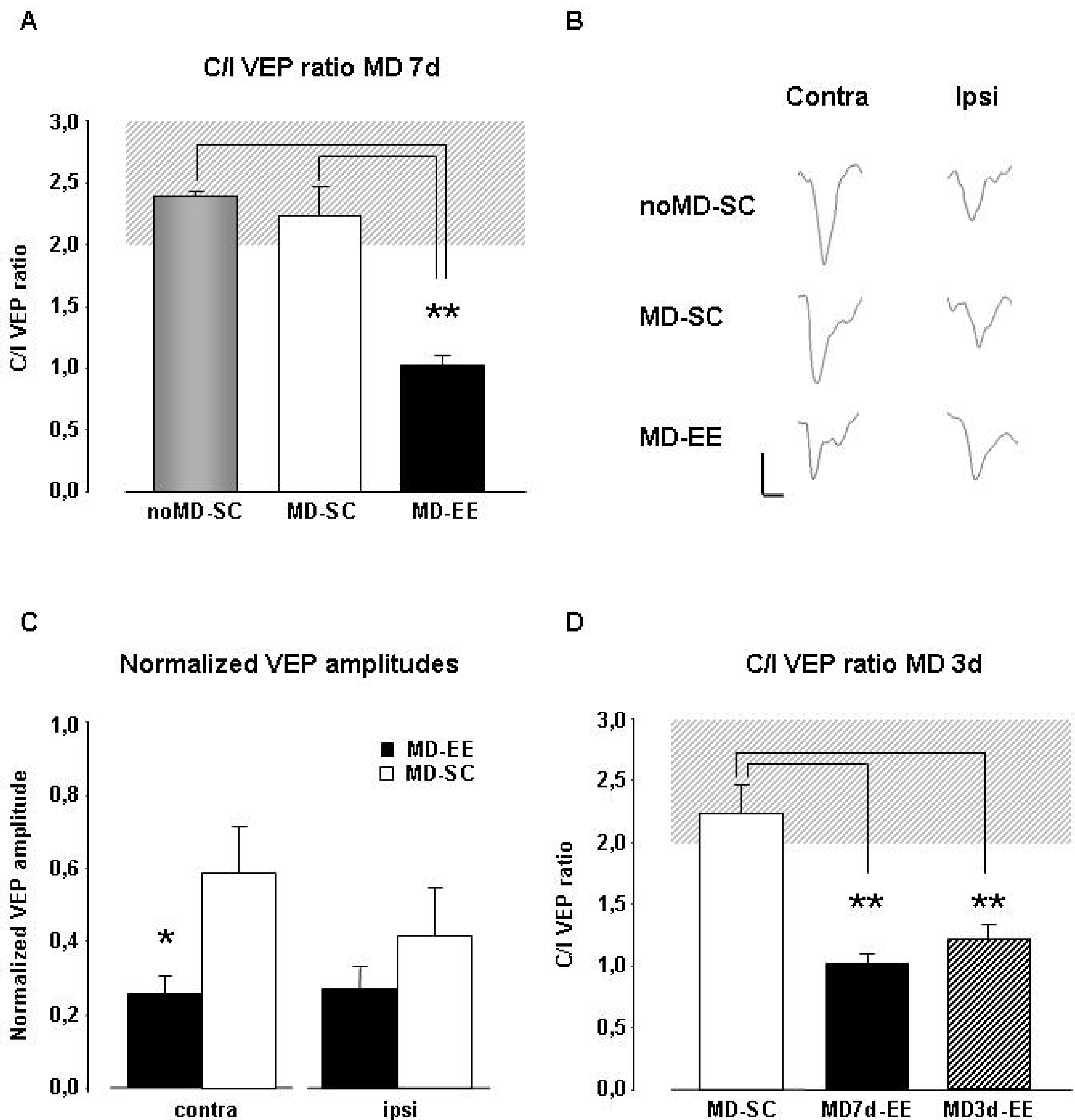


Figure 6: Environmental enrichment (EE) reactivates ocular dominance (OD) plasticity in the adult visual cortex: Visual Evoked Potentials (VEPs). (A) Contralateral to ipsilateral eye (C/I) VEP ratio mean values in noMD-SC, MD-SC and MD-EE animals. The grey box denotes the C/I VEP ratio range in adult normal animals. VEP recordings revealed that 7 days of MD did not affect the C/I VEP ratio in adult SC animals (noMD-SC: n = 4, VEP ratio = 2.39 ± 0.2; MD-SC: n = 10, VEP ratio = 2.23 ± 0.24; One-way

ANOVA, post-hoc Holm Sidak method, $p = 0.631$), whereas it led to a significant decrease in the C/I VEP ratio of EE animals, indicating the occurrence of a full OD shift in favour of the open eye, (n = 11, VEP ratio = 1.02 ± 0.07; One way ANOVA, $p < 0.001$, post hoc Holm-Sidak method). (**B**) Typical VEP responses to the stimulation of either contralateral or ipsilateral eye to the cortex in which the recording was performed in noMD-SC, MD-SC and MD-EE animals. Calibration bars: 50 μV, 100 ms (**C**) VEP amplitudes recorded in response to the stimulation of the contralateral and ipsilateral eye were compared in MD-EE and MD-SC rats. VEP amplitudes were normalized to the sum of the response to stimulation of the contralateral and ipsilateral eye (C + I) of control animals. The reduction of the C/I VEP ratio induced by MD in EE rats was entirely due to a significant depression in the response to stimulation of the occluded eye (MD-EE: n = 11, average VEP amplitude (normalized to C + I) = 0.25 ± 0.05; MD-SC: n = 10, average VEP amplitude (normalized to C + I) = 0.58 ± 0.13; t-test, $p = 0.025$). No difference in response to stimulation of the open eye was observed (MD-EE: average VEP amplitude (normalized to C + I) = 0.28 ± 0.05; MD-SC: average VEP amplitude (normalized to C + I) = 0.42 ± 0.13; t-test, $p = 0.751$). (**D**) C/I VEP ratio recorded in EE subjected to a 3 days MD. The grey box denotes the C/I VEP ratio range in adult normal animals. 3d MD in EE animals led to a significant decrease of C/I VEP ratio, which was comparable to that recorded after 7d of MD (n = 5, C/I VEP ratio = 1.21 ± 0.1; One way ANOVA, $p < 0.001$, post-hoc Holm-Sidak method; fig. 1c). Asterisks indicate statistical significance: (*) $p < 0.05$; (**) $p < 0.001$. Error bars represent SEM.

OD shift may be due to a strengthening of the response to stimulation of the non deprived eye, an event detected in both young and adult animals, or to a depression of the amplitude response to stimulation of the deprived eye, as observed only in the developing visual cortex. To distinguish between these two possibilities, I compared VEP amplitudes in response to stimulation of each eye after MD in EE and SC animals. To facilitate the comparison across animals, VEP amplitudes at each recording site were normalized to the sum of the responses to stimulation of the contralateral and ipsilateral eye (C + I) (He et al., 2006). I provide evidence showing that OD plasticity in EE animals acts through a juvenile-like mechanism. VEPs recorded in the visual cortex contralateral to the deprived eye revealed a marked decrease in the response to stimulation of the occluded eye in EE rats (MD-EE: n = 11, average VEP amplitude (normalized to C + I) = 0.25 ± 0.05; MD-SC: n = 10, average VEP amplitude (normalized to C + I) = 0.58 ± 0.13; t-test, $p = 0.025$; fig. 6c), whereas I did not notice any sign of difference in the VEP amplitudes recorded in response to stimulation of the open eye (MD-EE: average VEP amplitude (normalized to C

+ I) = 0.28 ± 0.05; MD-SC: average VEP amplitude (normalized to C + I) = 0.42 ± 0.13; t-test, $p = 0.751$).

If the plasticity restored by EE in adult animals is comparable to that present early in life (CP), a short period of MD should be sufficient to shift binocularity in visual cortical neurons. Remarkably, I found that 3d MD was able to effectively induce a marked OD shift, resulting in a mean C/I VEP ratio comparable to that obtained in MD-EE rats subjected to 7d of MD and significantly decreased with respect to both MD-SC rats and normal adult values (n = 5, C/I VEP ratio = 1.21 ± 0.1; One way ANOVA, $p < 0.001$, post-hoc Holm-Sidak method; fig. 6d).

Single-unit recordings

Spike activity represents the output of visual neurons and is not detected by VEPs. To make sure that EE plasticity is not only due to subthreshold modifications of postsynaptic potentials I also measured the OD distribution in response to MD in EE and SC rats by performing extracellular single-unit recording from the Oc1B contralateral to the occluded eye. OD of cortical neurons was assessed by quantitative evaluation of responsiveness to optimal visual stimulation of either eye and every single cell recorded was assigned to an OD class following the classic Hubel and Wiesel classification. Each distribution was represented as a single parameter, the contralateral bias index (CBI), which is an indicator of the degree to which the contralateral eye dominates cortical responses. The CBI of noMD-SC animals displayed the typical bias toward contralateral eye input (n = 5; CBI = 0.64 ± 0.04). I found that 7 d of MD were ineffective in inducing a change in eye preference in SC rats, being the CBI of these animals superimposable to that measured in non-deprived rats (n = 5; CBI = 0.62 ± 0.03; One-way ANOVA, post-hoc Holm Sidak method, $p = 0.721$; fig. 7a). In contrast, a prominent OD shift in favour of the open eye was

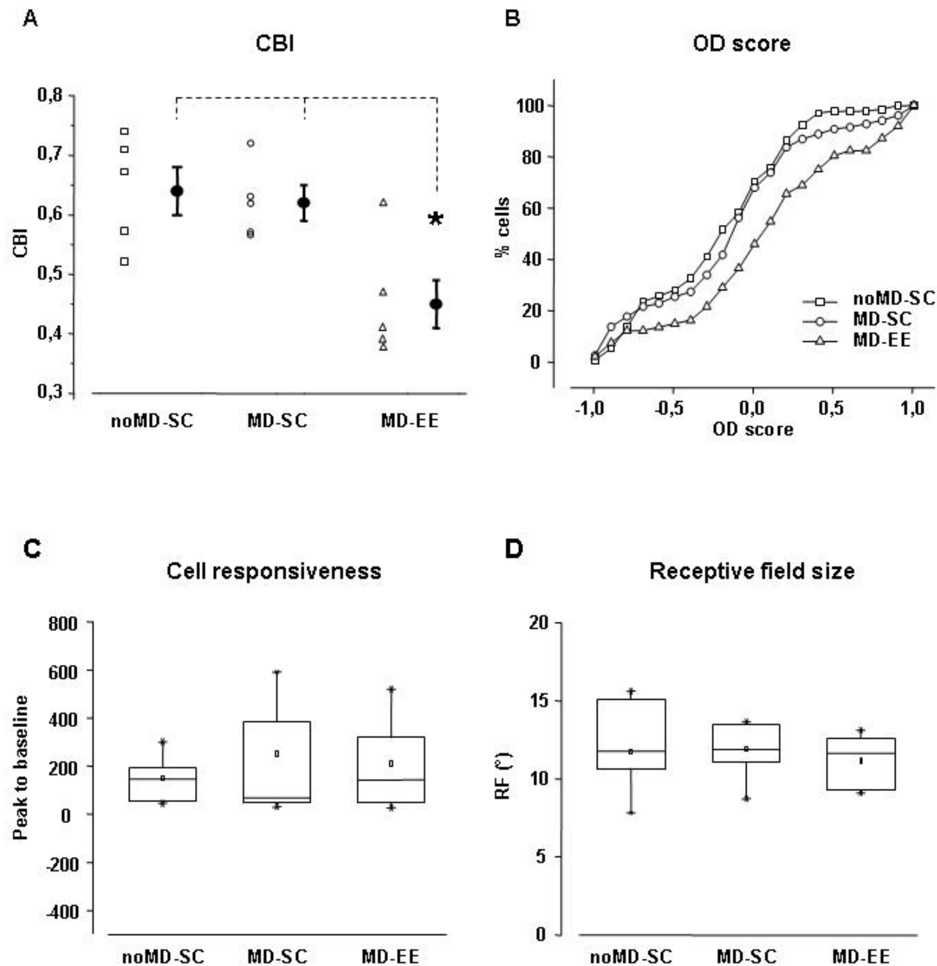


Figure 7: EE restores OD plasticity in the adult visual cortex: extracellular single-cell recordings. (A) CBI values for noMD-SC, MD-SC and MD-EE animals. Filled circles represent the average CBI ± SEM for each experimental group; open symbols represent individual CBIs for each animal. The CBI of MD-SC rats was not significantly different from that of noMD-SC animals (noMD-SC: n = 5, CBI = 0.64 ± 0.04; MD-SC: n = 5, CBI = 0.62 ± 0.03; One-way ANOVA, post-hoc Holm Sidak method, p = 0.721), whereas the CBI of MD-EE rats significantly differed from that of the two control groups (n = 5, CBI = 0.45 ± 0.04; One-way ANOVA p = 0.009, post-hoc Holm Sidak method). An asterisk indicates statistical significance: (*) p < 0.05. Error bars represent SEM. **(B)** OD score cumulative distribution for all different experimental groups. OD score distribution for noMD-SC (n = 5; 132 cells) and MD-SC animals (n = 5, 153 cells) did not significantly differ (Kolmogorov-Smirnov test, p > 0.05) between each other, whereas OD score distribution for MD-EE group (n = 5, 148 cells) was shifted towards the non deprived eye and statistically different from those of the two control groups (Kolmogorov-Smirnov test, p < 0.05). **(C) and (D)** Functional basic properties of visual cortical neurons were not affected in MD-SC and MD-EE animals. We analysed cell responsiveness and receptive field (RF) size in the same animals in which OD evaluation was performed. C: Cell responsiveness for each unit was expressed as the ratio between the peak response and the mean baseline activity obtained by optimal stimulation of the preferred eye. Data are represented as box charts. For each box chart, the central horizontal line represents the median value, the other two horizontal lines are the 25th and 75th percentiles;

error bars denote the 5th and 95th percentiles; square symbols denote the mean value. No statistical difference was present between all groups (One-way ANOVA, $p = 0.732$). D: RF size for each cell was calculated on the basis of the peristimulus time histogram obtained by optimal stimulation of the preferred eye and was expressed in degrees (deg) of visual angle. Data are represented as box charts. No statistical difference of RF size distribution was detectable between all groups (mean RF size: noMD-SC = 13.7° ± 1.8°; MD-SC = 11.9° ± 0.8°; MD-EE = 11.2° ± 0.8°; One-way ANOVA, $p = 0.361$).

evident in EE rats. Statistical testing demonstrated that CBI values were lower in MD-EE rats compared with normal and deprived standard animals (n = 5, CBI = 0.45 ± 0.04; One-way ANOVA $p = 0.009$, post-hoc Holm Sidak method; fig. 7a). In addition, to allow a finer and statistically more robust comparison of OD distributions I computed the normalized OD score of single neurons. The cumulative distribution of OD score did not differ between noMD-SC (132 cells) and MD-SC animals (153 cells; Kolmogorov-Smirnov test, $p > 0.05$), whereas it was statistically different in MD-EE animals compared to noMD-SC and MD-SC rats (148 cells; Kolmogorov-Smirnov test, $p < 0.05$; fig. 7b), indicating that MD is able to induce visual cortex plasticity only in EE animals.

I also checked that EE did not affect the physiological response properties of visual cortical neurons in the same animals in which CBI and OD score were recorded. EE exposure does not alter neither the visual responsiveness of cortical neurons, as assessed by measuring the signal-to-noise ratio for each recorded cell, neither the receptive field (RF) distribution of the cell population (One-way ANOVA, $p > 0.05$ for both comparison; fig. 7 c,d).

5.2 Binocular decorrelation impairs visual depth perception in EE animals

I assessed whether the binocularity shift observed in MD-EE animals was correlated with an impairment in visual depth perception, comparing the performance of EE and SC

animals on two different versions of the visual cliff task under binocular vision condition re-established after MD.

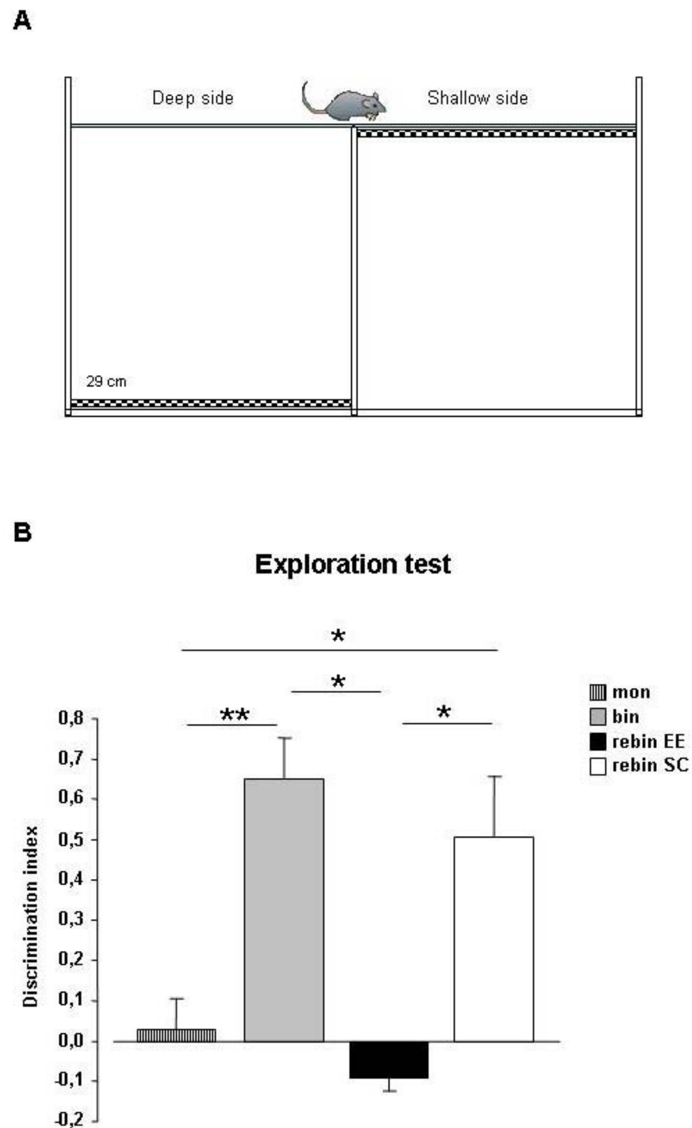


Figure 8: Binocular decorrelation impairs visual depth perception in adult EE animals: exploration test. (A) An illustrative diagram of the visual cliff apparatus used for the exploration test. Discrimination index (DI) = $(s - d)/(s + d)$, where s represents the time spent on the shallow side and d the time spent on the deep side. **(B)** Discrimination performance in the exploration version of visual cliff task for all the different experimental groups. Statistical analysis revealed that DI of BIN (n = 9, 0.65 ± 0.1) and MON rats significantly differed (n = 9, 0.03 ± 0.08; t-test, $p < 0.001$). DI of REBIN-SC animals (n = 10, DI = 0.51 ± 0.15) was similar to that of BIN rats, whereas DI of REBIN-EE animals (n = 6, DI = -0.09 ± 0.03) was comparable to that calculated for MON rats (One way ANOVA on Ranks, $p< 0.001$, post hoc Dunn's method). Asterisks indicate statistical significance: (*) $p < 0.05$; (**) $p < 0.01$. Error bars represent SEM.

I initially tested the animals by using a simple exploration test in which I observed the spontaneous preference of the rats for the deep or the shallow side of the arena, taking advantage of the assumption that animals with normal stereoscopic perception are tending to avoid the shallow part (for an illustrative scheme of the apparatus, see fig. 8a). For each animal I measured the time spent exploring the two side of the apparatus and a discrimination index (DI) was calculated as the difference between the time spent exploring the shallow and the deep side divided by the total time spent exploring the arena [$(s - d)/(s + d)$, where s represents the shallow side and d represents the deep side]. The spontaneous behaviour of rats was strongly influenced by their viewing condition (i.e. monocular or binocular). Since the absence of statistical difference, data from SC and EE animals in binocular (BIN; SC: n = 4, DI = 0.62 ± 0.12; EE: n = 5, DI = 0.68 ± 0.17; t-test, $p = 0.786$) and monocular conditions (MON; SC: n = 4, DI = 0.05 ± 0.04; EE: n = 5, DI = 0.01 ± 0.14; Mann-Whitney Rank Sum test, $p = 0.730$) were pooled together. I showed that BIN rats spent a significantly greater period of time on the shallow side of the arena (n = 9, DI = 0.65 ± 0.1), whereas MON animals exhibited no side preference on the visual cliff (n = 9; DI = 0.03 ± 0.08; t-test, $p < 0.001$), indicating that depth perception is compromised in absence of binocular cues (fig. 8b).

Remarkably, when the animals were tested after the restoration of binocular vision (i. e. the day after the reopening of the deprived eye, REBIN), REBIN-SC rats showed a significant preference for the shallow side of the visual cliff (n = 10, DI = 0.51 ± 0.15); on the contrary, REBIN-EE rats exhibited a behaviour completely comparable to that observed for MON animals, equally exploring the deep or the shallow side of the arena (n = 6, DI = -0.09 ± 0.03; Kruskal-Wallis One way ANOVA on Ranks, $p < 0.001$, post hoc Dunn's method) (see fig. 8b).

To further investigate the effects of MD on visual depth perception in adult animals, I tested the same four groups of animals with a different version of the visual cliff task. This procedure takes advantage of a natural tendency of rats to jump from an elevated board to planes situated below them. Rats were mildly food deprived and trained to associate a reward (i.e. a pellet of food) to the descent onto the shallow plane located under the board. Then, the differential depth (DD) between the two sides was made progressively smaller, as previously reported (Tees and Midgley, 1978) (for an illustrative scheme of the apparatus, see fig. 9a). A choice index (CI) for each DD was calculated as the difference between the number of correct choices and errors, divided by the total number of trials without falling [$(s - d)/(t - f)$, where s represents the number of descents to the shallow side, d represents the number of descents to the deep side, t represents the total number of trials and f represents the number of falls].

BIN animals were able to discriminate the differential depth, descending to the shallow side, when the depth difference between the two platforms was 24 cm (n = 16, CI = 0.96 ± 0.02) or 16 cm (CI = 1 ± 0), whereas they did a significantly worse performance at 8 cm of DD (CI = 0.27 ± 0.13; $p < 0.001$, One-way ANOVA, post-hoc Tukey test). A similar analysis revealed that MON vision allowed rats to discriminate the shallow side when the depth difference between the two platforms was 24 cm (n = 17, CI = 0.86 ± 0.04), but MON rats exhibit a significant decay in the percentage of descents onto the shallow side at 16 cm and at 8 cm of DD ($CI_{16cm} = 0.58 \pm 0.09$, $CI_{8cm} = 0.09 \pm 0.15$; One-way ANOVA, post-hoc Tukey test, $p < 0.05$, see fig. 9b). Since the absence of statistical difference, data from SC and EE animals in BIN (SC: n = 12; EE: n = 4; Two-way ANOVA, $p = 0.427$) and MON conditions (SC: n = 11; EE: n = 6; Two-way ANOVA, $p = 0.498$) were pooled together. A Two-way ANOVA test underlined a significant

discrepancy between the performances of BIN and MON rats at 16 cm and 8 cm of DD ($p < 0.001$, post-hoc Holm-Sidak method).

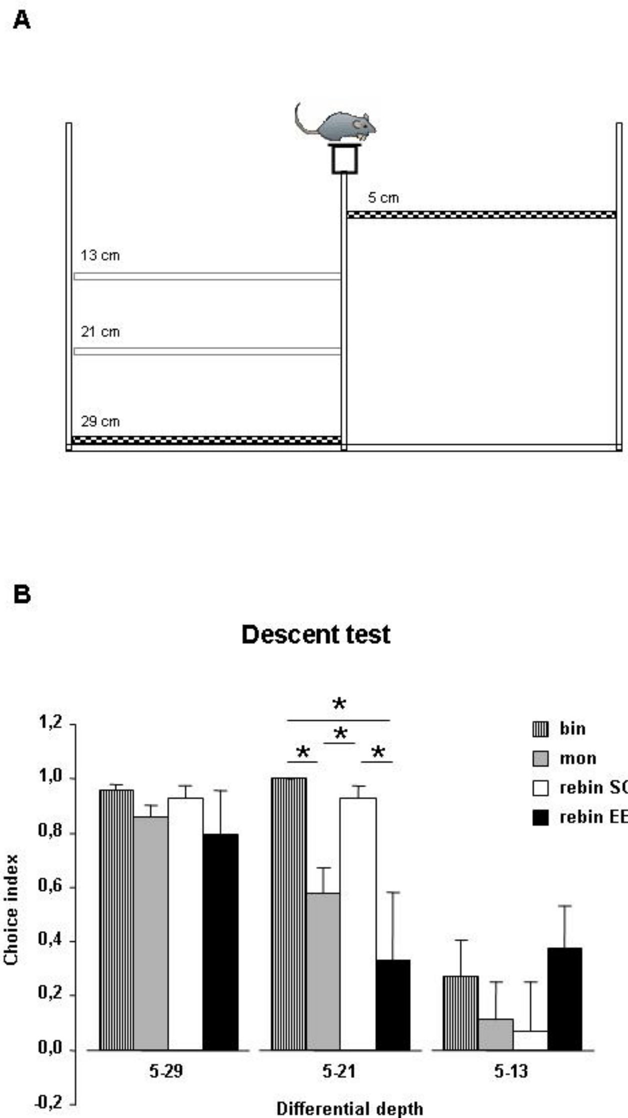


Figure 9: Binocular decorrelation impairs visual depth perception in adult EE animals: descent test. **(A)** An illustrative diagram of the visual cliff apparatus used for the descent test. Choice index (CI) = $(s - d)/(t - f)$, where s represents the number of descents to the shallow side, d represents the number of descents to the deep side, t represents the total number of trials and f represents the number of falls. **(B)** Depth discrimination performance in the descent version of visual cliff task for all the different experimental groups. Statistical analysis revealed that at 16 cm of DD the CI of BIN animals (n = 16, 1 ± 0) was significantly different from that of MON rats (n = 17, 0.58 ± 0.09; Two-way ANOVA, post-hoc Tukey test, $p < 0.05$). CI of REBIN-SC animals (n = 7, 0.93 ± 0.05) was comparable to that of BIN rats, whereas DI of

REBIN-EE animals (n = 10, 0.38 ± 0.17) was similar that calculated for MON rats (Two-way ANOVA, post-hoc Tukey test, $p < 0.05$). An asterisk indicate statistical significance: (*) $p < 0.05$. Error bars represent SEM.

Remarkably, the reinstatement of binocular vision after MD led to a performance completely comparable to BIN animals in SC rats (REBIN-SC; n = 7; $CI_{24cm} = 0.93 \pm 0.05$, $CI_{16cm} = 0.93 \pm 0.05$, $CI_{8cm} = 0.07 \pm 0.18$) and to a performance similar to MON animals in EE rats (REBIN-EE; n = 10, $CI_{24cm} = 0.88 \pm 0.10$, $CI_{16cm} = 0.38 \pm 0.17$, $CI_{8cm} = 0.25 \pm 0.12$; Two-way ANOVA, $p < 0.001$) (see fig. 9b): in particular, at 16 cm of DD, REBIN-SC rats exhibit a CI equal to that of BIN rats and statistically different with respect to the performance of MON and REBIN-EE rats, while REBIN-EE rats show a CI equivalent to that obtained by MON rats, but different from that calculated for BIN and REBIN-SC rats (Holm-Sidak method, $p < 0.05$).

5.3 5-HT is crucially involved in the reopening of OD plasticity

I tested whether serotonin (5-HT) has a fundamental role in restoring visual cortical plasticity in adult EE animals. I measured 5-HT in homogenates of the Oc1B of the visual cortex in adult rats. Visual cortex samples were collected from adult animals after 15 days of EE exposure (n = 8) or from animals reared in SC (n = 6) and 5-HT content was assessed by means of HPLC. I found a significant raise of 5-HT levels in the visual cortex of EE animals (EE = 54.4 ± 8.8 nM; SC = 24.5 ± 9.7 nM; t-test, $p = 0.043$); the ratio between EE and SC visual cortex levels was 2,22, corresponding to an enhancement in 5-HT production larger than 200% (Fig. 10a).

I then investigated whether the increase of 5-HT levels documented in the visual cortex of EE animals was causally linked to the restoration of OD plasticity. To address this issue I reduced serotoninergic transmission in EE rats, by employing intracortical microperfusion via osmotic minipumps of para-chlorophenylalanine (pCPA), a well known

inhibitor of the activity of 5-HT synthetic enzyme tryptophan hydroxylase which has been previously used to reduce 5-HT content in nervous tissue (Zetterström et al., 1999; Kornum et al., 2006; Hritcu et al., 2007).

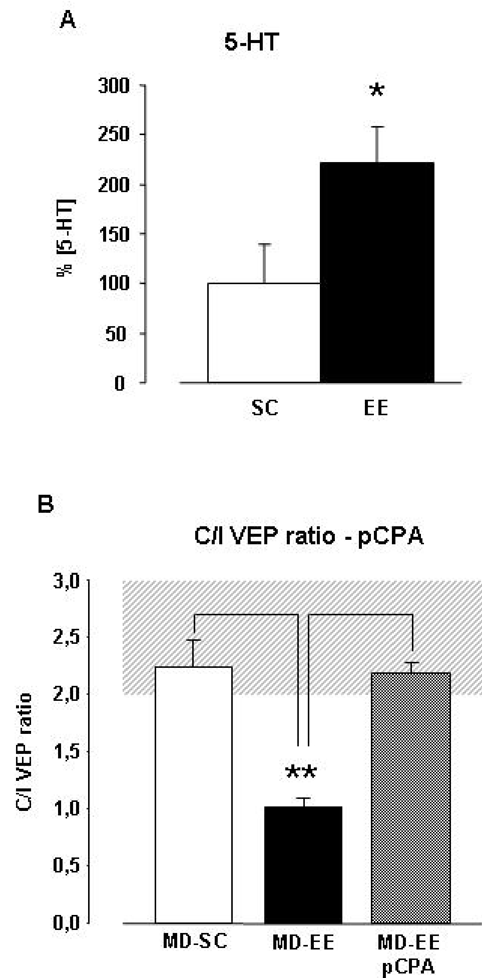


Figure 10: A crucial role for 5-HT in reopening EE-induced OD plasticity in adulthood. (A) EE strengthens serotoninergic neurotransmission in the visual cortex. 5-HT levels in the visual cortex of SC (n = 6; 24.5 ± 9.7 nM) and EE rats (n = 8; 54.4 ± 8.8 nM), assessed by *ex vivo* HPLC. 5-HT content in the EE visual cortex was normalized to the mean value in the control cortex. Statistical analysis revealed that 5-HT content was significantly increased in EE animals with respect to SC rats (t-test, p =0.043). **(B)** Chronic infusion of pCPA in the visual cortex prevents OD shift in MD-EE rats. VEP ratio mean values for all the different experimental groups. The grey box denotes the C/I VEP ratio range in adult normal animals. VEP recordings revealed that MD did not affect VEP ratio in MD-EE-pCPA animals. VEP ratio was statistically lower in MD-EE than in MD-SC and in MD-EE-pCPA rats (n = 7, VEP ratio = 2.19 ± 0.09). We found no difference in VEP ratio between MD-SC and MD-EE-pCPA animals (One-way ANOVA on ranks, post-hoc

Dunn's method, $p > 0.05$). Asterisks indicate statistical significance: (*) $p < 0.05$; (**) $p < 0.001$. Error bars represent SEM.

To check pCPA effectiveness in reducing 5-HT production I measured the 5-HT content in the visual cortex of treated animals by means of HPLC. The 5-HT content in the pCPA treated cortex was significantly reduced with respect to that observed in the visual cortex of EE untreated animals and returned to levels detected in control animals (One-way ANOVA on ranks, post-hoc Dunn's method, $p > 0.05$).

I evaluated OD plasticity in MD-EE animals treated with pCPA during the MD week by recording C/I VEP ratio in the Oc1B contralateral to the occluded eye. Our results showed that pCPA administration completely prevented the OD shift observed in MD-EE rats: I found no differences in C/I VEP ratio between MD-SC animals ($n = 10$, C/I VEP ratio = 2.23 ± 0.24) and MD-EE rats treated with pCPA (MD-EE-pCPA; $n = 7$, C/I VEP ratio = 2.19 ± 0.09; One-way ANOVA on ranks, post-hoc Dunn's method, $p > 0.05$; fig. 10b). EE rats infused with the vehicle solution exhibited a significant shift of OD distribution in response to MD ($n = 5$, C/I VEP ratio= 0.99 ± 0.07; One-way ANOVA on ranks, $p < 0.001$, post hoc Dunn's method).

5.4 Excitation-inhibition balance regulates plasticity

Because there is evidence that the maturation of cortical inhibitory circuits ends plasticity in the visual system (Hensch, 2005a), I investigated whether the restored plasticity under EE conditions was accompanied by a reduction of visual cortex inhibition. I quantified the extracellular levels of GABA and glutamate (GLU) in the Oc1B of EE and SC animals, using *in vivo* brain microdialysis and HPLC. Neurotransmitter basal release was assessed in adult animals after 15 days of EE exposure and in animals reared in SC. My results indicated that intracortical inhibition was markedly reduced in the visual cortex of EE

animals: extracellular GABA levels were diminished by a factor of three in the Oc1B of EE rats ($n = 5$, 1.53 ± 0.4 μM) compared to SC animals ($n = 8$, 4.62 ± 0.97 μM; t-test, $p = 0.041$) (Fig. 11a). On the contrary, I did not detect any difference in extracellular levels of GLU between EE ($2.73 \pm 0,3$ μM) and SC rats ($3.6 \pm 0,42$ μM; t-test, $p = 0.168$).

To further analyse the effects of EE on the GABAergic system, I assessed the expression of GAD67 in the visual cortex of EE and SC animals by means of semi-quantitative immunohistochemistry. GAD67 is the 67 kDa isoform of the glutamic acid decarboxylase (GAD), the enzyme responsible for GABA synthesis. I collected the brain samples from adult animals housed in EE for 15 d and animals reared in SC. We found that the number of GAD67-positive interneurons normalized to the area of Oc1B ($GAD67^+$) was lower in Oc1B of EE rats with respect to control rats (EE: $n = 6$, $GAD67^+ = 125.39 \pm 2.92$ cells/mm^2; SC: $n = 5$, $GAD67^+ = 150.56 \pm 9.00$ cells/mm^2; Mann-Whitney Rank Sum test, $p = 0,03$). The decrease of GAD67 expression was significant both in layers II-III (EE: $GAD67^+ = 139.19 \pm 6.04$ cells/mm^2; SC: $GAD67^+ = 175.19 \pm 10.37$ cells/mm^2) and V-VI (EE: $GAD67^+ = 85.56 \pm 6.43$ cells/mm^2; SC: $GAD67^+ = 98.12 \pm 8.33$ cells/mm^2) but not in layer IV (EE: $GAD67^+ = 147.85 \pm 8.26$ cells/mm^2; SC: $BDNF^+ = 172.53 \pm 13.54$ cells/mm^2; Two-way Anova, $p < 0.001$, post-hoc Holm-Sidak method).

It has been demonstrated in young and adult animals that the balance between excitatory and inhibitory neurotransmission is able to dynamically regulate the synaptic modification threshold of neuronal responses in the visual cortex (Kirkwood and Bear, 1994; Kirkwood et al., 1996; Huang et al., 1999). I assessed long-term potentiation of layer II-III field potentials induced by theta-burst stimulation from the white matter (WM-LTP) and long-term depression of layer II-III field potentials after low-frequency stimulation (LFS) from the white matter (LTD) in the visual cortex of EE and SC animals. Slices for *in*

vitro electrophysiological recordings were collected from adult animals exposed to EE for 15 d and SC animals.

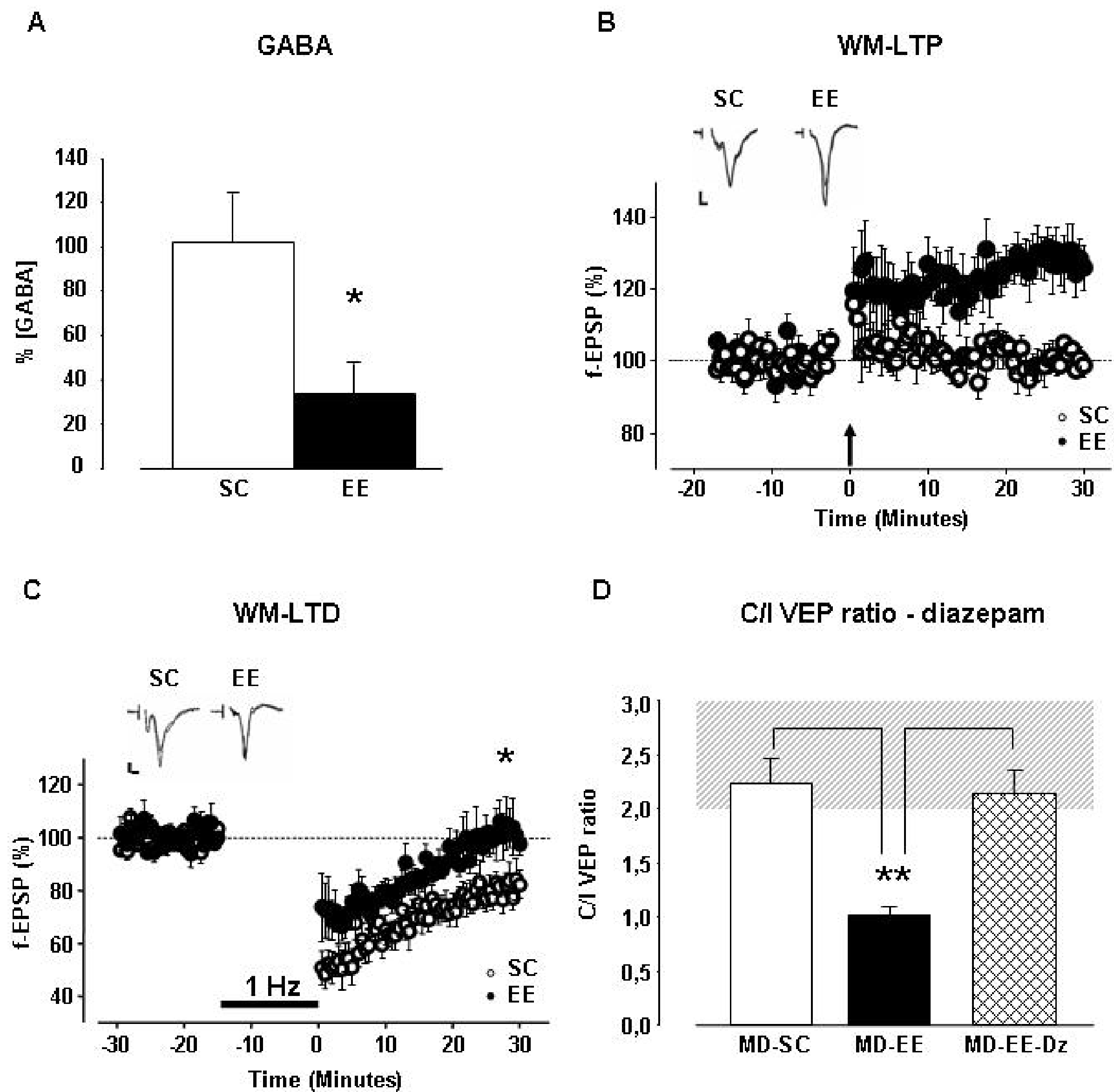


Figure 11: EE reduces GABAergic neurotransmission in the adult visual cortex. (A) EE reduces GABA release in the visual cortex. GABA levels in the visual cortex of SC (n = 8, 4.62 ± 0.97 μM) and EE rats (n = 5, 1.53 ± 0.4 μM), measured by in vivo microdialysis and HPLC. GABA content in EE visual cortex was normalized to the mean value of the control cortex. Statistical analysis revealed that GABA content was significantly decreased in EE animals with respect to SC rats (t-test, p = 0.041). **(B)** EE restores LTP synaptic plasticity in the adult visual cortex. Average time course of layer II-III field potential (FP) amplitude before and after Theta Burst Stimulation (TBS, arrow) delivered at the border of the white matter (WM). WM-LTP (measured 20–30 min after TBS) was significantly greater in adult rats given environmental enrichment (n =

6; 128.4 ± 6.7 % of pre-TBS baseline amplitude) than in those maintained in standard conditions (n = 5; 100.7 ± 3.1 % of pre-TBS baseline amplitude) (Two-way repeated measures ANOVA, $p < 0.05$). The averaged FP amplitudes after TBS were normalized to the mean value of the baseline recorded for each group. Sample traces before (thin line) and 25–30 min after (thick line) TBS are also shown. Scale bars are 100 μV and 2.5 ms. **(C)** EE reduces WM-LTD in the adult visual cortex. Average time course of layer II-III FP amplitude before and after Low Frequency Stimulation (LFS, 15 minutes, blue segment) delivered at the border of the WM. Slices from EE animals (n = 7) exhibited a significant decrease in the expression of WM-LTD (measured 20-30 min after LFS delivery; 101.7 ± 7.4 % of pre-LFS of baseline amplitude) with respect to control animals (n = 6; 80.4 ± 5.3 % of pre-LFS baseline amplitude) (Two-way repeated measures ANOVA, $p < 0.05$). The averaged FP amplitudes after TBS were normalized to the mean value of the baseline recorded for each group. Sample traces before (thin line) and 25–30 min after (thick line) LFS are also shown. Scale bars are 100 μV and 2.5 ms. **(D)** Osmotic infusion of diazepam in the visual cortex blocks OD shift in MD-EE rats. C/I VEP ratio mean values for MD-SC, MD-EE and MD-EE animals treated with diazepam. The grey box denotes the C/I VEP ratio range in adult normal animals. No difference was present between the C/I VEP ratio of MD-SC compared to MD-EE-Dz animals (One-way ANOVA on ranks, post-hoc Dunn's method, $p > 0.05$). Asterisks indicate statistical significance: (*) $p < 0.05$; (**) $p < 0.001$. Error bars represent SEM.

WM-LTP is not present in the adult as a result of the maturation of inhibitory circuits (Kirkwood and Bear, 1994; Huang et al., 1999), but it can be restored if GABA-mediated inhibition is reduced (Artola and Singer, 1987; Kirkwood and Bear, 1994). As expected, I observed that no WM-LTP was present in SC animals (n = 5; 100.7 ± 3.1 % of pre-TBS baseline amplitude), whereas WM-LTP was fully restored in the visual cortex of EE adult rats (n = 6, 128.4 ± 6.7 % of pre-TBS baseline amplitude; Two-way RM-ANOVA, $p < 0.05$) (Fig. 11b).

LFS of WM induced synaptic LTD in layer II/III of adult rat visual cortex (Kirkwood et al., 1993). I found that LFS of WM enabled the induction and the expression of LTD in control animals (n = 6, 80.4 ± 5.3 % of pre-LFS baseline amplitude), whereas LTD was strongly reduced in EE rats (n = 7, 101.7 ± 7.4 % of pre-LFS of baseline amplitude; Two-way RM ANOVA, $p < 0.05$) (Fig. 11c). These results indicate that also the electrophysiological evaluation of postsynaptic potentials confirms the reduction of intracortical inhibition in EE animals.

I next investigated whether the reduction of intracortical inhibition in the visual cortex induced by EE was related to the recovery of OD plasticity. To do this, I strengthen GABAergic transmission in a separate group of MD-EE rats by chronic infusion via osmotic minipumps of the benzodiazepine agonist diazepam. Diazepam enhances in a use-dependent manner specific GABA type A receptor-mediated currents and is a common tool used for enhancing inhibition in the cerebral cortex (Hensch et al., 1998, Fagiolini et al., 2004). I measured binocularity in MD-EE rats treated with diazepam during the MD week by recording C/I VEP ratio in the Oc1B contralateral to the deprived eye. I showed that diazepam infusion totally blocked the OD shift induced by MD in EE rats: no differences in C/I VEP ratio between MD-SC animals ($n = 10$, C/I VEP ratio = 2.23 ± 0.24) and MD-EE rats treated with diazepam (MD-EE-Dz; $n = 5$, C/I VEP ratio = 2.15 ± 0.23; One-way ANOVA on ranks, post-hoc Dunn's method, $p > 0.05$) were detected (Fig. 11d). EE animals intracortically infused with vehicle solution showed an OD shift in favour of the open eye after MD ($n = 5$, C/I VEP ratio = 0.95 ± 0.06; One-way ANOVA on ranks, $p < 0.001$, post-hoc Dunn's method).

5.5 A role for BDNF in the recovery of adult OD plasticity

I also measured BDNF protein content in the visual cortex of 15 d EE and SC animals, using a semi-quantitative immunohistochemistry protocol. The number of BDNF-positive cells normalized to the area of Oc1B ($BDNF^+$) was significantly increased in EE compared to SC rats (EE: $n = 6$, $BDNF^+ = 1621.58 \pm 73.41$ cells/mm^2; SC: $n = 5$, $BDNF^+ = 1422.95 \pm 79.84$ cells/mm^2; t-test, $p = 0.001$). The enhancement of BDNF expression in the visual cortex of EE animals was in the range of 15-20 %. This effect was significant both in layers II-III (EE: $BDNF^+ = 1736.84 \pm 101.47$ cells/mm^2; SC: $BDNF^+ = 1517.71 \pm 119.52$ cells/mm^2) and V-VI (EE: $BDNF^+ = 1713.72 \pm 77.86$ cells/mm^2; SC: $BDNF^+ = 1410.83 \pm$

56.79 cells/mm^2) but not in layer IV (EE: BDNF$^+$ = 1396.37 ± 65.13 cells/mm^2; SC: BDNF$^+$ = 1323.20 ± 65.42 cells/mm^2; Two-way Anova, $p < 0.001$, post-hoc Holm-Sidak method) (Fig. 12a).

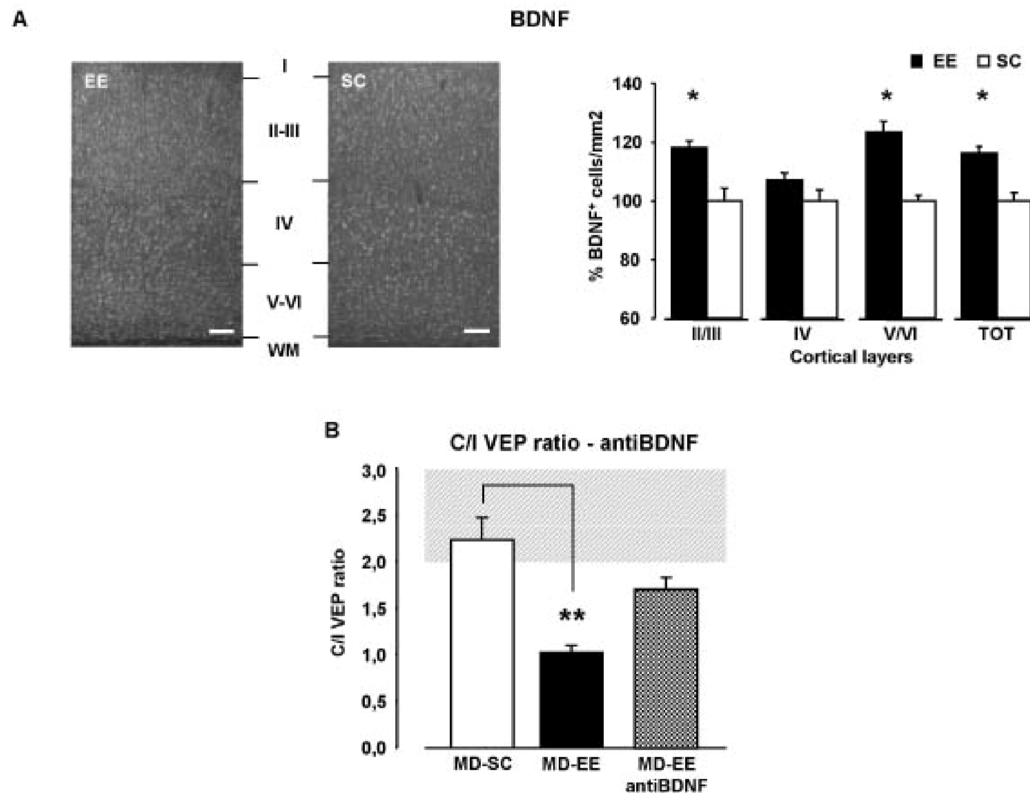


Figure 12: A Role for BDNF in the induction of EE-dependent induction of OD plasticity in the adult visual cortex. (A) BDNF expression is increased in the visual cortex of animals exposed to EE. Density of cells positive for BDNF (BDNF$^+$) in the II-III (EE: n = 6; BDNF$^+$ = 1736.84 ± 101.47 cells/mm^2; SC: n =5; BDNF$^+$ = 1517.71 ± 119.52 cells/mm^2), IV (EE: BDNF$^+$ = 1396.37 ± 65.13 cells/mm^2; SC: BDNF$^+$ = 1323.20 ± 65.42 cells/mm^2) and V-VI layers (EE: BDNF$^+$ = 1713.72 ± 77.86 cells/mm^2; SC: BDNF$^+$ = 1410.83 ± 56.79 cells/mm^2) and in the entire thickness of the visual cortex of SC (BDNF$^+$ = 1422.95 ± 79.84 cells/mm^2) and EE animals (BDNF$^+$ = 1621.58 ± 73.41 cells/mm^2). BDNF$^+$ cell density in EE visual cortex was normalized to the mean value in the control cortex. BDNF$^+$ cell density was higher in the visual cortex of EE compared to SC animals in layers II-III and V-VI, but not in layer IV (Two-way Anova, $p < 0.001$, post-hoc Holm-Sidak method). BDNF$^+$ cell density in the entire thickness of the cortex was higher in EE animals with respect to SC rats (t-test, p = 0.001). Micrographs (20x magnification) of EE and SC primary visual cortex (Oc1b) slices immunostained for BDNF are also shown. Calibration bar: 100 µm. **(B)** Administration of BDNF antisense oligonucleotides in the visual cortex partially prevents OD shift in MD-EE rats. The grey box denotes the C/I VEP ratio range in adult normal animals. The C/I VEP ratio of MD-EE-antiBDNF rats (n = 6, VEP ratio = 1.71 ± 0.13) did not different from that of either MD-SC or MD-EE animals (One-way

ANOVA on ranks, post-hoc Dunn's method, $p > 0.05$). Asterisks indicate statistical significance: (*) $p < 0.05$; (**) $p < 0.001$. Error bars represent SEM.

I then asked whether the rise of cortical BDNF levels had a critical role in the reinstatement of cortical plasticity in adult animals. I reduced BDNF levels in a different group of MD-EE animals during the time window of the eye occlusion by intracortical infusion via osmotic minipumps of BDNF antisense oligonucleotides (antiBDNF) as previously reported in the rat CNS (Ma et al., 1998; Landi et al., 2007a; Ploughman et al., 2009) and I tested plasticity, by recording the C/I VEP ratio in the Oc1B contralateral to the deprived eye. I observed that antiBDNF administration partially prevented the OD shift induced by MD in EE rats. Statistical analysis revealed that the C/I VEP ratio recorded in MD-EE animals treated with anti-BDNF (MD-EE-antiBDNF; n = 6, C/I VEP ratio = 1.71 ± 0.13) did not differ from that obtained either in MD-SC or in MD-EE rats (One-way ANOVA on ranks, post-hoc Dunn's method, $p > 0.05$) (Fig. 12b). EE rats infused with saline exhibited a full OD shift after MD (n = 5, C/I VEP ratio= 0.99 ± 0.07; One-way ANOVA on ranks, $p < 0.001$, post hoc Dunn's method).

I tested whether antiBDNF treatment was effective in reducing BDNF expression in a subset (n = 3) of the animals in which I assessed binocularity. I compared BDNF protein levels in the cortex implanted with the antiBDNF minipump (ABCtx) and in the untreated cortex (UNCtx) by means of immunohistochemistry. I found that BDNF expression was decreased by about 20% in the infused visual cortex of MD-EE-antiBDNF rats (ABCtx: $BDNF^+$ = 1077.88 ± 49.71 cells/mm^2; UNCtx: $BDNF^+$ = 1279.07 ± 16.81 cells/mm^2; paired t-test, $p = 0.032$). It is worth noting that the BDNF increase produced by EE was of the same order than the BDNF reduction induced by antiBDNF treatment, indicating that antiBDNF treatment completely prevented the modulation of BDNF expression in EE animals.

5.6 Reducing 5-HT levels prevents environmental modulation of GABAergic inhibition and BDNF expression

My results highlighted that serotoninergic neurotransmission, GABA-mediated inhibition and BDNF levels were sensitive to environmental stimulation and that their modulation was critically involved in the regulation of OD plasticity in adult EE animals.

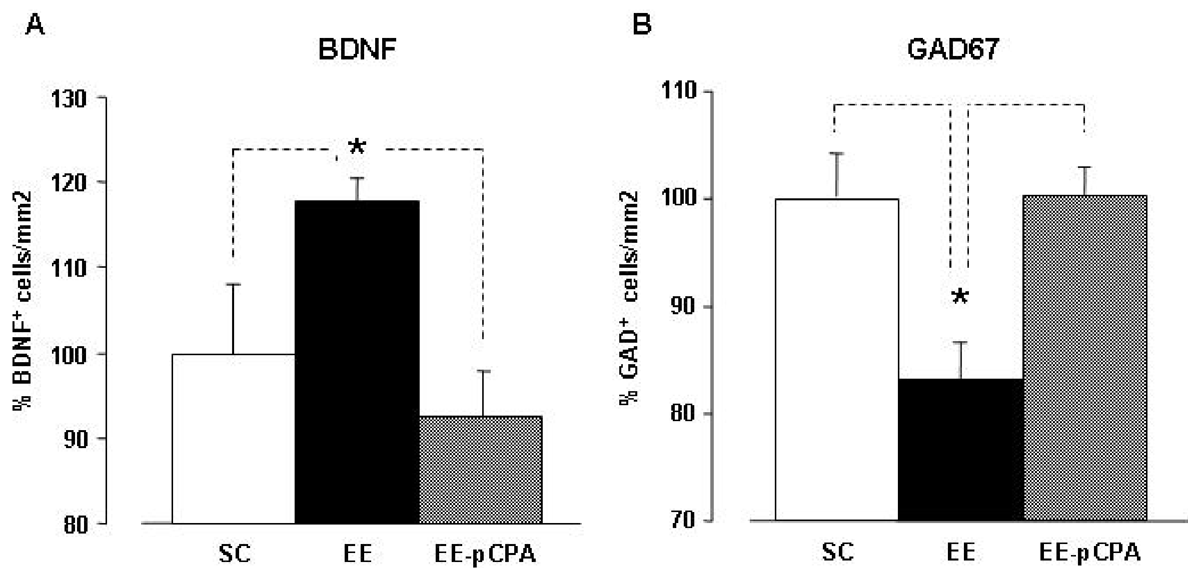


Figure 13: Reducing 5-HT levels in EE rats blocks the enhancement of BDNF expression and the decrease of intracortical inhibition. (A) BDNF expression is not increased in the visual cortex of animals exposed to EE and treated with pCPA. Density of $BDNF^+$ cells in the visual cortex of EE-pCPA (n = 4, $BDNF^+$ = 1012.92 ± 60.59 cells/mm^2) and SC animals (n = 3, $BDNF^+$ = 1096.13 ± 89.12 cells/mm^2). $BDNF^+$ cell density measured in EE-pCPA rats was normalized to the mean value in the control cortex. No difference in BDNF protein levels was present between EE-pCPA and SC animals (t-test, p = 0.458). **(B)** GAD67 expression is not changed in the visual cortex of EE-pCPA animals. Density of $GAD67^+$ cells in the visual cortex of EE-pCPA (n = 4; $GAD67^+$ = 122.83 ± 3.58 cells/mm^2) and SC animals (n = 3; $GAD67^+$ = 122.58 ± 4.93 cells/mm^2). $GAD67^+$ cell density in EE-pCPA visual cortex was normalized to the mean value in the control cortex. No difference in GAD67 was detected levels between EE-pCPA and SC rats (t-test, p = 0.967). An asterisk indicates statistical significance: (*) $p < 0.05$. Error bars represent SEM.

To clarify the interactions existing between these factors I analysed whether pharmacological reduction of 5-HT levels affected intracortical inhibition and neutrophic

factor expression. At this purpose I quantified by immunohistochemistry BDNF and GAD67 protein content in the Oc1B of a separate group of EE rats infused bilaterally with pCPA. I observed that pCPA administration in the visual cortex prevented the increase of BDNF expression produced by EE. Statistical analysis revealed that the density of $BDNF^+$ cells in EE rats treated with pCPA (EE-pCPA; n = 4, $BDNF^+$ = 1012.92 ± 60.59 cells/mm^2) did not differ from that measured in SC animals (n = 3, $BDNF^+$ = 1096.13 ± 89.12 cells/mm^2; t-test, p = 0.458; fig. 13a). Similar results have been obtained analysing GAD67 staining: no significant difference in the number of $GAD67^+$ cells was present between EE-pCPA (n = 4; $GAD67^+$ = 122.83 ± 3.58 cells/mm^2) and SC rats (n = 3; $GAD67^+$ = 122.58 ± 4.93 cells/mm^2; t- test, p = 0.967; fig. 13b).

5.7 EE housing promotes the recovery in adult amblyopic rats

The reactivation of experience-dependent plasticity induced by EE could be exploited to promote in adult animals the recovery of visual functions from amblyopia, normally restricted early during the development (Mitchell and MacKinnon, 2002). An abnormal visual experience during development, resulting from a functional imbalance between the two eyes (i.e. long-term MD starting during the CP in animal models), induces an OD shift of visual cortical neurons in favour of the normal eye and a loss of VA in the disadvantaged eye (Kiorpes, 2006).

I provide evidence that a two-three week exposure to EE induced a full recovery of VA and OD in adult rats that were rendered amblyopic by long-term MD and then reverse-sutured. I measured VA of each eye using electrophysiological recordings of VEPs from the primary visual cortex contralateral to the formerly deprived eye. As expected, in reverse-sutured rats housed in standard conditions (RS-SC, n = 5), VA of the deprived eye

remained significantly lower (0.62 ± 0.01 cycles per degree, c/deg) with respect to the other eye (1.06 ± 0.06 c/deg; paired t-test, p = 0.003).

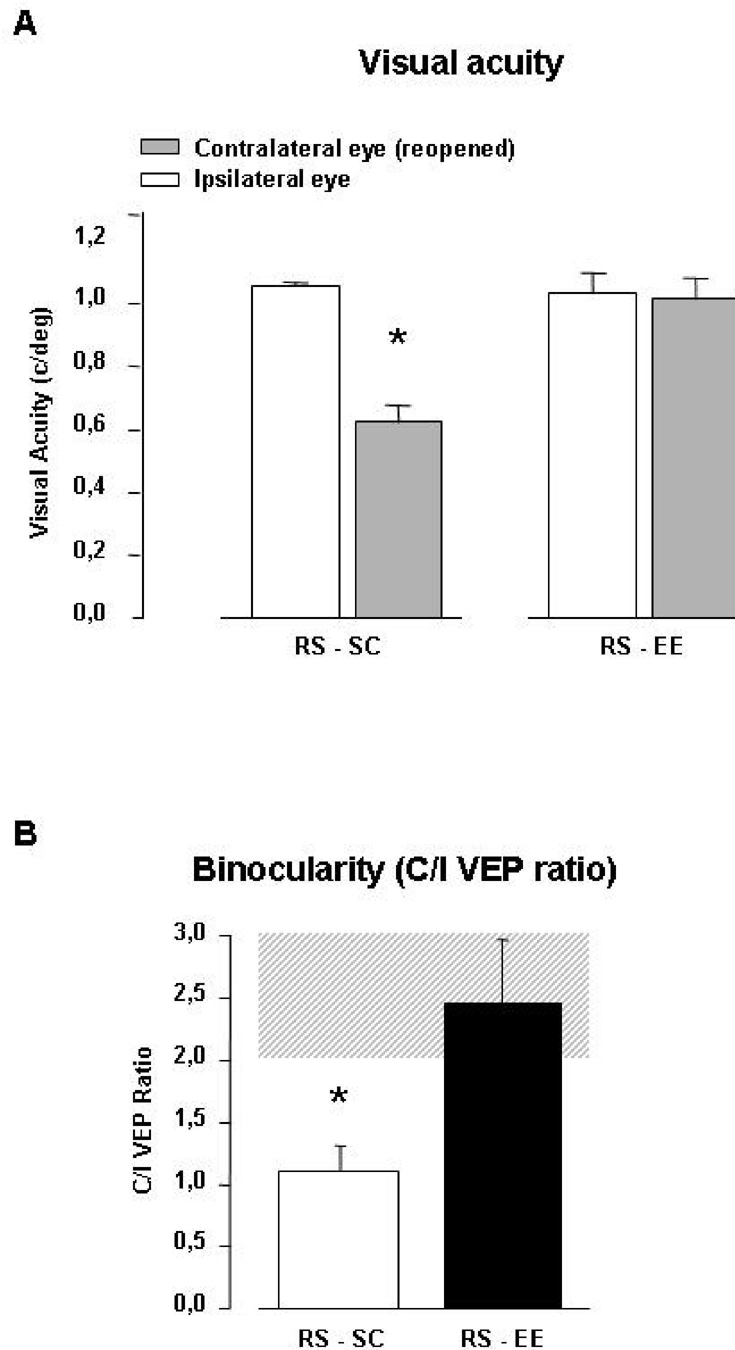


Figure 14: EE in adulthood promotes VA and OD recovery from amblyopia. (A) VA of the two eyes in both experimental groups, measured by electrophysiological recordings. The VA of the formerly deprived eye was significantly lower with respect to the other eye in RS-SC rats (n = 5; 0.62 ± 0.01 c/deg vs. 1.06 ±

0.06 c/deg; paired t-test, p = 0.003), but not in RS-EE rats (n = 5; 1.01 ± 0.07 c/deg vs. 1.03 ± 0.07 c/deg; paired t-test, p = 0.864). **(C):** VEP ratio mean values for all the different experimental groups. The hatched grey box represents the range of values for VEP ratio in adult normal animals. VEP ratio was statistically lower in RS-SC (n = 5; VEP ratio = 1.11 ± 0.20) than in RS-EE rats (n = 5; VEP ratio = 2.47 ± 0.50; t-test, p = 0.037), but was statistically undistinguishable between RS-EE and adult normal (not deprived) rats (n = 4, VEP ratio = 2.39 ± 0.2; t-test, p = 0.896). An asterisk indicates statistical significance: (*) p < 0.05. Error bars represent SEM.

In contrast, I found a full VA recovery of the long-term deprived eye in reverse sutured rats housed under EE conditions (RS-EE rats, n = 5): indeed, VA of this eye (1.01 ± 0.07 c/deg) resulted not different from that of the fellow (previously open) eye (1.03 ± 0.07 c/deg; paired t-test, p = 0.864) (Fig. 14a). I next asked whether the ability of EE to promote the electrophysiological recovery of VA was correlated to the reinstatement of normal spatial acuity discrimination skills functional to animal behaviour. Long-term monocularly deprived EE and SC animals were tested with a water-based two-choice visual-discrimination task (Prusky et al., 2000b). I measured VA of the open eye (not deprived) before performing RS; then, after RS and the period of differential rearing, I measured VA of the formerly deprived eye. The VA behavioural measure confirmed the electrophysiological data: a full recovery was evident in RS-EE animals (n = 5; VA of the formerly deprived eye: 0.9 ± 0.03 c/deg vs. VA of the fellow eye: 0.96 ± 0.02 c/deg; paired t-test, p = 0.115), whereas I did not detect any sign of plasticity in RS-SC rats (n = 5; VA of the formerly deprived eye: 0.7 ± 0.01 c/deg vs. VA of the fellow eye: 0.95 ± 0.02 c/deg; paired t-test, p < 0.001).

To control whether EE *per se* modified visual functions in adult animals, I assessed behavioural VA in a different group of normal (not deprived) adult rats reared SC (n = 4) or housed in EE (n = 4) in adulthood. VA values remained completely unaltered after two weeks of EE (before EE: VA = 0.92 ± 0.03 c/deg; after EE: 0.93 ± 0.04 c/deg; paired t-test, p = 0.867; SC: VA = 0.89±0.01 c/deg; t-test EE vs. controls, p = 0.406). To evaluate OD in

the visual cortex contralateral to the long-term deprived eye, I recorded the C/I VEP ratio in the same animals in which I estimated electrophysiologically VA recovery. In RS-SC rats there was no recovery of binocularity, since the OD distribution of this experimental group remained significantly shifted towards the open eye (n = 5; C/I VEP ratio = 1.11 ± 0.20). On the contrary, in RS-EE rats the OD of visual cortical neurons returned to be dominated by the contralateral eye, showing a mean VEP ratio into the range of normal adult values and predictive of a full recovery of binocularity (n = 5; C/I VEP ratio = 2.47 ± 0.50) (Fig. 14b). Statistical analysis revealed that the VEP ratio of RS-EE animals significantly differed from that of RS-SC rats (t-test, p = 0.037), whereas no differences were present between RS-EE and normal (noMD-SC) animals (n = 4, VEP ratio = 2.39 ± 0.2; t-test, p = 0.896).

CHAPTER 6

DISCUSSION

6.1 Rejuvenating the visual cortex

A classical assumption in Neuroscience is that experience-dependent plasticity is a prominent feature of the developing visual cortex and declines with age (Berardi et al., 2000; Hensch, 2005a; Morishita and Hensch, 2008; Spolidoro et al., 2009). However, functional and structural reorganizations of adult cortical circuits may occur as a consequence of a variety of manipulations, such as perceptual learning and retinal scotomas (Karmarkar and Dan, 2006). Moreover, several recent studies showed that specific experimental strategies promote neural plasticity in the adult visual cortex even well after the end of the critical period (Pizzorusso et al., 2002; McGee et al., 2005; He et al., 2006; Maya-Vetencourt et al., 2008; Harauzov et al., in press; for a recent review, see Spolidoro et al., 2009).

Here, I demonstrate that rearing adult animals in EE for a short time period (three weeks) restores juvenile-like plasticity in their visual cortex, allowing the induction of OD plasticity in response to MD.

Recent studies using VEP recordings, optical imaging or immediate early gene (Arc/c-Fos) induction have reported that OD plasticity can be observed in the visual cortex of adult mice, though after longer deprivation periods than what required for saturating plasticity during the CP (Sawtell et al., 2003; Pham et al., 2004; Tagawa et al., 2005; Hofer et al., 2006). The possibility to elicit OD plasticity in the mature brain has challenged the notion of CP in the visual system, leading to a still open controversy in the field (see Morishita and Hensch, 2008 for a review). It appears that the technique adopted for

measuring binocular cell activity is a critical factor determining the possibility to detect adult visual cortical plasticity (Morishita and Hensch, 2008). OD shift elicited by MD may be due to subtreshold processes of synaptic strenght changes and/or to alterations at the level of spike activity output of visual neurons in the binocular visual cortex. The most used recording methods can be subdivided into two main categories on the basis of the functional changes they are able to detect: single-unit recordings or flavoprotein imaging are used to measure spike-related events, whereas VEP recordings, immediate early gene expression or hemodynamic imaging are employed to detect primarily subthresold events. An inspection across the literature highlights that analysis of subthreshold synaptic changes can be used to unmask plasticity after the end of the CP, whereas modifications in spike-related signals from V1 reflect the effectiveness of MD only during the CP (Morishita and Hensch, 2008).

I first performed VEP recordings to compute the C/I ratio, showing that while seven days of MD were totally ineffective in shifting OD distribution in normal adult rats, a pronounced shift of OD toward the non-deprived eye was induced in rats housed in EE. To make sure that plasticity is not present only at the level of subthreshold modifications of postsynaptic potentials, I measured spike activity of visual neurons. Extracellular recordings of single-cell activity confirmed a marked shift in binocularity in the visual cortex contralateral to the deprived eye in EE animals.

A detailed comparison of the effects of visual deprivation on binocular cortical responses in adult and young animals revealed that adult OD plasticity differs qualitatively from that in the CP for several aspects (Sato and Stryker, 2008): in particular, the binocularity alteration in response to MD may be due to a depression of the response to stimulation of the deprived eye (followed by an increased open-eye response) as typically reported during the CP (Frenkel and Bear, 2004; Mrsic-Flogel et al., 2007), or only to a

potentiation of the response to stimulation of the non-deprived eye, as observed for adult residual plasticity in rodents (Sawtell et al., 2003).

I found that the OD shift was entirely due to a marked reduction in the deprived eye response, a strong suggestion that the mechanisms underlying EE-induced plasticity in the adult brain are similar to those implicated in the CP. This observation was further confirmed by data showing that a protocol of only 3 days of MD was equally able to induce a marked OD shift in the visual cortex of EE rats. Since it has been established that a period longer than 3 days of MD is required for saturating adult plasticity (Sawtell et al., 2003; Hofer et al., 2006; Sato and Stryker, 2008), our results indicate that EE reports the visual system to a juvenile condition of experience-dependent plasticity.

6.2 Monocular deprivation affects depth perception in adult EE animals

Stereoscopic depth perception, which is important for the visual perception of three-dimensional space, requires the coordinate use of the two eyes and the computation of subtle differences between the images received by each eye (binocular disparities). Cortical binocular neurons of V1, combining signals from the two eyes, have a crucial role at least in the earliest phases of visual depth perception (Parker, 2007).

It has been demonstrated that the development of normal binocular depth skills requires proper sensory experience. A total absence of visual experience leads to the degradation of stereoscopic perception abilities, probably due to the lack of precise development of tuning for binocular disparities in the visual cortex (Nealey and Riley, 1963; Kaye et al., 1981). In particular, a key role for concordant binocular visual experience has been reported. There are forms of visual deprivation, such as artificial strabismus or alternating monocular occlusion, that dissociate the input to the two eyes, producing a breakdown of binocularity among neurons of visual cortex to the extent that

only a very small proportion of cells can be excited by either eye. Two studies in kittens have documented that early alternating monocular occlusion (i.e. a condition of reduced sensory input obtained by MD of the two eyes in sequence) causes a complete absence of stereoscopic depth perception in adulthood (Blake and Hirsch, 1975; Packwood and Gordon, 1975). Similarly, Kaye et al. (1981) indicated that stereopsis failed to develop in kittens in which strabismus was induced by sectioning of an extraocular muscle. Tests for the presence of stereoscopic vision have been conducted also in monkeys reared with optically induced strabismus, showing that these animals display very inadequate depth perception abilities (Crawford and von Noorden, 1980; Crawford et al., 1996).

In addition to depriving one eye of patterned visual input, MD also causes the two eyes receiving discordant visual inputs. Indeed, the OD shift in favour of the open eye observed following MD is associated with a reduction of the proportion of binocularly-driven cells. It has been demonstrated that early MD profoundly affects the development of stereoacuity, since in deprived animals no differences of stereoscopic abilities were observed in binocular and monocular viewing conditions. Importantly, the susceptibility of binocular depth vision to the effects of MD declines with age, establishing the existence of a CP (Timney, 1983; 1990).

Here, I provided evidence that EE leads to the restoration of MD effectiveness in impairing visual depth perception in adult animals, well after the end of the CP. These behavioural results are strongly correlated with the modifications of OD distribution observed in V1 neurons of adult EE animals, suggesting that the decrease of binocular cells consequent to MD could be the physiological substrate of the functional deterioration in stereoscopic perception abilities. Given the recent results highlighting the involvement of higher visual cortex areas in computational processing of depth perception (Read, 2005;

Parker, 2007), I can not rule out, however, that EE restores the susceptibility to experience-dependent modifications of neural circuits also beyond the V1.

6.3 The crucial role of serotonin in EE-induced cortical plasticity

I found that 5-HT is a crucial molecular factor for triggering the restoration of juvenile-like plasticity in adult EE animals. HPLC analysis revealed that 5-HT was markedly increased in the binocular visual cortex of EE animals. I further investigated the role of 5-HT in EE-induced plasticity by infusing the visual cortex of MD-EE animals with pCPA, an inhibitor of 5-HT synthesis commonly used to decrease 5-HT levels in the cerebral cortex (e.g. Zetterström et al., 1999; Kornum et al., 2006; Hritcu et al., 2007). I observed that pCPA administration completely prevented the OD shift observed in MD-EE rats.

My results are in agreement with the recent finding that administration of the 5-HT uptake inhibitor fluoxetine restores neural plasticity in adult animals (Maya-Vetencourt et al., 2008). Besides, it is well known that the neurotransmitter systems characterized by diffuse projections to the entire brain are particularly sensitive to environmental stimuli. First studies by Rosenzweig et al. (1962a; 1967) reported an increase in acetylcholinesterase activity, indicating that EE affects the cholinergic system. Subsequently this initial observation has been extended to other neurotransmitter systems, showing that EE increases noradrenaline concentration and potentiates β-adrenoceptor signalling pathway in the cerebral cortex, cerebellum and brainstem (Escorihuela et al., 1995; Naka et al., 2002), and enhances mRNA expression levels of serotonin 1A receptor and serotonin concentration in the hippocampus (Rasmuson et al., 1998; Galani et al., 2007; Brenes et al., 2008).

Several possibilities may be considered for explaining the important role of 5-HT on OD plasticity. In vitro intracellular recordings obtained in cortical neurons

demonstrated that 5-HT promotes the depolarization induced by excitatory aminoacids (Nedergaard et al., 1987; Reynolds et al., 1988). Moreover, it has been reported that 5-HT facilitates the induction of synaptic plasticity in the visual cortex during development (Kojic et al., 1997; Inaba et al., 2009). These effects could be due to a reduction of membrane K^+ conductances in cortical neurons enhancing depolarization in response to excitatory inputs (Davies et al., 1987; Andrade and Chaput, 1991; Panicker et al., 1991; Fagni et al., 1992; Mlinar et al., 2006) or to an increase of second messenger intracellular levels which could in turn have synergistic interactions with down-stream responses induced by NMDAR activation (Hoyer and Martin, 1997). Thus, one possibility is that activation of serotonergic receptors could enhance the probability that retinal inputs drive cortical neuronal activity above the threshold needed for inducing activity-dependent modifications of synaptic connections.

GABAergic inhibitory neurons appear to be the principal cortical target of 5-HT fibers (DeFelipe et al., 1991; Hornung and Celio, 1992; Smiley and Goldman-Rakic, 1996), suggesting a potential role of GABAergic neurons in functioning of 5-HT system.

I observed that the increase in 5-HT levels is accompanied by a reduction of intracortical inhibition and an enhancement of BDNF protein content in the visual cortex of EE animals. These results are in agreement with those previously reported by Maya-Vetencourt et al. (2008) in adult rats chronically treated with fluoxetine.

Interestingly, I found that pCPA infusion not only was able to block plasticity in response to MD, but also counteracted the effects produced by EE on GABA-mediated inhibition and BDNF expression, preventing both the decrease of GAD67 and the increase of BDNF levels detected in EE rats. Therefore, I suggest that the potentiation of serotoninergic transmission is upstream the modulation of both the intracortical inhibition-excitatory balance and BDNF protein levels.

This result highlights that a reduction of intracortical inhibition could be an additional mechanism through which an enhanced serotoninergic transmission may facilitate OD plasticity, suggesting the possibility that 5-HT receptors on GABAergic interneurons may mediate the reduction of GABA release we observed in the adult visual cortex after EE housing. Consistent with this hypothesis, it has been reported that 5-HT inhibits GABA release from interneurons of several brain regions via a presynaptic mechanism mediated by 5-$HT_{1/2}$ receptor families (Schmitz et al., 1995; 1998; Koyama et al., 1999; Zhou and Hablitz, 1999; Xiang and Prince, 2003; Bramley et al., 2005; Lee et al., 2008), probably regulating the availability of transmitter vescicles (Wang and Zucker, 1998). Moreover, it has been recently observed a modification of visual cortex pyramidal neuron responses to input signals depending on the behavioural state, related to a bidirectional modulation of somatic inhibition (Kurotani et al., 2008). In layer V pyramidal neurons of rat visual cortex, repetitive firing from a depolarized membrane potential, which typically occurs during arousal, produced long-lasting depression of somatic inhibition; in contrast, slow wave oscillations with firing in the depolarized phase, mimicking slow-wave sleep, produced long-lasting potentiation of inhibition (Kurotani et al., 2008).

It is possible that increased serotonin levels might affect the animal arousal state, changing the excitatory-inhibitory balance, a factor crucially involved in the regulation of cortical plasticity (Maya-Vetencourt et al., 2008; Spolidoro et al., 2009).

In addition, a vast number of studies showed that 5-HT dramatically increases the expression of BDNF mRNA in the neocortex (Nibuya et al., 1995; Vaidya et al., 1997; Zetterström et al., 1999; Castren, 2004; Balu et al., 2008), suggesting that the effects induced by the potentiation of serotoninergic neuromodulation could be, at least in part, mediated by the enhancement of BDNF expression in the visual cortex of EE animals.

My results strongly demonstrate that 5-HT plays a critical role in the induction of plastic phenomena observed in adult EE animals. The importance of 5-HT in mediating EE effects is confirmed by a recent work showing that 5-HT depletion in the hippocampus of EE rats produced a significant decrease of neurogenesis in the dentate gyrus (Ueda et al., 2005). I suggest that the potentiation of serotoninergic transmission triggers OD plasticity in EE adult animals, acting directly on excitatory transmission, but also modulating both intracortical inhibition and BDNF protein levels. Given their sensitivity to environmental stimulation, however, we can not rule out a parallel contribution of other neuromodulators.

6.4 Intracortical inhibition regulates plasticity recovery in EE animals

Modulation of the excitatory-inhibitory balance of cortical activity is well known to be crucially involved in the regulation of plasticity during development (Hensch, 2005a; Spolidoro et al., 2009). Fagiolini and Hensch (2000) provided strong evidence that that development of intracortical inhibition is a major factor responsible for triggering the initial onset of the CP. Indeed, a premature enhancement of inhibition with diazepam in normal wild-type mice just after eye opening can promote precocious plasticity in the visual cortex. Similarly, the faster maturation of intracortical inhibition in BDNF overexpressing mice is accompanied by a marked acceleration both in the maturation of VA and in the time-course of the CP for OD plasticity (Huang et al. 1999). These striking results led to the proposal that the inhibitory tone surpasses two functional thresholds in the visual cortex: the first one corresponds to the point after which the level of inhibition is sufficient to allow OD plasticity to be expressed, a critical level of inhibition being probably necessary to detect activity differences between the two eyes; as development proceeds further, the inhibitory tone continues to increase until it crosses a second threshold, after which plasticity is reduced to the low levels typically found in adults.

The recovery of plasticity I found in adult EE animals was associated with a marked reduction of GABAergic inhibition and I provide evidence that visual cortex infusion of the benzodiazepine diazepam prevented the OD shift in the visual cortex of MD-EE rats. Thus, I confirm that intracortical inhibitory transmission plays a fundamental role in the decline of plasticity occurring in parallel to brain maturation and that its modulation is crucial for reopening plasticity in the adult nervous system.

My results are well supported by other works. Indeed, the possibility to restore plasticity in the mature visual cortex by decreasing inhibition levels has been directly demonstrated in a very recent study in which pharmacological reduction of intracortical GABAergic neurotransmission obtained through infusion of either MPA (an inhibitor of GABA synthesis) or picrotoxin (a GABAA antagonist) directly into the visual cortex reactivated OD plasticity in response to MD in adult rats (Harauzov et al., in press). Similarly, recent papers have shown that restoration of plasticity in adulthood obtained with different experimental paradigms is related to a reduction of cortical GABAergic neurotransmission (He et al., 2006; 2007; Maya-Vetencourt et al., 2008).

One possibility is that subthreshold changes elicited by sensory manipulation also in the adult brain can be translated into spike output changes, as normally occur in developmental plasticity, by resetting excitatory-inhibitory balance to a CP state.

The overall increase of cortical activity could also influence the expression of activity-regulated genes, in turn affecting cortical plasticity, such as neurotrophic factors (see the following paragraph). Moreover, the recent finding that a pharmacological reduction of intracortical inhibition is followed by a reduction in the levels of extracellular matrix components (Harauzov et al., in press) suggests the involvement of structural plasticity processes, such as an increase of spine motility and turnover, leading in the adult

to the reorganization of cortical circuitry, as observed during the CP (Mataga et al., 2004; Oray et al., 2004).

6.5 BDNF in EE-induced plasticity

The role of BDNF in visual cortex plasticity during development is still debated (e.g. Berardi and Maffei, 1999; Kaneko et al., 2008b), whereas it has been clearly established that BDNF expression controls the CP time course by promoting the maturation of intracortical GABA-mediated inhibition (Huang et al., 1999). In the adult visual system it has been recently shown that the intracortical administration of BDNF is *per se* able to reactivate OD plasticity (Maya-Vetencourt et al., 2008).

Consistently, I observed a 15-20% increase of BDNF protein levels in the visual cortex of adult animals exposed to EE and I provide evidence that visual cortex infusion of BDNF anti-sense oligonucleotides prevents the OD shift in the visual cortex of MD-EE rats. In this context it is worth noting that, even if antiBNDF treatment completely counteracts the BDNF increase in EE animals, it blocks only partially the MD-dependent binocularity modifications, suggesting that BDNF is not the main player in the restoration of adult plasticity and acts in parallel to other molecular factors, such as different members of the neurotrophin family.

BDNF has been shown to have a facilitatory action on excitatory synaptic transmission (Carmignoto et al., 1997; Levine et al., 1998; Sala et al., 1998) and intrinsic excitability of neocortical pyramidal neurons (Desai et al., 1999), thus indicating a pathway through which BDNF may affect plasticity in the adult visual cortex. However, the BDNF-dependent increase in CNS excitability may also be derived from a suppression of GABAergic inhibition, as reported for other brain regions (Tanaka et al., 1997; Frerking et al., 1998; Henneberger et al., 2002).

In the developing visual system it has been well documented that BDNF affects GABAergic neurotransmission both at the presynaptic and postsynaptic level, leading to the maturation and stabilization of inhibitory synapses (Rutherford et al., 1997; Huang et al., 1999; Gianfranceschi et al., 2003; Abidin et al., 2008). Since recent gene expression studies revealed that a distinct biochemical milieu characterizes the juvenile and adult cortex (Majdan and Shatz, 2006; Tropea et al., 2006), one possibility to explain the concomitant increase of BDNF levels and depression of intracortical inhibition is that the relationship between BDNF and GABAergic neurotransmission is different in the adult visual cortex with respect to what observed during development. Unpublished data obtained in my laboratory, showing that BDNF infusion could reduce GABA release in the adult visual cortex, are consistent with this hypothesis. A similar developmental switch of BDNF action on the inhibitory system, probably due to changes in the intracellular signalling pathway, has been reported in the hippocampus (Tanaka et al., 1997; Brunig et al., 2001; Mizoguchi et al., 2003). The direction of BDNF effects on inhibitory synapses could depend both on the preferred postsynaptic signalling cascade activated by the TrkB receptor (Mizoguchi et al., 2003) and the phosphorylation state of the GABA receptors (Jovanovic et al., 2004).

Taking into account the finding that antiBNDF oligonucleotide administration prevented only partially the shift in OD distribution in the visual cortex of MD-EE rats and the well-known modulation of neurotrophin synthesis and release provided by neuronal activity (Isackson et al., 1991; Castren et al., 1992; McAllister et al., 1999), however, it is also possible that the enhancement of BDNF levels occurs downstream the decrease of intracortical inhibition in the cascade of molecular events leading to the reopening of cortical plasticity. Indeed, the artificial reduction of intracortical inhibition by means of

MPA infusion in the visual cortex leads to a significant increase of BDNF expression (unpublished observation).

A possible explanation that may conciliate all the aforementioned data is that a reciprocal and iterative interaction occurs between BDNF (and eventually other neurotrophins) and GABAergic neurotransmission with BDNF contributing to the decrease of intracortical inhibition, in turn affecting the neurotrophic factor expression through the modulation of cortical activity.

In addition, BDNF might promote visual cortex plasticity also by modulating gene expression levels. Indeed, BDNF stimulates CREB phosphorylation and activation (Finkbeiner et al., 1997; Pizzorusso et al., 2000; Ratto and Pizzorusso, 2006) and it is well known the important role of CRE-mediated transcription in development and plasticity of the visual system, in particular at the cortical level (Pham et al., 1999; Mower et al., 2002; Cancedda et al., 2003).

6.6 A model of molecular mechanisms underlying EE-induced plasticity

A comprehensive model for explaining the molecular mechanisms underlying EE-induced plasticity is that environmental stimulation promotes the strengthening of serotoninergic transmission triggering the decrease of GABA-mediated intracortical inhibition and, in parallel or in series, the enhancement of BDNF expression (Fig. 15). Both the increase of overall cortical activity and BDNF intracellular signalling could in turn induce a transcriptional program that leads to activation of other genes promoting plasticity. Activity-dependent regulation of gene expression by means of posttranslational modifications of histones has been proposed to be important for hippocampal synaptic plasticity and learning and memory (Colvis et al., 2005; Levenson and Sweatt, 2005).

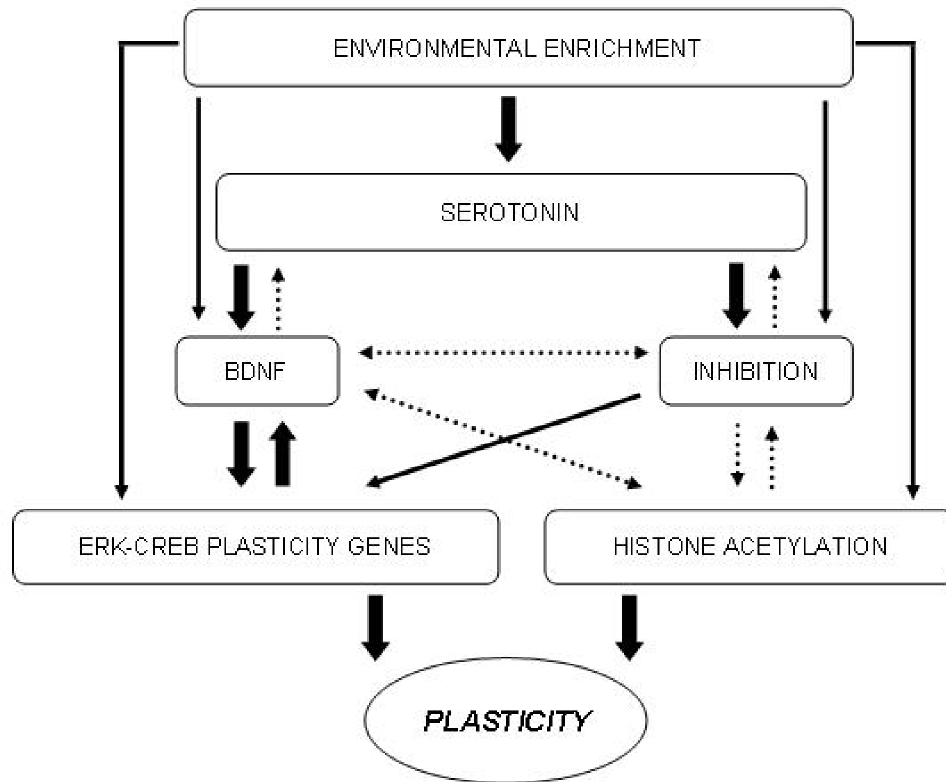


Figure 15: Schematic diagram showing key molecular events underlying restoration of plasticity in the adult visual system. A comprehensive model for explaining the molecular mechanisms underlying EE-induced plasticity is that environmental stimulation promotes the strengthening of serotoninergic transmission that triggers the decrease of GABA-mediated intracortical inhibition and, in parallel or in series, the enhancement of BDNF expression. Both the increase of overall cortical activity and BDNF intracellular signalling could in turn induce a transcriptional program that leads to activation of other genes promoting plasticity, for instance through the ERK-CREB pathway. Furthermore, an influence on the epigenetic control of gene transcription has been suggested for EE. *Continuous lines* represent well-documented interactions between *boxes*; *dashed lines* indicate likely interactions in the context of visual cortical plasticity deserving further experimental characterization.

Histone posttranslational modifications regulate chromatin susceptibility to transcription, with high levels of histone acetylation on a specific DNA segment being generally correlated with increased transcription rates (Mellor 2006). Interestingly, it has been recently demonstrated that EE housing leads to increased levels of histone acetylation in both hippocampus and cerebral cortex (Fischer et al., 2007). A similar relationship between histone acetylation and EE effects could also be enrolled in the adult visual

system. It has been recently demonstrated that visual experience activates histone acetylation in the visual cortex during the CP, but this capacity is downregulated in adult animals (Putignano et al., 2007). Moreover, trichostatin treatment, which promotes histone acetylation, also enhances plasticity in the adult visual cortex (Putignano et al. 2007). Thus, it is possible that the cellular and molecular mechanisms proposed to mediate the effects of EE on the adult visual system could ultimately regulate the pattern of histone acetylation, thus modulating the expression of genes critical for plasticity (Fig. 15).

Another way by which EE can regulate gene expression might be the activation of specific transcription factors. One possibility is that EE promotes the phosphorylation of ERK, a kinase acting as a convergence point between electrical activity and neurotrophins and appearing to be in a strategic position to translate the regulatory actions of activity and neurotrophins into modifications of cortical circuitry (Ratto and Pizzorusso, 2006). It has been shown that ERK is a key mediator of experience-dependent plasticity in the visual cortex during the CP (Di Cristo et al., 2001). ERK is promptly and potently activated by visual experience in visual cortical neurons, and its pharmacological inhibition prevents synaptic plasticity as well as the effects of MD on OD distribution in the developing visual cortex (Di Cristo et al., 2001; Cancedda et al., 2003; Suzuki et al., 2004). In addition, ERK action is necessary to mediate the effects of visual experience on the transcription of several genes (Majdan and Shatz, 2006).

ERK could act through the transcription factor CREB. Indeed, ERK is required for visually stimulated transcription mediated by CREB (Cancedda et al., 2003), and CREB plays a pivotal role in various forms of plasticity in the visual cortex (Pham et al., 1999; Mower et al., 2002; Pham et al., 2004) and other brain structures (Lonze and Ginty, 2002). Consistently with this hypothesis, it has been shown that EE in adulthood increases immunoreactivity to CREB in the hippocampus (Williams et al., 2001) (Fig. 15).

6.7 EE promotes the recovery of visual functions in amblyopic animals

Promoting plasticity in the adult nervous system could pave the way for novel therapeutic strategies for the treatment of brain injuries and neurological disorders in adulthood, when recovery and functional rehabilitation are very hard to achieve. In the visual system a widely diffused pathology (2-4% of incidence in the general population), for which no suitable treatment is still available in the adult, is amblyopia (Holmes and Clarke, 2006). This pathology derives from conditions of early abnormal visual experience in which a functional imbalance between the two eyes is predominant owing to anisometropia (unequal refractive power in the two eyes), strabismus (abnormal alignment of one or both eyes), congenital cataract or, in animal models, long-term MD starting from the CP (Levi and Li, 2009). The impairment produced by amblyopia is generally expressed in the clinical setting as a loss of VA in an apparently healthy eye, despite appropriate optical correction; however, there is a great deal of evidence showing that amblyopia results in a broad range of perceptual abnormalities, including stereopsis deficits (Lewis and Maurer, 2005; Levi, 2006). Similarly, in animal models the classic hallmarks of amblyopia are a permanent decrease of VA in the affected eye and a pronounced OD shift of visual cortical neurons in favour of the normal eye (Timney 1983; Kiorpes et al. 1998; Maurer et al. 1999; Prusky and Douglas 2003).

The traditional amblyopia therapy consists in patching or penalizing the fellow preferred eye, thus forcing the brain to use the visual input carried by the weaker amblyopic eye (Wu and Hunter, 2006). The success rate of the treatment is dependent on several factors, including seriousness of visual abilities disruption, type of amblyopia, occlusion dose, age of onset and patient compliance (Stewart et al., 2005). Although it is widely accepted that reinstatement of visual functions is possible only if corrective therapy is started early in development (Wu and Hunter, 2006), recent studies in rodents have

unmasked a previously unsuspected potential for promoting recovery well after the end of the CP (for a review, see Spolidoro et al., 2009). One approach is the infusion of chondroitinase ABC (chABC) to degrade chondroitin sulphate proteoglycans of extracellular matrix in the mature cortex. Adult chABC treatment coupled with reverse suture (i.e. the deprivation of the previously open eye and opening of the previously deprived eye, RS) in amblyopic rats produces a full recovery of both OD and VA (Pizzorusso et al., 2006). This previously unsuspected potential for adult brain plasticity can also be evoked by a brief period (ten days) of complete visual deprivation (He et al., 2007) and by the administration (one month) of fluoxetine, a serotonin reuptake inhibitor (Maya-Vetencourt et al., 2008).

Very interestingly, EE turned out to be very effective for treating amblyopia in adulthood. A brief exposure (two-three weeks) of adult amblyopic rats to EE has been demonstrated to promote a complete recovery of both VA and OD, an effect documented not only at the electrophysiological level, but also using behavioural assessments.

The promising results obtained with EE in rodents open new hopes for clinical application to human patients, given the completely non-invasive nature of this approach. Strikingly, an increasing number of clinical studies has reported that repetitive visual training based on sensory enrichment procedures may represent a very useful approach for the treatment of amblyopia, providing a substantial improvement in a variety of visual tasks (e.g. Levi and Polat, 1996; Levi et al., 1997; Polat et al., 2004; Levi, 2005; for a review, see Polat, 2008 and Levi and Li, 2009). One caveat to the therapeutic value of these visual practice procedures, however, is the narrow specificity of the achievable improvement, which is typically limited to the selected trained stimulus, condition or task (Fahle, 2005). Only in a few tasks (Vernier acuity, contrast sensitivity and detection) did

training lead, at least in some subjects, to generalization of the beneficial effects to other degraded visual functions, such as VA and stereoacuity (Levi and Li, 2009).

The study of experimental models of amblyopia has the advantage of enabling researchers to uncover the molecular mechanisms underlying the therapeutic value of the employed procedures. My results, showing that serotonin, inhibition and BDNF are crucially involved in plasticity recovery induced by EE, provide new insights into a potential molecular cascade that could regulate the reinstatement of visual function in response to environmental experience (see the previous paragraph). A previous suggestion that the strengthening of serotoninergic neurotransmission could mediate the recovery from amblyopia in animal models derives from the study of Maya-Vetencourt and colleagues (2008). Interestingly, neuromodulatory systems are known to regulate the arousal state of the brain (Gu, 2002) and to modulate attentional processes (Robbins, 1997; Coull, 1998; Boulougouris and Tsaltas, 2008). A recent study in non amblyopic subjects provides indirect support to the important role of visual attention in driving visual cortex plasticity, showing that normal-sighted people trained with action-based video games displayed robust improvements in basic visual functions (Li et al., 2009). The same effect was not observed after non-action video game playing (equally engaging and visually complex, but operating at a slower pace and not requiring precise visually guided actions), suggesting that allocation of attention is a fundamental component for the effectiveness of the training paradigm (Green and Bavelier, 2003; Caplovitz and Kastner, 2009).

Consistently with our observation that EE-plasticity is associated with a decrease of inhibitory transmission, the GABA receptor antagonist bicuculline has been reported to rapidly restore input from the deprived eye and reverse amblyopia in the visual cortex of cats (Duffy et al., 1976; Burchfiel and Duffy, 1981), prompting that intracortical inhibition is an important obstacle for removing the physiological abnormalities produced in visual

cortex by MD (Hensch, 2005b). It has been suggested that the balance between excitation and inhibition is impaired during development also in amblyopic human subjects and that cortical over-inhibition could underlie the degradation of spatial vision abilities (Polat et al., 1997; Polat, 1999; Levi et al., 2002; Polat et al., 2004; 2005; Wong et al., 2005).

6.8 Conclusions

The encouraging results obtained using EE as a strategy to reopen plasticity windows in the adult have shown that it is possible to control processes crucial for brain function in a totally non-invasive way, making this experimental paradigm particularly eligible for human application. Studies employing the EE paradigm have indicated a number of molecular hotspots that might emerge as possible ways of accession for a successful treatment of neuropathological conditions affecting the juvenile and the adult CNS.

An open issue is to what extent is EE in animal models relevant for the human living experience. EE is a complex paradigm, since an increased stimulation is provided at multiple sensory, motor, cognitive and social levels. Although most humans do experience a high degree of environmental complexity and novelty, levels of cognitive, social and physical stimulation vary greatly among individuals and in different periods of life. Strong correlative and epidemiological evidence shows that life style, including occupation, leisure activities and physical exercise has a direct effect on the risk of cognitive decline. The results indicate that a higher level and variety of mental and physical activity is associated with a lower cognitive decline and a reduced risk for dementia (e.g., Katzman et al., 1993; Laurin et. al, 2001; Fratiglioni et al., 2004; Marx, 2005; Podewils et al., 2005; Kramer and Colcombe, 2006; Kramer and Erickson, 2007). These results encourage stronger efforts in the application of EE paradigms, alone or in combination with pharmacological treatments, for the therapy of neurological disorders.

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