SCUOLA NORMALE SUPERIORE

PISA

CLASSE DI SCIENZE
CORSO DI PERFEZIONAMENTO IN NEUROBIOLOGIA
ANNI ACCADEMICI 2000-2002

TESI DI PERFEZIONAMENTO IN

NEUROBIOLOGIA

ENVIRONMENTAL ENRICHMENT PREVENTS THE EFFECTS OF DARK REARING AND SHORTENS THE CRITICAL PERIOD FOR MONOCULAR DEPRIVATION IN THE RAT VISUAL CORTEX

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The present work represents the accidental but successful application of an ancient stratagem:
adding wood to quench the flames.

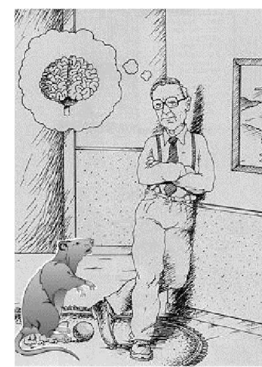
INTRODUCTION

Neuronal plasticity is a central theme in modern neurobiology; experience elicits plasticity through activity-dependent processes.

In 1949, the psychologist Donald O. Hebb formulated a modern conceptual framework for neuronal plasticity. In Hebb's words: *"Let us assume that the persistence or repetition of reverberatory activity (or "trace") tends to induce lasting cellular changes that add to its stability … when an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased"*. In other words, he postulated that when one cell excites another repeatedly, a change takes place in one or both cells such that one cell becomes more efficient at firing the other.

Hebb's view has since been extended to include the plasticity of many definable anatomical substrates, such as synapses, neurites or entire neurons. Obviously, changes on these levels are, in turn, based on changes at the biochemical and molecular levels.

In the late 1940s, Hebb was also the first to propose the "enriched environment" as an experimental concept. He reported anecdotally that rats that he took home as pets for his children



showed behavioral improvements over their litter mates kept in standard laboratory environment.

In the early 1960s, two experimental approaches were initiated to investigate the effects of experience on the brain. Hubel and Wiesel established a programme to examine the effects of selective visual deprivation during development on the anatomy and physiology of the visual cortex, and Rosenzweig and colleagues introduced enriched environments as a testable scientific paradigm.

However, no data are available on interactional effects of enriching the environment when being subjected to a visual deprivation paradigm. Surely, such observation could be of great interest both for basic biological research and for its possible application in the field of human clinical research.

In the present PhD thesis, we present an experimental work in which the effects of enriched environment were tested in a paradigm of total deprivation of visual experience (dark rearing) and in a paradigm of monocular deprivation.

In the introduction, the first chapter is a general description of the enriched environment paradigm, its applications and experimental results; the second chapter focuses on visual cortical plasticity paradigms, such as they have been applied to the model of rodent's visual system.

The third chapter exposes the original experimental work of this Thesis entitled "Environmental Enrichment prevents the effects of dark rearing and shortens the critical period for monocular deprivation in the rat visual cortex". In a first experiment, we tested whether environmental enrichment could counteract the loss of visual acuity and the lack of consolidation of visual cortical connections found in dark reared rats. In a second experiment, we compared the time course of the critical period for monocular deprivation in light reared rats raised either in enriched environment or in standard housing.

Following, Appendix A shows and discusses a few methodological results obtained by comparing the two main electrophysiological techniques used in recording the ocular dominance of visual cortex and is entitled "Measuring ocular dominance in rodents: correlation between single-cell recording and Visually Evoked Potentials (VEPs)".

CHAPTER 1

THE ENRICHED ENVIRONMENT PARADIGM (EE)

In 1924, two psychologists, Edward Tolman and Robert Tryon, selectively inbreeded rats on the basis of their performance on a behavioral task. The animals had to learn a pathway to find some food in a labyrinth. They obtained two strains of rats, a “clever” strain and a “stupid” one. Years after, these rats were reared either in an enriched or in an impoverished environment and then tested in the original labyrinth. The clever-impoverished rats became stupid whereas the stupid-enriched rats became intelligent!

1.1. ENRICHMENT, IMPOVERISHMENT AND NATURALISTIC ENVIRONMENTS

In an experimental setting, an enriched environment is “enriched” in relation to standard laboratory housing conditions (van Praag et al. 2000). Therefore, enrichment is often opposed to impoverished environment (IE), as the reverse condition. However, as we will see in this paragraph, EE only represents a less impoverished condition when compared to naturalistic environments, being merely a step up from standard laboratory impoverishment.

The standard definition of an enriched environment is “a combination of complex inanimate and social stimulation” (Rosenzweig and Bennett, 1996, review; Rosenzweig et al., 1978). In general, the enriched animals[1] are kept in larger cages and in larger groups with the opportunity of more complex social interaction. The environment is complex and is varied over the period of the experiments: tunnels, nesting material, toys and (often) food locations are changed frequently. In addition, animals are often given the opportunity for voluntary physical activity on running wheels (Fig I-1). Typically, littermates of the same sex are assigned to three different laboratory environments. The standard condition (SC; also called standard environment, SE), with 2-3 up to 5 animals in a standard laboratory cage provided with free access to food and water and standardized dark/light schedule (12 h dark/12 h dark). The enriched condition (EC or EE), containing a group of 10 to 12 animals with a variety of stimulus objects and typically one or more running wheels. Objects are changed in position or replaced daily or every 3-7 days. The impoverished or isolated condition (IC), with SC-size cages housing single animals.

[1] The elective animal model for EE paradigms is that of rodents of the Muridae family, though many authors have developed similar enriching conditions in insects, fishes, rabbits and other mammals such as cats (Harris et al., 2001; Sandeman et al., 2000; Scotto Lomassese et al., 2000) or bears (Renner and Plebani Lussier, 2002).

All those three conditions are very far from that of a naturalistic environment in which an organism itself must provide the resources for its survival and has free possibilities for movement and social interactions. However, in laboratory settings, EE is thought to mimic the naturalistic habitats, allowing to infer the effects of naturalistic environments on the brain.

Many different EE paradigms have been employed ranging from a constant "full time" enrichment to time spaced intervals of EE stimulation, during specific developmental time windows or in adult life. In a full time paradigm, animals are raised in a EC typically after weaning, whereas in the interleaved stimulation animals are took out from their cages and exposed to the EC for certain time periods every day (usually few hours).

In the initial studies on the effects of EE, the neurobiological and behavioral effects investigated included changes in gross parameters such as total brain weight, total DNA or RNA content, total brain protein, and various behavioral learning measures (Rosenzweig et al., 1969, 1967; Bennett et al., 1969). More recently, many studies have shown that EE elicits many plastic responses in developing or adult brains of experimental animals. These studies include effects observed in biochemical parameters, dendritic arborization, gliogenesis, neurogenesis and improved learning, memory and problem solving (van Praag et al., 2000, review; Rampon and Tsien, 2000, review; Bennett et al., 1976; Cummins et al., 1973).

Taking into account environmental effects when studying complex brain functions is crucial for good scientific practice and results in biomedical as well as cognitive research. In fact, many criticisms pointed out that interactions between genotype and housing conditions can lead to misleading results (Wurbel, 2001, review). Rodents used in biomedical research are typically reared in small cages that lack key features of their natural habitats. These conditions impose constraints on behavior and brain development, resulting in altered brain functions. Barren housing conditions may interfere with brain development and behavior in three different ways: early environmental deprivation, thwarting of behavioral response rules, and disruption of habitat-dependent adaptation processes.

Specific evidences come from research on early social deprivation. Depriving of social contact during the postweaning period leads to disruption of higher cognitive function such as inhibitory control in attentional selection (Robbins, 1996; Hall, 1998, review). Isolation-rearing disrupts the attenuation of the startle response, elicits hyperactivity in response to novelty and a tendency towards perseveration. Consistent with these behavioral effects are underlying neurochemical changes. Reduced dopamine turnover in the prefrontal cortex is paralleled by enhanced dopaminergic function in the dorsal and ventral striatum. This could explain why isolation-rearing and lesions to the rat medial prefrontal cortex share the same selective deficit in attentional

selection (Schrijver and Wurbel, 2001; Birrell and Brown, 2000). To appreciate the significance of these effects it might be important to mention that several of the concomitant symptoms are the same as those used to model key symptoms in schizophrenic patients (Geyer et al., 1993).

Lack of environmental stimulation is, however, not the only way in which housing conditions can selectively alter brain functions. Indeed, animals' responses are largely based on evolutionary rules, which critically depend on specific environmental features. When subjected to chronic thwarting of behavioral response rules, animal can develop stereotypic behaviors. Stereotypes are repetitive behaviors, with extreme performance levels, fixed in form and orientation (Garner and Mason, 2002). They are the most common form of abnormal behavior found in laboratory rodents and in other restricted and confined animals. Laboratory mice, for example, develop different forms of stereotypies as a result of attempts to escape from their cages (Wurbel and Stauffacher, 1996) (Fig I-2). Recent evidences[2] support the idea that stereotypes are caused by the chronic thwarting of behavioural response rules. Behavioural and neurochemical evidence indicates functional changes in the dorsal basal ganglia, structures implicated in the initiation and sequencing of movements (Albin et al., 1989; Ridley, 1994; Hauber, 1998). Stereotypes can be induced pharmacologically by either inducing dopamine release at the level of the ventral and dorsal striatum, or by sensitizing target cells to dopamine release (Cabib, 1993; Steiner and Gerfen, 1998). These findings are consistent with the hypothesis that stereotypy development depends on stress-induced sensitization of dopamine systems, possibly mediated by endogenous opioids (which are released in massive quantities during conditions of stress) (Cabib, 1993). Regardless of the underlying mechanism, perseverative responding in stereotypic animals could affect many standard behavioral tests that are based on behavioral flexibility (e.g. extinction, reversal, or conditional learning tasks).

Finally, a mismatch between the postnatal and adult environment can disrupt habitat-dependent adaptation processes that are mediated by the early postnatal environment. Recent studies in rats suggest that habitat-dependent variation in maternal care could influence the behavioral transmission of fearfulness and stress responses (Liu et al., 1997; Caldji et al., 1998; Francis et al.,

[2] In a series of beautifully designed experiments, Wiedenmayer (1996) showed how gerbils develop stereotypic digging when denied access to an appropriate shelter. In laboratory cages, gerbils first dig at the bedding when their eyes open, but increasingly direct digging to the corners of the cage. Corners resemble structures in the wild that would normally orient the beginning of a digging sequence in burrow construction. Stereotypic digging develops, even when gerbils have access to an arena filled with sand, showing that performance of the behavior fails to reduce the underlying motivation. By contrast, when reared without any digging substrate, but instead given access to a dark chamber, stereotypic digging does not occur. However, the chamber fails to prevent stereotypy development unless its entrance is tunnel-shaped, showing that behavioral response rules might crucially depend on highly specific stimulus conditions.

1999). A classical example is that of postnatal handling[3] which substantially decreases behavioral and endocrine responses to stress in adulthood and vulnerability to stress-related diseases (Meaney et al., 1996; Bhatnagar et al., 1996; Fernandez-Teruel, 2002). These effects persist throughout the life of the animals and recent studies have shown that they are caused by handling-induced changes in maternal care: mothers of handled pups have shorter, but more frequent, nest-bouts, resulting in higher overall levels of licking, grooming and "arched-back" nursing (Liu et al., 1997; Caldji et al., 1998). Moreover, the same research indicates that rats (and possibly other rodents) could become sensitive to stressors because of a mismatch between the early postnatal and the adult environment. Maternal influence on stress responses is likely to be adaptive in the wild, where rats tend to spend their adult life in an environment which resembles that of their mothers (Francis and Meaney, 1999). In the laboratory, however, the safety and stability of the rearing environment does not predict the future challenges involved in life as an experimental animal. In other words, when animals are reared under false pretences, the outcome of maternal programming might be maladaptive.

Maybe, one of the more striking feature underlining the differences between EE, IE and naturalistic environments, is the finding that the changes in the brain can be detected even when the enriched experience is provided to an adult or aged animal. This finding underscores the possibility that experimental enrichment is a reversal of the impoverishment generally found in the laboratory setting rather than an enrichment over a natural setting. One of the best examples of an attempt to address this issue were studies of birds captured from the wild and pulse-labelled[4] with ^{3}H-thymidine. Some were housed in an aviary and the rest returned to the wild (Barnea and Nottebohm, 1996, 1994). Some of the freed birds were recaptured and the recruitment of new neurons in the avian equivalent of the hippocampus in these birds was compared with that in the laboratory birds. More of the neurons that were born during the brief captivity period survived in the animals recaptured after six weeks from the wild than in those in the aviary. So, either the wild environment is enriched or the laboratory environment is relatively impoverished and stressful for the captured birds, which showed a decrease in new neurons.

Finally, the crucial importance of environmental condition on the development of neocortical circuitry was recently showed by an "extreme" experiment on the effects of space flight during cortical synaptogenesis (DeFelipe et al., 2002). The effect of 16 days of microgravity environment

[3] Postnatal handling is a brief (3–15 min) daily separation of the pup from the mother for the first 2-3 weeks postnatally. For a comparison of similarities and differences between EE and postnatal handling compare Fernandez-Teruel et al., 2002.

[4] Briefly, in the study of neurogenesis in the adult brain, there are two basic methods for identification of new born neurons: ^{3}H-thymidine (^{3}H-dT) and bromodeoxyuridine (BrdU) autoradiographic methods. Both markers are incorporated into nuclear DNA during the S phase of the cell cycle (Rakic, 2002a,b; Nowakowski et al., 1989).

from P14 to P30 on the neocortical representation of the hindlimb synaptic circuitry was investigated in rats. Development in microgravity leads to changes in the number and morphology of cortical synapses in a laminar-specific manner. In the layers II/III and Va, the synaptic cross-sectional lengths were significantly larger in flight animals than in ground control animals. Flight animals also showed significantly lower synaptic densities in layers II/III, IV and Va. The greatest difference was found in layer II/III, where there was a 15.6% decrease. All the changes observed only affected asymmetrical synapses, which are known to be excitatory. Furthermore, some alterations were transient, while some new differences also appeared, as demonstrated after a 4 month period of re-adaptation to terrestrial gravity. For example, significant differences in synaptic density in layers II/III and Va after re-adaptation were no longer observed, whereas in layer IV the density of synapses increased notably in flight animals. These results indicates that terrestrial gravity is a necessary environmental parameter for normal cortical synaptogenesis and highlights the extreme importance of taking account of the environmental conditions when we study “normal” ontogenesis or biological and behavioral effects of experimental manipulations.

1.2 ENRICHED BRAINS AND MINDS

1.2.1 NEUROANATOMICAL CHANGES

Initial studies on EE relied on gross neuroanatomical parameters, such as brain weight, for the quantification of experimental effects of EE. Indeed, since nineteenth century, it was known that domestication had strong influences on the brain of animals in captivity. Darwin (1868) was the first to point out that the size of the brain tends to be considerably reduced in animals as a result of domestication. The effects of an EE on brain weight appear to have relevance to the well-known observation that domesticated animals have smaller brains than the same species in the wild-naturalistic environment (Kruska, 1987, review). Furthermore, the differences in brain weight due to the exposure to a naturalistic environment are much grater then those reported between animals exposed to enriched and impoverished environments in the laboratory (Bennett et al., 1969; Rosenzweig at al., 1971, 1972; Walsh et al., 1971,1973; Cummins and Livesey, 1979). In domesticated birds, dogs, wolves, alpacas, llamas, sheep, pigs and donkeys the brain weight is 15-30% less then of those in the wild (Kruska, 1987, review). The cerebellum is reduced by about 15% and the medulla by 10% in animals in captivity. But the most marked effect is on the visual cortex, which may be reduced by 35% in domesticated animals compared with those in the wild. This may be related to the smaller size of the eye and retina in domesticated animals (Wigger, 1939). Moreover, the brain weight of the first generation of animals born in captivity is 10-20% less than that of the parents raised in the wild (Herre, 1966, review).

In parallel with domestication studies, early studies on EE confirmed the increase in the total brain weight and in the thickness and weight of cerebral cortex in rats (Altman, 1962; Bennett et al. 1969; Diamond et al., 1966, 1967, 1972; Rosenzweig and Bennett, 1969; Rosenzweig et al., 1969, 1971, 1972, 1978; Walsh et al., 1971, 1973; Cummins and Livesey, 1979). Again, the most striking effect occurs in the occipital zone, the visual cortex. Noticeably, this effect also occurs in blinded rats raised under EE (Krech et al., 1963).

Dendritic length and morphology, neurite branching, spine density and synapse changes are also parameters affected by enrichment (Fig I-3). Holloway (1966) first reported that enriched rats had more branching of basal dendrites of stellate neurons in layer II of the visual cortex as compared to rats raised in isolated laboratory cages. Furthermore, early studies (Volkmar and Greenough, 1972; Globus et al., 1973; Greenough et al., 1973) demonstrated that EE increased dendritic branching of pyramidal neurons in layers II, IV, and V of medial occipital and temporal cortices in rats. Increase in dendritic length and size, and in spine density and branching were also observed in the occipital cortex (Mollgaard et al., 1971; West and Greenough, 1972; Diamond et al., 1975; Uylings et al., 1978; Juraska et al., 1980; Green et al., 1983), somatosensory cortex

(Johansson and Belichenko 2002), hippocampus (Wenzel et al., 1980; Moser et al., 1994; Rampon et al., 2000), striatum (Comery et al., 1995), and cerebellum (Floeter and Greenough, 1979) after enrichment.

The synaptic density and morphology of visual cortex of cats raised in EE was further analyzed in two classical studies using electron microscopy (Beaulieu and Colonnier, 1987, 1988). These authors demonstrated that the numerical density of symmetrical synaptic contacts formed by boutons containing flat vesicles is nearly twice as large in the visual cortex of cats raised in the impoverished condition as in animals raised in the enriched condition. Since the vast majority of flat vesicles contacts are GABAergic, authors expected that the change in the numerical density of flat synapses was accompanied by a change in the number of boutons containing GABA. In spite of the large increase in the number of flat synapses in impoverished cortex, they found no obvious change in the apparent amount of labeled GAD terminals. This surprising result led to the hypothesis that EE could conceivably affect the number of contacts formed by the flat boutons without affecting the number of boutons. Indeed, for the total cortical thickness, the numerical density of flat boutons was only 17% lower in enriched than in impoverished cats but the diameter of boutons was 6% larger in the enriched cortex because the flat boutons became fewer in number as they became larger in size. In average, they found 2 contacts per flat bouton in enriched and 3 contacts per flat bouton in impoverished visual cortex. Thus, EE did not affect the number of flat bouton per neuron, but only the number of contacts established by each bouton resulting in smaller but more numerous contacts in the impoverished cortex. Principal cellular differences in the morphology of occipital cortex between rats raised in EE and IE are summarized in Tab I-1.

In a recent paper, the attention was focused on the effects of pre-weaning polysensorial enrichment on the development of the basal dendritic field of neurons in medial and lateral parts of parieto-occipital cortices in rats (Fernandez et al., 2003). By analyzing the geometrical complexity and territorial expansion of cortical pyramids using Golgi staining, authors compared the effects of early (from P2 to P22) EE between the medial part of the parieto-occipital cortex, that contains the representation of the foot, paw and trunk, and the lateral sector of parieto-occipital cortex, that has the representation of face and vibrissae. Authors detected two types of dendritic configuration: simple fields, that show well defined geometrical shapes such as triangles and squares, and complex fields, which are geometrically irregular. Enriched pups showed a larger number of complex dendritic fields compared to standard pups in both medial and lateral parts of parieto-occipital cortices. Within EE pups group, the lateral part of parieto-occipital cortex contained higher number of complex fields. Furthermore, percentages of complex or simple dendritic fields correlated directly with larger or smaller territorial expansion of the dendrites.

Recently, the focus of research has shifted towards EE effects on neurogenesis, gliogenesis, synaptogenesis. After few initial findings (e.g. Altman, 1962; Kaplan and Hinds, 1977, 1980; Kaplan, 1981), it is now accepted that EE effects on adult neurogenesis[5] can be found at least in two brain regions: the olfactory bulb and the hippocampus (Altman, 1962; Kempermann et al., 2001; Rochefort et al., 2002; Kozorovitskiy and Gould, 2003). Recently it has been claimed that new neurons could be generated in the adult cerebral cortex (Gould et al., 1999, 2001; but see Ehninger and Kempermann, 2003). In the early 1980s, Kaplan (1981) has reported a similar observation in the adult visual cortex of the rat. However, these findings were not taken up by other researchers and remained unconfirmed until now.

The hippocampus is of particular interest because of its importance for higher cognitive functions, especially learning and memory, and its pivotal involvement in degenerative disorders of the aging brain. Adult hippocampal neurogenesis in the dentate gyrus seems to reflect both survival and proliferative effects of EE on neurons and glia and this model has been widely used as an efficient indicator to dissect the differential effects of single environmental factors acting in the EE stimulation (Van Praag et al., 1999; Gould et al., 1999; Greenough et al., 1999; Ehninger and Kempermann, 2003), such that effects of complex environments have become a part of the scientific debate on adult neurogenesis.

1.2.2 NEUROTRANSMITTERS

Pioneering experiments on EE focused on changes in the brain chemistry of enriched rats and tried to correlate such changes with behavioural performance (e.g. Krech et al., 1960; 1962; Rosenzweig and Bennett 1969). Rosenzweig and colleagues found significant correlations between levels of the enzyme acetylcholinesterase (AChE) in the cerebral cortex of rats and their ability to solve spatial problems after behavioural training. Littermates were either trained on a difficult problem or left untrained and the cortical AChE activity of trained rats was significantly higher than that of their untrained littermates. Starting from these results the same authors tested the effect of differential informal learning by comparing AChE levels between EE and IE rats finding that informal enriched experience led to an increased cortical AChE activity.

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[5] One of the past century neurobiological dogma was that of the inability of the central nervous system (CNS) of an adult animal to generate new neurons. Between 1970s and 1980s a few works generated considerable interest when they first reported that neurons and glial cells continued to be produced in the dentate gyrus of the rodents' hippocampus throughout adult life (Kaplan and Hinds, 1977, 1980; Bayer, 1982; Bayer et al., 1982). This initial excitement was tempered by the failure to detect neurogenesis in adult macaques (Eckenhoff and Rakic, 1988), but since the end of 1990s the field has been revised by reports of neurogenesis in the dentate gyrus of marmosets (Gould et al., 1998), macaques (Fallah et al., 1998) and even humans (Eriksson et al., 1998). But see Rakic, 2002a,b.

Other small-molecule transmitters such as biogenic amines and amino acids are also implied in neuroplastic changes due to EE. Monoamines such as serotonin (5-HT) and noradrenaline (NA) can facilitate the formation and maintenance of synapses in the central nervous system (Matsukawa et al., 1997; Niitsu et al., 1995; Okado et al., 1993). Since 5-HT together with acetylcholine plays an important role in spatial learning (Matsukawa et al., 1997), it is possible that the effects of EE are mediated, in part, through the actions of monoaminergic neurons. Indeed, intact NA projection is required for environmentally dependent changes in cortical morphology (Benloucif et al., 1995).

The hypothesis that NA is critically involved in neural plasticity was tested by administering xylamine, a noradrenergic neurotoxin, to young rats prior to environmentally enriched housing (Benloucif et al., 1995). In saline-treated rats, exposure to enriched conditions significantly increased the weight of occipital, dorsal, and ventral cortices and the remaining brain compared to individually housed rats. In xylamine-treated rats, only the weight of dorsal cortex increased with exposure to enriched conditions. Xylamine treatment did not reduce brain weights of individually housed animals.

In another experiment (Dalley and Stanford, 1995), levels of extracellular NA in the frontal cortex of rats were examined using in vivo microdialysis during exposure to a novel naturalistic environment. NA efflux increased when rats were transferred to a novel cage but this was not significant when compared with either basal efflux or with changes after equivalent handling in their home cage. However, a sustained and significant increase in NA efflux was found when an unfamiliar conspecific was also present in the novel cage.

A more recent work (Naka et al., 2002) using high performance liquid chromatography demonstrated that EE increases NA concentration in the mouse brain. After being exposed to an enriched environment for 40 days, NA content was increased significantly in the parieto-temporo-occipital cortex, the cerebellum and the pons/medulla oblongata. In contrast, no changes were observed in serotonin (5-HT) or dopamine (DA) levels in these same regions. Tab I-2 summarizes the effects of EE on monoamines and their metabolites concentrations in the mouse brain.

The amino acid content has been studied in six discrete CNS areas of mice exposed to EE or IE (Cordoba et al., 1984). Differences between enriched and impoverished groups were found for different amino acids. A significant increase for aspartate was found in the spinal cord, whereas glutamate (Glu) significantly decreased in colliculi and cerebral cortex of EE animals. Similarly, glycine increased in cerebral cortex and decreased in colliculi and pons-medulla, and gamma-aminobutyric acid (GABA) increased in spinal cord, pons-medulla and cerebellum and decreased in thalamus-hypothalamus. In adult rats, EE has been shown to selectively increase the glutamate

agonist ^{3}H-AMPA binding in the hippocampus but not in the cerebral cortex (Gagne et al., 1998; Foster et al., 1996).

1.2.3 NEUROTROPHINS

Environmental enrichment induces changes in neurotrophin expression. Neurotrophins (Nerve Growth Factor, NGF; Brain-derived Growth factor, BDNF; Neurotrophin 3 and 4/5, NT-3 and NT-4/5) are a class of molecules strongly implied in neuroplasticity during development and adult life. Neurotrophic factors are special endogenous signalling proteins that promote survival, division and growth, as well as differentiation and morphological plasticity of neural cells. In the developing, adult and ageing nervous system, neurons are nourished and maintained by neurotrophic factors. Neurons sustain their inputs to targeted cells by producing and releasing neurotrophic factors mainly in a retrograde manner (Vicario-Abejo et al., 2002, review).

The best-characterized neurotrophic factor is the NGF, first described by Levi-Montalcini and Hamburger (1953). NGF was originally purified from the mouse submandibular gland as a complex of three dissimilar subunits: α, β and γ. Only the β subunit appears to be the neuroactive component. The NGF gene that codes for the pro β-NGF protein precursor is highly conserved across species. More recently, other members of the neurotrophin family of growth factors were identified. Using polymerase chain reaction (PCR) and hybridisation screening, it was found that the BDNF had 50% sequence homologue with NGF (Hohn et al., 1990; Barde, 1994). Other new members that were found include NT-3 and NT-4/5 (Ernfors et al., 1990; Ibanez, 1996). In the brain, NGF mRNA levels are highly expressed in the hippocampus, the cerebral cortex and the olfactory bulb (Korsching et al., 1985; Whittemore et al., 1986; Ayer-LeLievre et al., 1988). These are the major sites of the basal forebrain cholinergic innervation. The cholinergic neurons in the basal forebrain (the medial septum, the nucleus basalis of Meynert and the diagonal band of Broca) provide primary action site for NGF. As a target-derived factor, NGF supports cholinergic neurones in the brain by retrograde transport to the targeted sites (Seiler and Schwab, 1984; Hefti, 1986; Krommer, 1987).

The neurotrophins bind to two types of transmembrane receptor protein on presynaptic nerve terminals to elicit biological responses: the tyrosine kinase Trk (tropomyosin receptor kinase) receptors and the p75 receptor. NGF is the preferred ligand for TrkA, whereas BDNF and NT4 bind preferentially to TrkB, and NT3 to TrkC, although this neurotrophin can also activate TrkA and TrkB in some cellular contexts (Huang and Reichardt, 2001). They can all

bind to the low affinity p75, but it has not been shown clearly whether this receptor is involved in central synapse development (Yang et al., 2002). High levels of both p75 and TrkA receptors are localized in the cholinergic neurons of the medial septal nucleus (MSN) and ventral diagonal band (VDB).

Available evidence indicates that at least three members of the NGF family of neurotrophic factors, NGF, BDNF, and NT-3 are involved in neuroplasticity related to environmentally conditions. The research group by Mohammed and colleagues widely explored the effects of EE on neurotrophic factors (Mohammed et al., 1990, 1993; Ickes et al., 2000; Pham et al., 1999a,b, 2002 review). Rats raised in EE have higher hippocampal NGF levels than isolated rats. Increased expression of hippocampal mRNAs for NGF, BDNF and NT-3 is also observed following EE (Falkenberg et al., 1992; Torasdotter et al., 1996, 1998; but see Ickes et al., 2000 for NT3).

Long-term exposure (1 year) to EE has been employed to examine effects on NGF levels in the hippocampus, the visual cortex, the entorhinal cortex and the hypothalamus, and the NGF receptors (NGFr), p75 and TrkA, in the cholinergic neurons of the MSN (Pham et al., 1999b). The NGF levels in all brain regions examined except the hypothalamus were significantly higher after a year of enriched housing compared to isolated housing. The highest NGF levels were detected in the hippocampus and relatively low levels were seen in the hypothalamus (Fig I-4). Enriched animals had significantly higher overall staining densities for both p75 and TrkA-immunoreactive cell bodies in the MSN. There was also a difference between the p75 and TrkA antibody staining distribution. The p75 receptor appeared to be expressed along the entire cell membrane of cell bodies and neurites, while the TrkA receptor was expressed primarily in the cytoplasm of cell bodies and varicosity of neurites.

The comparison of various brain regions of animals housed in an enriched environment to isolated animals with respect to NGF, BDNF and NT-3 levels showed that enriched brains have higher neurotrophin levels in all regions examined (Ickes et al., 2000) (Fig I-5). Higher levels of BDNF, NGF and NT-3 were found in the medial basal forebrain of animals raised in an EE compared with isolated animals. In the cerebral cortex, was also found that BDNF and NT-3 levels were significantly higher in the enriched than in the isolated animal (Fig I-6). As anticipated before, significantly higher BDNF and NGF was detected in the hippocampal formation and in the hindbrain of enriched animals. In contrast with a previous study (Torasdotter et al., 1996) the levels of hippocampal NT-3 were found relatively low. It has been suggested that NT-3, as opposed to NGF and BDNF, appears to be regulated independently of neuronal activity, its mRNA expression and secretion are dependent on hormonal levels and on BDNF levels (Hyman et al., 1994;

Lindholm et al., 1994). Consequently, EE effects on BDNF levels in the brain might indirectly affect NT-3 levels.

BDNF is also up-regulated by physical exercise in running wheels in mice (Oliff et al., 1998; Berchtold et al., 2001, 2002; Cotman and Berchtold 2002). Hippocampal full-length BDNF mRNA expression was rapidly influenced by physical activity, showing significant increases in expression levels as soon as after 6 and 12 h of voluntary wheel running. This up-regulation of hippocampal BDNF mRNA results from an interaction between estrogen and exercise. The exercise effect on BDNF up-regulation is reduced in the absence of estrogen, in a time-dependent manner. In addition, voluntary activity itself is stimulated by the presence of estrogen.

1.2.4 GENES

The expression of a large number of genes changes in response to EE; many of these genes can be linked to neuronal structure, synaptic plasticity, transmission, and play important roles in modulating learning and memory.

In a study by Rampon and colleagues (2000) oligonucleotide array probes for more than 11.000 (13.069 probe sets) mouse genes were used to examine the early and late gene expression patterns of EE and IE groups of mice. Early gene expression was analyzed after exposing adult mice (4 months) to an EE for 3h or 6h. Late gene expression was analyzed after exposure to EE for 2 days or 14 days. Control condition was represented by littermate mice housed under standard laboratory condition.

In the short-term EE condition only 78 of the 13,069 probe sets on the arrays were changed by more than 1.5-fold. Almost half (46%) of the environmentally responsive, differentially expressed genes coded for proteins involved in macromolecule synthesis and processing, including transcription factors, translational regulatory enzymes, and enzymes involved in DNA, RNA, and protein processing. Among these, the highest level of induction was in DNA methyltransferase (over 10-fold increase), the major function of which is to maintain DNA methylation during DNA replication. Indeed, it has been shown that DNA methyltransferase activity is critical for neuronal cell differentiation induced by NGF (Persengiev and Kilpatrick, 1996), but also that nonproliferating neurons express high levels of DNA methyltransferase, suggesting it may perform functions such as DNA mismatch repair in the adult brain (Brooks et al., 1996). Many genes encoding transcription factors were also up-regulated (e.g. myelin gene expression factor and an estrogen-responsive finger protein), with the notable exception of retinoid X receptor (RXR) alpha, whose expression decreased dramatically (226 and 216-fold at 3 and 6 h, respectively). It has been shown that expression of RXR alpha is also decreased by elevated intracellular calcium during T

cell activation (Ishaq et al., 1998), suggesting that down-regulation of RXR alpha in neurons may also involve calcium signaling. A group of genes encoding proteolytic proteins involved in signaling and apoptosis were also found to be differentially expressed after enrichment (e.g. prolyl oligopeptidase, caspase-6, protease 4). The Bcl-2 associated protein Bax was also down-regulated, indicating a possible antiapoptotic effect of EE (Chan and Mattson, 1999). The expression of genes involved in formation of new synapses and reorganization or strengthening of existing synapses also showed changes. For example, the gene encoding integrin alpha-4 and the expression of GTPase RhoA are increased after short-term EE. Changes in the expression of these genes clearly suggest that presynaptic processes are being modified by enriched experiences. A number of genes whose products are associated with neuronal excitability also changed their expression levels after 3 and 6 h of enrichment (e.g. the seizure-related protein, the 78-kDa, neurokinin A, Lissencephaly-1 gene).

In the long-term EE condition approximately 100 transcripts changed by at least 1.5-fold; most are different from those whose expression levels changed at the short-term EE condition, and most are involved in neuronal transmission and structural changes. The mRNA level of the transcription factor X-box binding protein-1 (XBP-1) increased 2.4-fold after 2 days of training. XBP-1 interacts with cAMP-responsive elements of many genes and activates their expression (Clauss et al., 1996) and the cAMP pathway is essential for memory formation (Mayford and Kandel, 1999).

A number of genes associated with NMDA receptor function were affected by enrichment. For example, the expression level of postsynaptic density 95 (PSD-95) increased after 2 days (7-fold) and 14 days (2.3-fold) of enrichment. PSD-95 participates in the anchoring of the NMDA receptor. The PSD-95 protein complex seems to be important in coupling the NMDA receptor to pathways that control synaptic plasticity and learning. Environmental enrichment was also associated with changes in expression of molecules downstream of the NMDA receptor including the up-regulation of calmodulin or down-regulation of neurogranin. Many proteolytic proteins and many genes associated with structural changes that occur during neuronal growth (e.g. the cytoskeletal protein dynactin, cortactin) are up-regulated after long term enrichment. The protein defender against cell death 1 (DAD1), and apolipoprotein E (apoE), mutation of which has been linked to Alzheimer's disease, are also increased after long EE.

However, also physical exercise has been demonstrated to influence gene expression in a EE-like manner. Exercise can promote changes in neuronal plasticity via modulation of neurotrophins. Rodents exposed to voluntary wheel running for 3 or 7 days were assessed for

changes in their lumbar spinal cord and soleus muscle. Exercise increased the expression of BDNF and its receptor, synapsin I (mRNA and phosphorylated protein), growthassociated protein (GAP-43) mRNA, and cyclic AMP response element-binding (CREB) mRNA in the lumbar spinal cord (Gomez-Pinilla et al., 2002).

In conclusion, EE affects the expression levels of a number of genes involved in neuronal structure, synaptic signaling, and plasticity. Some of these genes are known to be associated with learning, memory, apoptosis and neurodegenerative disorders.

1.2.5 SPONTANEOUS BEHAVIOR, LEARNING AND MEMORY

Behavioral effects of EE were the starting point for subsequent neurobiological investigation on enrichment (Hebb, 1949). Initial studies focused on the superior learning, explorative and spatial abilities of EE animals. To summarize, a frequent effect of EE treatment on spontaneous activity/exploration in novelty situations is a long-lasting increase in exploratory activity, as mentioned, in the open-field test (Denenberg et al., 1978; Ferchmin and Eterovic 1980; Ray and Hochhauser, 1969; Widman and Rosellini, 1990; Larsson et al., 2002) and in the hole-board test (Escorihuela et al., 1994a,b; Fernandez-Teruel et al., 1992.). Furthermore, EE-treated animals show a different (i.e. a more complex and diverse) organization of exploratory behavior, as reported by Renner and Rosenzweig (Renner, 1987; Renner and Rosenzweig, 1986, 1987; Widman and Rosellini, 1990) than standard or impoverished rats. EE improves acquisition and retention in several learning tasks, ranging from spatial and problem-solving tasks (e.g. Bennett et al., 1970; Cooper and Zubek, 1958; Denenberg et al., 1968; Denenberg and Morton, 1962; Forgays and Read, 1962; Freeman and Ray, 1972; Juraska et al., 1980; Kempermann et al. 1997; Liljequist et al., 1993; Mohammed et al., 1990; Paylor et al., 1992; Ray and Hochhauser, 1969; Smith, 1972; Venable et al., 1988; Woods, 1959; reviewed by Escorihuela et al., 1994b; Renner and Rosenzweig, 1987) to the acquisition and long-term retention of two-way active avoidance (Escorihuela et al., 1994a). Likewise, EE has been shown to also improve retention in nonspatial tasks and object recognition tests (Escorihuela et al., 1995c; Rampon et al., 2000). Nevertheless, there are also reports of no effects of EE when the tasks used are relatively simple, as for instance habituation (but see also Larsson et al., 2002), passive avoidance or some tasks of visual discrimination (e.g. Bernstein, 1973; Davenport, 1976; Freeman and Ray, 1972; Krech et al., 1962; Lore, 1969; Sjoden, 1976; Renner and Roseznweig, 1987, review) although recently Prusky and colleagues found ameliorative effects of EE on the visual acuity in mice (Prusky et al., 2000). It appears, therefore, that EE treatment improves learning most consistently when the tasks used have a relatively high level of aversiveness and complexity, as do some spatial tasks (the most commonly used being the Morris

water maze) and the two-way active avoidance task. Noticeably, when tested on a different spatial memory task (a T-maze), enriched rats did better than isolated rats with a running wheel (Bernstein, 1973). In addition, the influences of EE on learning ability appear to be lifelong (e.g. Meaney et al., 1988, 1991; Fernandez-Teruel et al., 1997; Renner and Rosenzweig, 1987).

Present thesis begun with the statement that EE only represents a step ahead the standard laboratory rearing conditions. A recent paper by van der Harst and colleagues (2003) strongly lights up the behavioral meaning of rearing animals in standard laboratory conditions compared to EE. The sensitivity to rewards in standard or enriched housed rats changes dramatically, clearly showing that the enriched environment paradigm has rewarding properties for rats. The sensitivity to rewards of the differentially housed rats is determined by focusing on the anticipatory response (expectation), an early component of appetitive behavior, to an announced reward (e.g. sucrose). Standard housed rats were more sensitive[1] to the reward than the enriched animals, indicating that SE leads to a deprivation of the ability to satisfy behavioral needs in these impoverished housing conditions and that EE partly compensate for this deprivation. This rewarding property is likely to be a result of the ability to display a more extensive natural behavioral repertoire. In this light, standard housing condition encompasses many properties of what in human psychology has been called “frustration”.

On the opposite site, EE has been demonstrated to influence the behavioral stress response. In a classical experiment (Klein et al., 1994) rats were randomly assigned to either an enriched or standard environment for 30 days prior to behavioral testing. The predator stress testing consisted of placing the rat in a cat avoidance apparatus so that the rat’s behavioral response to a natural stressor, the cat, could be assessed. The rats were subsequently exposed to a partial predator stimulus (cat urine) that was placed in the home cages for 7 days. Results indicated that the enriched rats engaged in less defensive behavior than the standard rats during behavioral testing and that female rats demonstrated more defensive behavioral patterns compared to male rats.

In another study (Larsson et al., 2002), rats raised in differentially housing conditions were exposed to differing degrees of stress intensity levels and the effects on basal and post-stressor behavior were analyzed. Animals were exposed to a mild stress condition or a powerful stress condition and later behaviourally tested. The mild stress condition consisted of placing animals in a passive avoidance box without shock exposure, while in the powerful stress condition, the animals were exposed to the box and shock. Results indicated that enriched animals showed less anxiety-related behavior. In the open-field test the enriched animals explored more initially and then

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[6] The anticipatory increase in activity is measured by observing animals by means of an ethogram grid describing the frequency or transitions of different behavioural elements (mobile exploration, walking, rearing, sniffing, freezing, etc).

habituated faster and also reduced defecation, an index of fearfulness. In the Morris water maze task enriched performed better in comparison to impoverished animals. These findings are also confirmed by prior works (Freeman and Ray, 1972; Manosevitz and Joel, 1973; Fernandez-Teruel et al. 1992, Mohammed et al., 1986, 1990; Nilsson et al., 1993; Falkenberg et al., 1992; Torasdotter et al., 1996; Pham et al., 1999a,b). The enriched animals also showed less locomotion scores than the impoverished animals during a second test in the open-field given 24 days after the first test, indicating that the enrichment effects on emotionality and exploratory behavior is long-lasting and also persists after extensive training and testing. Stress exposure prior to training in Morris water maze revealed distinct learning and memory effects in the differentially housed animals. While exposure to stress prior to test enhanced escape performance in enriched animals, the same treatment impaired performance in impoverished animals. These environmentally induced differences were seen following different stress intensity levels indicating that mild stress significantly enhanced performance in the enriched animals, but had no impact on escape latency in the impoverished animals; however, after exposure to high-intensity stress, performance on escape latency were severely impaired in the impoverished animals from the first day of training, while in the enriched animals escape performance were only impaired during the last day of training. In the open-field test, stress pre-exposure reduced locomotion counts in both the differentially housed animals, but the enrichment effect on emotional reactivity in the open-field is long-lasting and persists even after extensive training and housing in standard laboratory conditions. As before, it seems that EE animals, being less frustrated, have stronger abilities to deal with stressing events. How these effects can be translated into useful indications for human psychology has been recently demonstrated by a longitudinal study showing a less incidence of psychopathological disorders in human subjects "enriched" during childhood (Raine et al., 2003).

However, it is worth to note that improved spatial abilities after EE could also interact with motivational and emotional aspects of behavior. Indeed, novelty preference and the complexity of exploratory behavior in which animals engage, appears to be clearly enhanced in EE rats (e.g. Escorihuela et al., 1995c; Fernandez-Teruel et al., 1992a; Larsson et al., 2002; Renner 1987; Renner and Rosenzweig, 1986, 1987;Widman and Rosellini, 1990), although faster habituation to novelty (i.e. faster within-session decrease of activity and exploration of novel objects) has also been found as a consequence of EE, thus suggesting that improved spatial abilities (due to EE) could explain at least some of the findings of EE effects on behavior under novelty conditions (Zimmermann et al., 2001).

In this light, consequences of EE on emotionality and anxiety are good indicators to settle this debate. EE reduces fearfulness, as suggested by reductions of defecation in open-field-like tests

(Fernandez-Teruel et al., 1992a; Freeman and Ray, 1972; Manosevitz and Joel, 1973; Larsson et al., 2002) and by increases in the number of entries into (although not in the time spent in) the open arms of the elevated plus-maze (Escorihuela et al., 1994b; Fernandez-Teruel et al., 1997). In tasks involving classical fear conditioning, there is some evidence of a lesser expression of fear-related responses in EE-treated rats (e.g. Nikolaev et al., 2002; Larsson et al., 2002), and long-lasting improvements of two-way active avoidance acquisition have also been found in EE rats (Escorihuela et al., 1994a; Ray and Hochhauser, 1969). Taken together, these findings suggest that EE may induce some ‘‘anxiolytic’’ effects.

Using general behavioral and psychological constructs rather than specific behavioral measures, we could summarize EE effects on behavior as follows: EE increases spontaneous activity and specific exploratory behavior in tests involving novelty (e.g. open-field, hole-board, etc.), moreover influencing the quality and the organization of exploratory behavior in a more complex direction as compared to standard or impoverished housing; emotionality and fearfulness (as measured, for instance, by novelty-induced defecation, dark–light tests and elevated plus-maze) is clearly reduced by EE; learning abilities and spatial orientation are clearly enhanced by EE, providing more evidences to the fact that standard laboratory housing represents a “standard experimental condition” surely for the researcher, maybe not for the experimental animals. EE animals seem to be less frustrated, less stressed and more motivated.

1.3 DISSECTING THE ENRICHING FACTORS

All measures affected by an enriched environment depend on, and have not been dissociated from, an increase in voluntary motor behavior or exercise. The importance of single factors on EE effects have been tested particularly for the effects of socialization, inanimate environment, learning and general activity.

Several experiments were conducted to test whether, mere group living (social stimulation) can account for the significant differences in measures of brain anatomy and brain chemistry that develop between rodents housed in groups in enriched environments and rodents housed singly in restricted environments (Rosenzweig et al., 1978; Ferchmin and Bennett, 1975; Bernstein, 1973). To test the relative effectiveness of direct vs. indirect social interaction with an EE, some rats were housed in groups of 12 in large EC cages while littermates "observers" (OC) rats were placed singly in small wire-mesh cages within EC (Ferchmin and Bennett, 1975). A third group was housed singly in an IC where stimulation was minimal. After 30 days, the brain differences were measured. In both experiments the usual pattern of EC-IC differences in brain weights appeared, whereas OC showed no significant differences from IC. On measures of exploratory behavior taken during the last 2 days of the second experiment, IC fell significantly below EC, and OC was somewhat below IC. Thus EC differed from both IC and OC in brain and in behavior. Active contact with an enriched environment appears necessary for development of EE effects.

Other authors tested the alternative hypothesis that features of the inanimate environment could significantly affect brain measures of animals living in a social group (Rosenzweig et al., 1978). In these experiments, groups of 12 male rats were assigned for 30 days to several types of environmental conditions: (a) large cage without stimulus objects, (b) large cage containing varied stimulus objects, (c) large cage containing a maze whose pattern of barriers was changed daily, and (d) a semi-natural outdoor environment; in each experiment, littermates of rats in the social conditions were housed in isolation in small colony cages. At the end of the 30-day period, measures were taken of weights of brain regions, RNA and DNA contents of regions of cerebral cortex, and acetylcholinesterase activities of brain regions. Although the number of rats housed together was constant for conditions a-d and cage size was constant for conditions a-c, the magnitudes of the cerebral measures varied significantly as a function of the inanimate stimulus conditions. The differences from isolation-housed littermates was greatest in condition d and smallest in condition a. Thus, social grouping alone is inadequate to explain the cerebral effects of enriched environments and the inanimate stimulus conditions seems to be an indispensable variable to account for EE effects. Summarizing, neither observing an enriched environment without being allowed active participation nor social interaction alone can elicit the same brain effects of enriched environments.

Completely separating different behaviors in a complex environment is difficult and can depend on the measure used to assess the changes in the brain. Assessment of neurogenesis, especially in

the hippocampal formation, has recently become one of the most important measures to account for EE effects on the brain. Van Praag and colleagues (1999) demonstrated that both proliferation and survival of newly formed neurons can be affected by EE. Furthermore, they attempted to separate the differential effects of enriching components by investigating the contribution of learning and physical activity on neurogenesis. They compared the rate of proliferation and survival in the dentate gyrus neurons of adult female mice for a short-term exposed to a variety of experiences, including housing in an enriched laboratory environment, hidden-platform spatial navigation learning in the Morris water maze (MWM), forced swimming exercise, voluntary-exercise in a running wheel, as well as standard-living (control) conditions. Enriched environment increased the survival of newly born granulate cells in the dentate gyrus but did not affect proliferation, as assessed by the bromodeoxyuridine (BrdU)-positive cell number, thus confirming a previous finding by the same group (Kempermann et al., 1997a,b) (Fig I-7b). However, more interesting is the fact that voluntary exercise in a running wheel increased cell proliferation and cell survival (Fig I-7). Indeed, runners mice had more proliferation than any of the other groups examined and same rate of survival as that observed in the EE group.

Those findings strongly suggest that physical activity is sufficient to enhance several aspects of adult hippocampal neurogenesis and raises parallels, but also questions, on the enriching factors acting in an EE. However, things might be more complicated. Other researchers (Gould et al., 1999) examined the rate of neuronal survival in the dentate gyrus of adult male rats after training in four different learning tasks[7] and found that training on associative learning tasks that require hippocampus doubled the number of adult generated neurons in the dentate gyrus, thus indicating a specific effect of hippocampal-dependent learning on neurogenesis. Taking also into account that van Praag's study was carried out on female mice whereas Gould's was on male rats, the differences between the rate of neurogenesis observed after learning experience can be explained by

[7] The first task was "trace" associative eyeblink conditioning, in which a brief time interval separates the conditioned stimulus (CS; a burst of white noise) from the unconditioned stimulus (US; a shock to the eye). Performance on this task is impaired by damage to the hippocampal formation. They compared this with "delay" conditioning, which is similar to trace conditioning except that the US overlaps and coterminates with the CS; this task can still be learned after hippocampal lesions. In a second experiment, they compared spatial learning in a water maze, in which the rat must learn to use distant cues to find the hidden platform, with a local cue task, in which the rat learns to recognize the platform directly. As above, learning the first task requires an intact hippocampus, whereas learning the second task does not. In both experiments, the authors found that the survival rate of labeled neurons was more than two times greater in animals that had learned the hippocampus-dependent task than in those learning a similar but hippocampus-independent task.

the different labeling protocols used and the time interval between labeling and behavioral training (Greenough et al., 1999)[8].

More important seems to be the specificity of the neurogenic effect, as demonstrated by investigating the effects of long-term exposure to EE on the senescent dentate gyrus neurogenesis (Kempermann et al., 1998; 2002) (Fig I-8). After long-term environmental enrichment of 10 months (and 1 day after the last injection of BrdU), as many BrdU-labeled cells were found in the dentate gyrus of mice living under EE as compared with controls. However, this comparison was not statistically significant indicating no clear difference in proliferative activity in the subgranular zone. Survival of BrdU-labeled cells was assessed 4 weeks later. Again, no statistically significant difference was found for the total number of BrdU-positive cells, regardless of their phenotype. Taken together, these data indicate that long-term enrichment and activity do not have a clear measurable effect on the number of BrdU-labeled cells early or late after BrdU administration. However, analysis of phenotypes showed that, 4 weeks after BrdU in EE mice, 26% of the BrdU-labeled cells were also NeuN-positive, but only 8% in controls. Conversely, the control group produced relatively more astrocytes. There was no difference for the cells displaying neither phenotype. Therefore, the net neurogenesis (surviving BrdUlabeled cells ratio of NeuN/BrdU-positive cells) was five times higher in EE than in control mice. Thus, the induction of adult neurogenesis occurred at the expense of gliogenesis, but in absolute numbers no significant decrease in hippocampal gliogenesis occurred. Taken together, these data can be interpreted as a sustained survival promoting effect acting selectively on new neurons and induced by long-term exposure to an enriched environment.

In a subsequent experiment by the same group (Ehninger and Kempermann, 2003), after confirming that the experimental manipulations of EE and running led to the same type of enhancement in numbers of BrdU-labeled cells in the hippocampal dentate gyrus, the effects of EE on cortical neurogenesis were investigated. All the cortical regions examined revealed no difference in the number of BrdU-labeled cells between runners, EE and control mice. However, if cortical regions and cortical layers were analyzed individually, differences in absolute numbers of BrdU-labeled cells emerged. At day 1 (one day after the last BrdU injection), runners mice had

[8] Van Praag et al. administered BrdU daily between days 1 and 12 and counted labeled neurons after one day (day 13, assumed to reflect proliferation) or four weeks (day 43, assumed to reflect survival). Mice were trained daily for 30 days beginning at the start of the labeling period, with the platform location reversed at day 24. Most of the initial learning occurred early in the labeling period, and learning of the reversed platform condition occurred two weeks after labeling. Gould et al. administered BrdU for one day only, and counted neurons on day 11 or 17 (reflecting survival in both cases). Training began one week after labeling, and continued for one week. The discrepancy between the two studies could be explained if we assume that newly formed neurons go through a transient period of sensitivity to the survival-promoting effect of learning (Greenough et al., 1999).

significantly more BrdU-labeled cells in layer 1 of the cingulated cortex (as compared to control mice). This significant difference in layer 1 of the cingulate cortex could also be seen at 4 weeks (ibidem). Additionally, at 4 weeks runners mice had significantly more BrdU-labeled cells in layer 1 of the motor and visual cortex and fewer BrdU-labeled cells in layers 2–6 of the insular cortex. In the comparison EE versus control mice only layers 2–3 of the visual cortex showed a significant difference (statistically), with more cells under enriched conditions. The regions in which a significant increase on the level of BrdU counts was found were selected for further phenotypic analysis to investigate whether the BrdU-labeled cells would acquire a neuronal, astrocytic or oligodendrocytic phenotype[2]. No new neurons (BrdU/NeuN double-labeled cells) were found in the cortex. Astrocytes accounted for a large percentage of BrdU-labeled cells at 4 weeks. Only in layer 1 of the motor cortex, however, did the effects of running and EE on the numbers of BrdU-labeled cells translate into a net increase in astrogenesis, whereas only very few BrdU-labeled oligodendrocytes could be found and neither running nor EE had an obvious effect on this number. Labeling microglia revealed that the regions specific increases in the number of BrdU-marked cells in the running condition were due to significant increases in microglia. However, this effect was not seen under EE conditions.

Summarizing, analysis of enriching factors converge towards the importance of increased voluntary exercise as an indispensable variable to account for EE effects. However, the greater opportunity for physical activity cannot account for the effects entirely, e.g. the enriched environment significantly increases cortical weight, whereas exercise does not (Huntley and Newton, 1972). Indeed, as we saw describing the behavioral effects of EE, enriched rats have better behavioral performances than isolated rats with a running wheel (e.g. Bernstein, 1973) indicating that the interaction between socialization, inanimate environment, learning and general activity is probably the most important variable.

[2] Multipotent neural stem or progenitor cells can be isolated from the adult rodent cortex that give rise to neurons, astrocytes and oligodendrocytes (Palmer et al., 1997). One of the most used neuronal marker is NeuN.

1.4 AGEING, NEURODEGENERATIVE DISORDERS AND BRAIN REPAIR

That intellectual activity is neuroprotective maybe is more than a popular adage. Biomedical and psychological research support the idea that our daily behaviors can affect the molecular composition and cellular structure of our brain during developmental, mature and aging life, influencing the risk for neurodegenerative disorders and their outcome. Neurodegenerative disorders like Alzheimer disease (AD), Parkinson disease (PD), Huntington disease (HD), and stroke (occlusion or rupture of a cerebral blood vessel) are more common nowadays because of advances in the prevention and treatment of cardiovascular disease and cancer and mean life span elevated beyond the age of 70. At the same time, is well documented that risk for progressive neurodegenerative cognitive decline, e.g. in AD, has an inverse relationship with educational attainment such that more educated persons are at reduced risk (Evans et al., 1997). To explore the association between physical activity and the risk of cognitive impairment and dementia, Laurin and colleagues (2001) measured the co-occurrence of cognitive impairment and dementia with the levels of physical activity. High levels of physical activity were associated with reduced risks of cognitive impairment, AD and dementia of any type in elderly persons, suggesting a neuroprotective role for regular physical activity on mental decline.

In this light EE might be seen as an increased mental and physical activity that can counteract adverse effects of aging on the brain (Mattson et al., 2001, 2002). Indeed, in addition to its effects on neurogenesis, EE can reduce the rate of spontaneous apoptosis (Young et al., 1999). In the rat hippocampus cells containing fragmented DNA (a method used to assess spontaneous apoptotic cell death), was reduced by about 45% in enriched animals compared to SE rats. Furthermore, EE can prevent the synaptic density decreases in hippocampus during aging in rats (Saito et al., 1994) and living in an EE have both acute (after short-term EE exposure) and sustained (after long-term EE exposure) effect on neurogenesis in the dentate gyrus of senescent mice (Kempermann et al., 1998; 2002). The increased complexity at the neuroanatomical level of the enriched animals may function as a functional reserve that increases the resistance of neurons to the physiological age-related decline (Black et al., 1989; Kleim et al., 1997), thus extending their functionality in time.

This neuroprotective role of EE in facilitating neuronal survival during postnatal and aging life is also extended to the prevention of the apoptosis and cell death that is associated with exogenous cerebral insults. Effect of differential housing were assessed in response to kainate-induced[3] seizures and neuronal injury (Young et al., 1999). Intraperitoneally administration of kainic acid shows a characteristic progression through the behavioral seizure stages of immobility and staring, “wet-dog-shakes” (WDS), facial clonus, unilateral and bilateral forelimb clonus and leads to the development of a prolonged seizure disorder called status epilepticus. No enriched rat showed any substantial WDS or motor seizures, at least at the dose active for standard housed rats. The suppression of kainate-induced seizures by enrichment exposure is also confirmed by the absence of signs of seizure-induced neuropathology in the hippocampi of EE animals.

The induction of status epilepticus, affecting hippocampal functioning, determines cognitive functions impairments in experimental animals. EE can counteract the effects of lithium-pilocarpine-induced status epilepticus in rats (Faverjon et al., 2002). Rats with pilocarpine-induced status epilepticus were then exposed to EE and their visual-spatial memory was evaluated in the Morris water maze. Animals exposed to the EE performed significantly better than the non-enriched

[3] Kainic acid activates glutamate receptors and induces a seizure syndrome with associated excitotoxic neuropathology in rodents characteristic of temporal lobe epilepsy (Sperk, 1994). Systemic administration of this toxin mainly affects the hippocampus, and induces apoptosis.

group in the water maze. Furthermore, after behavioral testing, there was a significant increase in neurogenesis and phosphorylated cyclic AMP response element binding protein (pCREB) immunostaining in the dentate gyrus, suggesting that EE resulted in no change in status epilepticus induced histologic damage.

Epidemiological studies in humans have shown that environmental factors such as socioeconomic environments may influence the injurious effects on the brain of a neurotoxin such as lead (Bellinger, 2000; Bellinger et al., 1988). Schneider and colleagues (2001) used an animal model of lead poisoning to study the effects of EE. Young rats were raised in either EE or IE and drank either normal water or water with lead. Lead-exposed rats raised in the IE had spatial learning deficits and significantly decreased neurotrophic factors gene expression in the hippocampus. On the contrary, animals raised in the EE were significantly protected against the behavioral and neurochemical toxicity effects of lead. Similar results were found in another study (Guilarte et al., 2003) in which is reported that EE reverses long-term deficits in spatial learning produced by developmental lead exposure in rats. Enhanced learning performance of lead-exposed animals reared in an EE was associated with recovery of deficits in N-methyl-D-aspartate receptor subunit 1 (NR1) mRNA and induction of BDNF mRNA in the hippocampus. Authors conclude proposing EE as therapeutic strategy for the treatment of childhood lead intoxication.

The most common animal model to reproduce the human stroke is that of focal ischemia induced by transient or permanent middle cerebral artery occlusion in the rat (Coyle, 1982; Bedersen et al., 1986). Environment influences the functional outcome of experimental cerebral infarction in rats stimulating mechanisms that enhance brain plasticity after focal brain ischemia (Ohlsson and Johansson, 1995; Grabowski et al., 1995). The functional outcome of brain ischemia was investigated both after preoperative and postoperative enrichment of the environment. Rats kept in an EE before and after or only after the cerebral artery occlusion performed significantly better than rats kept only preoperatively in EE in a leg-placement test, beam walking, walking on a rotating pole, and climbing (Ohlsson and Johansson, 1995). Noticeably, even if delayed for 15 days after the ischemic event, EE can significantly improve functional outcome after an experimental focal brain infarction (Johansson, 1996).

Mechanisms responsible for these protective EE effects again require the activation of neurotrophic factors, in particular related to the neurotrophic activity of BDNF in the post-ischemic phase (Zhao et al., 2000, 2001; Dahlqvist et al., 2003). However, in contrast to what could be expected, BDNF gene expression in rats post-operatively housed in an enriched environment was lower than in standard rats at 2-12 days after the stroke in peri-infarcted frontal cortical regions, and in the hippocampus (Zhao et al., 2000). In another experiment (Zhao et al., 2001), showing that

housing conditions after permanent middle cerebral artery ligation leads to differential regulation of BDNF protein levels in the forebrain regions, tissue levels of BDNF protein were studied using enzyme immunoassay in different forebrain regions in the ipsi and contralateral hemispheres of rats housed under enriched or standard conditions after the middle cerebral artery ligation. BDNF levels ipsilaterally to ligation side was significantly higher only in the frontal cortex of standard as compared to enriched rats. However, BDNF overall was more abundant in standard than in enriched group. In addition, BDNF levels detected in the hippocampus and frontal cortex on the ischemic side of standard rats was higher as compared to contralateral side. Some other potential mediators of improved functional recovery are the inducible transcription factors nerve growth factor-induced gene A (NGFI-A) and NGFI-B (Dahlqvist et al., 2003). Rats housed in EE showed significantly higher mRNA expression of NGFI-A and NGFI-B in cortical regions outside the lesion and in the CA1 region of the hippocampus compared with IE rats with or without a running wheel. Furthermore, NGFI-B mRNA expression in cortex and in CA1 was significantly correlated to the degree of functional outcome. Serotonin system was also involved as 5-Hydroxytryptamine receptor subunit 1A ($5\text{-}HT_{1A}$) mRNA expression and binding were increased, as well as $5\text{-}HT_{2A}$ receptor mRNA expression were decreased in the hippocampus of the running wheel rats.

Moreover, EE has been successfully employed as a model in studies on neural tissue transplantation in the damaged adult brain (Dobrossy and Dunnett, 2001, review; Lakatos and Franklin, 2002, review). Embryonic tissue grafts can alleviate symptoms, at least partially, in PD (Dunnett et al., 2001; Lindvall and Hagell, 2001), and same strategies are under evaluation for HD, spinal cord injury, stroke and other disorders of the central nervous system (Bachoud-Levi et al., 2000; Falci et al., 1997; Kondziolka et al., 2000). Experience and training are important in the plasticity of functional recovery provided by neural grafts. Indeed, to aid spontaneous recovery, or after surgical repair of damage in the adult brain, patients and animals need appropriate rehabilitation, and they benefit from experience, practice and retraining in reacquiring lost function (Turbes, 1997; Wilson, 1998; Robertson and Murre, 1999).

Grafts that are rich in cholinergic neurons derived from the embryonic basal forebrain can reverse various cognitive deficits that are associated with cholinergic depletion in the rat cortex and hippocampus, a common method to model and study the neurodegenerative processes associated with ageing and Alzheimer's disease (Dunnett et al., 1982, 1986; Dunnett, 1990, 1991; Kelche et al., 1988, 1995). Cholinergic fibre outgrowth from basal forebrain tissues, implanted as dissociated cell suspension grafts into the dorsal neocortex, was greater in rats that were housed in an enriched environment than in rats kept in standard cages (Dunnett et al., 1986). Basal forebrain grafts can strongly alleviate learning deficits in rats with hippocampal cholinergic lesions (Kelche et al., 1988,

1995). Rats housed for 10 months in an enriched environment after receiving lesions and grafts showed significant attenuation of the profound lesion-induced deficit in relearning a Hebb-Williams maze task. However, things might not be so linear. A paradoxical result of EE stimulation on a mice model of AD was recently obtained (Jankowsky et al., 2003). Mice co-expressing mutant amyloid precursor protein (APP) and presenilin 1 (PS1) were housed either in EE or SE. Enriched APP/PS1 transgenic mice developed a higher amyloidal burden with commensurate increases in aggregated and total A beta. These results suggest that A beta deposition can be paradoxically exacerbated by the neuronal changes associated with EE, at least in a mouse model of AD.

In the cerebral artery occlusion model of stroke in the rat, after cortical grafts implanting into the infarcted area, behavioural improvements can be observed when grafted animals have been given the added benefit of housing in EE, in particular on simple tests of motor asymmetry, such as rotation, postural position and balance on a rotating rod (Grabowski et al., 1995; Mattson et al., 1997). However, recovery is not observable on more complex tests of motor skill learning, in which cortical and basal ganglia connections are necessary (Montoya et al., 1990; Dunnett, 1995). So, the behavioural benefits that were observed by combination of cortical grafts and EE in the ischemic cortex seem to depend upon a secondary protection against thalamic atrophy rather than primary cortical reconstruction (Mattson et al., 1997).

Finally, EE has been successfully employed to increase the effects of nigral grafts in the striatum in the rodent parkinsonian models. Enhanced graft survival in enriched animals comparatively to standard housed animals is likely to be attributable to induced neuroprotective changes of EE in the host cellular environment (Barker et al., 1996; Döbrössy et al., 2001).

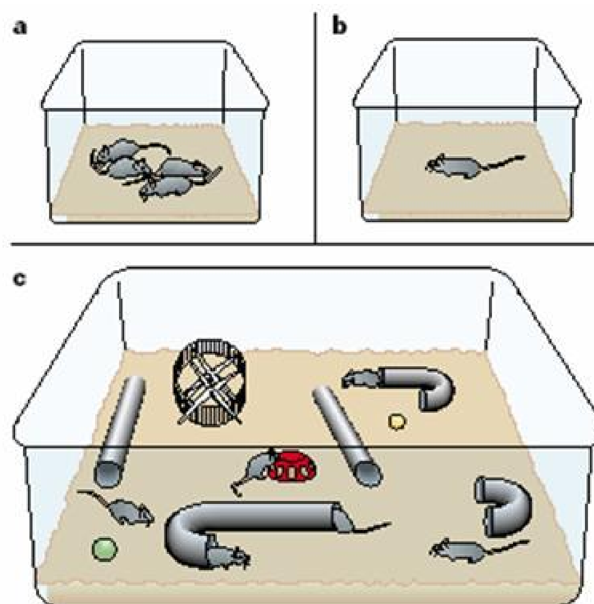

Fig I-1. The EE condition. **A** Standard housing cage containing up to 4-5 animals. **B** Impoverished condition housing isolated animals. **C** Typical EE cage housing up to 10-12 animals containing tunnels, nesting material, toys and a running wheel for voluntary physical exercise.

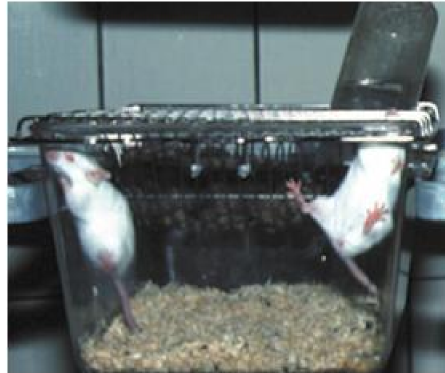

Fig I-2. Stereotypic behavior in mice. Although energetically costly, time consuming, and ineffective, mice persist jumping up-and-down along the cage wall or in gnawing the bars of the cage lid. These behaviors can account for up to 50% of their total daily activity. (*From* Wurbel, 2001).

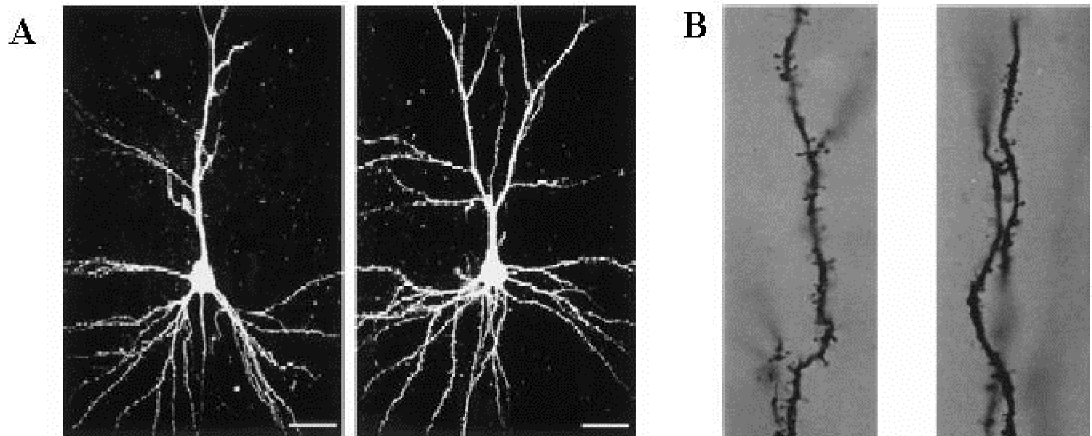

Fig I-3. A Dendritic branching of pyramidal neurons in layer III of the somatosensory cortex in a rat housed in standard (left) and enriched (right) environments, as viewed in confocal imaging after microinjection of Lucifer yellow into the neurons. Bar = 25 µm. (*From* Johansson and Belichenko, 2002). **B** Dendritic spines of neurons of EE (left) and IE (right) rats.

Cellular variable	Environment	Reference
Neuron size	EC > IC	Diamond et al., 1967
Neuron density	IC > EC	Turner and Greenough, 1985
Dendritic branching	EC > IC	Vokmar and Greenough, 1972
Dendritic spine density	EC > IC	Globus et al., 1973
Number of unmyelinated axons in splenium of corpus callosum	EC > IC	Juraska and Kopcik, 1988
Size of unmyelinated axons in splenium of corpus callosum	EC > IC	Juraska and Kopcik, 1988
Number of synapses per neuron	EC > IC	Turner and Greenough, 1985
Size of synaptic contact	EC > IC	West and Greenough, 1972
Synaptic plate perforations	EC > IC	Greenough et al., 1978
Capillary vessels	EC > IC	Black et al., 1990
Astrocytic nuclei	EC > IC	Sirevaag and Greenough, 1987
Oligodendrocytic nuclei	EC > IC	Sirevaag and Greenough, 1987
Mitochondria	EC > IC	Sirevaag and Greenough, 1987

Tab I-1. Synopsis of principal effects of EE on various cellular variables and relative references.

Monoamines	Group	Whole brain	Frontal cortex	Parieto-temporo-occipital cortex	Hippocampus	Cerebellum	Pons/medulla
NA (ng/g)	C	366.3±16.4	135.3±7.4	117.1±6.4	141.3±7.6	134.7±11.4	398.6±21.2
	E	405.6±12.2	165.6±13.2	149.1±8.3*	162.5±11.3	185.2±10.9**	466.2±22.4*
5-HT (ng/g)	C	651.1±29.4	370.2±24.9	408.7±26.3	255.9±12	142.8±21.7	537.1±39.3
	E	642.2±17.6	404.4±22.7	421.1±33.0	274.1±14	197.1±26.2	588.6±29.4
5-HIAA (ng/g)	C	373.6±17.2	148.2±10.6	165.0±15.6	273.8±10.7	77.8±8.5	358.0±31.6
	E	367.9±16.5	153.1±6.8	169.9±11.2	275.5±18.9	95.1±9.7	390.5±17.7
5-HIAA/5-HT	C	0.575±0.014	0.403±0.018	0.399±0.019	1.084±0.059	0.598±0.051	0.663±0.023
	E	0.574±0.023	0.382±0.012	0.413±0.024	1.001±0.035	0.514±0.044	0.669±0.025
DA (ng/g)	C	1246.5±51.6	580.8±91.8	59.3±13.4			27.8±3.8
	E	1258.9±35.1	617.2±66.6	66.0±10.9			40.1±4.9
DOPAC (ng/g)	C	146.5±7.7	110.4±15.4	17.8±2.8			23.9±3.3
	E	144.5±4.6	115.1±8.9	18.1±2.7			31.1±2.7
HVA (ng/g)	C	287.6±13.5	151.2±15.8	29.7±9.8			28.2±7.7
	E	283.0±16.6	157.4±11.0	25.5±5.8			41.4±6.7
DOPAC/DA	C	0.118±0.005	0.197±0.014	0.332±0.082			0.859±0.061
	E	0.115±0.004	0.194±0.01	0.273±0.041			0.825±0.050
HVA/DA	C	0.232±0.01	0.294±0.047	0.373±0.118			0.879±0.234
	E	0.224±0.01	0.267±0.015	0.393±0.103			0.965±0.155

Tab I-2. Effects of EE on monoamine concentrations and their metabolites (ng/g brain weight). C=control group; E=enriched group. (*From* Naka et al., 2002).

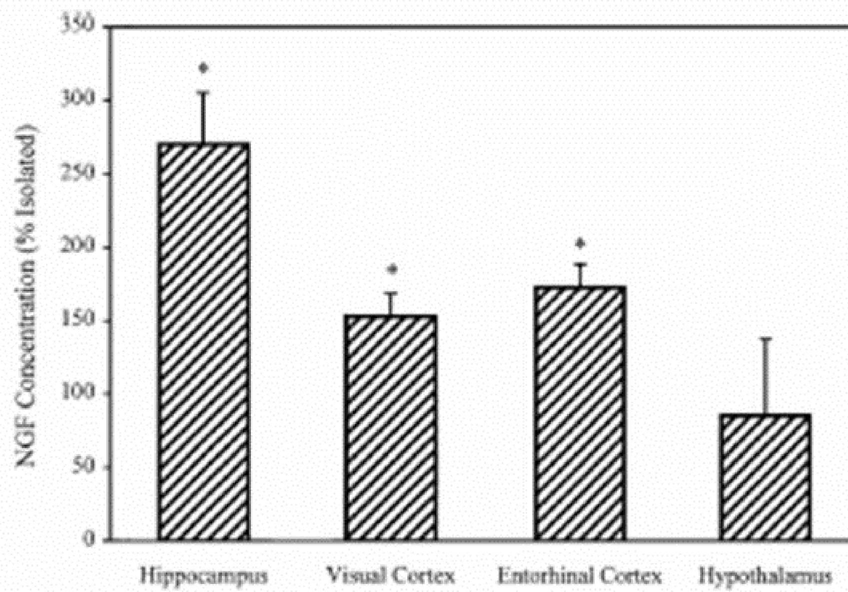

Fig I-4. Differential NGF levels expressed in various brain regions of rats after long-term (1 year) exposure to EE. (*From* Pham et al., 1999b).

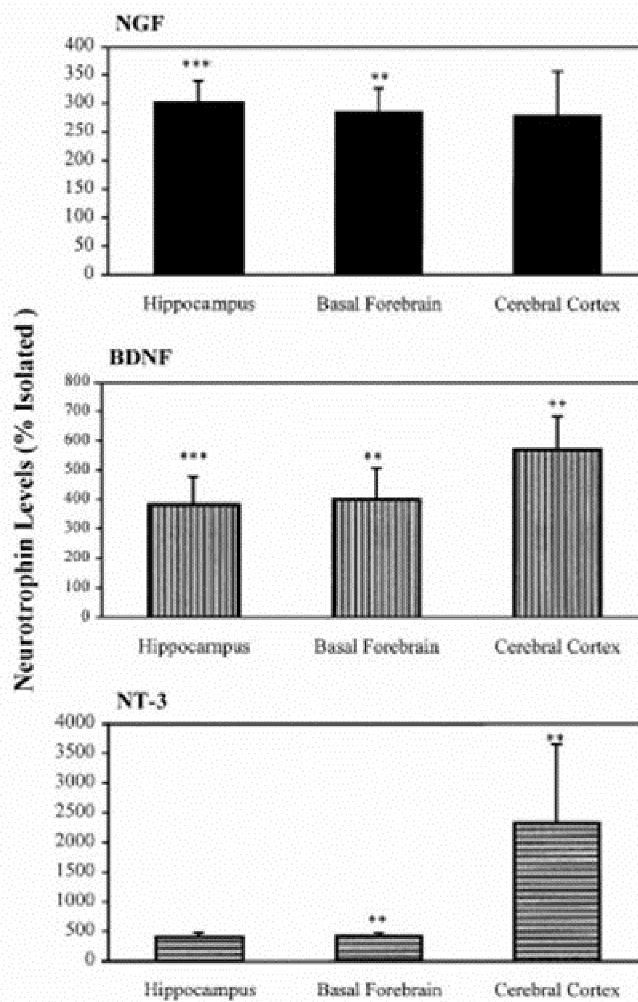

Fig I-5. Neurotrophin levels expressed in various brain regions after long-term (1 year) exposure to EE. (*From* Pham et al., 1999b).

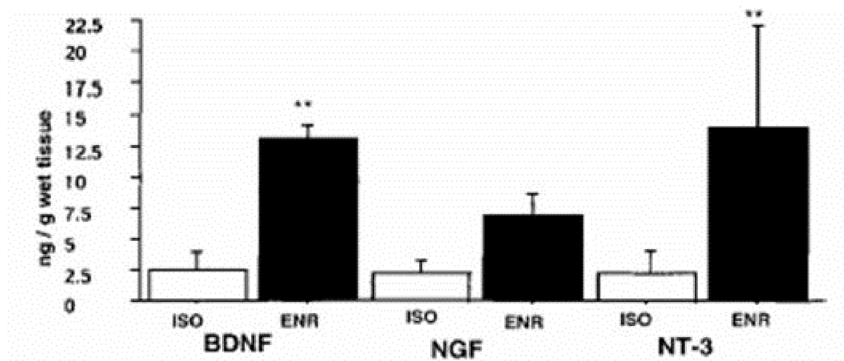

Fig I-6. Comparison between BDNF, NGF and NT-3 expression in the cerebral cortex of EE and IE rats after long-term (1 year) exposure to EE. ISO=IE rats; ENR=EE rats. (*From* Ickes et al., 2000).

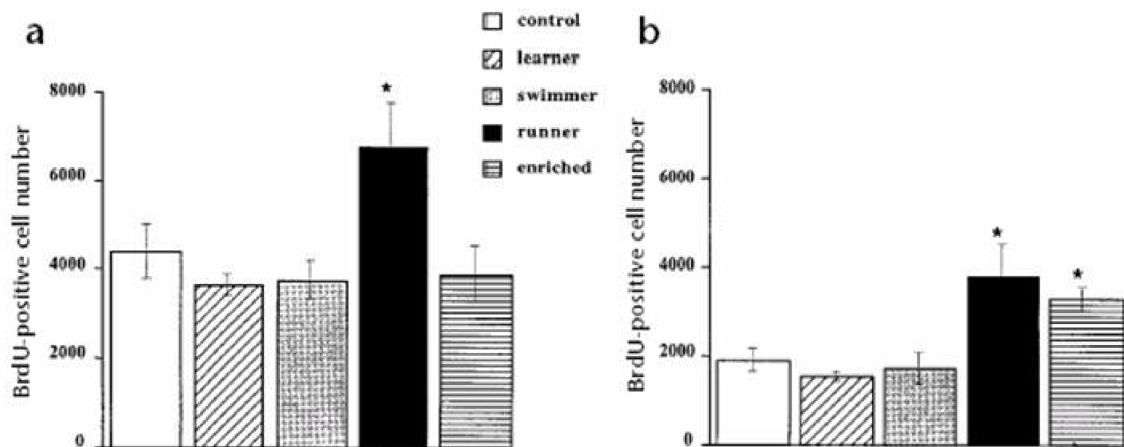

Fig I-7. Neurogenesis in mice dentate gyrus assessed by BrdU-positive cell number. **A** Proliferation. Total number of BrdU-positive cells per dentate gyrus one day after the last BrdU injection, to estimate ongoing proliferation. Significantly more cells were labeled in the runners as compared to the other groups. **B** Survival. Total number of BrdU-positive cells per dentate gyrus four weeks after the last BrdU injection, to estimate survival of labeled cells. Enrichment and running significantly increased the survival of newborn cells. (*From* van Praag et al., 1999).

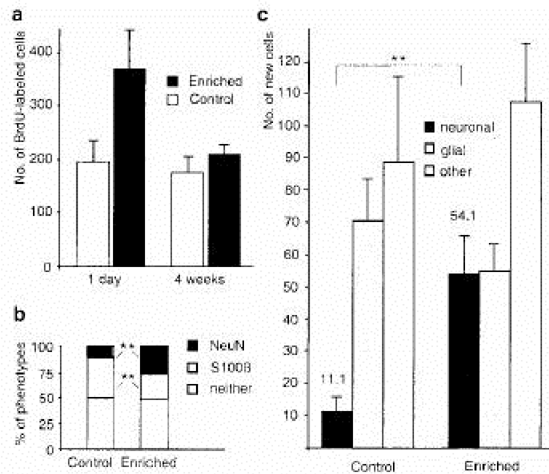

Fig I-8. Increased hippocampal neurogenesis in aging mice living in an enriched environment for 10 months. **A** Number of bromodeoxyuridine (BrdU)-labeled cells per dentate gyrus at 1 day and 4 weeks after the last of 12 injections of BrdU. Numbers are means ± SEM. **B** The distribution of phenotypes among the BrdU-labeled cells at 4 weeks after injection of BrdU. There is a relative phenotypic shift from glial cells (BrdU/S100-positive) to neurons (BrdU/NeuNpositive). **C** Net neurogenesis, gliogenesis, and production of undetermined phenotypes were calculated by multiplying the numbers of BrdU-labeled cells at 4 weeks after injection by the ratio of phenotypes for each animal. (*From* Kempermann et al 2002).

CHAPTER 2
THE VISUAL CORTICAL PLASTICITY PARADIGMS

2.1 VISUAL PATHWAYS PHYSIOLOGY[1]

Summarizing the complexity of the neuroanatomy and physiology of the mammal visual system in one chapter of a PhD thesis is a hard task and is likely to result in some hypersemplification. We will focus only on those features that allow to raise parallelisms with rodent's visual system in areas no further than the primary visual cortex (abbreviated V1), of particular interest for the experimental work presented in this thesis.

Containing over one million fibers (in human), the optic nerve renders the visual system as the most complex neural circuitry of all the sensory systems. The flow of visual information can be divided in two stages: first from the retina to the midbrain and thalamus, then from the thalamus to the primary visual cortex and superior visual cortices. From the primary visual cortex neural processing follows two pathways, the ventro-temporal and dorso-parietal ones, respectively responsible for the processing of the *what* (object vision) and the *where* (motion vision) features of visual

FROM DOTS TO LINES: THE MORSE ALPHABET OF THE VISUAL CORTEX

It is anecdotally narrated that the basic stimulus able to elicit a visual cortical cell response was discovered, as often happens, by serendipity (Hubel, 1988). In 1950 Stephen Kuffler first succeeded in recording from retinal ganglion cells of a mammal, the cat. He used a modified ophthalmoscope, planned by Samuel Talbot, that was able to project small light dots on the retina. This stimulus allowed Kuffler to make extracellular recordings by electrodes inserted directly in the retina. Moving a light dot on the retina he found a region, the receptive field, whose stimulation increased or inhibited the spontaneous discharge of a ganglion cell. But the light dot seemed to be ineffective on visual cortical cell; even a *ganzfield* light stimulus was useless! Visual cortical cells appeared to be silent to any of these light stimuli and only exhibited a spontaneous discharge. This was the situation in 1958, when David Hubel and Torsten Wiesel were engaging in a recording session from the visual cortex of a cat. They were using light and dark dots stimuli created by mounting on a light projector a transparent slide with a dot drawn in the center or a plate with a hole. Both stimuli failed in producing a response of visual neurons but occasionally, when changing slide into the instrument, they observed a brisk response. At the end, Hubel and Wiesel realized that the stimulus responsible for the neuronal response was the linear shadow created by the edge of the slide while inserting it into the projector. It was the basic discovery that led to understand the elementary stimulus of visual cortical cells: a segment of line, static or moving, oriented parallel to the long axis of the receptive field of a given cell and moving in a direction perpendicular to it. Responsible for this mechanism has been suggested to be a spatial integration process as that schematized in Fig II-1.

[1] If not elsewhere specified, informations concerning general physiology of visual system are taken from Kandel ER, Schwartz JH, Jessell TM. Principles of Neural Science. 4th edition, McGraw-Hill, 2000.

stimuli.

The visual field (the portion of visual space seen by the two eyes without head movements) is divided into three zones: the right monocular visual hemifield, the left monocular visual hemifield and the binocular zone (Fig II-2).

The left visual hemifield projects onto the nasal hemiretina (medial to the fovea) of the left eye and onto the temporal hemiretina (lateral to the fovea) of the right eye; conversely, the right visual hemifield projects onto the nasal hemiretina of the right eye and onto the temporal hemiretina of the left eye. Light coming from the central region (binocular zone) of the visual field enters both eyes. Upper visual field projects to lower hemiretina and vice versa.

Along the visual pathway we consider the different orders of neurons and synapses as different representations of the visual field[2]. Starting from the retina, the first order of neurons consists of photoreceptors, rods and cones; the second order neurons, bipolar cells and the third, retinal ganglion cells. Two classes of interneurons, horizontal and amacrine cells, modulate the connections respectively between photoreceptors and bipolar cells and between bipolar and ganglion cells.

The majority of ganglion cells are of two types and convey different kind of information. Magno (M) ganglion cells project to the magnocellular layers (layer 1, 2) of the lateral geniculate nucleus (LGN) of the thalamus and convey information about stimulus features such as high temporal frequency, luminance contrast and low spatial frequency, whereas Parvo (P) ganglion cells project to the parvocellular layers (layer 3, 4, 5, 6) of the LGN and convey stimulus features such as low temporal frequency, luminance contrast, high spatial frequency and color opponency.

In primates, including humans, the LGN is organized into 6 cellular layers (numbered 1 to 6, ventral to dorsal) separated by intralaminar layers of axons and dendrites. Each individual layer receives input from one eye only: contralateral nasal hemiretina fibers contact layers 1, 4 and 6; ipsilateral temporal hemiretina fibers contact layers 2, 3 and 5. In this way, each LGN contains a representation of the complete contralateral visual hemifield. Magnocellular and Parvocellular pathways project through optic radiation to V1, maintaining the information exclusively from the contralateral part of the visual field (Fig II-3).

In the striate[3] cortex, M and P pathways make synaptic contacts in different sublaminae of cortical layers. The principal layer for inputs from LGN is layer 4, predominated by spiny stellate

[2] The ratio of the area in the lateral geniculate nucleus or in the primary visual cortex to the area in the retina representing one degree of the visual field is called *magnification factor*.

[3] The primary visual cortex, V1, corresponding to Brodmann's area 17, is also called striate cortex from *Gennari's stria*; Gennari was an italian anatomist who first observed a prominent white line appearing in perpendicular sections of the visual cortex at about mid-thickness (in layer 4) of the cortical gray matter, composed largely of tangentially disposed intracortical myelinated axons.

cells, but while the axons of M cell terminate principally in sublamina 4Cα, the axons of most P cells terminate in sublamina 4Cβ.

Similarly to the ocular segregation observed in the LGN, in the primary visual cortex the inputs coming from the two eyes are segregated in a columnar-like way. This cortical eye specific segregation leads to various degrees of ocular dominance in different cells and underlies the perception of depth.

Since Hubel and Wiesel discovery of the ocular dominance (OD) preference properties of cortical neurons (Hubel and Wiesel, 1962, 1963), this property has become one of the most investigated among the physiological patterns in the cerebral cortex and a considerable amount of work has been dedicated to unraveling both the detailed structure and the neural mechanisms which underlie the formation and development of ocular dominance columns.

Briefly, ocular dominance columns consist in cortical modules vertically arranged which process visual information coming predominantly from one or other eye. They take part of a more complex modular organization of V1 consisting in a small region of cortex (~ 2mm square in human) called hypercolumn in which zones of visual field are processed for stimuli visual informations such as luminance contrast, color, orientation, etc. The importance of this discover has been honored by Nobel prize for the important applicative outcome in the treatment of congenital amblyopia and strabismus. The classical Hubel and Wiesel subdivision of V1 visual cortical neurons into arbitrary classes of OD responses has been applied to other mammals. Typically, due to the prominent contra lateralization of visual input from the retina, adult mammals show OD distributions strongly binocularly biased (Fig II-4a).

Prior to this stage of anatomical organization, the primary stimulus able to elicit a visual response is a luminance difference within the receptive fields, such as light dots or lines on dark background or dark dots or lines on a light background. Indeed, the receptive fields (RF) of retinal ganglion cells, LGN neurons, layer 4Cβ neurons and some layer 4Cα neurons are concentric, with an antagonistic center-surround organization that allows to measure the contrast in light intensity between their RF center and the surround (Fig II-5). Once afferents from LGN enter V1 in layer 4, spiny stellate cells distribute the input upward and downward the cortical layers, mainly through pyramidal cells. At this stage, the functional physiology of cortical cells changes and neurons become responsive to many different attributes of the visual stimuli, above all, to their *linearity*. The main characteristic of V1 cortical cells is to integrate the information coming from lower stages into RFs which decompose the outlines of a visual image into short line segments of various orientation. A line or a bar influences the RFs of these cortical cell that belong to two major groups, simple and complex. Simple cell RFs are rectangularly shaped, representing a restricted region in

the visual field (.25 x .25 deg in fovea; 1 x 1 deg in periphery), have segregated *on* and *off* zones, respond to a bright or dark bar, respond best to a specific orientation and a non-optimally oriented stimuli will be ineffective in stimulating the neuron (Fig II-1). Complex cell have larger RFs than simple cells (.5 x .5 deg. in foveal region), are orientation tuned, their *on* and *off* zones are mixed in the RF, and their main attribute is to respond well to a moving bar in a direction-selective way (Fig II-6a). Finally, a third functional class, hypercomplex cell, also called end-stopping or complex with terminal inhibition, show similar to complex cell RFs (orientation and direction selective) but are selective for the length of the stimulus and selective for features of shape such as length and width of the bar of light (Fig II-6b).

These basic properties of RFs allow to use a particular kind of stimulus to study visual acuity called *grating*. A sine or square luminance profile over space constitutes a grating. The important parameters to take into account are the amplitude of luminance contrast, the spatial frequency (SF; measured in cycles/degrees), the orientation and phase. In sine wave grating, luminance profile is, as name suggests, described by sine wave. Square wave or any complex visual stimulus can be defined as series of sine waves.

We can distinguish two ways of measuring the visual acuity. The first is a common experience for most of us when we measure our vision through a standard eye testing chart with letters of various sizes. This is the Snellen eye chart and acuity measured with this letter recognition task is the so-called Snellen acuity. A second way is to measure the angle between adjacent bars in the highest frequency grating that can be distinguished. This is called grating acuity and is the most common method used in research studies. Grating and Snellen acuities correlate to a certain degree[4].

Grating acuity is strongly interconnected with the theorization of the visual system as a Fourier analyzer machinery (Campbell and Robson, 1968; Campbell and Maffei, 1970, 1974). Fourier's theorem shows that any waveform pattern can be fully described as the sum of a number sinusoidal components of different frequencies, amplitudes and phases. Fourier analysis can be applied in both the temporal and spatial domain.

In this light, sine wave, a periodic distribution of luminance, is the elemental unit of spatial vision. Advantages of using the simplest stimulus possible are to more precisely determine how

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[4] In a work by Arai and colleagues (1997) grating acuity and Snellen acuity were measured in patients with various ocular pathologies, including macular diseases, diffuse retinal degeneration, optic nerve diseases, glaucoma, and high myopia. The grating acuity and the Snellen acuity correlated to a certain degree. Discrepancies in acuity measures were found in certain diseases, with the highest disparity in patients with optic nerve disease. Indeed, grating acuity measures visual function at a much larger area of the visual field than visual acuity measured with optotypes. Therefore grating acuity values should never be converted to optotype acuity values.

stimulus exerts effect. Applying different contrast (i.e. different amplitudes of luminance) levels to grating of different spatial frequencies allows to define a contrast sensitivity function (CSF). Presenting sine waves of varying SF and reducing contrast until just seen, is a measure of the contrast threshold. The reciprocal of threshold is called contrast sensitivity. The CSF has some general characteristics: peaks at intermediate frequencies and has a roughly linear (on a logarithmic scale) drop with increasing SF (Fig II-7 and Fig II-8).

Many studies have supported the idea that the visual system behaves as a Fourier analyzer at ganglion, LGN, as well as cortical levels. A nerve cell in a visual pathway that responds to sinusoidal gratings of spatial frequency within a restricted range and exhibit a higher discharge for one spatial frequency is said to show spatial frequency tuning. Cells in the striate cortex are sharply tuned to particular spatial frequencies (i.e. they are spatial frequency filters) and this tuning gives rise to spatial frequency channels (e.g Brady, 1997).

During postnatal life visual acuity and ocular dominance develop gradually to reach adult levels. Normal development of the visual cortex is strongly influenced by visual experience during short periods of postnatal development called critical periods (CP), in which the cortical connections are still highly plastic and susceptible to changes induced by visual experience (Berardi et al., 2003). After birth and with normal visual input, the acuity of infants shows a rapid improvement (Maurer et al., 1999). If visual experience is abnormal, visual system development can go through an abnormal wiring of visual connections resulting in pathological conditions such as amblyopia or strabismus.

Indeed, early in life, experience is necessary for the development of different behaviors, abilities, and physiological functions, so that sensory deprivation during CPs has profound and devastating effects on the adult physiology. The CP is a defined time-window deputed to the postnatal refinement of neuronal connections by experience and neuronal activity, the duration of which depends on the function tested and the operational way that function is tested. Positive correlations exist among the duration of the CP for the effects of MD on OD in different species and brain weight and between CP duration and life span (e.g. Berardi et al., 2000). In visual system research, the most extensively studied CP is that for the effects of monocular deprivation (MD) on OD of cortical neurons, which has been characterized in the monkey, cat, rat, mouse, ferret and humans (Berardi et al., 2000). Closing one eye at birth for the total duration of the CP, or parts of it, leads to a cortical rearrangement of OD between the two eyes so that the opened eye takes over the neuroanatomical territory (cells) that would “normally” (under normal visual experience and without genetic anomalies) belong to the other eye (Fig II-4b). Dark rearing (DR, the complete absence of light) during the CP for OD also affects the maturation of visual functions, both

prolonging the sensitivity to MD after the end of CP and affecting the development of visual acuity. In laboratory animals, dark rearing and monocular deprivation have become the most used paradigms for the study of the developmental plasticity of the visual system.

2.2 THE RAT'S VISUAL SYSTEM[5]

The rat's visual system (Fig II-9) shares many similarities with other mammals that have more complex visual capabilities (Paxinos, 1995). First, even though the rat's eye may not accommodate, it has a considerable depth of focus with a binocular overlap estimated about 40-60 degrees. This binocular field image is situated above the snout and falls on the lower temporal retina. The number of adult retinal ganglion cells has been estimated to be around 110.000 although this number is much greater in the early postnatal life and is reduced to adult levels within the first 5-10 postnatal days. Despite the small size of ipsilateral projections from the optic nerve (it has been estimated that only 3-10 percent of axons of ganglion cells in pigmented rats projects ipsilaterally), activity of one eye in hooded rats induces a considerable response in the ipsilateral visual pathway along the principal retino-recipient regions (LGN and primary visual cortex) and contributes to the binocular representation of the visual field. Despite the absence of cyto-architectonic lamination, the rat's LGN is divided into a caudo-dorsal "outer shell" and a ventro-medial "inner core" which grossly receive inputs respectively from the contra and the ipsilateral eye.

From the LGN, projections are restricted ipsilaterally to the primary visual cortex (in the rat is abbreviated as Oc1, homologous to area 17). Rat's Oc1 is divided into a medial monocular zone and a lateral binocular zone (Oc1B) and is topographically organized (Schuett et al., 2002; Hubener, 2003), with the lower portions of visual field mapped antero-medially and the upper portions mapped dorso-laterally. Also in the rat, cells in Oc1 can be classified as simple, complex or end-stopped (hypercomplex).

Electrophysiological studies on the visual system of adult rats and mice found that cortical neurons have well defined functional properties (e.g. Fagiolini et al., 1994; Gordon and Stryker, 1996; Pizzorusso et al., 1997; Girman et al., 1999; Porciatti et al., 1999, 2002) and, as in cats and monkeys, are distributed in different classes of ocular dominance, with a high proportion of binocular cells (Parnavelas et al., 1981; Maffei et al., 1992; Berardi et al., 1993; Fagiolini et al., 1994; Gordon and Stryker, 1996) (Fig II-10). Conversely, the visual abilities of rodents are very poor comparatively to other much studied mammals (rabbits, cats, monkeys and humans), probably because their habitat constrains rely mostly on the use of olfactory and tactile abilities (Rossier and Schenk, 2003). The visual acuity of the primary visual cortex, extrapolated on the basis of visual evoked potentials (VEPs), corresponds to a resolution of about 25 min of visual angle in pigmented rats, corresponding to about 1.2 cycles/degree, and only about 0.6 c/deg in albino rats and mice (Fig

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[5] If not elsewhere specified, general rodent's visual system physiology refers to information from Paxinos G., The Rat Nervous System. 1995; 2nd edition, Academic Press.

II-11). The establishment of adult levels of visual acuity is gradually achieved during the first postnatal month.

As in other mammals, the rat visual cortex is sensitive to monocular deprivation and dark rearing during an early period of postnatal development (Domenici et al., 1991; Maffei et al., 1992; Berardi et al., 1993; Fagiolini et al., 1994) so that patterned vision is necessary for correct development of the visual cortex. In this light, rodent's visual system has become an easy, economic and elective animal model to study the molecular basis of development and plasticity in the visual cortex (Berardi et al., 2003, for a recent review), and monocular deprivation and dark rearing the most used visual deprivation paradigms.

2.2.1 DARK REARING (DR)

Before Hubel and Wiesel's studies, a conspicuous amount of research had implemented the compete lack of light stimulation during development as a paradigm to study the behavioral effects of sensory stimulation (reported in Hubel, 1988). However, the knowledge and *zeitgeist* of the time interpreted the resulting visual deficits in terms of the impossibility of *learning to see*.

Actually, perceptual learning[6] (e.g. Matthews et al., 1999; Crist et al., 1997; Poggio et al., 1992; Vogels et al., 1985; Fiorentini and Berardi, 1980) is an important process of visual system development but works as corollary to a more basic development of visual system features such as those of visual acuity, ocular dominance or orientation tuning. This means for example that, after a proper practicing, even at the neuronal level of V1 it is possible to change the characteristics of orientation tuning of individual neurons (Schoups et al., 2001), but that those neurons must have previously achieved a specific tuning curve during development!

In rodents, DR paradigm has usually been implemented by raising the animals in total darkness since birth in standard laboratory conditions (with 2-3 up to 5 animals in a standard laboratory cage provided with free access to food and water). This treatment is a mainly *visual* manipulation that affects the development of visual physiology. When animals are DRed from birth to the end of CP (in rats the CP ends at about 6 weeks of life) (Berardi et al., 2000) two main effects are observable: first, all neuronal properties (such as receptive field size, peak to baseline discharge ratio, orientation preference, ocular dominance, visual acuity) remain immature, not developed to adult levels; second, the visual cortical (VC) connections remain plastic, that is, still sensible to the effects of visual input manipulations such as monocular deprivation (Hubel, 1988). The most striking effect of rearing an animal in the dark during CP is the loss (actually, the incomplete development) of visual acuity (Fagiolini et al., 1994). The delay of CP closure and the impaired development of functional properties of visual cortex caused by DR has allowed to characterize not only the time course of post-natal development of visual functions but also to study in an easier and more economic animal model, like that of rodent's visual system, the molecular basis of plasticity

[6] A recent finding by Schoups and colleagues (2001) demonstrated by electrophysiological recording that behavioral improvement (perceptual learning) in an orientation identification task in monkeys has a physiological correlate in the functional reorganization of the primary visual cortex neurons.

in the visual cortex and the role of biochemical treatments to counteract the disruptive effects of such sensorial deprivation (Berardi et al., 2003). As a matter of fact, these two kinds of sensory deprivation paradigms are no longer allowed by the scientific community in higher mammals used in experimental research (cats, dogs, non-human primates), so that, implementation of DR and MD in rodents is economic and molecularly productive, but is also a forced choice.

Because the most common treatment is to couple DR with MD (i.e. DR during CP followed by MD), molecular basis of this kind of plasticity will be described in the following paragraph.

2.2.2 MONOCULAR DEPRIVATION (MD)

Depriving one eye of patterned vision during CP determines an irreversible reduction of visually driven activity in the visual cortex through the deprived eye, which is reflected by a dramatic shift in the ocular dominance distribution of cortical neurons in favor of the non-deprived eye in all mammals tested (e.g. Wiesel and Hubel, 1965; Fagiolini et al., 1994; Berardi et al., 2000, 2003). Moreover, following MD, visual acuity and contrast sensitivity for the deprived eye, tested either behaviourally or electrophysiologically (Prusky et al., 2000a, b, c, d; 2002, Prusky and Douglas, 2003; Parnavelas et al., 1981; Domenici et al., 1991, 1992, 1994; Maffei et al., 1992; Berardi et al., 1993, 1994; Pizzorusso et al., 1994, 1997; Fagiolini et al., 1994; Fiorentini et al., 1995; Berardi and Maffei, 1999; Porciatti et al., 1999, 2002; Caleo and Maffei, 2002; Berardi et al., 2000, 2003), develop poorly (amblyopia) and there is a loss of depth perception. The loss of depth perception has been directly related to the loss of binocular cells in the visual cortex, whereas the loss of visual acuity has been attributed both to the total decrease of neurons driven by the deprived eye and to a loss of those neurons with the smallest receptive fields (Kiorpes et al., 1998, 1999).

It is now clear that maturation of the visual cortex during CP relies on many molecular bases at different cellular levels (Berardi et al., 2003). One of the first studied mechanisms was that of the so-called "2B-to-2A" switch. NMDA (N-methyl-D-aspartate) receptors of the visual cortex contain two subunits, 2A and 2B, the relative presence of which is developmentally regulated. Immunostaining studies showed that visual cortical neurons shift from a dominant presence of receptors containing the subunit 2B to a high presence of receptors containing the subunit 2A, with a time course paralleling that of functional visual cortical development and critical period, and that expression of the 2A subunit correlates with the progressive shortening of NMDA current. Experience-dependent plasticity during CP is influenced by NMDA receptors. Visual experience decreases the proportion of NMDA-2B receptors (Philpot et al., 2001a), [but transgene over expression do not significantly alter in vitro synaptic plasticity of the visual cortex (Philpot et al., 2001b)], and in adult mice, depriving the dominant contralateral eye of vision leads to a persistent,

NMDA receptor-dependent enhancement of the weak ipsilateral-eye inputs (Sawtell et al., 2003). Blocking of NMDA receptors blocks the effects of monocular deprivation during CP (Bear et al., 1990), and DR delays the 2B-to-2A switch. However, deletion of the NMDA-receptor 2A subunit does not alter the sensitivity to monocular deprivation, that is still restricted to the normal critical period (Fagiolini et al., 2003), thus suggesting that expression of the 2A subunit is not essential to delineate the time course of the CP for ocular-dominance plasticity, or that compensatory mechanisms can arise in 2A knock-out mice (as well as in all knock-out models).

Another widely investigated area of visual cortical plasticity is that of neurotrophins. Many studies support a role for neurotrophins in regulating the refinement and precise pattern of synaptic connections during development. There is also evidence that activity is required for the neurotrophin mediated regulation of synaptic transmission and plasticity (Poo, 2001). Indeed, in the visual cortex, it has been reported that the dendritic-growth-promoting effects of neurotrophins require activity (McAllister et al., 1996; Maffei, 2002).

Three approaches have been implemented to study neurotrophic effects on developmental visual plasticity: exogenous supply of neurotrophins, antagonizing of endogenous neurotrophins and transgenic models. Early, it was shown that endogenous neurotrophins (with the exception of NT3) can counteract the disruptive effects of MD and DR during CP (Domenici et al., 1991, 1992, 1994; Maffei et al., 1992; Berardi et al., 1993, 1994; Pizzorusso et al., 1994; Fiorentini et al., 1995; Cellerino and Maffei, 1996; Berardi and Maffei, 1999; Caleo and Maffei, 2002; Berardi et al., 2000, 2003). The first neurotrophin showing a strong modulating effect in counteracting MD and DR damages was NGF (Domenici et al., 1991). Intracortical infusion of excess BDNF disrupts OD columns but can counteract the effects of MD (Cabelli et al., 1995; Galuske et al., 1996; Lodovichi et al., 2000). However, exogenous neurotrophin supply alters other basic properties of visual cortical neurons, and their positive actions are accompanied by a significant alteration of cortical neurons functional properties such as discharge patterns and orientation selectivity (Gillespie et al., 2000; Lodovichi et al., 2000).

A second approach also showed the basilar importance of endogenous neurotrophins in regulating visual cortical development and plasticity (e.g. Berardi et al., 1994; Cabelli et al., 1997).

More recently, transgenic mice have demonstrated to be powerful heuristic models and the focus of attention has shifted toward BDNF, which has been also linked to the intracortical inhibition functioning in controlling visual cortical plasticity during CP (e.g. Huang et al., 1999; Gianfranceschi et al., 2003). In transgenic mice, visual cortex is rescued from the effects of dark rearing by overexpression of BDNF (Gianfranceschi et al., 2003). In DRed BDNF +/+ transgenic mice, visual acuity, receptive field size of visual cortical neurons, critical period for OD plasticity,

and intracortical inhibition were indistinguishable from those observed in normal light-reared mice, therefore indicating that BDNF overexpression is sufficient for the development of many aspects of visual cortex functions even in the absence of visual experience. Moreover, these BDNF +/+ mice are susceptible to the effects of MD, but their critical period terminates precociously (Hanover et al., 1999; Huang et al., 1999), showing that BDNF accelerates the development of visual acuity and the time course of ocular dominance and synaptic plasticity. In contrast, heterozygous knock-out mice for BDNF (BDNF +/-) show a pathway-specific impairment of long-term potentiation (LTP) but normal CP for MD (Bartoletti et al., 2002).

As mentioned, a strong link between BDNF and intracortical inhibition has been recently suggested by the finding that development of intracortical GABA-mediated inhibition is accelerated in BDNF-overexpressing mice (Huang et al., 1999; Gianfranceschi et al., 2003; Berardi et al., 2003), suggesting that BDNF controls the time course of the critical period by accelerating the maturation of GABA-mediated inhibition (Hensch, 2003). The intracortical inhibition maturation effects on visual cortex development and plasticity have been evaluated in transgenic mice lacking the 65-kDa isoform of the GABA-synthesizing enzyme GAD (GAD 65) (Hensch et al., 1998). In these mice, plasticity induced by MD during CP is deficient, but can be restored if GABA transmission is enhanced by means of exogenous supply of benzodiazepines. The same group (Fagiolini and Hensch, 2000; Iwai et al., 2003) demonstrated that diazepam administration into the visual cortex accelerates the opening of the CP for MD and that, interestingly, even two days of diazepam infusion during DR also closes the critical period.

Other mechanisms important for developmental visual cortical plasticity include intracellular and extracellular environments.

Recently, at least three kinases have been identified to influence intracellular signaling of cortical plasticity: cAMP-dependent protein kinase (PKA), extracellular-signal-regulated kinase (ERK) and αCa^{2+}/calmodulin-dependent protein kinase II (αCaMKII). In the visual cortex, PKA, ERK and αCaMKII are under the control of visually driven activation and exert their role at two different levels: the cytoplasm and the nucleus (Di Cristo et al., 2001; Beaver et al., 2001, 2002; Taha et al., 2002). The interference on each of the three pathways mediated by these kinases reaches the same effect, that is the suppression of ocular dominance shift after MD during CP. Furthermore, inhibition of PKA reduces orientation selectivity in MD animals but not in normal animals (Beaver et al., 2002). In other words, PKA inhibition by itself does not affect orientation selectivity, but in combination with MD, PKA pathway affects orientation selectivity. At the initial steps of plasticity, the changes in synaptic efficacy do not require new protein synthesis. However, stable changes require nuclear involvement through gene expression of new proteins so that the

pattern of kinases activation is translated into a pattern of gene expression. Ocular dominance plasticity requires cortical protein synthesis (Taha and Stryker, 2002). A pivotal role is played by camp-response-element-binding (CREB) phosphorilation (Mower et al., 2002). CREB is activated during MD and viral deactivation of CREB determines the blockade of ocular dominance plasticity. CRE-mediated gene expression is controlled by neurotrophins and kinases pathways (Berardi et al., 2003).

At least two mechanisms are responsible for extra cellular environment involvement in visual cortical plasticity: proteolytic activity and extracellular matrix (ECM). The extracellular proteolytic activity induced by the tissue plasminogen activator (tPA) immediate-early gene is increased during MD (Mataga et al., 2002) and its pharmacological inhibition attenuates the OD shift induced by MD (Mataga et al., 1996). After reaching a complete maturation at the end of CP, the ECM is inhibitory for experience-dependent plasticity in the visual cortex (Pizzorusso et al., 2002). It is possible to reactivate ocular dominance plasticity in the adult visual cortex by degrading pharmachologically the ECM. Pizzorusso and colleagues (2002) examined the effect of some components of the ECM: the chondroitin sulphate proteoglycans (CSPG). CSPG condense around the soma and dendrites of subset of neurons in the form of perineuronal nets (PNN) (Koppe et al., 1997; Bruckner et al., 2000), gradually develop during CP (Lander et al., 1997), and are inhibitory for axonal sprouting and growth (Fawcett and Asher, 1999). DR from birth inhibits the developmental maturation of PNN specifically in visual cortical areas, mainly in superficial and deeper layers, not affecting layer 4. One week of normal light vision after DR is sufficient to restore PNN to normal levels. Degrading CSPG by means of chondroitinase-ABC (chABC), prevents the inhibitory activity of ECM and restores susceptibility to the effects of MD reactivating OD plasticity in adult rats.

In summary, one might infer that monocular deprivation during development has its strongest effects on correct functionality of object vision. However, the down-stream effect of MD has a strong impact also at higher levels of vision. A striking example is that of face processing in humans (Le Grand et al., 2003). Face processing is the build up of an expert system for a particular aspect of object vision. In adults, right hemisphere areas of the occipito-temporal cortex (including the fusiform face area) are more active in viewing faces versus non-faces objects, comparatively to left hemisphere. This hemispheric specialization, however, is not completely under genetic control and requires early visual experience during infancy to reach adult expertise in face processing, as demonstrated by the lower capability of face processing in children deprived monocularly of visual experience to the left eye (that in young infants is predominantly contralateral to the right hemisphere).

As mentioned, DR and MD paradigms have been conceived to be *visual* manipulations. Next chapter results suggest to place first the adverb *mainly*.

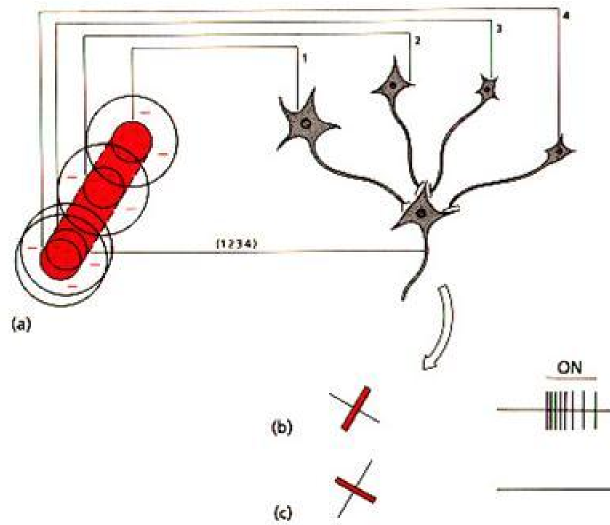

Fig II-1. Hypothetical anatomical organization responsible for the receptive field of a simple cell in V1. a) Receptive field of a single cell. The stimulus able to elicit a response is a line segment of light of a given orientation (b), whereas a stimulus with different orientation (c) determines no response. The receptive field is created by the spatial integration of receptive fields of different LGN or layer IV neurons with center-surround receptive fields.

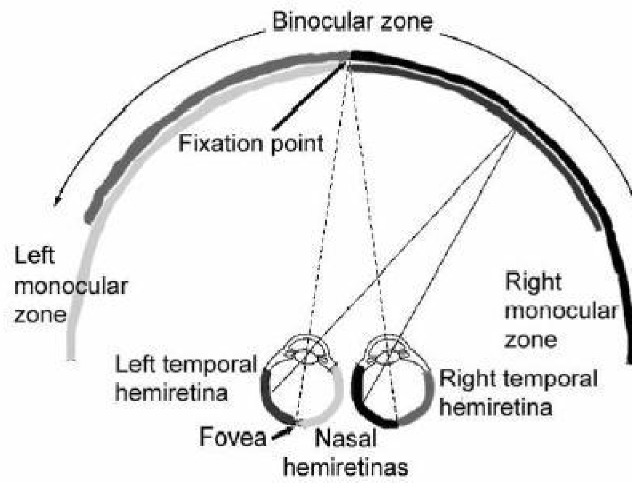

Fig II-2. Human visual field.

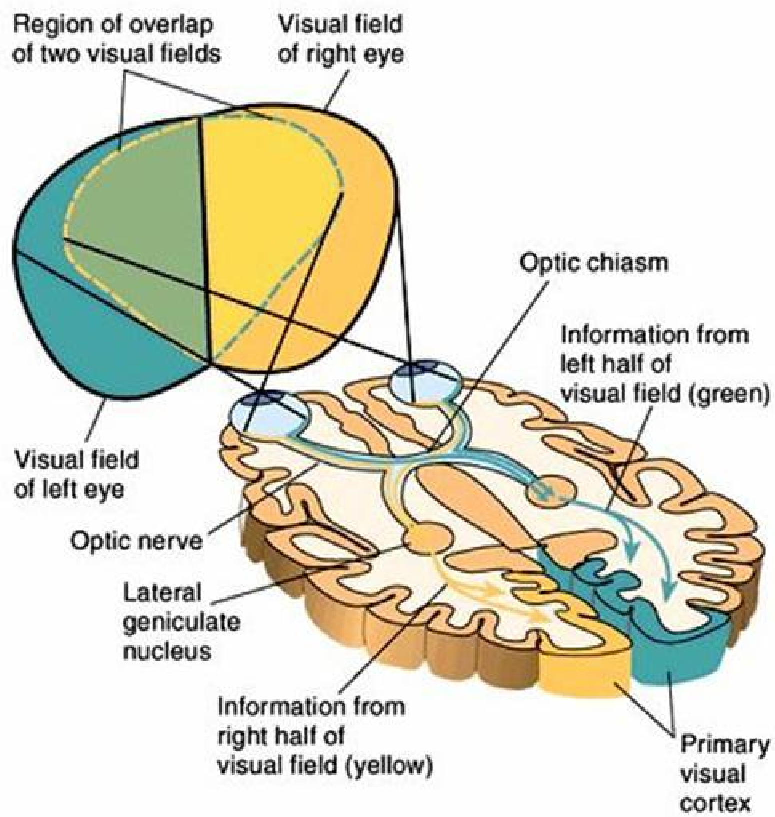

Fig II-3. Primary visual pathway.

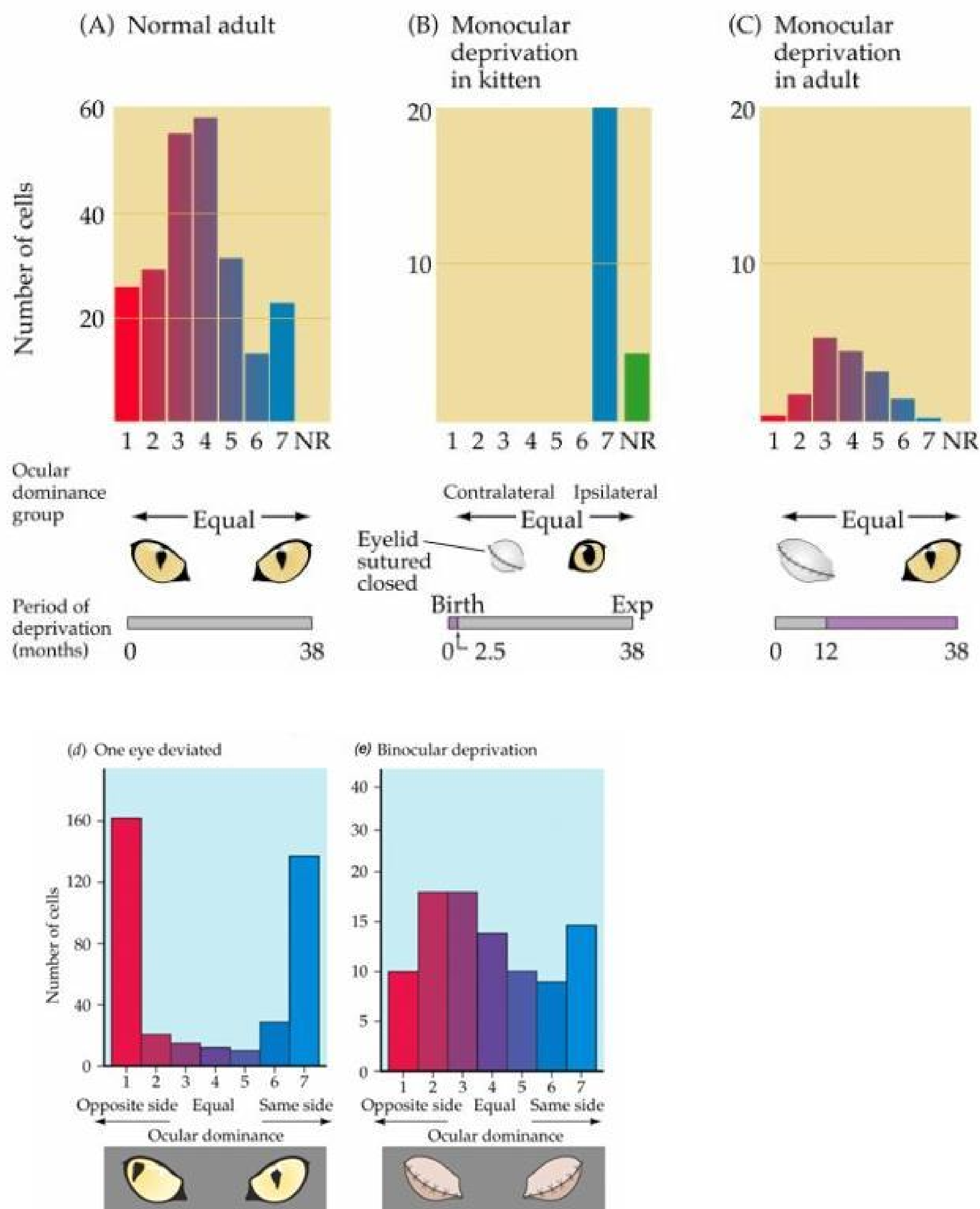

Fig II-4. Ocular dominance (OD) distributions of neurons in cat visual cortex and physiological effects of visual experience deprivation or manipulation. Classes 1 to 7 refer to neurons dominated respectively by contra or ipsi eye; NR = non responsive neurons. a) OD distribution in adult animal is strongly contra-lateralized. Monocular deprivation (MD) during the developmental CP (b) results in a strong OD shift toward the open eye, whereas MD during adulthood (c) have small or none effects on OD distribution. Strabismus (c) during CP determines a loss of binocular responsiveness of neurons and a "bipolarization" of neuronal responsiveness toward contra or ipsi eye. Binocular deprivation (e) during CP determines most neurons to become responsive to both eyes.

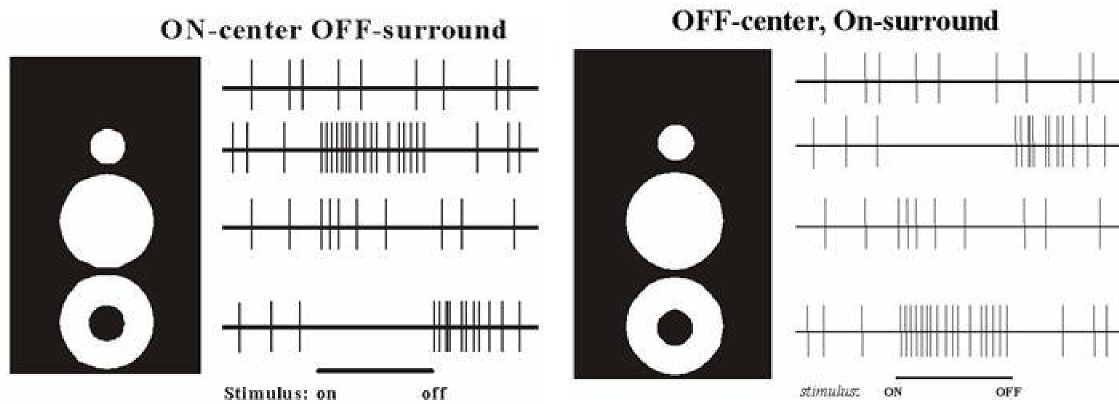

Fig II-5. Center surround interactions of concentric RFs (from Kuffler, 1953).

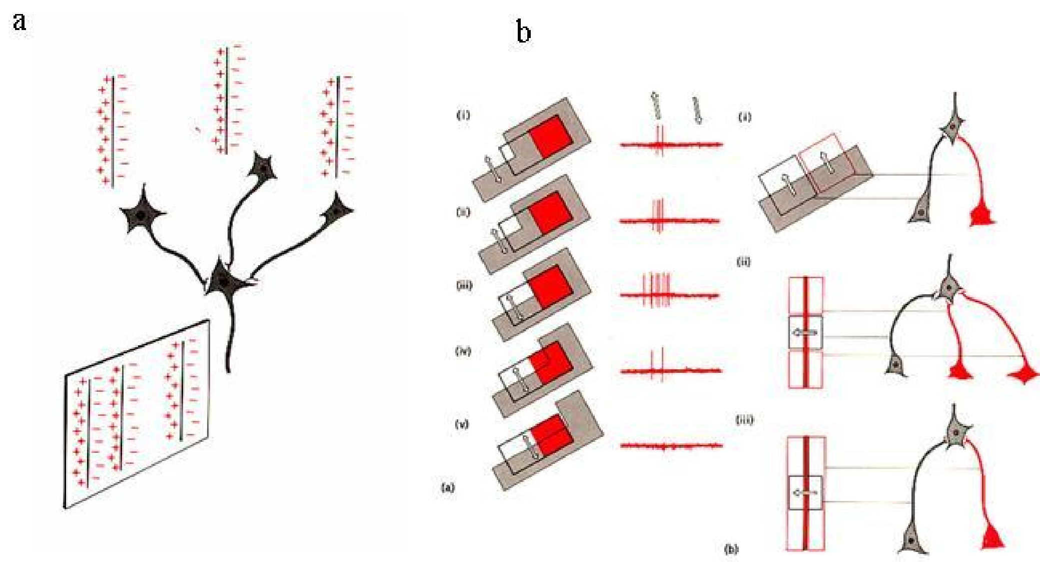

Fig II-6. Receptive fields of complex (a) and hypercomplex (b) cells.

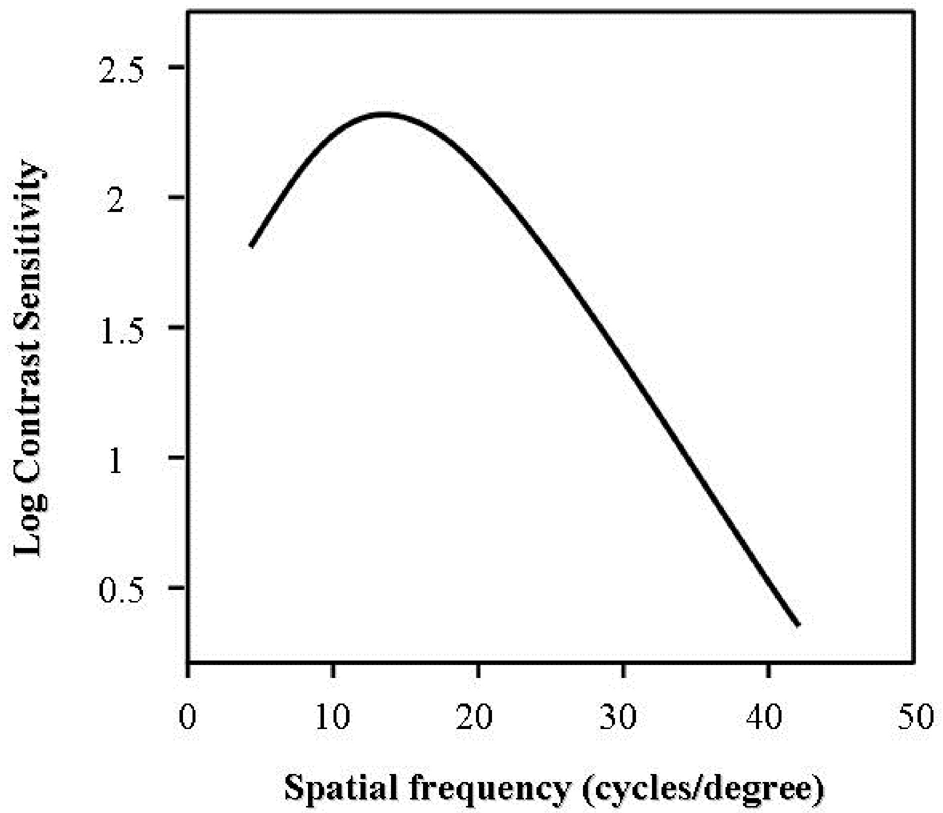

Fig II-7. Contrast sensitivity function.

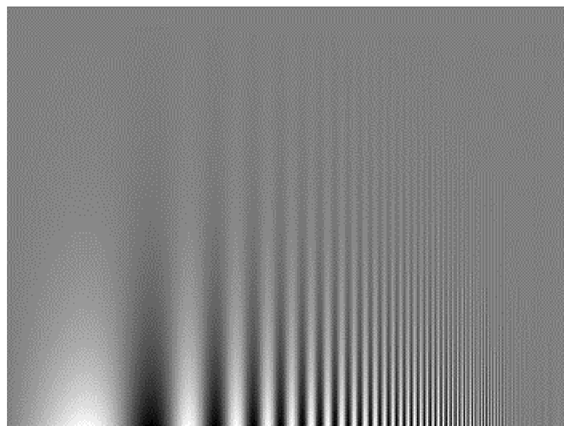

Fig II-8. Analogical representation of the contrast sensitivity function.

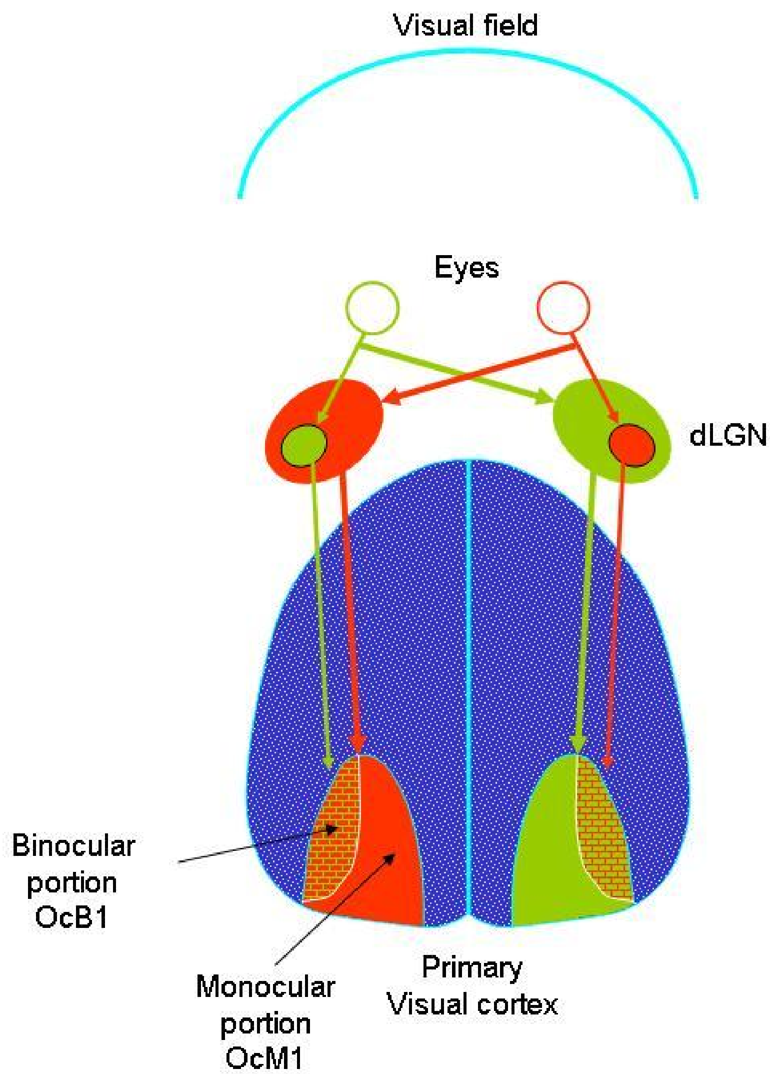

Fig II-9. Schematized rat's primary visual pathway. Visual information is strongly contralateralized. Rat's primary visual cortex (Oc1) is divided into a medial monocular zone and a lateral binocular zone (Oc1B); both are topographically organized (lower portions of visual field are mapped antero-medially whereas upper portions are mapped dorso-laterally).

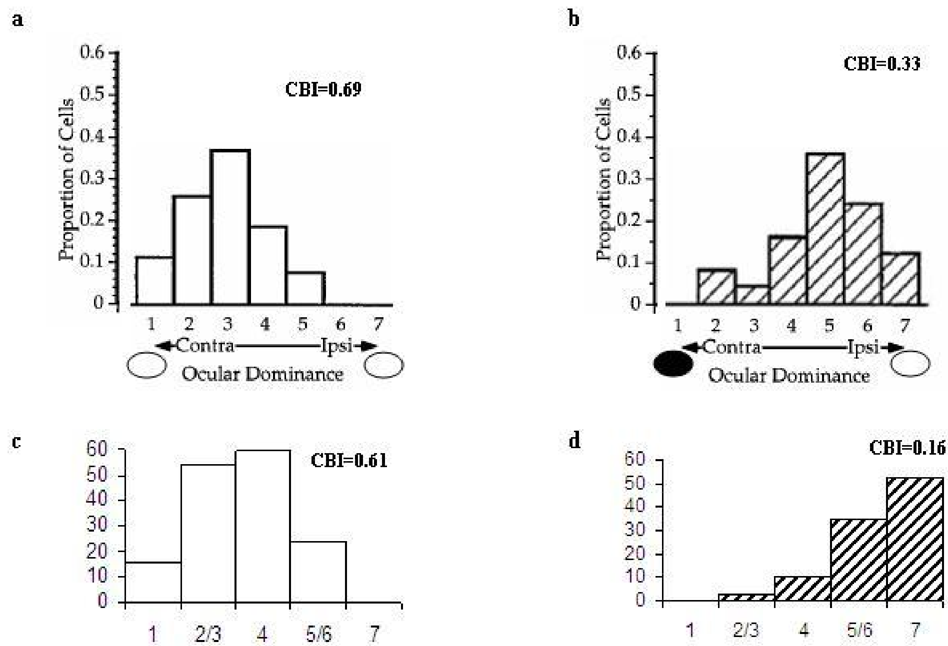

Fig II-10. Ocular dominance distribution of visual cortical neurons in non-deprived adult mice (a) and rats (c). OD distribution in mice (b) and rats (d) monocularly deprived in the contra eye for 4 (mice) or 7 (rats) days during the CP. (a. and b. are *modified from* Gordon and Stryker, 1996).

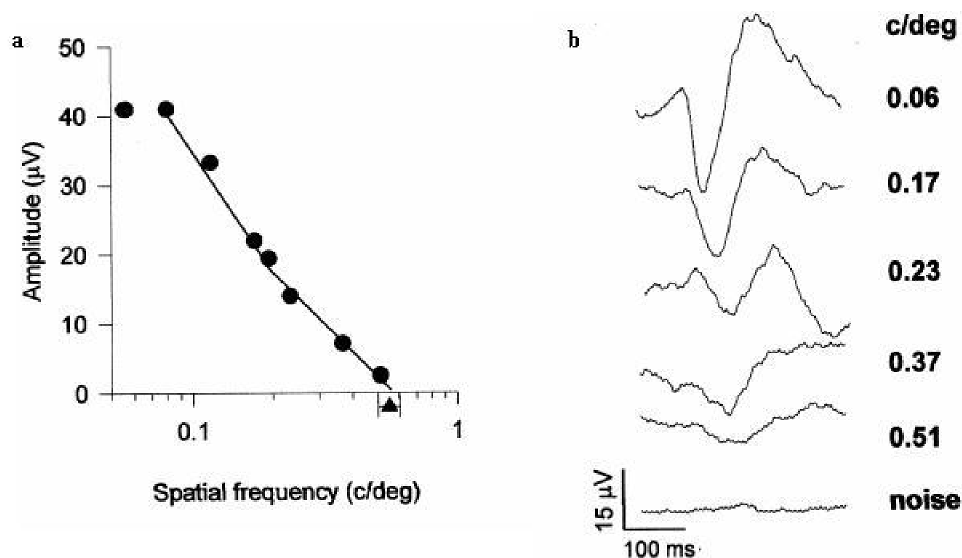

Fig II-11. Visual acuity determined by pattern Visual Evoked Potentials (VEPs) in a mouse. a) Example of visual acuity measurement by extrapolating response to noise level. b) Examples of VEP responses at stimulating patterns of different spatial frequencies. In mice, visual acuity reaches about 0.6 c/deg; in rats, about 1.2 c/deg.

CHAPTER 3

ENVIRONMENTAL ENRICHMENT PREVENTS THE EFFECTS OF DARK REARING AND SHORTENS THE CRITICAL PERIOD FOR MONOCULAR DEPRIVATION IN THE RAT VISUAL CORTEX.

... scientific revolutions are inaugurated by a growing sense ... that an existing paradigm has ceased to function adequately in the exploration of an aspect of nature to which that paradigm itself had previously led the way (Thomas Kuhn, 1962).

3.1 INTRODUCTION. *Enriching the visual system*

The enriched environment (EE) paradigm is thought to mimic in laboratory animals the naturalistic conditions of animals raised in the wild such as inanimate and social environmental complexity (van Praag et al., 2000). EE during development or in adulthood strongly influences neural plasticity, enhancing acquisition and consolidation of memory traces (Cotman and Berchtold, 2002).

Neural plasticity during critical periods (CP) of sensory cortices development is fundamental to cortical circuit maturation and sensory function acquisition (Berardi et al., 2003). This developmental plasticity declines after completion of this task. In the visual cortex, maturation and consolidation of connections is prevented by dark-rearing, so that visual acuity (VA) remains low and the CP for monocular deprivation (MD) after DR is prolonged. Other visual experience manipulations, such as MD strongly influence the outcome of the physiological development, leading to a shift in ocular dominance distribution (OD) towards the open eye.

However, the matter of how these neural plasticity paradigms interact with each other has not been clearly established, and reports studying effects on visual cortical development and plasticity are scanty and limited to the anatomical or behavioral levels (Beaulieu and Colonnier, 1987, 1988; Prusky et al., 2000a). As previously reported, the visual cortex is influenced by EE at the biochemical level of synaptic contacts formed by boutons containing flat vesicles, among others. The numerical density of symmetrical contacts is nearly twice as large in the visual cortex of cats raised in the impoverished condition (IE) as in animals raised in EE condition. More recently, Prusky and colleagues demonstrated that the visual acuity of an enriched group of C57B6 mice, behaviorally measured by a simple computer-based visual discrimination task (Prusky et al., 2000b), was 18% higher than the IE group. At a more direct level, no data are available on

interactional effects of enriching the environment for animals subjected to a visual deprivation paradigm, i.e. no electrophysiological data are present in the literature.

As often happens in the history of science, we just used the "hand-reaching tools at our disposal in a given time" (Khun, 1962). We mean by this that we lied on a rather common technical tool, namely dark rearing. But also that, in association with electrophysiological recording, dating 1929 (Berger), this was enough to change the acquired scientific meaning of intending dark rearing: DR and MD paradigms have been conceived to be *visual* manipulations; here we suggest to place first the adverb *mainly*.

Thus, we have studied by in-vivo electrophysiology, the effects of EE on the developmental plasticity of the visual cortex in a darkened or lit visual experience paradigm.

1st experiment. ***The darkened enriched environment***

In a first experiment, we tested whether environmental enrichment could counteract the loss of visual acuity and the lack of consolidation of visual cortical (VC) connections found in standard dark reared rats. Based on a considerable amount of previous data showing a remarkable positive effect of a number of neurobiological treatments, from exogenous neurotrophins supply to transgenic manipulations (Berardi et al., 2003), in preventing the disruptive effects of total absence of light during CP on visual system development, we asked if a non-pharmacological, neither genetic, treatment, such as EE, could counteract the absence of light and lead to a normal development of vision.

In addition we have examined the visual cortex status of the developmental organization into perineuronal nets (PNNs) of Chondroitin Sulphate Proteoglycans (CSPGs), components of the extracellular matrix recently shown to be non permissive factors for visual cortical plasticity (Pizzorusso et al., 2002), and the status of cortical inhibition (quantifying the expression of GAD65), as there is growing evidence that maturation of inhibition in the visual cortex is an important determinant of critical period (Berardi et al., 2003).

2nd experiment. ***The lit enriched environment.***

In a second experiment, we compared the time course of the critical period for monocular deprivation in light-reared rats raised either in enriched environment or in standard housing. Transgenic manipulations, e.g. that of BDNF overexpressing mice (Huang et al., 1999), demonstrated that it is possible to modify the duration of the critical period for MD. We asked therefore if EE could speed up the closure of CP for MD.

3.2 MATERIALS AND METHODS

Animal treatment

1st exp. Animals (Long Evans hooded rats) were dark-reared from birth (post-natal day, P0) in a climatized light-proof environment following a protocol approved by the Italian Ministry of Health. At P18 littermates were divided (under infrared viewing) in two groups, one assigned to enriched housing (DR-EE group) and one to standard (DR-non EE group) housing and left in the dark. Standard housing was provided by standard rat cages (25x35x18 cm) containing 2-3 littermates; enriched environment housing was provided by large (45x70x40 cm) cages containing 5-7 animals and a running wheel, toys, tunnels, ladders and boxes. Objects were displaced every few days and completely changed once a week. All manipulations were done with infrared viewers. Litter and food were the same in the two types of cages; food and water provided *ad libitum*.

For experiments on visual acuity (Fig 1a**), animals were left in the dark up to P60 and then subjected to electrophysiological recordings. For experiments on MD effects (**Fig 1b**), animals were left in the dark up to P50 and then anaesthetized with avertin (1ml/hg, Sigma) and monocularly deprived by means of eye lid suture. Animals were then brought back to a normally lit environment (12:12 light:dark) for a week. At P57 they were subjected to electrophysiological recordings.**

2nd exp. Rats have been reared in a normally lit environment (12:12 light:dark) and placed either in EE or non EE after weaning (P18). MD was performed for 1 week at different postnatal days (P20, 25, 31, 34, 40) and then animals were subjected to electrophysiological recordings (**Fig 2**). Enriched or impoverished conditions were the same as for the first experiment.

<u>**In vivo electrophysiology**</u>

VEP (Visually Evoked Potentials) recordings. Visual acuity determined with Visual Evoked Potentials (VEPs) is a sensitive measure of the state of visual cortical development and predicts visual behaviour in humans (Campbell and Maffei, 1970; see Teller and Movshon, 1986), monkeys (Boothe et al., 1988), cats (see Teller and Movshon, 1986), rats (Silveira et al., 1987; Fagiolini et al., 1994; Birch and Jacobs, 1979) and mice (Porciatti et al., 1999; Huang et al., 1999; Gianfranceschi et al., 1999; Prusky et al., 2000). Therefore, we used visual acuity assessed with VEPs to test the state of functional development of the visual cortex (**Fig 3a,b**). VEPs were recorded by means of a micropipette (2-2.5 MOhm impedance) inserted into the binocular portion of the primary visual cortex (Oc1B). Only penetrations where single cell receptive fields were

within 20 degrees from the vertical meridian were used to assess VEP acuity. To record VEPs the electrode was positioned at a depth of 450-500 microns; at this depth, VEPs had their maximal amplitude. Signals were band-pass filtered (0.1-100 Hz), amplified and fed to a computer for analysis as previously described (Fagiolini et al., 1994; Huang et al., 1999). Briefly, at least 400 events were averaged in synchrony with the stimulus temporal modulation period (250 msec, 4 Hz). Steady state VEPs in response to sinusoidal temporal modulation were evaluated in the frequency domain by measuring the second harmonic amplitude (frequency 8 Hz) of each record. Visual stimuli were horizontal sinusoidal gratings of different spatial frequency and contrast, generated by a VSG2/2 card running a custom software (kindly provided by C. Orsini) and presented on a monitor (20x22 cm, luminance 15 cd/m^2) positioned 20 or 30 cm from the rat eyes and centered on the previously determined receptive fields. Visual acuity was measured as the extrapolation to noise level of the linear regression through the last 4-5 data point in a curve where VEP amplitude (normalized to the value recorded for the lowest spatial frequency employed, 0.2 c/deg) is plotted against log spatial frequency. Recordings were performed blind to the rearing conditions.

Single cell recordings. Spiking activity recordings were performed under urethane anaesthesia (0.7 ml/hg i.p., Sigma, 20% solution in saline). For each animal, eight to ten cells were recorded in each of at least three tracks spaced evenly (>200 μm) across the binocular primary visual cortex (Oc1B), to avoid sampling bias. Visually driven cells were recorded at an interdistance of 100 μm from each other in normal animals as well as in all treatment groups. Cells were sampled across all six cortical layers. The position of receptive fields of single units was mapped using a hand-held stimulator. Only cells with receptive fields within 20° of the vertical meridian were included in our sample. Spontaneous activity, peak response and receptive field (RF) size were determined from peristimulus time histograms (PSTHs) recorded in response to computer-generated bars, averaged over at least 20 stimulus presentations as described in Lodovichi et al. 2000 (**Fig 4).**

Ocular dominance (OD) was quantitatively evaluated from PSTH according to the classification of Hubel and Wiesel. Cells were distributed in Hubel and Wiesel ocular dominance classes attributed following these criteria: if no response was obtained from the ispilateral eye the unit was classified as 1, if the contralateral peak response was >1.5x the peak response of the ispilateral eye the unit was classified as 2-3. When the ipsilateral eye was exclusively or predominantly driving the response, units were classified as 7 or 5-6, respectively, with the same criteria described above. When the peak response of dominant eye was less than 1.5x the response of the other eye the cell was classified as 4.

In addition, to obtain a finer and statistically more robust comparison of OD distributions we computed for each neuron also the normalized OD score of single neurons (Rittenhouse et al., 1999)

and plotted the cumulative distribution for each experimental group. OD score was computed on cells with complete PSTH analysis of peak and baseline spiking activity after closure of either eye. OD score was defined as:

{[Peak(ipsi)-baseline(ipsi)] – [Peak(contra)-baseline(contra)]} / {[Peak(ipsi)-baseline(ipsi)] + [Peak(contra)-baseline(contra)]}.

This score is -1 for class 1 cells, +1 for class 7 cells and around 0 for class 4 cells.

For each animal the OD distribution was summarized with the CBI (Contralateral Bias Index). The CBI is:

CBI={[N(1)-N(7)] + 1/2[N(2/3)-N(5/6)] + N(Tot)}/2N(Tot),

where N(tot) is the total number of recorded cells and N(i) is the number of cells in class(i).

Recordings were performed blind to the rearing conditions.

Anatomy

Perineuronal networks (PNN) and GAD65. For the first experiment, 26 rats were sacrificed at P50 either after dark rearing in a climatized environment following a protocol approved by the Italian Ministry of Health or after rearing in a normally lit environment.

The group of dark reared rats was further divided in two groups at P18: one group assigned to enriched environment (DR-EE) and one group left in standard housing (DR-non EE). All manipulations of the animals during dark rearing were performed in complete darkness using infrared viewers. At the time of the experiment, dark reared animals were anaesthetised while still in complete darkness and immediately perfused. Chondroitin sulphate proteoglycans (CSPG) organization into perineuronal nets was assessed at P50 by Wisteria Floribunda Agglutinin staining (Pizzorusso et al., 2002) (**Fig 5**) and GAD65 by monoclonal antibodies anti GAD65 (Huang et al., 1999). 4 normal animals, 5 DR-EE and 4 DR non EE were used to assess the developmental state of PNN; 4 normal, 5 DR EE and 4 DR non EE were used to assess the expression of GAD65.

Perfusion of all animals was done with 4% paraformaldehyde in phosphate buffer and brains were post-fixed for two hours before being placed in 30% sucrose in PBS. 50 µm coronal sections from the occipital cortex were cut on a sledge microtome and collected in PBS. Free-floating sections were incubated for 30 minutes in a blocking solution composed of 3% BSA, 0,1 % Triton X-100 in PBS, pH 7.4. Sections were incubated overnight at 4°C in the monoclonal antibodies anti GAD65 (clone Gad 6, 1:500 Sigma), Neu-N (1:500 Chemicon) or in a solution of biotin-conjugated lectin Wisteria Floribunda (WFA) (Sigma, 10 µg/ml). Primary antibodies were then revealed with biotinylated anti mouse (1:200, Vector), followed by Cy3-conjugated streptavidin (1.0 µg/ml, Sigma), incubated for 2 hours and 1 hour respectively. WFA was stained with a 1h incubation in

FITC-conjugated streptavidin (10 μg/ml, Sigma). For each immunohistochemical staining, sections of the different experimental groups were reacted together. Sections were acquired blind to the treatment using an Olympus confocal microscope making a stack of 8 optical sections taken every 3 μm. At least ten 20x fields were acquired for each animal and cells were counted in 180 μm x 200μm fields located in layers 2/3, 4 and 5/6. Layer boundaries were established by NeuN laminar density pattern. For each field the ratio of WFA-positive/NeuN positive cells was calculated.

GAD 65 puncta ring quantification. For each animal at least ten 120x (oil immersion objective) layer 2/3 fields were acquired blind to the treatment. For each field a maximum projection was obtained of three contiguous 1μm-spaced optical sections, centered on the Z value where mean pixel intensity was higher. Acquisitions were made using the same instrument settings (laser intensity, confocal aperture, PMT values) estimated analyzing all sections before acquisitions so that pixel saturation was avoided. For each field, at least five somas were outlined and for each cell a perisomatic ring of about 2 μm was outlined. Mean gray level intensity of each soma (B value, background) and of its perisomatic ring (P value, puncta ring) was calculated using Metamorph analysis of TIFF files. For each cell a P-B value was calculated as in Huang et al., 1999.

3.3 RESULTS

Visual acuity develops normally in DR-EE rats

Our data show that environmental enrichment promotes the development of visual acuity in dark-reared rats. Summary of visual acuities in all DR animals, DR-EE and DR-nonEE groups, is shown in **Fig 6**. Visual acuity estimation from Oc1B by means of VEPs showed that in P60 DR-nonEE rats (n = 8) visual acuity was low (0.58 ± 0.04 c.p.d), as expected (Fagiolini et al., 1994); on the contrary, in P60 DR-EE rats (n = 11) visual acuity was normal (1.03 ± 0.02 c.p.d) and did not statistically differ from normal (nonEE neither DR) adult rats (one-way ANOVA, $P < 0.001$, post-hoc Tukey's test). Shadowed rectangle is the range of visual acuity in normal adults (1.05 ± 0.1c.p.d). We conclude that EE during dark-rearing promotes a lit-environment-like visual acuity development, in rats.

Critical period closes normally in DR-EE rats

Environmental enrichment promoted the consolidation of visual cortical connections in dark-reared rats (**Fig 7**). As expected, MD (1 week) from P50 was still effective in shifting OD of visual cortical neurons in favor of the ipsilateral non-deprived eye in DR-nonEE rats (n=6, 138 cells) (**Fig 7a,b**). However, MD from P50 was ineffective in DR-EE rats (n=7, 159 cells) littermates of DR-nonEE rats, placed in EE at P18, as in adult rats with normal visual experience (n=5, 115 cells) or adult rats subjected to 1 week MD (n=5, 117 cells).

The OD distribution of each animal, summarized with the CBI index (**Fig. 7c**), confirmed that only in DR-nonEE+MD rats there was a significant ODD shift towards the open eye: mean CBI for DR-nonEE rats+MD was significantly lower (0.29 ± 0.03) than for DR-EE rats+MD (0.54 ± 0.04) and for normal undeprived (n=5, 0.6 ± 0.05), or MD (n=5, 0.61 ± 0.06) adult rats; CBIs in these latter three groups did not differ. These results suggest that EE promotes the consolidation of developing visual connections, allowing a normal critical period closure in dark-reared rats.

Perineuronal nets develop normally in the visual cortex of DR-EE rats

We have found that EE greatly reduced the effects of dark-rearing on the development of PNNs in all cortical layers examined (**Fig 8d**). In P50 DR-EE animals, which no longer show OD plasticity in response to MD, the density of neurons surrounded by PNNs in Oc1B was normal in

layer IV and close to normal in layer II/III and layer V/VI. On the contrary, in DR-nonEE animals, which are still susceptible to MD effects, density of PNNs-surrounded neurons was much lower than normal as previously shown (Pizzorusso et al., 2002).

Inhibitory circuitry maturates in DR-EE rats

We quantified the expression of GAD65 in the presynaptic boutons of GABAgergic interneurons around the soma of target neurons in P50 normal (n = 4), DR-nonEE (n = 4) and DR-EE (n = 5) animals (**Fig 9**). We found that dark-rearing decreased GAD65 expression (mean GAD65 fluorescence intensity 54 ± 1.2 pixel-value/unit-area in DR-nonEE animals and 63 ± 1.01 pixel-value/unit-area in normal animals). In DR-EE animals, however, GAD65 expression was normal (mean GAD65 fluorescence intensity 64.8 ± 0.96 pixel-value/unit-area). We conclude that EE is able to stimulate the maturation of cortical inhibition that is prevented by dark-rearing.

EE accelerates the closure of the critical period in rats reared in a normally lit environment

In the lit experiment, we found that EE shortens the critical period for monocular deprivation by stirring up the CP closure earlier than in non-EE reared rats. We tested the difference sensitivity to MD effects all along the time course of CP in EE and non-EE rats (**Tab 1**). Results showed that EE efficacy in preventing the effects of MD during CP was already present at the very beginning of CP (P20, but not statistically significant) and continued all wide the CP duration, bringing the EE rats' sensitivity to MD at the adult levels already at P30, comparatively to the P40-45 end of CP for MD of non-EE rats (**Fig 10**). Statistically significant differences between EE and non-EE rats emerged at P25, P31 and P34 (one-way ANOVA, $P<0.05$, post-hoc Tukey's tests).

We therefore conclude that EE can speed up the closure of CP for MD shortening its duration and that moreover this effect is active since the earlier stages of CP for MD.

3.4 DISCUSSION

Who runs away today lives to fight tomorrow.
(from the "36 Ancient Chinese Stratagems of War")

Here we show that environmental enrichment can promote physiological maturation and consolidation of visual cortical connections in dark-reared rats, leading to critical period closure and normal development of visual acuity. Moreover, we show that in rats reared in a normally lit animal house the environmental enrichment shortens the CP for MD.

To justify the present experimental results, at least two different interpretative lines are eligible of interest. The first refers to all those plastic phenomena related the cross-modal plasticity concept, or better, to a new emerging meaning of it (Shimojo and Shams, 2001). A second interpretation refers to the concept of supra-modal developmental plasticity, by stressing the role of an increased neuroprotection under certain "conditions" (e.g. Carro et al., 2003; Maffei et al., 1992). The first interpretation applies to EE+dark-rearing experiments only; the second may also apply to EE+light-rearing experiment.

Traditionally, in the study of perception, the concept of cross-modal plasticity has included all those plastic neuronal rearrangements in which a part of the brain "shifts" from its usual specificity in processing one sensory modality to a new ability in processing stimuli coming from a different sensory modality (Bavelier and Neville, 2002; Kandel et al., 2000). This ability is particularly present during the developmental periods and gradually decreases throughout life. Indeed, typical findings by surgical cross-modal rewiring and/or afferent sensorial lesion paradigms in animals or humans (Bronchti et al., 2002; Kahn and Krubitzer, 2002; Newton et al., 2002; von Melchner et al., 2000; Sur et al., 1990; Neville et al., 1983), and clinical studies on humans (Chen et al., 2002; Sadato et al., 2002; D'Angiulli et al., 2002; Finney et al., 2002; Kujala et al., 1997; Sadato et al., 1996; Uhl et al., 1991; Neville et al., 1983), repeatedly demonstrated that sensory deprivation in one modality starting from an early period of life causes a cortical area that is normally devoted to one modality to be used by some other modality.

Whether this applies also to rats after dark rearing has never been experimentally explored. One of the few observations that mimic a similar experiment was done in the blind mole rat (Bronchti et al., 2002)[4], but the results must be interpreted as a cross-modal evolutionary adaptation rather then a cross-modal plasticity effect.

[1] The mole rat (Spalax ehrenbergi) is a subterranean rodent whose adaptations to its fossorial life include an extremely reduced peripheral visual system. It was shown that in this blind rodent the dorsal lateral geniculate nucleus is activated by auditory stimuli and that this cross-modal adaptation parallels a cross-modal compensation at the cortical level. Cyto-architectural analyses and electrophysiological recording of the occipital area showed that, despite the almost total blindness of the mole rat, this area has retained the organization of a typical mammalian primary visual cortex, but is activated by auditory stimuli.

However, an increased amount of recent data have started to challenge the stringent version of this approach, i.e. the phrenology-like specialized brain. (Shimojo and Shams, 2001).

Recent behavioral and brain imaging studies challenged this view suggesting that, in perception, cross-modal *interactions* are the rule and not the exception. Of course, that different sensory modalities operate not completely independently of each other is demonstrated by the fact that cortical pathways previously thought to be sensory-specific can be modulated by signals coming from other modalities, but the idea of a necessary interaction between modalities is a rather newly stressed hypothesis. (The typically human experience of synesthesia might help the reader to immediately catch this concept of interactional perception).

Two lines of observations will better clarify this idea. An interesting information comes from a series of experiments conducted in chicks by the group of Lickliter. When bobwhite quail chicks are prenatally exposed to an auditory, visual, tactile or vestibular stimuli, their postnatal auditory and visual responsiveness are enhanced, irrespective of the modality of prenatal stimulation (Honeycutt and Lickliter, 2003; Foushee and Lickliter, 2002; Carlsen and Lickliter, 1999; Casey and Lickliter, 1998; Sleigh and Lickliter, 1998, 1997). This means that, during development, sensory plasticity "pushes" development in a cross-modal way; an interesting parallel has been observed in infants (Bahrick et al., 2002; Bahrick and Lickliter, 2002, 2000), in which was shown the importance of polysensorial versus unimodal activation during infancy on successive development of higher cognitive processes.

The second line comes from human psychology and is exemplified by citing few perceptual "paradoxes". The "common scientific sense" has divided perception into distinct perceptual modalities. If we look at phenomena such as the McGurk effect, the ventriloquist effect, the bouncing motion effect, the illusory double flash, just to cite few of them, we might doubt that this assumption is the rule in perception and we might start to suspect that the distinction is a researcher's *ad usum Delphini* demand, rather than perceiver's. When we "listen" as "da" a sound "ba" coupled with a visual lip movement associated with "ga", we are experiencing the McGurk effect, a perceptual phenomenon in which vision alters speech perception (McGurk and MacDonald, 1976). Similarly, in every day life we experience location source of voices as originating "truly" by actors, even when originating from a TV screen, or when coming from a ventriloquist puppet (Alais and Burr, 2004; Soto-Faraco et al., 2002). In other terms, humans, as primarily vision-dominated animals, are "captured" by visual location. However, visual perception can also be altered by other modalities: a simple model of optimal *combination* of visual and auditory information, rather then one sense capturing the other, best fits experimental data (Alais and Burr, 2004). In other terms, visual and auditory information is combined by minimizing

variance, leading to an improvement in discriminating bimodal spatial location. Recently it has also been demonstrated that ventriloquism effect is dissociated from deliberate or automatic direction of visual spatial attention, appearing on the contrary to reflect the natural way of sensory interaction during perception (Vroomen et al., 2001; Bertelson et al., 2000). In the bouncing motion effect (Sekuler et al., 1997), two objects move across each other. Observers typically perceive the object as streaming with an X-shaped trajectory when there is no accompanying sound or an accompanying sound that is not synchronous with the visual crossing. But when there is a sound (or a flash or a tactile vibration) synchronized with visual coincidence the perceptual dominance reverses and most observers see the objects as bouncing against each other. In the illusory double flash, a small continuous (and peripherally viewed) light source seems to pulse when viewed together with a pulsing sound source (Shams et al., 2002, 2000). Furthermore, the presence of the clicks do not only make the light appear to flash, but can actually improve performance on visual discrimination tasks (Berger et al., 2003; Morein-Zamir et al., 2003).

The very important feature to highlight, within these reports, is that cross-modal alterations are not limited to the situations of ambiguity in the visual or auditory context. Cross-modal interaction rather appears to be a founding characteristic of the perceptual system!

Thus, these cross-modal perceptual paradoxes are truly “paradoxes” only if we persist in the vision of perception as a segmented sensorial ability. But if we accept the idea of a necessary integration among modalities in the ecology of an organism’s life, then there is no more paradox, just perception. Accepting this idea might be simplified by citing other primary sensory cortices paradoxes. Primary “auditory” cortex is activated when a “talking” face is viewed in the absence of sound (Calvert et al., 1997). [With the obvious result that integrating information across the senses can enhance our ability to detect and classify stimuli in the environment (Calvert et al., 1999)]. Tactile stimulation of a hand enhances activity in the primary visual cortex (Macaluso et al., 2000) when the touched hand is on the same side as the visual stimulus. Likewise, an ERP study also suggests that activity in the visual cortical areas is modulated by sound (Shams et al., 2001). Maybe, we should better reverse the problem and say that cross-modal plasticity exists, because perception itself is a cross-modal phenomenon.

How this interpretation applies to our dark-rearing experiments?

To summarize, we have seen that cross-modal interaction among sensory modalities is the rule and not the exception, at least in higher mammals as humans, and that a developmental sensory specific stimulation can have a cross-modal effect. This latter effect seems not completely surprising if we consider the adaptive profit of parallel development of systems evolved to interact during adulthood. What is surprising in our EE+dark-rearing experiment is the fact that half a

century of visual system research has stressed the notion of the *necessary* presence of visual experience, either by permissive or by instructive role, for a correct development of vision. At least in humans and higher mammals, afferent visual information[2] has been considered the *conditio sine qua non* to correctly shape the maturation of cortical circuitry during development (Berardi et al., 2003). In other terms, even though a similar prominent role has been assigned to endogenous factors such as retinal spontaneous activity (Galli and Maffei, 1988; Shatz, 1990; Penn et al., 1998; Stellwagen and Shatz, 2002) and more recently to endogenous genetic programming (Crowley and Katz, 1999, 2000), visual experience has been considered as an inalienable factor for correct development. Exceptions emerged in visual system animal models, typically in mice, rats, ferrets and cats, in which endogenous or exogenous modifications at the biochemical level (e.g. endogenous or exogenous supply of neurotrophic factors, transgenic mice lacking or over-expressing inhibitory circuitry-related molecules) have shown to strongly affect the outcome of visual experience manipulations (e.g. Maffei et al., 1992; Hensch et al., 1998; Huang et al., 1999).

Effects of EE during dark-rearing, determining a normal VA development and normal CP closure, strongly challenge the necessity of visual experience for correct visual development, in absence of any biochemical and/or transgenic experimental alteration. In terms of cross-modal plasticity, we can affirm that EE in DR animals enhanced activity of other, than visual, sensory modalities: motor, tactile, olfactive, auditory. It is plausible that this enhancement “pushed” by cross-modal interactions the development of visual system.

It is basilar to emphasize that these EE effects observed in dark-reared rats may not necessarily apply in the same measure to other species with much higher visual acuities, as monkeys and humans, in which lack of visual experience determines more dramatic effects on visual performance: in human infants deprived of all patterned visual input by cataracts from 1 week to 9 months of age, immediately after cataract removal, acuity is no better than that of normal newborns (Maurer et al., 1999). And we assume that, during their developmental visual deprivation, they had not been reared in impoverished conditions! Moreover, the importance of early visual input in human infants is amplified by the fact that human brain is strongly lateralized, neuroanatomically and functionally, so that visual information coming from left eye mainly reaches right hemisphere and vice versa. Up to six months, an early MD of the right eye rather than of to the left eye does not produce the same consequences: expert face processing requires visual input to the right hemisphere during infancy (Le Grand et al., 2003).

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[2] In this light, classical cross-modal plasticity studies themselves, showing that one sensory cortical area can substitute for another after a switch of input modality during development, strongly suggested that afferent inputs direct the formation of their own processing circuitry.

However, we think important to have shown in a mammalian species that it is possible to modulate the outcome of visual deprivation by varying the environmental - non visual! -conditions. This suggests that factors which are not under the control of visual experience may contribute to visual cortical development. Which molecular factors might be involved introduce us to the next line of interpretation.

The use of a "supra-modal" developmental plasticity concept, in explaining our results, stresses the role of increased "neuroprotection" under certain experimental "conditions" (e.g. Carro et al., 2003; Maffei et al., 1992). As introduced in chapter II, maturation of the visual cortex during CP relies on many molecular basis at different cellular levels (Berardi et al., 2003), and many of these mechanisms are similar in EE and visual system development.

A particular intriguing mechanism is that involving the activation of neurotrophic factors. Indeed, it is known that EE increases NGF (Nerve Growth Factor) and BDNF (Brain-Derived Neurotrophic Factor) expression in the visual cortex (Pham et al., 2002) and that these neurotrophins are important determinants of visual cortical development and plasticity (Berardi et al., 2003). In particular, BDNF overexpression and NGF supply prevent dark-rearing effects (Maffei et al., 1992; Huang et al., 1999; Berardi et al., 2003; Gianfranceschi et al., 2003).

A particularly good new candidate is insulin-like growth factor I (IGF-I), a member of the family of growth factors whose most representative member is insulin, with potent neurotrophic activity (Carro et al., 2003; Trejo et al., 2002); IGF-I is of particular interest in our argumentation because of its recently discovered role that links this molecule directly to physical activity (Carro et al., 2000). IGF-I is produced peripherally in the serum, crosses the blood-brain barrier and, binding to neurons bearing IGF-I receptors, determines a sustained increase of their electrical activity, leading to the production of factors important for neural plasticity (Carro et al., 2003). Indeed IGF-I is important during brain development, by regulating neuronal differentiation and survival (Feldman et al., 1997), and by mediating functional plasticity in the adult brain (Torres-Aleman, 1999).

In rats, running induces brain uptake of serum IGF-I, with the effect that neurons accumulating IGF-I show increased spontaneous firing and a protracted increase in sensitivity to afferent stimulation (Carro et al., 2000). Since IGF-I receptors are present in the occipital cortex (Frolich et al., 1998), that IGF-I could influence the expression of molecules relevant for visual cortical plasticity, is more than a possibility. Increased levels of brain IGF-I, enhanced by EE-induced physical activity, may play a role in accelerating the time course of CP for MD in EE+light-rearing rats and in the correct development of visual properties in EE-dark-reared rats.

Finally, I like to remind one of the most quoted statement in the history of comparative psychology: the Lloyd Morgan's canon. In his classic textbook, Morgan (1894) outlined his famous

canon that an animal's behavior should be interpreted in terms of the psychologically simplest processes consistent with the data: “In no case is an animal activity to be interpreted in terms of higher psychological processes, if it can be fairly interpreted in terms of processes which stand lower in the scale of psychological evolution and development”. If we apply this, not to our experimental problem, but to our interpretative theoretical thinking of our results, we might reformulate the principle and say: “In no case is an experimental effect to be interpreted in terms of higher theoretical hypothesis, if it can be fairly interpreted in terms of theories which stand lower in the scale of scientific evolution and development of that given discipline”. This could lead us to interpret our data as in a friend of mine words: “the more natural-like we allow an organism to develop, the less deficits will it have”.

This view is fortified by an ancient anecdote. The pharaoh Psamtik, founder of the twenty-sixth Egyptian dynasty, was the first “experimenter” to try discerning how children acquire language (Harris, 1986). He ordered to put a couple of newborn brothers to be raised in isolation without any contact with nurses and without listening any talking during all the infancy period. In this way he wished to discover the most ancient human language. We now know why he could only listen silence from those poor chaps. His result also reminds us an ancient taoist master’s aphorism: “let a horse rear in a cage, it will act like a pig”.

But this incongruence also put in a new light our efforts to disclose nature secrets (“nature loves hiding” said Heraclitus). In the oriental strategic thinking, instead of toiling to actively change a situation, it is common to wait the flow of events to find the point of less resistance and, once identified, press it. Applied to our experimental results and to the issue of how environmental factors might contribute to visual system development this could be translated into a different experimental protocol.

It could be for example interesting to note how evolutionary conditions shaped the post natal behavioral habits of rearing in rats. An experimental device could be implemented to leave the possibility to the litter to chose a darkened or lit environment. Their “innate” preference for darkened or lit rearing environments during the first postnatal period, and the relative measurement of their developmental curves for visual system parameters, would give a complementary information to that of animals “actively” put in the dark or in the light, in EE or IE, during CP. Rodents are indeed nocturnal animals. It would be bizarre to find a dark preference, associated with motor hyperactivity, in the first postnatal weeks of development! Then, the apparent paradox of DR-EE rats visual development would be solved.

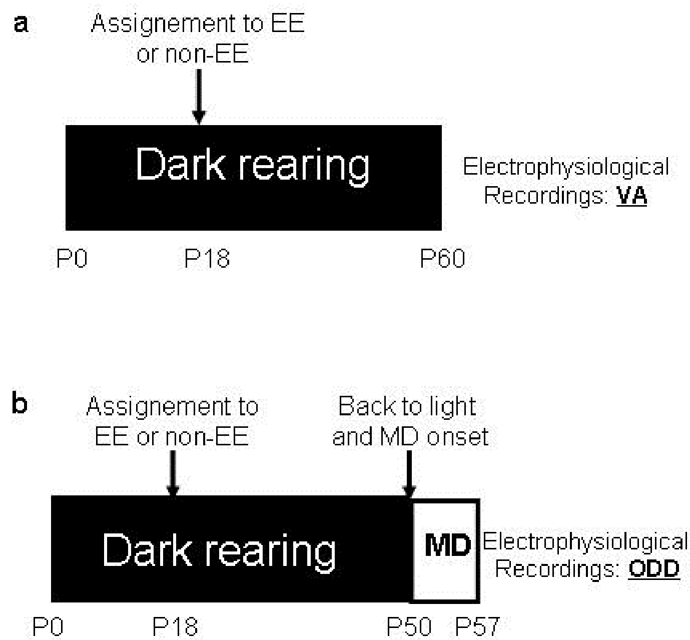

Fig 1. The "darkened enriched environment" experimental protocols. **a:** Visual acuity (VA) assessment. Rats have been DR from birth to P60, which is beyond CP end. After weaning (P18), they have been placed either in EE (DR-EE rats) or standard housing (DR-nonEE rats). VA was measured at P60. **b:** Ocular dominance assessment. Animals were DR from birth to P50. After weaning (P18), rats have been placed either in EE (DR-EE+MD rats) or standard housing (DR-nonEE+MD rats). They were left in the dark up to P50 and monocularly deprived by means of eye lid suture. Animals were then brought back to a normally lit environment (12:12 light:dark) for a week. At P57 they were subjected to single cell electrophysiological recordings performed from binocular primary visual cortex Oc1B to measure ocular dominance distribution (ODD).

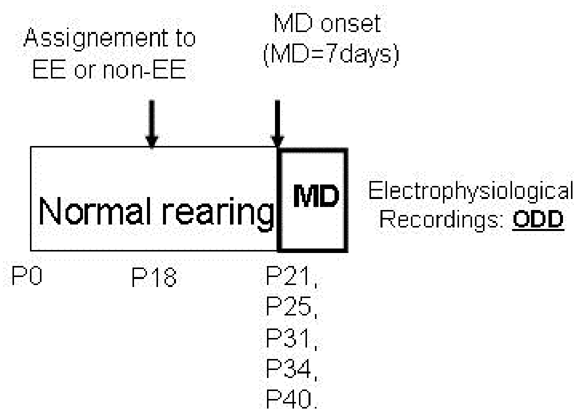

Fig 2. The "lit enriched environment" experimental protocol. Ocular dominance assessment. Animals were light-reared (12:12 light:dark) from birth up to P40. After weaning (P18), rats have been placed either in EE (EE+MD rats) or standard housing (nonEE+MD rats). The two groups were then monocularly deprived at different post-natal ages (P21, P25, P31, P34, P34, P40) by means of eye lid suture, and brought back to a normally non-EE lit environment for a week. At the end of MD they were subjected to single cell electrophysiological recordings performed from binocular primary visual cortex Oc1B to measure ocular dominance distribution (ODD).

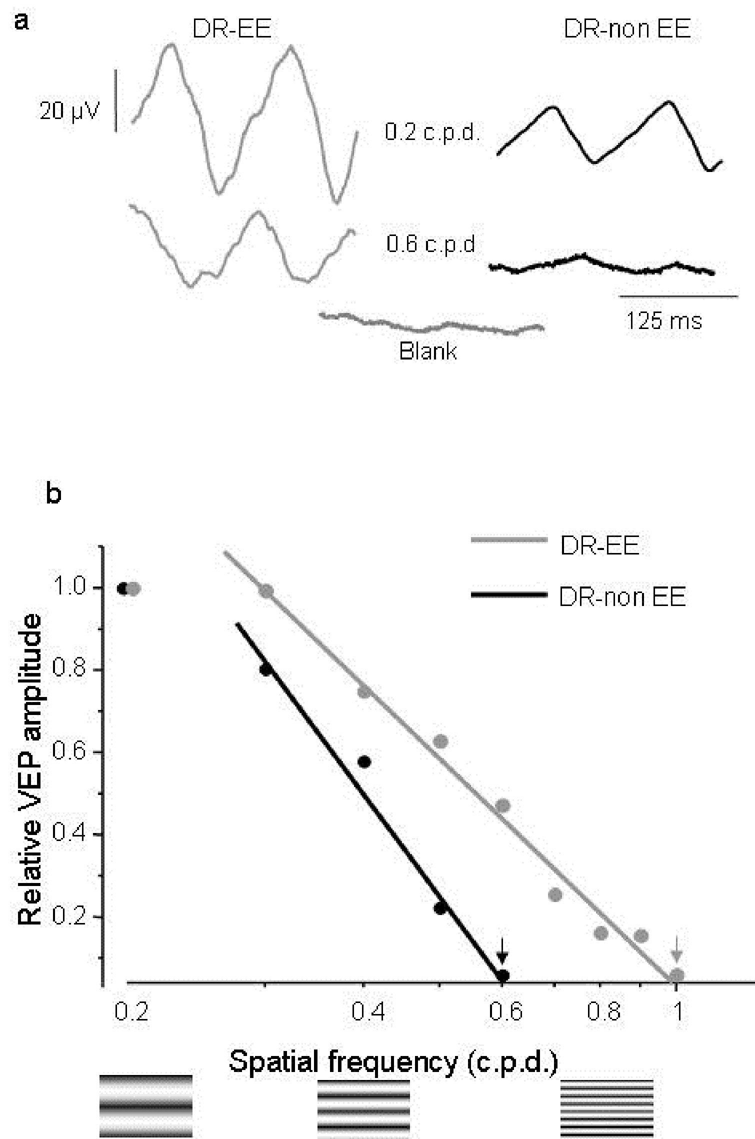

Fig 3. Visual acuity estimation by VEPs recording. **a:** Examples of Visual Evoked Potentials (VEP) recorded from Oc1B in response to visual stimulation with gratings of spatial frequencies 0.2 and 0.6 c.p.d, in P60 DR-nonEE and DR-EE rats. Gratings are temporally modulated at 4 Hz (period 250 msec); the principal component of the VEP response is on a temporal frequency twice the stimulus temporal frequency (second-harmonic). VEP response to a blank field reported to show noise level. **b:** Example of visual acuity estimate in a DR-EE and a DR-nonEE rat. Experimental points are normalized VEP second-harmonic amplitudes; thick lines are linear fits to the data. Estimated visual acuities are indicated by arrows. The gratings in figure have spatial frequencies in the ratio 1:2:4.

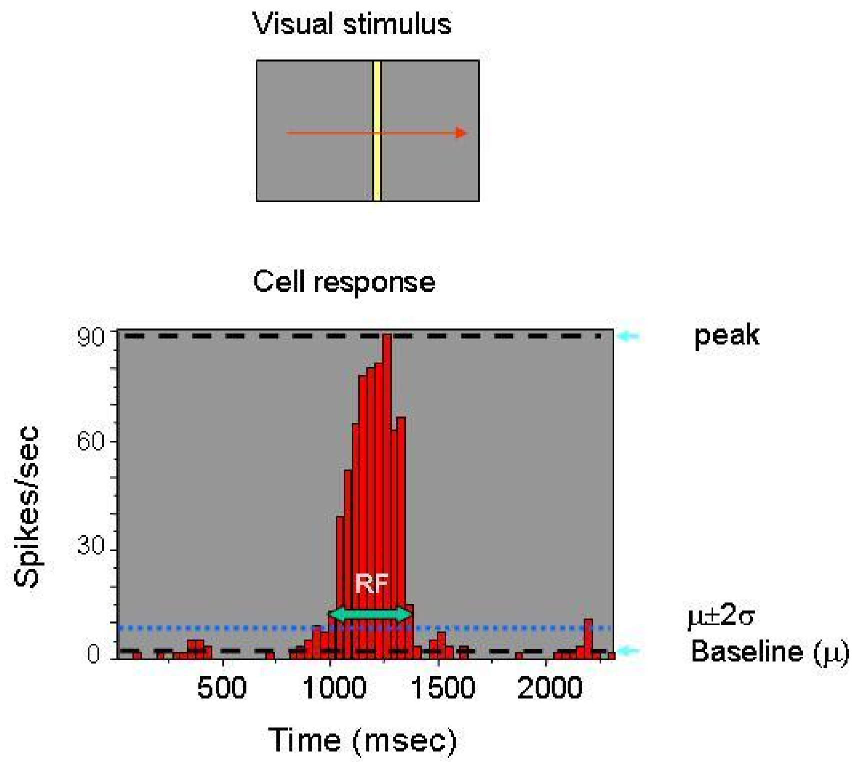

Fig 4. Single cell recording for ocular dominace distribution (ODD) estimation. Example of a peristimulus time histogram (PSTH) in response to computer-generated visual stimuli (moving bars displayed on a screen with the possibility to change velocity and direction of a bar's movement). For each neuron, peak response, spontaneous activity, responsiveness (peak-to-baseline ratio) and receptive field (RF) size were derived from PSTH.

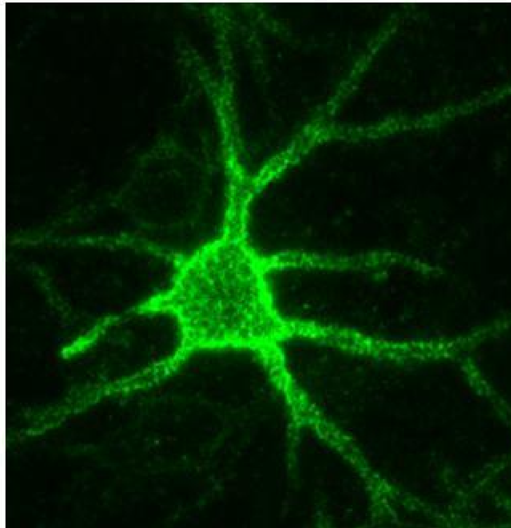

Fig 5. Example of WFA staining of perineuronal nets. Extracellular matrix (ECM) is an intercellular net of molecules responsable for structural and functional characteristics of the cortex. In the visual cortex, components of the ECM, such as the chondroitin sulphate proteoglycans (CSPGs), condense around the soma and dendrites of subset of neurons in the form of perineuronal nets (PNN). CSPGs can be observed by Wisteria Floribunda Agglutinin (WFA) staining.

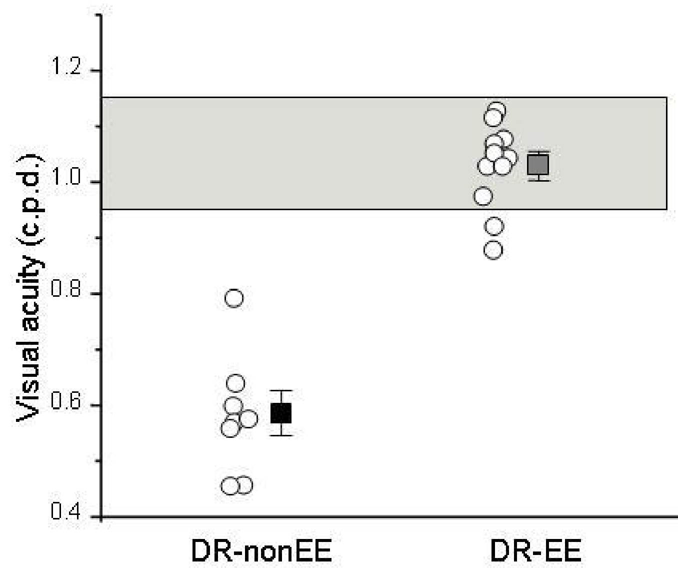

Fig 6. Environmental enrichment promotes development of visual acuity in dark-reared rats. Summary of visual acuities in all DR animals; shadowed rectangle is the range of visual acuity in normal adults (1.05 ± 0.1c.p.d, not different from DR-EE rats, one-way ANOVA, $P<0.001$, post-hoc Tukey's test).

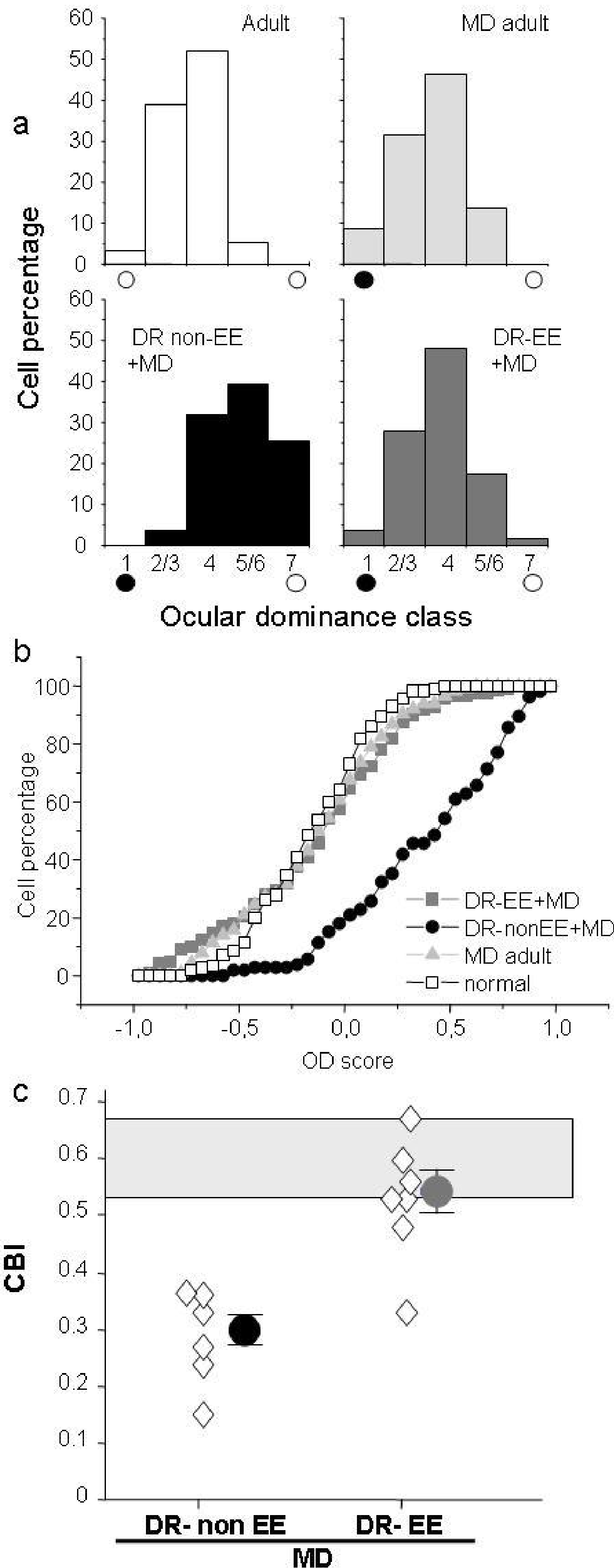

Fig 7. DR-EE rats are no longer vulnerable to MD at the end of CP.

a. Ocular dominance distributions (ODD) of visual cortical cells recorded from binocular primary visual cortex Oc1B. Adult normal rats (Adult; *n*=5, 115 cells) (***up-left***) have a contralateral biased distribution of cortical neurons. One week MD (MD Adult; *n*=5, 117 cells) in adult (P50) is no longer effective in shifting ODD toward non-deprived eye (open circle) (***up-right***). In dark-reared animals (DR-nonEE+MD; *n*=6, 138 cells) (***down-left***), the critical period for monocular deprivation is prolonged and MD still produces an ocular dominance shift toward the non-deprived eye in the visual cortex of dark-reared animals at ages (P50) for which it is ineffective in normal animals. EE during DR (DR-EE+MD; *n*=7, 159 cells) (***down right***) is able to promote a normal CP closure so that one week MD from P50 is ineffective in shifting ODD.

b. Cumulative fractions for OD scores. For each cell an OD score was computed. Number of animals as for Fig 7a, cells 114, 115, 122 and 105 for normal, adult+MD, DR-EE+MD and DR-nonEE+MD, respectively. Only the curve for DR-nonEE animals significantly differs from that in normals (Kolmogorov-Smirnov test, $P<0.05$).

c. Summary of MD effects in all DR animals. CBIs in DR-nonEE+MD rats differ from CBIs in DR-EE+MD rats, which do not differ from CBIs in normal adults (normal CBI range shadowed rectangle) (one-way-ANOVA, $P < 0.001$, post-hoc Tukey's test).

We conclude that environmental enrichment promotes consolidation of visual cortical connections in dark-reared rats.

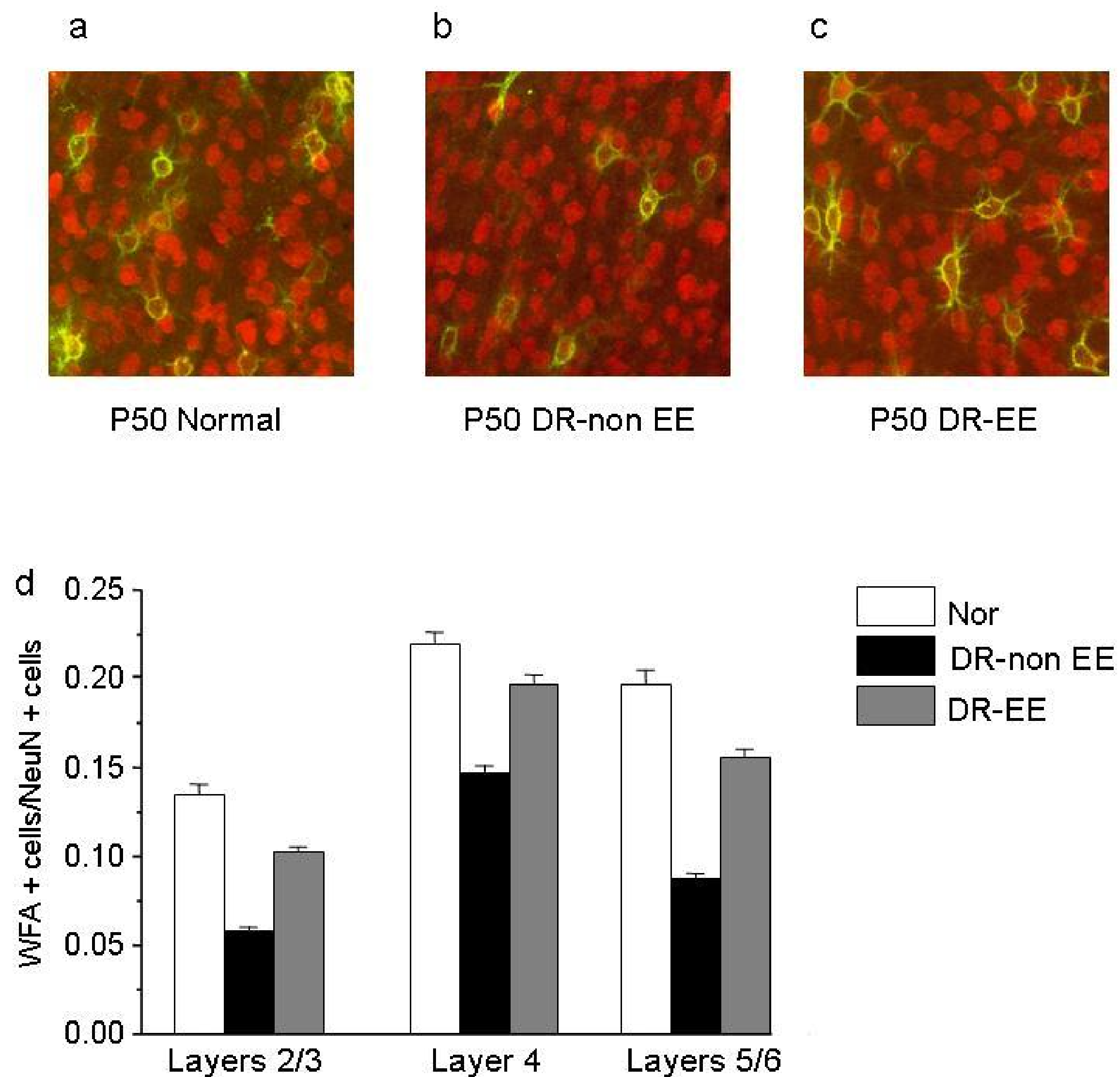

Fig 8. Environmental enrichment prevents dark-rearing effects on CSPG developmental organization into perineuronal nets (PNNs) in the visual cortex. **a, b and c.** Examples of staining for WFA(Wisteria Floribunda Agglutinin, which labels PNNs, green) and NeuN (neuronal marker, red) in Oc1B of a normal, a DR-non EE and a DR-EE rat at P50. The decrease caused by dark-rearing in the number of PNNs surrounded neurons is reduced in EE-DR animals. Calibration bar: 100 μm. **d.** Quantification of the density of PNNs surrounded neurons in layers II/III, IV and V/VI of normal (*n*=4), DR-non EE (*n*=4) and DR-EE (*n*=5) animals. The reduction in PNNs density caused by dark-rearing and the increase in PNNs density in DR-EE with respect to DR-nonEE animals is significant in all layers (one-way-ANOVA on ranks, Dunn's post-hoc test, $P < 0.001$).

The density of PNN-surrounded neurons is expressed as the ratio of total neuronal density (NeuN staining). DR-EE: layer II/III 0,1 ± 0,002; layer IV 0,2 ± 0,005; layer V/VI 0,16 ± 0,004. DR-nonEE animals: layer II/III 0,058 ± 0,002; layer IV 0,147 ± 0,003; layer V/VI 0,088 ± 0,003 Normal animals: layer II/III 0,13 ± 0,006; layer IV 0,219 ± 0,006; layer V/VI 0,197 ± 0,007. Statistical comparisons (one-way ANOVA on ranks, post-hoc Dunn's test) show that the density of PNN-surrounded neurons in DR-EE rats is significantly higher than in DR-nonEE rats in all layers ($p<0.001$) and reaches the normal level in layer IV (density of PNN-surrounded neurons in DR-EE rats in layer IV is not statistically different from that in normal animals ($p>0.05$)); density of PNN-surrounded neurons in DR-non EE animals significantly lower than normal in all layers ($p<0.001$).

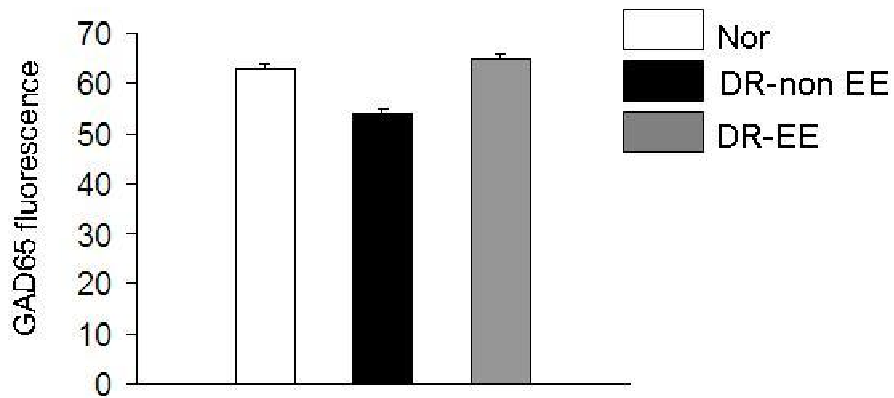

Fig 9. Inhibitory circuitry maturates in DR-EE rats.

We have also examined EE effects on the status of cortical inhibition, as there is growing evidence that maturation of inhibition in the visual cortex is an important determinant of critical period. We have quantified the expression of GAD65 (Glutamic Acid Decarboxylase, the biosynthetic enzyme for the inhibitory transmitter GABA) in the presynaptic boutons of GABAgergic interneurons around the soma of target neurons (perisomatic puncta-rings; Huang et al., 1999) in P50 normal (*n* = 4), DR-nonEE (*n* = 4) and DR-EE (*n* = 5) animals. We found that, as known for other markers of GABAergic function, dark-rearing decreased GAD65 expression (mean GAD65 fluorescence intensity 54 ± 1.2 pixel-value/unit-area in DR-nonEE animals and 63 ± 1.01 pixel-value/unit-area in normal animals). In DR-EE animals, however, GAD65 expression was normal (mean GAD65 fluorescence intensity 64.8 ± 0.96 pixel-value/unit-area).

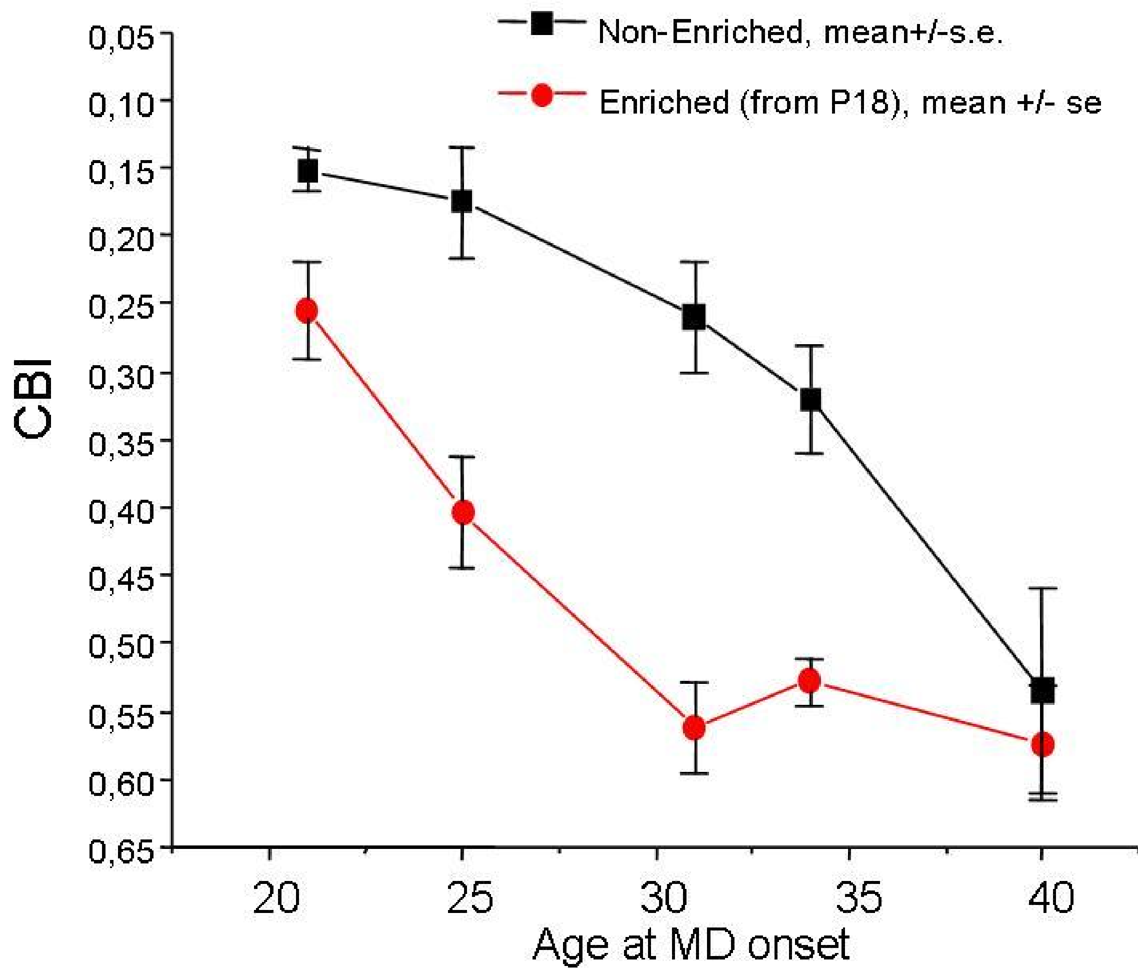

Fig 10. EE shortens the critical period for MD in rats reared in lit environment. Developmental curves relative to MD effects in EE and non-EE rats. Rats have been reared in a normally lit environment and placed either in EE or non EE after weaning (P18). MD was performed for 1 week at different postnatal days (P20, 25, 31, 34, 40) and then animals were subjected to electrophysiological recordings. Statistically significant differences between EE and non-EE rats emerged at P25, P31 and P34 (one-way ANOVA, $P<0.05$, post-hoc Tukey's tests).

		MD	
		Non-EE	EE
P21	N animals	**4**	**3**
	CBI	**0.15±0.02**	**0.25±0.04**
P25	N animals	**4**	**3**
	CBI	**0.18±0.04**	**0.40±0.04**
P31	N animals	**3**	**3**
	CBI	**0.26±0.02**	**0.56±0.03**
P34	N animals	**3**	**4**
	CBI	**0.32±0.02**	**0.53±0.01**
P40	N animals	**2**	**3**
	CBI	**0.54±0.08**	**0.57±0.04**

Tab 1. Summary table of CBIs (mean ± SE) and number of rats deprived at different postnatal ages between P21 and P40. The two groups of rats, EE and non-EE, were subjected to MD for 1 week at different postnatal ages. Ocular dominance distributions (ODD) of visual cortical cells were recorded from binocular primary visual cortex Oc1B and summarized with the CBI index.

APPENDIX A

Measuring ocular dominance in rodents: correlation between single-cell recording and Visually Evoked Potentials (VEPs).

Electrophysiological recording is still the most reliable technique to evaluate ocular dominance (OD) in laboratory animals. It is commonly assumed that single-cell distributions and VEPs (Visually Evoked Potentials) ratios are grossly interchangeable methods, and that the former is preferable being a more objective measure of the OD. Aim of the present study was to correlate measures of OD obtained by single-cell and VEPs recordings from rats and mice.
We found a high positive correlation between single-cell distributions and VEPs ratios (R^2=0.89), concluding that VEPs ratios are reliable measures of OD and give the advantage of larger and quicker sampling of the cortex comparatively to single-cell recording.

The main electrophysiological methods to evaluate ocular dominance (OD) in laboratory animals contemplate: 1) the measure of OD distribution, based on the assignment of each cortical neuron to an ocular dominance class (Hubel and Wiesel) or on the computation, for each neuron, of an ocular dominance score (Rittenhouse et al., 1999); 2) the measure of OD ratio resulting from relative ipsi/contra eye amplitudes of VEPs in response to transient gratings (Porciatti et al., 1999, 2002). Despite their longstanding application to the study of visual cortical OD, no effort has been made to systematically compare the inter-techniques reliability. Rather, it is commonly assumed that single-cell distributions and VEPs ratios are grossly interchangeable methods, and that the former is preferable being a more objective measure of the OD. Unfortunately, single-cell recording is much more time consuming comparatively to VEPs and large numbers of recorded cells are needed to obtain a representative sample of cortical OD.

We aimed to correlate measures of OD obtained by single-cell and VEPs recordings from rats (N=5) and mice (N=12). Single-electrode tracks (N=26) were recorded in primary visual cortex perpendicularly (<30 deg) to the pial surface. Cells were collected from each track though the all cortical depth and VEPs were measured in the same track at

maximum amplitude cortical depth (see Methods). Contralateral bias index (CBI) of each track OD distribution was calculated and correlated with corresponding VEPs ratios. We found a high positive correlation between CBIs and VEPs ratios (R^2=0.89) (**Fig 1**).

We conclude that VEPs ratios are reliable measures of OD and give the advantage of larger and quicker sampling of the cortex comparatively to single-cell recording. The *conditio sine qua non* to obtain a reliable correlation between CBIs and VEPs ratios seems to be a cortical penetration performed perpendicularly (<30 deg) to the pial surface.

Furthermore, the strong correlation found between VEPs and CBIs raises an important question of functional anatomy: do rodents share with other mammals a columnar-like functional organization of ocular dominance in the primary visual cortex? Classical neuroanatomical methods failed to identify OD columns in rodents. And so did imaging techniques, being only able to delineated the monocular and binocular zones of area 17 (Antonini et al., 1999; Schuett et al., 2002; Hubener et al., 2003). Hints of a columnar-like organization in rodents comes from a physiological observation previously made in our laboratory (Caleo et al., 1999). Authors observed that, in each track recorded, there is a high probability (>90%) that a class 2/3 neuron is followed by another class 2/3 or class 4 neuron, and that the probability that a class 5/6 neuron is followed by another class 5/6 cell is 68%.

Because of the strong correlation between the signals coming from pools of adjacent cells (VEPs) and OD distributions of cells in single perpendicular tracks, present data reinforce the idea of a non-random distribution of ocular dominance even in rodents and points toward the existence of a patch-like organization.

MATERIALS AND METHODS

Animal treatment.

Data were collected from rats (N=5) and mice (N=12). Animals used in this study underwent different experimental manipulation. Mice employed were of different genetic strains (wild type C57BL/6J; BDNF+/-), different ages (from P26 to adult) and few underwent monocular deprivation (MD, typically 4 days). Rats were pigmented (Long Evans) hooded rats of different ages (from P25 to adult) and few underwent monocular deprivation (MD, typically 7 days).

In vivo electrophysiology.

Single-cell recording. In vivo electrophysiological recordings were performed from the primary visual cortex. Anesthetized rats (urethane 20% in saline, 7 ml/kg, Sigma) and mice (8ml/kg) were mounted in a stereotaxic apparatus. In mice, the eyes were not restrained in a fixed position, nor were they kept artificially opened, since the eyelids remain open and eyes do not diverge significantly during anaesthesia. In rats, eyes were kept open and fixed by means of a metal ring positioned at the equatorial portion of the eye bulb. Body temperature was monitored with a rectal probe and maintained at 37°C with a heating pad. ECG and EEG were also continuously monitored. A large portion of the skull overlying the binocular visual cortex of the right side was drilled and removed, and the dura was removed in rats. Using a computer-driven three-axis motorized manipulator, the recording electrode (a glass micropipette with inner filament, filled with 3 M NaCl, 1–2 M impedance, 3µm tip) was inserted in the the binocular portion of the primary visual cortex (binocular area 17 or Oc1B). For single-units recording signal was amplified 25000 times, filtered (band pass 300-8000 Hz) and converted through A/D converter. Single-electrode tracks were recorded in primary visual cortex perpendicularly (<20 deg) to the pial surface. A total of 26 tracks were recorded. For each track no less than 10-12 cells were recorded.

The positions of receptive fields of single units were mapped using a hand-held stimulator. Single units were recorded after checking that their receptive field corresponded to the location of the vertical meridian (only cells with receptive fields within 20° from the vertical meridian were included in our sample). For each animal, eight to ten cells were recorded in each tracks spaced across Oc1B. Care was taken to equally sample cells across the whole cortical depth (in rats, distance was not less 70 µm from each other; in mice, not less 50 µm) so that all layers contributed to the analysis of the ocular dominance.

For each neuron, peak response, spontaneous activity, responsiveness (peak-to-baseline ratio) and receptive field (RF) size were derived from peri-stimulus time histograms (PSTH). PSTH were recorded in response to computer-generated visual stimuli (moving bars displayed on a screen with the possibility to change velocity and direction of a bar's movement). In rats, the screen was positioned 27 cm from the eye, in such a way as to include the binocular field. In mice, the display was placed 14 cm in front of the animal and centered on its midline in, thereby covering 81x86 deg of the visual field.

For each track, ocular dominance was evaluated according to the classification of Hubel and Wiesel (1962). Cells in ocular dominance class 1 were neurons driven only by the contralateral eye; cells in ocular dominance classes 2-3 were binocular, preferentially driven by the contralateral eye (in rats, ratio of contra to ipsi peak response >1.5; in mice, ratio >1.3); neurons in ocular dominance class 4 were equally driven by the two eyes; neurons in ocular dominance classes 5-6 were binocular and preferentially driven by the ipsilateral eye (in rats, ratio of ipsi to contra peak response >1.5; in mice, ratio >1.3) and neurons in ocular dominance

class 7 were driven only by the ipsilateral eye. For each animal the bias of the OD distribution towards the contralateral eye was evaluated using the CBI (Contralateral Bias Index) that is defined as CBI={[N(1)-N(7)] + 1/2[N(2/3)-N(5/6)] + N(Tot)}/2N(Tot), where N(tot) is the total number of recorded cells and N(i) is the number of cells in class (i). CBI of each track was calculated and correlated with corresponding VEPs OD ratios.

Visually evoked potentials (VEP). Transient VEPs were measured (in the same track of single cell recording) at cortical depth yielding maximal VEP amplitude (500 μm in rats, 400 μm in mice). Electrical signals were amplified (50 000 fold), band-pass filtered (0.3–100 Hz, -6 dB/oct), digitized (12 bit resolution) and averaged (at least 200 events) in synchrony with the stimulus contrast reversal. Typical visual stimuli were horizontal sinusoidal (or square) gratings (spatial frequency 0.1 c/deg; contrast 90%, mean luminance 15-25 cd/m2) abruptly reversed at a temporal frequency of 1Hz. Transient VEPs in response to abrupt contrast reversal (1 Hz) were evaluated in the time domain by measuring the peak-to-trough amplitude of the major component. OD ratios were obtained recording a series of responses (at least ten) from each eye alternatively, whose average amplitude was used to evaluate the contralateral/ipsilateral VEP amplitude ratio.

Due to the curvature of the brain (expecially in rats) during recordings, all tracks resulted somehow angled (about 20 deg) as compared to the cortical layers. This small angle in electrode penetration was adopted in previous studies and mantained in the present study in order to address the relationship between CBI extracted from OD of cells in all layers of the perpendicular penetration and VEP ratio of the same track. At 400 μm depth, with 20 deg angle, the lateral shift of the electrode is 145 μm. At 500 μm depth, with 20 deg angle, the lateral shift of the electrode is 181 μm.

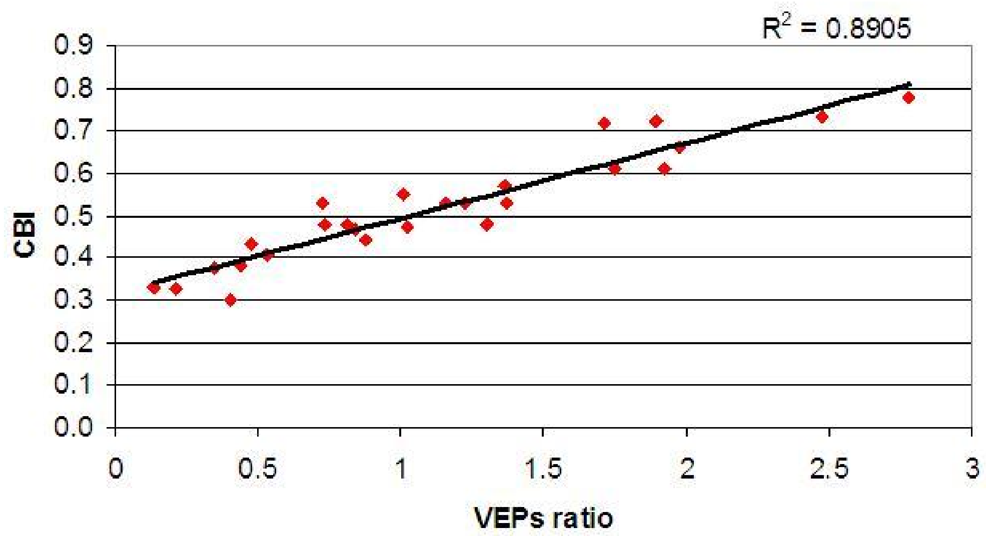

Fig 1. Single-cell distributions of OD and VEPs ratios are highly correlated.
A total of 26 tracks were recorded from rats (N=5) and mice (N=12). For each track no less than 10-12 cells were recorded and ocular dominance was evaluated using the CBI (Contralateral Bias Index). VEPs were measured in the same tracks at maximum amplitude cortical depth.

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ACKNOWLEDGEMENTS

I would like to thank all the people working at the CNR Neuroscience Institute of Pisa for the friendly and emotionally healthy climate that has surrounded me for three years.

A particular acknowledgment goes to Vittorio Porciatti, for his ability in transmitting the passion and the enthusiasm for electrophysiology, prior to techniques; to Nicoletta Berardi for her endless patience, and just for "being" Nicoletta Berardi, a precious resource; to Tommaso Pizzorusso, for unclosing me the giant world of the mice small brain; to Gimmi Ratto, for his gorgeous musicality and for the very relaxing Spring Parties in that of Calci; to Matteo Caleo, for his capacity of "active" listening; to Paolo Medini, for the electrifying moments while working together; and to Alessandro Cellerino, a friend.

Finally, I'm deeply grateful to Professor Lamberto Maffei who, although wrote that *"the prefix psycho is a declaration of ignorance"*, allowed me to be the first psychologist admitted to the Neurobiology PhD program at Scuola Normale Superiore.

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