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PLASTICITY OF THE MAMMALIAN RETINA DURING DEVELOPMENT

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INTRODUCTION

Understanding the effects of experience on neural circuitry development and the underlying mechanisms is an important issue in neurobiology. A large literature documents the plasticity of nervous structures such as the cortex or hippocampus in response to experience during development (reviewed in Berardi et al., 2003; Mangina and Sokolov, 2005); the retina on the contrary has generally been thought of as unresponsive to experience. It was well known, indeed that while the visual cortex responds to paradigms of visual deprivations, such as monocular deprivation (MD) or dark rearing (DR) with dramatic functional and anatomical alterations, retinal development was not substantially modified in cats, rats and humans by these rearing conditions (Baro et al., 1990; Fagiolini et al., 1994; Fine et al., 2003). Recently, there has been the first indication of retinal response to experience in mice by Tian and Copenhagen (2001 and 2003). Their studies have shown that retinal development is sensitive to DR; in particular, retinal ganglion cell (RGC) development is affected by DR both at electrophysiological and anatomical level.

In this work, we have chosen to investigate the actual sensitivity of the developing retina to experience; in particular, we have used a paradigm of complex sensory-motor stimulation as that provided by environmental enrichment (EE). EE seemed to us a valid tool to probe retinal plasticity, since we have recently found that it affects visual cortical development and plasticity (Cancedda et al., 2004) and prevents DR effects (Bartoletti et al., 2004). We have studied the anatomical and functional maturation of Rodent retina, analyzing the development of RGC dendritic stratification and of retinal acuity, and the molecular factors activated by EE and capable to trigger these changes.

We find a remarkable response of RGC developmental remodelling of dendritic stratification to EE, both in normal and in DR animals and a response of functional retinal development to EE as robust as shown by the developing visual cortex. Both effects of EE requires retinal BDNF action.

The Rodent visual system

In the Mammalian visual system, visual information are processed in the retina and sent to different structures of the CNS through RGC axons, which represent the output of the retina. RGCs project to the visual centres of the brain that are located in the midbrain and in the thalamus. The pattern of retinal projections varies considerably from species to species. In Rodents, the vast majority of RGCs project to the superior colliculus (SC) and the

pretectal nuclei, with about 30% of them sending collaterals to the dorsal-lateral geniculate nucleus (dLGN) in the thalamus (Sefton and Dreher, 1985). RGC axons from each eye project to both sides of the brain, however the major afferents to the SC and dLGN arise from the contralateral eye and only 5% of optic axons project ipsilaterally. Within the dLGN ganglion cell axons are not intermixed; in cats, ferrets and primates they terminate in a set of separate, alternate eye-specific layers that are strictly monocular (Hickley and Guillery, 1974). In Rodents there is not a proper lamination of the dLGN; however, even if the rat dLGN is not clearly laminated, ipsilateral and contralateral retinal fibers are segregated in a patchy fashion originating two eye-specific territories in the dLGN: the ipsilateral patch or inner core and the contralateral patch or outer shell (Reese and Jeffery, 1983; Reese, 1988). The geniculate body provides ascending input to the visual cortex via thalamo-cortical connections that terminate in the layer IV of the primary visual cortex. In carnivores and primates, afferents from the dLGN segregate by eye within the cortical layer IV into alternating, equal-sized stripes called ocular dominance columns (Hubel and Wiesel, 1963; Shatz and Stryker, 1978). This functional and structural organization has been found also in Rodents, although at a very primitive state; Thurlow and Cooper (1988) observed hints of patchy organization of ipsilateral and contralateral inputs in the visual cortex of hooded rats, using a functional marker as deoxiglucose and this has been confirmed with electrophysiological techniques by Caleo et al., 1999.

The target of this study: the retina

Architecture of the mature retina

Unlike other sensory structures, such as cochlea or somatic receptors in the skin, retina is not a peripheral organ but part of the CNS and its synaptic organization is similar to that of other central neural structures. The identification and classification of retinal neurons begun more than 100 years ago by Santiago Ramon y Cajal and is nearing completion (Masland et al., 2001).

Briefly, light is focused by the cornea and the lens, onto the photoreceptor layer in the retina. The retina lies in front of the pigmented epithelium that lines the back of the eye; these cells contain melatonin that adsorbs any light not captured by the retina and this prevents the light from being reflected off the back of the eye to the retina again -which would degrade the visual image. To allow light reach the photoreceptors without being adsorbed or greatly scattered, the axons of neurons in the proximal layers of the retina are unmyelinated so that these layers of cells are relatively transparent. Moreover, in one

region of the retina, the fovea, the cell bodies of the proximal retinal neurons are shifted to the side, enabling the photoreceptors there to receive the visual image in the least distorted form; the shifting is more pronounced in the foveola, the center of the fovea. Fovea, as a specialized retinal region, is not present in the retina of Rodents.

Although much remains to be learned, the fundamental structural principles are now becoming clear giving a bottom-up view of the strategies used in the retina's processing of visual information.

Retina consists of many parallel, anatomically equipotent microcircuits; it presents an intricate pattern of connections in spite of a layered anatomical rearrangement. Mammalian retina contains a huge diversity of neuronal types; it is composed approximately by 55 distinct cell types, each with a different function.

In particular, five different layers can be identified: ONL -outer nuclear layer- with cell bodies of photoreceptors, OPL -outer plexiform layer- with cone and rod axons, horizontal cell dendrites, bipolar dendrites, INL -inner nuclear layer- with nuclei of horizontal cells, bipolar cells, amacrine cells and Müller cells, IPL -inner plexiform layer- with axons of bipolar cells and amacrine cells, dendrites of ganglion cells and GCL -ganglion cell layer- with the soma of ganglion cells and displaced amacrine cells.

Photoreceptors

In nocturnal Rodents the number of rods is many-fold that of cones; in particular, in mice cones are about 3% of photoreceptors (Jeon et al., 1998). A typical mammalian retina contains 9-11 types of cone-driven bipolar cells. Everyone of these assortments of pathways from cones to the inner retina is responsible for a different type of information and this characteristic initially attributed to the diverse cell structure and to the different proteins expressed, is now known also at a functional level.

First, the output of the cone photoreceptors is separated into ON and OFF signals. All cone synapses release glutamate, but bipolar cell types respond to glutamate differently. Some bipolar cells have ionotropic glutamate receptors: glutamate opens a cation channel, and the cell depolarizes. Other bipolar cells have a sign-inverting synapse mediated by metabotropic glutamate receptors, mainly mGluR6; these bipolar cells hyperpolarize in response to glutamate (Nawi et al., 1991). Photoreceptor cells hyperpolarize after light absorption, causing their synapses to release less glutamate. When the retina is stimulated by light, one type of bipolar cell hyperpolarizes and the other type depolarizes. OFF and ON bipolar cells occur in approximately equal numbers. The distinction, created at the first retinal synapse, is propagated throughout the visual system.

Bipolar cells

The classes of ON and OFF bipolars are each further subdivided; there are three to five distinct types of ON and three to five types of OFF bipolars. The purpose of the subdivision is, at least in part, to provide separate channels for high-frequency (transient) and low-frequency (sustained) information. Thus, there are separate ON-transient, ON-sustained, OFF-transient and OFF sustained bipolar cells (Kaneko et al., 1970; Awatramani et al., 2000). An elegant series of experiments shows that the distinction is caused by different glutamate receptors on the respective OFF bipolar cells; they recover from desensitization quickly in the transient cells and more slowly in the sustained cells (DeVries et al., 2000). An important point is that there are no dedicated cones -cones that provide input, say, only to ON bipolars or only to OFF bipolars. Instead, the output of each cone is tapped by several bipolar cell types to provide many parallel channels, each communicating a different version of the cone's output to the inner retina.

Most amacrine cells and all ganglion cells receive their main bipolar cell synapses from cone bipolars, but retinas work in starlight as well as daylight, and this range is created by a division of labor between cones (for bright light) and rods (for dim light). Signals originating in rod photoreceptors reach the RGCs via an indirect route using as its final path the axon terminals of the cone bipolar cells (Famiglietti and Kolb, 1975; Strettoi et al., 1990 and 1992). That a single set of ganglion cells is used for both starlight and sunlight represents an obvious efficiency, long known from electrophysiological findings. However, it was not obvious *a priori* that rod-driven information would reach the ganglion cells by an indirect path. Furthermore, rod photoreceptors far outnumber cones in most mammalian retinas; it was a surprise to learn, when quantitative methods became available, that cone bipolars outnumber rod bipolars in all but a few mammalian retinas (Strettoi et al., 1995). The reason is that more rods converge onto a single rod bipolar than cones onto cone bipolars; the rod system trades acuity for sensitivity, and the circuitry associated with rods is simpler than that of cones. Because rods evolved after cones, the likely scenario is that the rod circuitry was grafted onto the cone pathways.

Only one kind of rod photoreceptor exists, and rods drive only a single type of bipolar cell. It synapses on a specialized amacrine cell, termed AII, which then transmits the output of rod bipolar cells to ganglion cells. This occurs largely via synapses (chemical or gap junctional) by AII onto axon terminals of cone bipolar cells, which then excite the ganglion cells. It may seem strange that rod bipolar cells would not simply drive retinal ganglion cells directly, but seems less strange when one appreciates the complexity of the pre-

existing inner retinal circuitry of the cone pathways. By synapsing on the axon of the cone bipolar cell, the rod pathway gains access to the elaborate circuitry of the cone pathway, including its associated amacrine circuitry. For example, the directionally selective type of ganglion cell retains its function in very dim light, even though it receives no direct synapses from the rod bipolar cells. The rod system piggybacks on the cone circuitry rather than re-inventing it (Masland et al., 2001).

Horizontal cells

All rods and cones receive feedback from horizontal cells, but these cells are a numerically small proportion of the retina's interneurons, generally less than 5% of cells of the inner nuclear layer (Jeon et al., 1998). In most mammals, there are two morphologically distinct types of horizontal cells, while Rodents have only one. In monkeys, these have different numbers of synapses with different types of cones. The reason for this biasing is not yet certain; it may involve chromatic opponency in the red–green system. Traditionally, horizontal cells are said to enhance contrast between adjacent light and dark regions. Excitation of a central cone causes feedback inhibition of both the excited cone and a ring of neighbouring ones. Because each cone -both the central one and its neighbours- transmits a signal to the inner retina, the upshot is that a small stimulus excites those ganglion cells that lie directly under the stimulus, but inhibits neighbouring ganglion cells. This is the classical 'center–surround' organization, in which a ganglion cell is excited or inhibited by stimuli falling in its receptive field center, whereas stimulation of the surrounding region has an opposite effect. An alternate formulation of the same facts is that horizontal cells adjust the system's response to the overall level of illumination; they measure illumination across a broad region and subtract it from the signal that is transmitted to the inner retina about a local image. In effect, this reduces redundancy in the signal transmitted to the inner retina. The mean luminance across a large region of retina is shared by many cones and contains little information.

Amacrine cells

All retinal ganglion cells receive input from cone bipolar cells, but direct synapses from bipolar cells are a minority of all synapses on the ganglion cells; most are from amacrine cells (Freed et al., 1988; Calkins et al., 1994). The exact fraction varies among different functional types of ganglion cells, ranging from roughly 70% for alpha cells (large, movement-sensitive ganglion cells found in most mammals) to 50% for the midget ganglion cells located in the monkey central fovea. Amacrine cells also make inhibitory synapses on the axon terminals of bipolar cells, thus controlling their output to ganglion

cells. In contrast to horizontal cells, which have a single broad role, amacrine cells have dedicated functions since they carry out narrow tasks concerned with shaping and control of ganglion cell responses. Amacrine cells outnumber horizontal cells by amounts that range from 4:1 to 10:1 (depending on the species) and can outnumber ganglion cells by 15 to 1. The different amacrine cells have distinct pre- and postsynaptic partners, contain a variety of neurotransmitters, survey narrow areas of the visual scene or broad ones, branch within one level of the inner synaptic layer or communicate among many. Both the specific molecules expressed and their morphology point to diverse functions.

Those amacrine cells with functions that are more precisely understood do remarkably specific jobs. The dopaminergic amacrine cells globally adjust the retina's responsiveness under bright or dim light. Dopamine affects many elements of the retina's circuitry; it alters the gap-junctional conductance between horizontal cells and between amacrine cells (Hampson et al., 1992), potentiates the responses of ionotropic glutamate receptors on bipolar cells, and ultimately affects the center–surround balance of ganglion cells. Remarkably, retinal dopamine can even cause pigment migration in cells of the retinal pigment epithelium, a neighbouring non-neural tissue. In the latter case (and very likely some of the former as well), this is mediated non-synaptically, via a diffuse, paracrine release of the neurotransmitter. In contrast, the starburst amacrine cells seem to be narrowly associated with a particular computational circuit. They arborize in thin (2-4 μm) strata within the IPL, where they make excitatory cholinergic synapses on certain RGCs, notably those particularly sensitive to moving stimuli. By feed forward excitation and/or inhibition (these neurons release both acetylcholine and GABA), starbust cells are important for direction selectivity (Masland et al., 1986).

Retinal ganglion cells

RGCs process and convey information from the retina to visual centres in the brain. These output neurons comprise subpopulations with distinct structure and function (Sernagor et al., 2001). The morphology of RGCs is highly disparate; their somata and dendritic field vary in size, and they exhibit strikingly varied dendritic architecture (Cajal, 1893; Wassle and Boycott, 1991; Rodieck, 1998) and axonal projection patterns (Garraghty and Sur, 1993; Yamagata and Sanes, 1995a,b). Functionally, RGCs differ in their response to light in a variety of ways (reviewed by Wassle and Boycott, 1991; Rodieck, 1998; Dacey, 1999). Their response to light may be transient or sustained, brisk or sluggish, tonic or phasic. Some RGCs are good motion detectors and may prefer a specific direction of stimulus movement, whereas others are sensitive to the orientation of the stimulus but not

to its direction. In addition, RGCs show different contrast sensitivity, visual acuity, and color-coding. Despite the enormous diversity in structure and function, combined anatomical and electrophysiological studies have revealed a close correlation between the morphology and function of RGCs in vertebrates (Saito, 1983; Stanford and Sherman, 1984; Amthor et al., 1984, 1989a, b; Dacey, 1999).

Within a species, structure and function studies have enabled classification of RGCs into broad subclasses (Rockhill et al., 2002). In Primate retina, RGCs fall into two functional classes, M (for *magno* or large) or midget cells and P (for *parvo* or small) or parasol cells. Each class includes both on-center and off-center cells. M cells have large receptive fields (reflected in their large dendritic arbors) and respond relatively transiently to sustained illumination. They respond optimally to large objects and are able to follow rapid changes in the stimulus; on the contrary, the smaller P cells, which are numerous, have small receptive fields, respond specifically to certain wavelengths and are involved in the perception of form and color. P cells are thought to be responsible for the analysis of fine details in the visual image, although some M cells may also be involved in this function.

In the well-studied cat retina, small-field beta RGCs are the anatomical correlate of physiologically identified brisk-sustained or X-RGCs, and large-field alpha RGCs are correlated with brisk-sustained or Y-RGCs (reviewed by Wassle and Boycott, 1991). Major subclasses of RGCs, such as the alpha and beta cells in cat, can be further divided into subtypes, notably those which are depolarized (ON RGCs), or hyperpolarized (OFF RGCs), by light. In general, within a species, each subtype of RGC shares key features: (i) their dendritic branching patterns and arbor size are similar at any fixed retinal location; (ii) their dendritic fields overlap forming mosaics that cover the retinal surface effectively (Wassle et al., 1983; Cook and Chalupa, 2000); (iii) they receive the same complement of presynaptic inputs; (iv) they project to common regions within targets in the brain. But not all RGC subclasses defined within one species are present in all species. However, in all species studied thus far, the IPL, the plexus within which RGCs form intraretinal connections, is organized into structurally and functionally distinct sublaminae. Irrespective of RGC subclass, ON RGCs have dendritic arbors that stratify in the inner region (sublamina b) of the IPL, whereas OFF RGCs stratify in the outer sublamina (sublamina a) of the IPL (Famiglietti and Kolb, 1976; Nelson et al., 1978). Cells with arbors in both sublaminae have ON and OFF responses (e.g. Amthor et al., 1984). The diverse morphological and physiological properties of RGCs have presented an enormous challenge to investigators seeking to understand how the visual image is encoded and relayed to the brain. For

developmental neurobiologists, the rich diversity of RGC structure and function make these neurons ideal for studies of cell-fate determination (reviewed by Cepko et al., 1996; Harris,1997; Rapaport and Dorsky, 1998) and axonal and dendritic development (Goodman and Shatz, 1993; Wong and Wong, 2000).

Mammalian retina and its development

The eye originates as a bilateral organ from a single field in the anterior neural plate. The primordial eye field is separated into two regions by anterior migration of diencephalic precursor cells along the midline. Proliferation and evagination give rise to the optic vesicles. Their infolding into optic cups and their progressive determination originates the optic stalk, the neural retina and the retinal pigment epithelium. In the retina, cell differentiation from retinal precursors is initiated in the inner layer of the central portion of the optic cup to progress concentrically in a wave-like fashion towards the peripheral edges of the retina (Isenmann et al., 2003).

Neurones seems to be generated in the same sequence during the first phase of ventricular cytogenesis in all species analyzed apart from minor differences: RGC, displaced amacrine cells, horizontal cells and cone photoreceptors. Progenitor cells in the neurepithelium lining the surface of the neural tube, later become the ventricular zone of the optic vesicles, optic cup and early retina. Postmitotic cells leave the ventricular zone to migrate to one of three cell layers in the retina remaining attached radially from one side of the retina to the other, as noted by Cajal a century ago. The neural cells lie at different levels in the retina and when in correct position lose their anchoring radial connections. Then, polarity of the differentiating cells occurs and dendrites and axons grow out appropriately. Actually, ultrastructural studies (Olney, 1968; Fisher, 1979a; Blanks et al., 1974) suggest that synaptogenesis between the major neuronal classes of the vertebrate retina occurs in three major steps. Retinal ganglion cells and amacrine cells are the first cell classes to differentiate and form the earliest functional circuits in the IPL of the developing retina. Shortly after, horizontal cells and photoreceptors differentiate and contact each other in the outer retina, forming the OPL. The vertical networks in the inner and outer retina are later interconnected when bipolar cells are born and connections with ganglion cells are established. This sequential pattern of retinal circuit development is common across species, although the separation in time between inner and outer retinal circuit development varies, ranging from a few hours in animals such as the zebrafish to many days and weeks in Mammals.

Finally, bipolar cells, rod photoreceptors and Muller cells are generated throughout the second phase. Therefore, Muller cells, so important in guiding optic nerve formation and in organizing plexiform and nuclear layers, are evident in two different waves of propagation by using different types of labelling. After the cytogenesis in the ventricular zone, cell proliferation continues in the sub-ventricular zone with the generation of macroglia (Ichikawa et al., 1983), microglia (Kitamura et al., 1984) and certain classes of intrinsic neurons. A fundamental process in retinal development is cell loss by apoptosis; 54-74% of axons initially present in the mammalian optic nerve are eliminated during development and so a corresponding number of RGCs and amacrine cells undergo this fate (Dreher and Robinson, 1988).

There is good evidence that neurotransmitters can be found at the earliest stages of retinal development and these neurotransmitters can function in the absence of traditional synapses (Redburn and Rowe-Rendleman, 1996). For instance, cholinergic neurons can be observed by means of antibodies against choline acetyltransferase and acetylcholine esterase in the neuroblastic layer as early as embryonic day 3 in chick and P0 in the ferret and mouse (Feller et al., 1996; Bansal et al., 2000), the developmental period during which amacrine cells are being generated. These cells are presumably starburst amacrine cells, the only source of acetylcholine (ACh) in the adult retina. By monitoring intracellular calcium concentrations using fluorescence imaging, Wong (1995) showed that during these initial stages of retinal development muscarinic acetylcholine receptor (mAChR) agonists cause substantial increases in intracellular calcium of many cells in the neuroblastic layer.

M-AChRs are cGMP gated channels that lead to increases in intracellular calcium by causing a release of calcium from internal stores, as opposed to influx through ligand- or voltage-activated channels. After the cells were postmitotic and began to migrate out of the ventricular zone, this responsiveness to mAChR agonists was reduced. Amacrine and ganglion cells still had responses to cholinergic agonists, but they were mediated via nicotinic receptors, as they are in the adult. Hence, it seems possible that even before cholinergic neurons have left the ventricular zone, and long before these neurons have formed synaptic connections, they could be inducing signalling that is important for early phases of neurogenesis and also cell migration. Another neurotransmitter, γ–aminobutyric acid- GABA is expressed in more cells during development than during adulthood. This exuberance of GABA positive neurons suggests that like ACh, GABA may play a transient role in circuit formation (for review, see Sandell, 1998). For instance, GABA is thought to play a role in synaptogenesis between cones and horizontal cells early in postnatal

development of the OPL in rabbit retina (Messersmith and Redburn, 1993). GABA has a particularly high and transient expression in the ganglion cell layer during the first few postnatal days of rabbit. In addition, markers for enzymes involved in the synthesis of GABA can be found on either side of the IPL early in development in ferret retina (Karne et al., 1997).

The first synaptically connected circuits that appear in the developing IPL are between amacrine and ganglion cells (Greiner and Weidman, 1981; Karne et al., 1997). Prior to photoreceptor maturation and eye opening, RGCs periodically fire bursts of action potentials. This spontaneous rhythmic activity was first measured in fetal rat pups. This activity was found to be highly correlated among neighbouring ganglion cells (Galli and Maffei, 1988). Both extracellular recording using a multielectrode array (Meister et al., 1991) and imaging of calcium transients associated with bursts of action potentials (Feller et al., 1997; Wong et al., 1995) have revealed that these spontaneous bursts propagate from one cell to the next in a wave-like manner. Recent experiments demonstrate that blockade of spontaneous retinal activity disrupts the normal pattern of retinal ganglion cell axons in its primary target, the lateral geniculate nucleus of the thalamus (Penn et al, 1998), indicating that spontaneous activity in the retina plays a critical role in the normal development of the adult visual system.

Retinal waves are a characteristic phenomenon, observed in a large variety of vertebrate species, including chick, turtle, mouse, rabbit, rat, ferret and cat (Wong, 1999). Though wave periodicity and velocity in all species are comparable, the circuitry underlying the propagation may be substantially different. Waves are first seen around the time that neurons residing in the inner retina are starting to form circuits while the outer retinal neurons have not made synaptic connections, and photoreceptors are not yet functional (Greiner and Weidman, 1981; Mey and Thanos, 1992). At this stage of development, ganglion cells have migrated into the ganglion cell layer and their axons have reached their primary targets, the lateral geniculate nucleus in mammals, and the tectum in chick.

In postnatal ferret and mice retinas, chemical synaptic transmission is a prerequisite for wave propagation, as indicated by several experimental results. First, simultaneous whole cell voltage clamp recordings from ganglion cells demonstrate that increases in $[Ca^{2+}]_i$ correlated across cells are driven by compound synaptic inputs (Feller et al., 1996). Second, the compound postsynaptic currents measured from ganglion cells are blocked by bath application of Cd^{2+}, a blocker of voltage-activated calcium channels, including those associated with transmitter release (Feller et al., 1996). Third, the periodic Ca^{2+} increases,

action potential, and compound postsynaptic currents associated with waves can all be blocked by a variety of nAChRs antagonists (Feller et al., 1996; Penn et al., 1994). Although in the adult retina, acetylcholine acts as a modulator of ganglion cell firing, while glutamate is the primary excitatory transmitter at the earliest ages studied, glutamatergic blockers do not affect wave generation (Wong, 1995).

The synaptic circuitry that drives retinal waves changes postnatally. Though cholinergic neurotransmission is required and GABA contributes to the depolarization of cells during retinal waves early in development in the ferret, recent studies in older ferrets indicate that waves are insensitive to cholinergic antagonists and can be blocked by glutamate receptor antagonists (Wong, 1999). This switch in the requisite transmitter occurs at the age that bipolar cells are making their initial synaptic connections with ganglion cells and when conventional synapses between amacrine and ganglion cells become morphologically mature and numerous.

GABA has a modulatory role in retinal waves. Imaging of the spontaneous increases in Ca^{2+} associated with waves has shown that GABA-A receptor antagonists can dramatically alter the amount of wave-induced depolarization (Fischer et al., 1998). GABA is the primary transmitter of most amacrine cells in the retina, and, at the youngest ages studied, it provides excitatory input for ganglion cells (Fischer et al., 1998). Unlike ACh, however, GABA does not influence wave periodicity, since GABA blockers do not alter either the frequency of the cholinergic barrages that are associated with waves measured in ganglion cells (Feller et al., 1996) or other properties of wave propagation at ages less than P10 (Fischer et al., 1998). Waves persist after GABA becomes inhibitory (Fischer et al., 1998). However, these changes in the circuitry mediating waves with development leads to changes in the frequency of events occurring in different subsets of ganglion cells.

Until few years ago, it was largely assumed that retinal function was mature by the time of eye opening (Tian et al., 2001). Consistent with this idea, most morphological and neurochemical systems of the retina as the number of cells, the number of ribbons and conventional synapses, the expression of synthetizing enzymes, of transporters and receptors for neurotransmitters have reached adult levels at the age of eye opening (Fisher, 1979a; Greiner and Weidman, 1981; Pow and Barnett, 2000; Sassoe-Pognetto and Wassle, 1997). Nonetheless, some studies show a continued maturation of visual responsiveness in retina after eye opening. In RGCs of cat and rabbit, the centers of the receptive fields shrink and the surrounds become much more prominent with age (Bowe-Anders et al., 1975; Rusoff and Dubin, 1977). Moreover, light-evoked response amplitudes of RGCs are larger

and the latencies are shorter after eye opening in cat (Tootle, 1993) and ferret (Wang et al., 2001).

Light responses emerge as the photoreceptor-bipolar pathway begins to mature shortly before eye opening (Dacheux and miller, 1981a, 1981b; McArdle et al., 1977; Tootle, 1993). Electrophysiological recordings from retinal ganglion cells show several major trends in the maturation of their responses to light. The early response of RGCs to light stimulation is weak and the cells adapt rapidly (Masland, 1977; Tootle, 1993). But when robust responses to light become detectable a few days later, the concentric center-surround organization of the receptive fields, as well as ON and OF center responses, are already present (Bowe-Anders et al., 1975; Masland, 1977; Tootle, 1993). Whether the connectivity that underlies these physiological properties is established before photoreceptors are present is unknown. Determining how surround inhibition appears in the RGCs has not been straightforward. Some immature rabbit RGCs have silent surrounds, that when stimulated, can suppress the response to center stimulation, but direct stimulation of the surround does not evoke a response (Masland, 1977). In the cat, however, the strength of the antagonistic surround relative to that of the center does not seem to change with postnatal maturation (Tootle, 1993). Recordings from ferrets, however, clearly demonstrate that connectivity in the inner retina is remodelled with maturation. In the postnatal ferret, α and β–like RGCs have convergent ON and OFF inputs prior to maturity (Wang et al., 2001). In these cells, maturation of the receptive field center responses thus involve the loss of one type of input. Specialized receptive fields properties such as direction selectivity also develop before eye opening (Masland, 1977; Sernagor and Grzywacz, 1995), although the synaptic basis for this property remains to be determined. How is the RGC receptive field established during development? Visual experience after eye opening does not appear to alter the receptive field properties of Mammals that were raised in an environment with unidirectionally moving stimuli (Daw and Wyatt, 1974). But this may be because, in rabbits, ganglion cell receptive fields are fairly mature by the time of eye opening (Masland, 1977). In contrast, the peak firing rate of RGCs in response to light stimulation is decreased in DR mice (Tian and Copenaghen, 2001). Cells responds more sluggishly in DR animals. The spatial organizations of the receptive fields have not yet been assessed after DR of mice. In turtles, which become light responsive prior to hatching, DR causes an increase in receptive field size (Sernagor and Grzywacz, 1996). However, this study suggests that spontaneous activity rather than visual stimulation regulates the receptive field size. Clearly, much

remains to be done to fill our knowledge gaps concerning how the light responses of RGCs are established in ways that are characteristic of each cell type.

Little is known of structural plasticity in the mature mammalian retina. Light adaptation is an archetypal plasticity that effects a functional transition from scotopic to photopic vision. In fishes and amphibians, both graphic structural and subtle molecular events attend light adaptation, including photomechanical movements of the RPE and photoreceptors, neurite extension and retraction by horizontal cells, and alterations in bipolar cell synaptic terminal structure. More subtly, but perhaps more physiologically evident, several molecular switches are invoked by light-adaptation, e.g. reduction of homologous coupling between horizontal cells (reviewed in Witkovsky, 1991 and Dearry, 1991; Weiler et al., 2000) and reduction of spike firing frequency and truncation of firing episodes in ganglion cells (Vaquero et al., 2001). Many adaptive processes are apparently gated by dopamine, presumably released by amacrine-like cells driven by cone-dominated circuits (Marc, 1995, 2003). Light adaptation attenuates coupling between cone horizontal cells, gated at least by dopamine in most vertebrates, including mammals (He et al., 2000; Weiler et al., 2000) and is presumed effected through a D1-type PKA-dependent pathway. Other adaptive mechanisms are gated by nitric oxide signaling, which is more complex, but nevertheless potent (Blute et al., 2000). Both dopamine and nitric oxide appear involved in mammalian network adaptation of glycinergic AII amacrine cells. Dopamine selectively attenuates homologous AII-AII gap junctional coupling while exogenous nitric oxide donors attenuate heterologous AII-cone bipolar cell coupling (Mills and Massey, 1995). These network plasticity will become unregulated when photoreceptor drive is removed in retinal degenerations and, glossing the details, retinal degenerations should effectively convert the retina to a perpetually or at least sporadically photopic network.

Intrinsic and environmental cues shaping retinal neurons during development

There are at least two features of the structure of retinal neurons that impact their connectivity and therefore the circuits that they form. The first relates to the lateral branching patterns of their axons and dendrites. The lateral organization of the pre- and postsynaptic arbors of retinal neurons determines the spatial coverage of the cell, and possibly the density of input and output that they form. Second, as mentioned earlier, the pre- and postsynaptic arbors of retinal neurons are highly restricted to laminae in the inner and outer retina. This laminar organization in structure reflects the connectivity between specific subsets of cells. Thus, one way to gain a better understanding of how retinal

circuits form appropriately, is to determine the mechanisms that regulate the lateral and vertical organization of the axonal and dendritic arbors of retinal neurons. As aforementioned, there are many types of RGCs, each with characteristic branching patterns, arbor size and amount of overlap with neighbours of the same subtype. Both intrinsic and environmental cues appear to shape the branching patterns of retinal neurons, at least for ganglion cells (reviewed in Sernagor et al., 2001).

Cell-cell interactions

When RGCs are isolated in culture without contact with other cells, they elaborate a dendritic arbor that is complex in branching and resembles that in vivo. Stereotypic organization of the branching patterns of the various cell types (for example, alpha cells and beta cells in the cat retina) also argue that cell intrinsic mechanisms help to define their branching patterns. However, the shape and size of the ganglion cell dendritic arbor changes when the density of neighbouring RGCs is altered during development. Laser ablation or axon section results in a local lesion depleted of RGCs (Eysel et al., 1985). This manipulation results in cells at the edge of the lesion projecting their dendrites into the ganglion cell-free region. Experimental manipulation of eye size that causes an increase in the spacing between cells is paralleled by an increase in their arbor size (Troilo et al., 1996). However, for certain subtypes of ganglion cells in the mouse retina, a reduction in ganglion cell density does not affect the size and patterning of their dendritic arbors (Lin et al., 2004). These differences raise the possibility that the balance between cell-intrinsic and cell-extrinsic cues in shaping the lateral organization of ganglion cell dendritic arbors may vary from one cellular subtype to another, or perhaps even across species. It's important to ask what factors shape dendritic stratification of retinal ganglion cells during development. In Mammals, retinal ganglion cells initially project dendrites throughout the depth of the IPL. Stratification occurs progressively and becomes precise by maturity. How immature amacrine cells elaborate their processes after neuronal differentiation has been described by Golgi technique (Prada et al., 1987) and electron microscope (Hinds and Hinds, 1978; 1983) studies a few decades ago. From observations of retinas fixed at different ages, it is thought that cells in the differentiating inner nuclear layer that are multipolar in shape are immature amacrine cells. Such cells extend neurites in many different directions prior to reaching the border of the INL and IPL. Thereafter, amacrine cells elaborate processes within the IPL to form their arbors. Time-lapse studies of amacrine cells labeled by expression of fluorescent protein in stable transgenic zebrafish lines have provided some

insight into the lamination of amacrine cell neurites (Kay et al., 2004). In a transgenic line in which subpopulations of ON and OFF amacrine cells express GFP, it is possible to visualize the emergence of a plexus of processes from these amacrine cells. Initially, it appears that GFP-positive amacrine cell processes contributing to the nascent IPL are diffusely arranged, as no sublamination is observed. However, over time, two distinct bands of bright fluorescence resolve, corresponding in depth to sublamina a (OFF) and sublamina b (ON) of the IPL. This is because it is impossible to differentiate between closely apposed, but segregated, arbors in the IPL from arbors that are intermingled in depth. It is evident that even after amacrine cell neurites have attained their correct sublamination, their arbors still undergo significant reorganization in the lateral dimension prior to achieving their mature form. Starburst amacrine cells in the mammalian retina show a distinctive radial morphology early in development, but their detailed branching pattern alters with maturation. Like RGCs, the morphological changes primarily involve the loss of small protrusions, and the emergence of bouton-like structures at the distal branches (Wong and Collin, 1989) that in the mature cell, are sites of presynaptic transmitter. RGCs, although a major postsynaptic target of amacrine cells, are not required for lamination of amacrine cell arbors. Optic nerve section in Rodents that leads to ganglion cell death, does not alter the stratification of starburst amacrine cells (reviewed in Chalupa and Gunhan, 2004). In mutant animals in which ganglion cells do not differentiate (Math5 knockout mice as in Brown et al., 2001 and in zebrafish in Wang et al., 2001a), amacrine cell lamination also occurs. Since bipolar cells differentiate only after amacrine cell lamination has taken place, it is unlikely that these neurons influence the initial stratification of amacrine cells. It is possible that interactions between amacrine cells that are specific to each subtype result in the formation of their separate laminae. Alternatively, lamination cues may arise from Muller glial cells or their precursors. Such cues may be diffusible, creating a gradient of permissive or repulsive factors with retinal depth, or are contact-mediated. Because amacrine cells express neurotransmitters even before their cell bodies reach the INL/IPL border, it also remains plausible that communication between these cells via secretion of transmitters influences their arborization. Golgi studies of the chick retina suggest that the axonal and dendritic arbors of developing bipolar cells elaborate from vertical processes terminating in the inner and outer limiting membranes (Quesada et al., 1981). Stratification of the axonal arbors of bipolar cells may be influenced by interactions with amacrine cells. AII amacrine cells, which are the postsynaptic targets of rod bipolar cells, have a bistratified arbor, each with stereotypic morphology. The outer arbor (OFF sublamina)

comprises lobular appendages whereas the inner arbor (ON) is finely branched. AII amacrine cells express Disabled1, an adaptor protein involved in the reelin pathway (Rice et al., 2001). In the reeler knock-out mouse in which reelin is absent, the distribution of lobular and non-lobular appendages of the AII amacrine cells are imprecise. Likewise, some rod bipolar cells are found to have axonal stratification defects. Also in the lakritz mutant, bipolar axonal terminals are similarly perturbed in their organization in local regions of disrupted amacrine cell lamination. ON and OFF bipolar cell lamination persists in the absence of ganglion cells (Gunhan-Agar et al., 2000; Wang et al., 2001; Brown et al., 2001). Together, these observations raise the possibility that amacrine cells, rather than ganglion cells, provide lamination cues for bipolar axon terminals. Early Golgi studies and immunolabeling for GAD67 (Schnitzer and Rusoff, 1984), one of the two synthetic enzymes for GABA, suggest that horizontal cell processes undergo dramatic progression from a radial to lateral organization during development. Horizontal cells achieve this lateral organization in the absence of cone photoreceptors (Reese et al., 2005). However, abnormal lamination patterns in horizontal cells are observed in the Rb mouse during development in regions where rods are absent (Donovan and Dyer, 2004). In the mature retina, horizontal cells do not contact cones uniformly within their dendritic fields. The number of dendritic terminals that contact cones decreases as a function of distance from the cell body. Interestingly, in developing horizontal cells, the number of terminals does not vary greatly with distance from the cell body. Because the overlap of dendritic fields of neighbouring horizontal cells is relatively unchanged after birth in the mouse, horizontal cells appear to lose peripheral terminals but gain central terminals with maturation. The terminal branching pattern of horizontal cell dendrites is altered in the coneless mouse (see Reese et al., 2005). This suggests that contact with cones is either important in the initial organization, or in the maintenance, of these structures. Structural changes in the morphology and axonal targeting of photoreceptors have been assessed using immunolabeling methods. In a variety of species, these studies reveal that the terminals of a large number of rods (80% of the population) and some cones project beyond the forming outer plexiform layer, reaching the IPL (Johnson et al., 1999; reviewed by Reese, 2004). With maturation, all photoreceptor terminals become restricted to the OPL. The transient projection of photoreceptor terminals to the IPL is not perturbed when RGCs are ablated. However, pharmacological deletion of amacrine cells, for example with VAChT-saporin, results in these photoreceptor terminals ending at various depths of the IPL, and even

extending into the GCL. This implicates the amacrine cells as targets of the immature photoreceptor terminals (see Johnson et al., 2001).

Neurotransmission

It has long been known that neurotransmitters such as acetylcholine affect neurite outgrowth of RGCs in culture (Lipton et al., 1988); the blockade of cholinergic transmission in vivo results in RGCs with reduced total dendritic length and branch numbers. Likewise, dendritic arbors of the embryonic chick retina simplify in the absence of glutamatergic transmission during the period of bipolar synaptogenesis. Moreover, neurotransmission influences dendritic filopodial motility and remodelling (Wong et al., 2000); antagonists to NMDA and non-NMDA receptors affect the rate and extent of filopodial movements. These movements are not activity-dependent and TTX injections in the kitten eye don't prevent the normal loss of dendritic filopodia with maturation (Wong et al., 1991). Early studies with intraocular injections of 2-amino-4-phosphonobutyrate, APB (a mGluR6 receptor agonist) prevented the emergence of stratified alpha and beta ganglion cell dendritic arbors in cat (Bodnarenko and Chalupa, 1993; Bodnarenko et al., 1995) and ferret (Bodnarenko et al., 1999) retina. This is supported by pharmacological studies in turtles and chick whereby blockade of cholinergic or glutamatergic transmission, respectively, resulted in ganglion cells with relatively smaller, and less branched arbors (Wong et al., 2000; Sernagor and Mehta, 2001). If direct bipolar to RGC transmission is important, the segregation of arbors into ON and OFF laminae may be the result of competition between ON and OFF bipolar cells for synaptic targets. Studies by Wang et al. (1999b) demonstrate that during development, alpha and beta RGCs initially receive convergent ON and OFF inputs. RGC dendritic restructuring occurs during the time when bipolar cells make synaptic contacts with RGCs (Maslim and Stone, 1986 and 1988). The blockade of glutamate release by ON-center bipolar cells without affecting OFF-bipolar cell properties by means of APB can suggest that the restriction of multistratified RGC dendritic processes and the establishment of bipolar cell RGC synaptic contacts may be causally related (Chalupa and Gunham, 2004). Indeed, the reorganization of ON and OFF inputs onto RGCs may require little restructuring of the bipolar axon terminals because these terminals are stratified even before ribbon synapses appear in the IPL (Miller et al., 1999; Gunhan-Agar et al., 2000). Interestingly, bipolar terminals are stratified even in the absence of RGCs (Gunhan-Agar et al., 2000). As proposed by Bodnarenko et al., 1995, regions of the dendritic tree that receive little innervation by bipolar cells may eventually be lost whereas relatively well innervated parts of the arbour may be maintained and continue

to elaborate. We can't forget that also cholinergic amacrine cells are important in contributing in RGC stratification; in vivo blockade of cholinergic transmission with curare results in relatively smaller dendritic arbors and ON-OFF stratification of RGCs is perturbed in animals lacking the β2 nicotinic receptor subunit (Bansal et al., 2000).

Neurotrophins

Neurotrophins and their receptors partecipate in the development of visual connectivity at multiple levels, from guiding the morphological differentiation of neurons to controlling the functional plasticity of visual circuits (Huang and Reichardt, 2001; Cohen-Cory and Lom, 2004). During development, RGCs are characterized by two coincident events: the axon extension, growth cone pathfinding and target recognition at one side and growth of dendritic arborisation on the other (Holt, 1989). Many environmental factors modulate these different aspects of RGC development as neurotrophins, ephrin ligands, ephrin receptors, semaphorins, cell adhesion molecules; neurotrophins especially can retrogradely influence the development of presynaptic neurons and anterogradely the development of presynaptic cells (von Bartheld et al., 2001; Caleo et al., 2000 and 2004). In particular, studies in vitro and in vivo have repeatedly shown that BDNF is an important neurotrophic signal that influences multiple phases of vertebrate RGC development including survival, morphological differentiation of axons and dendrites, synapse formation and regeneration (Bahr, 2000; Frost et al., 2001; von Bartheld et al., 1998). BDNF and its receptor Trk B are highly expressed in the visual system of most vertebrate species examined, from fish to Mammals (Cellerino and Kolher, 1997, Cohen-Cory et al., 1996; Frost et al., 2001; Herzog and von Bartheld, 1998). It was first characterized for its ability to promote survival of cultured RGCs (Di Polo et al., 1998). BDNF is expressed by target neurons in the tectum of Xenopus tadpoles, where RGC axons arborize and locally by retinal neurons in regions where RGC dendrites arborize. Indeed, most but not all neurons in the RGC layer express BDNF and TrkB, indicating that these cells are capable to produce and respond to BDNF (Cohen-Cory et al., 1996; Cohen-Cory and Fraser, 1994; Hallbook et al., 1995; Perez and Caminos, 1995). As shown by Cohen-Cory and coworkers (1996), BDNF released by RGC stimulates also a subset of amacrine and bipolar cells, that express TrkB receptor. Branching and refinement of RGC axon terminals seems to be controlled by activity (Cohen-Cory et al., 1999).

Neural plasticity

In the malleable young brain, neurons readily adapt to new experiences by changing which cells they connect to and how to communicate with those partners. This situation is present during a 'critical period' after which brain loses most part of its plasticity. This phenomenon is phylogenetically conserved, as it is present in mice (Gordon and Stryker, 1996), rats (Fagiolini et al., 1994), ferrets (Issa et al., 1999), cats (Hubel and Wiesel, 1998), monkeys (Blakemore et al., 1978) and humans (Ellemberg et al., 2000). Sensory experience during the postnatal critical period is essential for the normal maturation of visual cortical circuits and function. Since Hubel and Wiesel's studies demonstrating the influence of visual experience on ocular dominance columns, much effort has been focused on determining how experience shapes neuronal architecture and connectivity in ways that impact their physiology and behavior. Technical advances in live-imaging studies and molecular approaches have contributed significantly to our current understanding of developmental plasticity. Visual and somatosensory systems of Mammals represent an elective model to understand the mechanisms of plastic changes in CNS because of the detailed knowledge of their anatomical and physiological organization, as well as to the ease of manipulating the visual/external environment (Berardi et al., 2000). Then, rats and mice, initially neglected in this type of studies, are nowadays the preferred model for their simplified CNS and the possibility to study single gene function by using transgenic models (Hubener et al., 2003).

In particular, the role of visual experience has typically been studied by raising animals in the dark or by depriving one (monocular deprivation, MD) or both (binocular deprivation, BD) eyes of patterned vision by lids suturing. MD is accompanied by a dramatic degradation of spatial vision through the deprived eye if vision is not restored before critical period closure, a phenomenon known as amblyopia (reviewed by Mitchell and MacKinnon, 2002). The developmental decline of plasticity is evident when attempting the rescue from sensory deficits established during infancy. Indeed, recovery from the loss of visual acuity that occurs when one eye is deprived of patterned vision during infancy is extremely limited in adults (Mitchell and MacKinnon, 2002; Fine et al., 2003). Similarly, deep neural deafness can be effectively cured by cochlear implants only when surgery is performed in the first years of life (Rauschecker and Shannon, 2002).

It's clear that there are experience-independent and experience-dependent processes; these roughly correspond to two stages of development: the initial formation of anatomical and physiological maps and the subsequent maturation or refinement, respectively, of these

maps to produce a mature visual system. Examples of intrinsic, experience-independent processes include the formation of layers in the LGN and of ocular dominance bands in layer 4 of the primary visual cortex (reviewed by Sengpiel and Kind, 2002). Although these features form prior to the onset of visually evoked activity, they could require spontaneously generated activity.

Visual cortical plasticity

The cellular and molecular mechanisms that control the developmental plasticity of visual cortical connections and restrict experience-dependent plasticity to short critical periods are still little known, though intensely investigated. In general, the first steps of neural plasticity, which are changes in synaptic efficacy, that do not require new protein synthesis, are followed by long-lasting changes in neuronal circuitry that require gene expression and protein synthesis. These molecular determinants have been summed up in a review by Berardi and colleagues (2003).

- *NMDA receptors*

The first modifications induced by experience in visual cortical circuits are likely to be changes in synaptic efficacy. Ever since the discovery of NMDA receptors, these synaptic receptors have been implicated in experience-dependent plasticity. Their characteristic of being both transmitter and voltage-dependent, and their coupling via Ca^{2+} influx to plasticity-related intracellular signalling, has led to the notion that they might be a neural implementation of Hebbian synapses.

Involvement of NMDA receptors in developmental visual cortical plasticity has been initially suggested by the observation that block of NMDA receptors blocks the effects of MD (Bear et al., 1990). A difficulty with pharmacological block of NMDA receptors can be that it significantly affects visually driven activity, but the use of different NMDA receptor antagonists (Daw et al., 1999) or antisense oligonucleotides to reduce expression of the NMDAR1 subunit has overcome this problem, showing that it is possible to block the effects of MD without affecting visual responses (Roberts et al., 1998) and confirming NMDA-receptor involvement in visual cortical plasticity.

NMDA receptors are developmentally regulated and their expression is modified by electrical activity. In particular, their subunit composition varies in the visual cortex, 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 the critical period. Expression of the 2A subunit correlates with the progressive shortening of NMDA current. DR, which delays critical period closure and impairs development of functional properties of the visual cortex and of visual acuity, delays the developmental shortening of NMDA-receptor currents and of subunit 2A expression, suggesting that the 2B-to-2A switch is related to visual cortical development and, possibly, to the closure of the critical period (Berardi et al., 2000). However, recent results have shown that in mice with deletion of the NMDA-receptor 2A subunit, the sensitivity to monocular deprivation is restricted to the normal critical period, thus suggesting that expression of the 2A subunit is not essential to delineate the time course of the critical period of ocular-dominance plasticity (Fagiolini et al., 2003) and might be related to other features of visual cortical plasticity.

- *Neurotrophins*

Several observations have suggested that neurotrophins control visual cortical plasticity during the critical period. Initially, it was shown that exogenous supply of neurotrophins in the visual cortex strongly affects the ocular dominance plasticity induced by MD. In these studies, the effects of neurotrophins on ocular dominance plasticity were sometimes accompanied by alteration of other properties of visual cortical neurons, such as their pattern of discharge and orientation selectivity (Gillespie et al., 2000; Lodovichi et al., 2000), possibly owing to the high concentration of exogenous neurotrophins. Other studies, which followed the opposite course of antagonizing the action of endogenous neurotrophins, have clearly shown that neurotrophins are important for normal visual cortical development and plasticity (Berardi et al., 1994; Cabelli et al., 1997; Patz and Wahle, 2004; Wirth et al., 2005). Then, Huang et al. (1999) generated a mouse overexpressing brain-derived neurotrophic factor (BDNF) in the visual cortex, maintaining a normal cellular pattern of BDNF expression and release. In this mouse, BDNF overexpression accelerates both the development of visual acuity and the time course of ocular dominance and synaptic plasticity. Neurotrophin production and release depend on electrical activity and, in particular, depend on visual activity (Berardi et al., 2003). In turn, neurotrophins can modulate electrical activity and synaptic transmission at both presynaptic and postsynaptic levels (Poo, 2001). They can have both fast actions, for instance by increasing transmitter release (Sala et al., 1998) or by directly depolarizing neurons (Kafitz et al., 1999), and slow actions, by modulating gene expression. BDNF also enhances visual cortical synaptic plasticity (Berardi et al., 2003). This reciprocal regulation between

neurotrophins and neural activity might provide a means by which active neuronal connections are selectively strengthened.

Indeed, neurotrophins seem to require the presence of electrical activity to exert their actions. In fact, it has been demonstrated that the coincidence between weak synaptic activity and localized BDNF application, which by themselves do not lead to long lasting changes in synaptic efficacy, induces long-lasting potentiation of synaptic transmission, suggesting that neurotrophins operate in synergy with electrical activity in promoting synaptic plasticity (Kovalchuk et al., 2002). It is interesting to note that, although BDNF can promote the phosphorylation of the transcription factor cAMP response-element-binding protein (CREB), it evokes only weak CREB-mediated gene expression unless it is coupled with electrical activity (Hu et al., 1999). Several studies on neurotrophin-receptor expression and on the effects of neurotrophins on visual cortical neurons or afferents to the visual cortex have then indicated that different neurotrophins act on different neuronal targets. Therefore, the synergy between neurotrophins and activity has to be considered to be specific for each neurotrophin and the neuronal populations that are its targets. For example, 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, suggesting that BDNF controls the time course of the critical period by accelerating the maturation of GABA-mediated inhibition.

- *Intracortical inhibition*

Inhibition is not only a 'brake' for excitation but also has an important role in sculpting the pattern of electrical activity. This action contributes to the detection of imbalance of activity between the afferents to a cortical neuron. A failure of the postsynaptic neuron to evaluate the timing of arrival of its synaptic inputs is bound to be a failure in plasticity.

Indeed, Hensch and colleagues have shown that inhibitory interactions are necessary for the manifestation of experience-dependent plasticity (for a review see Hensch., 2005). In transgenic mice lacking the 65-kDa isoform of the GABA-synthesizing enzyme GAD (GAD65), experience-dependent plasticity in response to monocular deprivation is deficient. Normal plasticity in these animals can be rescued if GABA transmission is enhanced in the visual cortex by means of benzodiazepines (Hensch et al., 1998). Development of inhibition seems also to be a determinant of the critical period (Hanover et al., 1999). The results obtained in mice with precocious BDNF expression clearly show that accelerated development of GABA mediated inhibition results in an early opening and

closure of the critical period. This point is further strengthened by the work of Fagiolini and Hensch (2000) showing that precocious enhancement of inhibitory tone by early administration of diazepam to the visual cortex accelerates opening of the critical period.

- *Intracellular signalling*

Another important question is how central neurons integrate electrical activity and neurotrophin signalling to control plasticity of cortical circuitry. A flurry of recent experiments has identified three kinases that are necessary for shift of ocular dominance during monocular deprivation: cAMP-dependent protein kinase (PKA), extracellular-signal-regulated kinase (ERK) and αCa^{2+}/calmodulin-dependent protein kinase II (αCaMKII) (Taha et al., 2002; Di Cristo et al., 2001). Each kinase is activated by a specific pattern of extracellular signals and is tightly woven within a network of mutual interactions. The possible targets of PKA, ERK and αCaMKII after visually driven activation are at two different levels: the cytoplasm and the nucleus. In the first case, we can envisage a local and rapid action of these kinases and that, upon their activation, they phosphorylate substrates that are crucial for synaptic transmission, neuronal excitability and morphological stabilization. The list of possible targets is continuously expanding, underlining the complexity of the action of these kinases on neuronal function. Because the PKA, ERK and αCaMKII pathways vary in the signal integration that leads to their activation and in their downstream targets, it is somewhat surprising that interfering with the activation of any of these pathways causes the same end result: the suppression of the ocular dominance shift after MD. This could be due to the extensive overlap and cross talk of these pathways, so that the blockade of a single kinase reverberates on the entire network. It is easy to see how the block of any of these kinases can lead to a depression spreading through the entire signalling network. It is now clear that this is true also for ocular-dominance plasticity in the visual cortex (Mower et al., 2002).

Thus, the pattern of kinase activation has to be translated into a pattern of gene expression, probably through the activation of transcription factors. How can the crucial kinase -transcription-factor interactions be individuated? Several transcription factors, such as early-growth-response 1(egr1/zif 268), are regulated by visual activity (Caleo et al., 1999). However, the condition of being visual-activity-dependent does not necessarily imply that the activation of a specific transcription factor is necessary for ocular-dominance plasticity, as exemplified by egr1/zif 268: mice with this factor knocked-out exhibit a normal response to monocular deprivation (Mataga et al., 2001). An important hint leading

to the molecular identity of the transcription factors necessary for plasticity is offered by the recent finding that the activation of CREB is necessary for ocular-dominance plasticity (Liao et al., 2002; Pham et al., 1999). To cause CREB phosphorylation, activated kinases must translocate to the nucleus, where they start the expression of genes under the cAMP-response-element (CRE) promoter, with the consequent production of gene transcripts essential for establishment and maintenance of plastic changes (Silva et al., 1998). Both PKA and ERK are well-characterized activators of CREB (Impey et al., 1996), although the ability of αCaMKII to translocate into the nucleus and directly activate CREB is far less certain. Another activator of CREB is Ca^{2+}/calmodulin-dependent protein kinase IV (CaMKIV) but the role of this factor in the visual system is unknown. What is the pathway responsible for CRE-mediated gene expression activated by visual stimulation? This question can only be answered by in vivo studies on behaving animals because the details of the PKA–ERK interaction depend strongly on the cellular context (Grewal et al., 1999). Recently, it has been shown that patterned vision is a powerful activator of ERK in neurons of the visual cortex. Visually induced ERK activation relies, at least partially, on the cAMP-PKA system, and pharmacological block of ERK phosphorylation completely suppresses CRE-mediated gene expression after visual stimulation (Cancedda et al., 2003). This is a strong indication that ERK is the final effector linking extracellular signals with gene expression in the visual system during the critical period. A rough scheme could be designed with the activation of αCaMKII by means of NMDA action, possibly helped by the co-occurring activation of PKA and the consequent inhibition of the αCaMKII phosphatase, protein phosphatase 1 (PP1). Locally activated αCaMKII acts on local targets, such as AMPA receptors (Benke et al., 1998), contributing to further depolarization. Finally, ERK detects the simultaneous and stabilized activation of PKA and αCaMKII, integrates these signals with those of the neurotrophin signalling cascades, and controls CRE-mediated gene expression and the induction of long-lasting modification of cortical circuitry.

A recent paper by Majdan and Shatz (2006) points out about the effects of visual experience on activity-dependent gene regulation in cortex trough DNA microarray technique. In particular, these authors suggest that sensory experience is needed for the sequential acquisition of age-specific, but not ‘common’ gene sets (present throughout development and common to all age groups), comparing the different level of gene expression both before, during and after the critical period for MD. Between the different signalling pathways identified as visually experience regulated, it emerges still MAP

(Mitogen-Activated Protein) kinase signalling pathway; visual deprivation leads to a sustained, rather than transient, downregulation of the MAP kinase pathway and these observation expands on earlier findings that visual stimulation enhances MAP kinase activity (Cancedda et al., 2003; Di Cristo et al., 2001) and that MEK1/2 is required for OD shifts induced by MD during the critical period (Di Cristo et al., 2001).

Thus, a dynamic interplay between experience and gene expression drives activity-dependent circuit maturation.

- *Extracellular environment*

Downstream effectors that implement the program initiated by the signalling mechanisms described in the preceding section are largely unknown; however, recent results indicate that removal of factors present in the extracellular environment is necessary for the experience-dependent modification of visual cortical circuits. The extracellular protease tissue plasminogen activator (tPA) is induced by electrical activity as an immediate-early gene (Qian et al., 1993) and its proteolytic activity in the visual cortex is increased during monocular deprivation (Mataga et al., 2002). The first investigations on the role of tPA in visual cortical plasticity indicated that its pharmacological inhibition attenuates the ocular dominance shift induced by MD (Mataga et al., 1996) and prevents the effects of reverse suture in kittens (Muller et al., 1998). The implications of these pharmacological studies have been deepened by analysis of the effects of MD on tPA-knockout mice. These mice displayed an impaired ocular-dominance shift that could be rescued by exogenous tPA. tPA has a wide spectrum of possible molecular targets, including extracellular-matrix proteins (Wu et al., 1999), growth factors (Yuan et al., 2002), membrane receptors (Nicole et al., 2001) and cell adhesion molecules (Endo et al., 1999), and the available information is not sufficient to dissect which of these actions of tPA are relevant for inhibition of plasticity. tPA has been recently implicated in the regulation of dendritic spine dynamic after brief periods of MD in two converging work (Oray et al., 2004 and Mataga et al., 2004). Oray and colleagues applied tPA on visual cortical slices and observed a dramatic increase of spine motility in all cortical layers. Then, tPA was applied to slices obtained from MD animals and it was found that the effects of tPA were not additive with the effects of MD, suggesting that tPA is a mediator of MD action on spine motility. Moreover, Mataga et al. have shown that tPA is needed for MD-induced changes in spine density. Counting spines on dendrites of layer III pyramids, the authors find that the decrease of spine density caused

by 4 days of of MD is not present in tPA knockouts and this effect could be rescued by exogenous tPA.

Recent data then suggest that at least part of the inhibitory action of the extracellular environment could reside in components of the extracellular matrix (Pizzorusso et al., 2002; 2006), the glycoproteins chondroitin-sulfate proteoglycans (CSPGs), comprising a core protein and chondroitin-sulfate glycosaminoglycan (CS-GAG) chains. CSPGs are abundantly expressed in the CNS, where they are used mainly to create barriers.

Thus, in the developing nervous system, barriers between the two sides of the brain contain large amounts of CSPGs injury they are upregulated in the CNS, with the effect of blocking axon regeneration (Bradbury et al., 2002). In the adult CNS, CSPGs are typically condensed in lattice-like structures, designated perineuronal nets (PNNs), which completely ensheath neuronal cell bodies and dendrites. PNNs are fenestrated at sites of synaptic contact, where they assume a perisynaptic localization (Celio et al., 1998). In the visual cortex, the process of condensation of CSPGs into PNNs begins during late development and is completed after the end of the critical period. Dark rearing, which is known to prolong the critical period for ocular-dominance plasticity, also prevents PNN formation, as assessed by staining for CS-GAG chains with Wisteria Floribunda Agglutinin, and by immunostaining for neurocan and with antibodies which recognize glycovariants of aggrecan (Lander et al., 1997). The correlation between CSPG maturation and critical period closure (Pizzorusso et al., 2002) suggested that CSPGs could hinder ocular-dominance plasticity in the adult visual cortex. The mechanisms by which CSPGs inhibit plasticity in the adult visual cortex are still unknown.

However, the inhibitory action of CSPGs on axonal sprouting suggests that the degradation of PNNs could restore plasticity by removing substrates that are non-permissive for the generation or rearrangement of synaptic connections. Experiments in the somatosensory cortex have suggested that plasticity of dendritic spines is at the core of plasticity of the somatotopic map during development (Stern et al., 2001; Lendvai et al., 2000). In the adult somatosensory cortex, dendritic spines are still dynamic and changes in spine turnover can be activated during experience-dependent plasticity. Indeed, long-term two-photon imaging of dendritic spines coupled with electron microscopy has shown a change in the dynamics of synaptic contacts in whisker-deprived mice (Trachtenberg et al., 2002). Surprisingly, this highly dynamic scenario seems not to be present in the adult mouse visual cortex. It is tempting to speculate that the developmental maturation of the

extracellular matrix, that is non-permissive for synaptic rearrangement, could cause the remarkable structural stability of the adult visual cortex.

Is the retina plastic?

In spite of its complexity, the retina has generally been thought of as a fixed circuitry where experience plays little role in shaping connections. Indeed, as reviewed in the preceding section, it exists a large literature about the plasticity of other nervous structures as the cortex.

Circuit assembly in the mammalian retina involves significant postnatal refinement, including improved high spatial frequency cut-offs of RGCs at about P30 in cats (Rusoff and Dubin, 1977). There is increasing evidence that light history impacts maturation.

In a remarkable study Maffei and Fiorentini (1976) reported that the early exposure to periodic gratings is found to affect the spatial frequency characteristics not only at cortical level but also at the level of LGN suggesting that deprivation of visual stimuli might also affect the spatial frequency responses of geniculate neurones. A similar result has been obtained by Ikeda and Wright in the same year; they demonstrated that the spatial resolutions of sustained LGN neurons driven by an amblyopic eye in strabismic kittens were poorer than those cells driven by the normal eye. They concluded that such effect was likely precedent the visual cortex and represented reorganization of pathways in the LGN or, provocatively, the retina.

The dominant model of amblyopia was then and today remains focused on cortical reorganization, and it is not surprising that this hypothesis was criticized and then ignored. It was well-known, that while the visual cortex responds to paradigms of visual deprivations, such as MD or DR with dramatic functional and anatomical alterations, retinal development is not substantially modified in cats, rats and humans by these rearing conditions (Baro et al.,1990; Fagiolini et al., 1994; Fine et al., 2003). Recently, it has been shown that light deprivation affects the properties of mouse RGCs (Tian and Copenhagen, 2001) and the maturation of retinal circuitry. In particular, a wave of spontaneous EPSPs and IPSPs emerges around P25, increasing RGC activity over 4-fold and subsiding by P60, even though light-driven responses are also maturing. This suggests a maturation epoch that may tune presynaptic efficacy or circuitry itself. Moreover, light deprivation trough DR influences the developmental process of stratification of RGC dendrites in the IPL, delaying it (Tian and Copenaghen, 2003). Taken together with observations from Fisher (1979b) and Sosula and Glow (1971) that postnatal light deprivation statistically increases

the number of amacrine cell synapses in the IPL, one might conclude that refinement of retinal circuitry, like that of virtually all other mammalian CNS networks, requires some level of visually driven activity to achieve normal status.

Intrinsic, light-independent processes also may participate in shaping the retina and ganglion cell arbors in particular. The work of Feller, Wong, Shatz and colleagues on the propagation of excitation waves across the mammalian retina prior to and during the emergence of mature retinal circuitry demonstrates that multiple endogenous sources of focal and global excitation are activated. In particular, large waves of Ca^{2+} influx are mediated by cholinergic signaling at P0-14 in mouse, precede the maturation of glutamatergic synaptic drive in the IPL, but are not required for it (Bansal et al., 2000). However, the absence of acetylcholine-gated waves in mice lacking $\alpha 2$ or $\beta 3$ nicotinic receptors does lead to a delay in both the pruning of RGC dendrites and their refined lamination into narrow sublayers of the IPL. It has long been suspected that pruning of RGC arbors is a key process in the refinement of their function (Rusoff and Dubin, 1977; Rusoff and Dubin, 1978) and these data suggest that cholinergic waves play a role in that refinement. Further insights into the developmental plasticity of RGCs were provided by an elegant experiment by Perry and Linden (1982). A small optic fiber layer lesion at birth led to loss of a strip of ganglion cells by 3-6 months of age. Axotomy is fatal to mammalian RGCs. However, surviving ganglion cells surrounding the lesion sent the majority of their dendrites to one side of the soma into the depopulated zone, unlike normal ganglion cells that have a roughly elliptical dendritic convex hull centred around the soma. The orientation distribution of the longest dendrite from each cell's arbor was nearly random in cells far from the lesion, while cells near the depopulated zone excluded 235° of orientation. The authors proposed that the phenomenon arose from imbalanced dendritic competition among ganglion cells, with those bordering the depopulated zone having preferential access to inputs from the IPL.

Environmental enrichment as a tool to unmask retinal plasticity

The experimental protocol

Environment plays an important role in remodelling the nervous system both during development than in adulthood. Genes and environment together define what we are in a complex interplay still little understood. It is still difficult to establish the different contribution of genes or environment in developing living beings; until the second half of the last century there was a strong debate between Behaviourists and Ethologists, the so-

called ‘nature versus nurture’ debate. The central question was “what is the contribution of genes to overt and covert behaviour, and what is the contribution of the environment to the same behaviours?” (for review, see Krubitzer and Kahn, 2003). Nobel prize Konrad Lorenz solved this debate introducing for the first time the concept of ‘innate predisposition to learn’ (1961). In this context ‘innate’ and ‘learned’ are the two ways through which information is available to the organism. As species are morphologically and physiologically different and these differences are subjected to the genetic laws of selection and heredity, the potentiality to acquire new information through learning is also regulated and programmed under species-specific constraints; so, genetic inheritance during phylogenesis and learning and memory during ontogenesis are intermingled in the construction of the individual personality.

In the same 1960s, after a period during which brain had been considered immutable, a group of neuroscientists guided by Rosenzweig, introduced a paradigmatic experimental protocol to test the effects of experience on the nervous system and behaviour.

Since that, environmental enrichment (EE) for laboratory animals has come to be viewed as a potential method for improving animal well-being in addition to its original sense as a paradigm for learning how experience molds the brain (Benefiel et al., 2005).

Hebb (1949) first described how increasing the complexity of a laboratory rodent’s environment from a typical laboratory setting improved its subsequent behaviour in learning tasks. Hebb brought laboratory rats to his home, where they where treated as family pets. Subsequently, students of Hebb or others he inspired repeated the basic finding that a more stimulating rearing environment enhanced performance on complex learning tasks (Bingham and Griffiths, 1952; Forgays and Read, 1962). Yet, the firsts to coin the definition of EE were Krech, Rosenzweig and Bennett in 1960. They referred to this paradigm as a ‘combination of complex inanimate and social stimulation” and they found biochemically changes in the brains of enriched rats. Enriched animals are reared in large groups (usually 8-12 individuals) in cages of great dimensions, where a variety of toys, tunnels, nesting material, stairs are present and changed frequently. In addition, an essential component of EE is the opportunity for animals to perform increased levels of voluntary physical activity on running wheels. In contraposition, in the standard housing condition normally used in laboratory, animals are reared in little groups of 3-5 individuals in small cages where no particular objects than food and water are present.

Many attempts have been done to separate the different contributions exerted by these various components to the effects of EE on brain and behaviour, but the prevailing

consensus is that no single variable can completely account for the consequences of enrichment (see van Praag et al., 2000). In particular, it has been early established that a direct interaction with the richness of the environment is essential (TV rats which are given the possibility to observe, but not to experience the enriched environment, are not "enriched", Ferchmin and Bennett, 1975) and that the component of increased sociality alone is not sufficient for all the effects of the enrichment (Rosenzweig et al., 1978). It is interesting to note, however, that the single variables included in the enriched condition can act in an addictive manner, as suggested by morphological studies reporting that synaptic density, number of synapses per neuron and maximum length of synaptic contact zone are highest in enriched rats, intermediate in socially reared rats and lowest in isolates (Turner and Greenough, 1985; Sirevaag and Greenough, 1985). Surely, an important aspect of EE is physical activity. It improves cognitive function in rats and aging humans (Fordyce and Farrar, 1991; Kramer et al., 1999; Churchill et al., 2002), attenuates motor deficit (Klintsova et al., 1998), increases neurogenesis (van Praag et al., 1999) and is neuroprotective, ameliorating neurological impairment in different neurodegenerative processes (Arkin et al.,1999; Petajan and White, 1999; Larsen et al., 2000; Mattson, 2000; Carro et al., 2000 and 2001). Furthermore, physical exercise increases angiogenesis (Black et al., 1990; Isaacs et al., 1992) and enhances neurotrophin levels in the brain (Neeper et al., 1996; Oliff et al., 1998; Carro et al., 2000; Johnson et al., 2003; Farmer et al., 2004; Klintsova et al., 2004). It becomes difficult to separate effects produced by EE from that produced by physical exercise alone. While enrichment including exercise seems to be more effective than exercise alone in enhancing memory functions (Bernstein, 1973), adult neurogenesis is more strongly affected by exercise than by enriched living. We could postulate that only together these aspects are important and synergic to determine EE effects, while exercise alone is not able to activate 'learning and memory' pathways to improve cognitive functions. Indeed, new results suggest that, in the absence of social interaction, a normally beneficial experience as physical exercise can exert a potentially deleterious influence on the brain (Stranahan et al., 2006). Indeed, it has been found that individual housing precludes the positive influence of short-term running on adult neurogenesis in the hippocampus of rats and, in the presence of additional stress, suppresses the generation of new neurons. These is accompanied by influences on corticosterone levels (runners in both housing conditions had elevated corticosterone during the active phase, but individually housed runners had higher levels of this hormone in

response to stress). Lowering corticosterone levels it was possible to convert the influence of short-term running on neurogenesis in individually housed rats from negative to positive.

Environmental enrichment and its influence on adult brain

Rosenzweig and colleagues introduced the concept of EE as a way to investigate the influence of environment on brain, showing that the morphology, chemistry and physiology of the brain can be artificially manipulated by modifying the quality and intensity of environmental stimulation. Initial experiments by this group in 1964, in temporal parallel with those performed by the Nobel prizes Hubel and Wiesel on the effects of monocular deprivation on the anatomy and physiology of the visual cortex, first put on evidence that the cortex -entire dorsal cortex comprehending frontal, parietal and occipital cortex- of rats living in enriched conditions for 30 days increased robustly in thickness and weight compared with that of standard reared rats. Since that, other diffuse anatomical effects were found:

- an increment in the size of the soma and of the nucleus of nerve cells (Diamond, 1988),
- increased dendritic arborisation (Holloway, 1966; Globus et al., 1973; Greenough et al., 1973),
- increased length of dendritic spines, synaptic size and number (Mollgaared et al., 1971; Turner and Greenough, 1985; Black et al., 1990),
- increased postsynaptic thickening (Diamond et al., 1964) and gliogenesis (Diamond et al., 1966).

Almost four consecutive days of enrichment were necessary to produce these changes (Wallace et al., 1992), while thirty days of EE caused long-lasting effects persisting even after 30 days of housing in individual cages (Camel et al., 1986). Same effects were found for pyramidal cells of CA1 and CA3 and for dentate granule neurons (Walsh et al., 1969; Walsh and Cummins, 1979; Rosenzweig and Bennett, 1996; Rampon et al., 2000b).

Another anatomical effect of EE is on hippocampal neurogenesis (Kempermann et al., 1997). Studies about this problem have begun when it has been shown that Rodent brain has neurogenesis even after sexual maturity (Gueneau et al., 1982; Kuhn et al., 1996) like monkeys' and humans' brain (respectively, Gould et al., 1999; Eriksson et al., 1998). A fundamental component of EE is an increment of voluntary physical exercise through running wheels in enriched cages; exercise alone in standard cages is responsible in both proliferation of neural precursors and survival of new-generated neurons, while EE causes an increased survival of newborn neurons (vanPraag et al., 1999b; van Praag et al., 2005).

In particular, it has been shown that EE influences neurogenesis, it reduces apoptotic cell death in the rat hippocampus under both natural or pathological conditions (Young et al., 1999).

These anatomical effects are supported by various molecular changes. It has been seen that EE affects:

- the functioning of cholinergic, serotoninergic and noradrenergic systems; in particular, EE determines an increase of acetylcholinesterase activity (Rosenzweig et al., 1962 and 1967), augmented mRNA expression levels of 5-HT1A receptor for serotonin (Rasmuson et al., 1998) and an increase of beta-adrenoceptor transduction system (Escorihuela et al., 1995; Naka et al., 2002), respectively. All these neurotransmitters are capable to influence learning and plasticity in adult brain (vanPraag et al., 2000) and are involved in the arousal state of the brain (Hobson et al., 1975; Berridge and Waterhouse, 2003).
- the production and action of neurotrophins, involved in neural circuits rearrangements both during development than in adult plasticity (reviewed in Bonhoeffer, 1996; Caleo et al., 2004; Berardi et al., 2003); in particular, EE determines higher levels of mRNA for NT-3 and NGF in the visual cortex and hippocampus (Torasdotter et al., 1996 and 1998), of the early candidate-plasticity gene NGFI-A (also Zif268, Pinaud et al., 2002) and increased levels of NGF, BDNF and NT-3 in several rat brain regions (Ickes et al., 2000; Pham et al., 2002),
- cyclicAMP response element binding protein (CREB) expression, which regulate BDNF expression,
- brain uptake of IGF-I, another trophic factor considered to mediate BDNF expression and c-fos activation (Carro et al., 2000), augmented hippocampal neurogenesis (Trejo et al., 2001) and neuroprotective effects against lesions (Carro et al., 2001),
- various genes analyzed through gene chip technique, activated already after three hours of EE with persisting expression until two weeks from the beginning of the enrichment (for mice, Rampon et al., 2000a; for rats, Keyvani et al., 2004).

Undoubtedly, these anatomical effects are reflected in behaviour (Renner and Rosenzweig, 1987), principally in the improvement of learning and memory tasks in EE animals. Morris water maze test is better executed by EE both in normal animals, independently on their gender and age (Pacteau et al., 1989; Tees et al. 1990; Falkenberg et al., 1992; Paylor et al., 1992; Moser et al., 1997; Kempermann et al., 1998; Tees, 1999; Williams et al., 2001) than in aging (for review, Winocur 1998) or pathological models. Moreover, EE mice are better than standard ones also in non-spatial tasks (Rampon et al.,

2000b) like object recognition test, a test of visual recognition memory, contextual fear-conditioning and cued fear-conditioning (Rampon et al., 2000).

The other behavioural consequence of living in an enriched environment is a diminished level of stress reaction (Isgor et al., 2004; Sandi, 2004; Lupien et al., 2005); for example, BALB/c mice, a pathological model of anxiety, display decreased levels of anxiety after rearing in EE. Studies in this field seem to be quite contradictory, even if EE appear to have a general "anxiolitic" outcome and EE subjects are more resilient to cope with stress (Chapillon et al., 1999) with reduced level of activation of the hypothalamic-pituitary-adrenal axis and correspondent reduced levels of basal corticosterone.

Wolfer and colleagues (2004) have tried to verify how much EE rearing paradigm can be considered standardized and replicable in different laboratories and they have concluded that EE increases neither individual variability in behavioural tests nor the risk of obtaining conflicting data in replicate studies. Taking female mice (male could be more aggressive and dominant in EE) of two inbred strains, they have valuated their response to four commons used behavioural tests (elevated-O-maze, open field test, novel-object test and place navigation in the water maze) in three distinct laboratories. These results can be applicable to animal's anatomy and physiology which are in any case less sensitive then behaviour to environmental perturbations.

Environmental enrichment and visual system development

Until few years ago, most studies about the influence of the enriched environment on living beings were focused on the understanding of the EE effects on adult animals enriched either after weaning or in adulthood, in particular in the rescue from pathological conditions. This represents a conspicuous field of research still topically in progress. However, recently in our laboratory we have begun to be interested in the EE effects during development, using Rodent visual system as a paradigmatic system to observe the influence of a complex environment on the nervous system. We have recently found that EE affects visual cortical development and plasticity (Cancedda et al., 2004; Sale et al., 2004) and prevents DR effects on the closure of critical period for MD (Bartoletti et al., 2004). As demonstrated by Cancedda and coauthors, mice reared in a EE show a precocious eye-opening, the accelerated development of visual acuity tested both electrophysiologically and behaviourally, the precocious developmental decline of WM-LTP, higher levels of BDNF protein and GAD65/67 expression in enriched pups [about 55% of BDNF at P7; higher GAD 65/67 levels at P7 (about 112%) and at P15 (about 37%)]. Moreover, in EE

mice we see an acceleration of the developmentally regulated CRE-mediated gene expression with a peak around P20 and correspondently, if we treat standard mice with rolipram, a specific inhibitor of the high-affinity phosphodiesterase type IV that activates the cAMP system via inhibition of cAMP breakdown, resulting in an increased phosphorylation of the transcription factor CREB (Tohda et al., 1996; Kato et al., 1998; Nakagawa et al., 2002), we can partially mimic EE effects on CREB pathway and so on visual system development. In Cancedda et al., 2004 and Sale et al., 2004 we underline that the effects of EE on visual system development could be explained by different levels of maternal care, since BDNF and GAD65/67 variations are found in a period after birth during which pups spend most of their time in the nest. Indeed, a detailed analysis of maternal care behaviour brought us to think that different levels of maternal care in different environmental conditions could act as an indirect mediator for the earliest effects of enrichment on visual system development.

In another work (Bartoletti et al., 2004), we have shown that post-weaning EE is capable to counteract the DR effects on visual system development. As said before, during early postnatal development, cortical connections are highly plastic. They consolidate progressively and become less modifiable by experience, in parallel with visual functional maturation. The absence of visual experience from birth prevents this maturation. In particular, in DR animals visual connections do not consolidate, remaining plastic well after the normal critical period and visual acuity do not develop (Fagiolini et al., 1994). We have seen that EE promotes the consolidation of visual cortical connections both studying the ocular dominance distribution and analyzing visual acuity development in animals monocularly deprived and or DR or DR reared in a EE. Moreover, we have shown on the anatomical plane that EE also prevents DR effects on CSPG developmental organization into perineuronal nets in the visual cortex.

EE promotes the expression of several factors that could control visual system development and plasticity. A particular good candidate is insulin-like growth factor-I (IGF-I); its receptors are present in the occipital cortex (Frolich et al., 1998) and IGF-I could influence the expression of molecules relevant for visual cortical plasticity such as NGF and BDNF. Indeed, EE increases NGF and BDNF expression in the visual cortex (Pham et al., 2002) and BDNF overexpression and NGF supply prevent DR effects (Gianfranceschi et al., 2003; Berardi et al., 2003).

Recently, it has been seen that both BDNF and IGF-I genes are regulated by MD and DR sharing the common PI3K pathway (Zheng et al., 2004) and demonstrating the existence of

a coordinated sets of molecules and pathways that transduce input activity during development into cortical connectivity and function (Tropea et al., 2006), but a causal relationship between BDNF and IGF-I has been properly demonstrated only in models of neurodegenerative diseases (Carro et al., 2001).

In our laboratory, more recently, it has been seen also that maternal enrichment during pregnancy accelerates retinal development of the fetus, influencing the migration of neural progenitors and the dynamics of natural cell death. These effects are under the control of IGF-I: its levels, higher in enriched pregnant rats and in their milk, are increased also in the retina of their offspring, its neutralization abolishes the action of maternal enrichment on retinal development and chronic insulin-like growth factor-I injection to standard-reared females mimics the effects of enrichment in the fetuses (Sale et al., 2004b).

Then, a remarkable report (Pinaud et al., 2002) suggests that three weeks spent in an enriched, complex visual environment in young adult rats can significantly increase expression of immediate early gene products NGFI-A (nerve growth factor-induced gene A) and Arc (a synaptic cytoskeleton- associated protein) in the retina, as well as the late gene products synapsin and GAP-43, implying significant synaptic reorganization, if not outright synaptogenesis. Moreover, it has been seen that this gene expression is activated as basal level in monkey retina, where it depends verisimilarly on normal visual processing (Pinaud et al., 2003). Given that the visual environments of two control groups in the study were likely different only in the behavioural salience of the visual scenes they experienced, this finding might suggest important roles for retinal efferents (Drager et al., 1984; Gastinger et al., 1999; Gastinger et al., 2001) as signalling elements in adult retinal plasticity.

Enrichment and maternal care

A possible source for the very precocious effects induced by EE on visual system development is maternal care influence. As reported in Sale et al., 2004, enriched pups experience higher levels of maternal care compared to standard pups. During the first two weeks of life, Rodents don't interact with environment; they spend their time in the nest, totally dependent on the mother that can be considered their most important source of sensory experience (Hofer et al., 1984; Liu et al., 2000). Moreover, the physical contact is both given by mother and filler females; there is a continuous tactile stimulation that can facilitate phenomenon as precocious eye-opening seen in enriched animals (Cancedda et al., 2004). In particular, it is important to observe the level of maternal care by analysing

certain stereotyped modules of the mother or adoptive mothers as grooming, licking, arched-back nursing typical of Rodents.

It is well documented that maternal influence is capable to alter stress responses exhibited by the offsprings when they become adult (Francis and Meaney, 1999). A brief daily maternal separation from the mother, called 'handling' during the first postnatal weeks decreases the magnitude of stress response in adulthood at both behavioural and endocrine level (Meaney et al., 1996), while longer periods (3-6 hours) of 'maternal separation' elicit the opposite effect, enhancing responses to stressors (van Oers et al., 1998; Ladd et al., 2000). Moreover, maternal separation has also been associated with adult cognitive deficits (Oitzl et al., 2000) and increase susceptibility to disease (Hofer, 1996), while handled animals show, as adults, a greater amplitude of LTP in the hippocampus (Wilson et al., 1986) and increase immune system function (Solomon et al., 1968).

It has been demonstrated that even one hour of maternal separation in rat produces a decrease in the activity of ornithine decarboxylase (Wang et al., 1996), which is an important enzyme necessary for normal growth (Marton and Morris, 1987) and this effect can be completely prevented through artificial tactile stimulation with a brush at a frequency resembling that of maternal licking (Pauk et al., 1986). Maternal behaviour seems to be highly variable in response to environmental demands with dams shifting from moderate licking levels when other females contribute to pup care to intermediate levels in standard conditions, to sustained licking in a complex environment where no social care is possible. Increased dam licking has been reported in handled animals, a result that has been interpreted as evidence that the long-lasting neurobehavioral changes induced by handling can be at least partially mediated by altered maternal care (Liu et al., 1997; Pryce et al., 2001). On the other hand, enriched mothers spend more time far from the nest exploring a more complex environment. It is known that maternal care can affect BDNF levels and neural development of the offsprings (Liu et al., 2000) and artificial manipulations and tactile stimulation in pups can influence eye-opening in Rodents (Barnett and Burn, 1967; Smart et al., 1990). Furthermore, tactile stimulation influences the expression of hormones implicated in the control of pup development (Kuhn and Schanberg, 1998; Schanberg et al., 2003) and can affect the adult pattern of cortical cell dendritic fields (Gibb and Kolb, 2005). These effects could explain our results about BDNF augmented levels at P7 in mice and the enhanced inhibitory levels observed at P7-15 (Cancedda et al., 2004; Sale et al., 2004). However, maternal behaviour could control also other factors important in visual system development such as growth factors crossing the placental barrier and present in

maternal milk, as for example IGF-1, capable to control the expression of NGF and BDNF. Nonetheless, the interaction between IGF-1 and BDNF has been clearly investigated in the adult (Carro et al., 2000; Thoenen and Sendtner, 2002), but not during development.

Aim of this work

We considered that EE, which so powerfully affects visual cortical development and which has been recently suggested to affect retinal early developmental events, was a paradigm suitable to probe the actual sensitivity of retinal development to experience and to gain insight on the factors involved.

In the first part of this work, I assessed the influence of EE on retinal circuitry development investigating the developmental remodelling of RGC dendritic arborisation and I identified a possible molecular factor involved in this process.

In the second part of my thesis, I explored whether RGC functional development, assessed recording P-ERG, is a target of EE and I studied the molecular factors.

The data exposed in this thesis work give a new idea of retinal development, both at anatomical and physiological level and underline the notion that it is sensitive to environment.

MATERIALS and METHODS

Animal handling and treatments

All experiments were performed on Rodents in accordance with the Italian Ministry of Public Health guidelines for care and use of laboratory animals. Rats (or mice) lived in an animal house with a temperature of 21 °C, 12/12 light/dark cycle and food and water available ad libitum.

- *Mice*

We have used line 21 of the transgenic mice expressing plasma-membrane marker green fluorescent protein under control of Thy-1 promoter [Thy-1-mGFP^{single} mice kindly provided by P. Caroni]. A sample of different cell types of RGCs expressed GFP in this transgenic line.

Female mice were put with males (one male for every mating cage) in standard cages for reproduction (26 X 42 X 18 cm). Pregnant mothers were assigned to four different rearing conditions:

1) standard condition (non-EE): dams with their offspring live in standard laboratory cages until pups surviving age;

2) enriched condition (EE): at least 7 days before delivery, pregnant females were transferred to an enriched cage, characterized by a large mesh cage (44 X 62 X 28 cm) containing several foodhoppers, a running wheel and differently shaped objects (tunnels, shelters, stairs) that were completely substituted with others once a week. At least two-three pregnant mothers were put into the enriched cage with four-five filler females.

3) dark rearing condition (DR): pregnant females were transferred in a dark air climatized lightproof environment at least 7 days before delivery and litters were dark-reared until postnatal day 30 (P30) All manipulations were done with infrared viewers.

4) dark rearing with enriched condition (EE-DR): pregnant females were transferred to an enriched cage in a dark room at least 7 days before delivery, litters were dark-reared until P30.

- *Rats*

Female subjects were put with males (one male for every mating cage) in standard cages for reproduction (60X40X20 cm). At least 7 days before delivery, pregnant

females were transferred to an enriched or standard cage; with this procedure, both enriched and standard females received equivalent levels of stress deriving from cage transfer during pregnancy. No difference in gestation time was detected in the two experimental conditions (Cancedda et al., 2004).

Enriched environment (EE) consisted of a large wire mesh cage (at least 60X50X80 cm) with two-three floors containing several foodhoppers, two running wheels (one bigger for adults, the other for postweaning pups) and differently shaped objects (tunnels, shelters, stairs) that were completely substituted with others once a week. At least two-three pregnant mothers were put into the enriched cage with four-five filler females. Cage for standard environment (non-EE) was a standard laboratory cage (30X40X20 cm) housing four adults or one dam with her pups until their postweaning age. After birth, all of the litters were housed with their mother until P45. From postnatal day 8, rat pups (N=45 pups for EE, N=33 pups for non-EE) were inspected for eye-opening twice a day at about 9 am and 7 pm. Eye-opening was defined as the initial break in the membrane sealing the lids of both eyes. The eyes of all animals in this study were clear and without obvious optical anomalies. Optics was checked with an ophthalmoscope and was completely transparent from P19, age of beginning of our electrophysiological recordings.

- *Animal treatments*

For the study of the role of the neurotrophin BDNF on RGC anatomical and functional development, mice or rats received intraocular injections of antisense or sense oligonucleotides (Eurogentec) under ether anesthesia.

Sequences of the BDNF antisense and sense oligonucleotides (targeted to the BDNF translation initiation codon) were 5´-CATCACTCTTCTCACCTGGTGGAAC-3´ and 5’-GTTCCACCAGGTGAGAAGAGTGATG-3´, which correspond to nucleotides 51–75 of the BDNF mRNA. Fully phosphorothioate oligonucleotides were dissolved in saline with stock solutions of 1mM for antisense and of 2 mM for sense oligos. Dilution from stock solution preserved at -80°C were made at the moment of utilization in saline.

Intraocular injections of BDNF oligos were performed by using a glass micropipette inserted at the ora serrata connected to an Hamilton syringe every 72 hr from P6 to P12 in mice and at P6 and P9 in rats.

The concentration was according the increasing size of the eye to maintain a final intraocular concentration of the oligos equal to 25 μM (Menna et al., 2003); the

volume of oligos that we injected was 500 nl at P6 and 1 µl at P9 in rats, while in mice 250 nl at P6, 500 nl at P9 and 750 nl at P12.

Thy-1-mGFP mice were sacrificed at P16 (antisense-treated mice: N= 5; sense-treated mice, N=5) and their retinas were dissected and processed to study the pattern of RGC stratification, while rats were recorded at P25-26 (antisense-treated rats, N=4; sense-treated rats, N=3) by P-ERG according the procedure reported in this Material and Methods section.

For the analysis of the role of the growth factor IGF-I on retinal acuity development in standard reared animals, rats received intraocular injections of IGF-I (kindly provided by Torres-Aleman) or saline (0.9% NaCl) under ether anesthesia at P1, P4 and P7. The concentration was according the increasing size of the eye camera to maintain a final intraocular concentration of the factor equal to 100 ng/µl (Sale et al., 2004); the injected volume was 250, 500 and 750 nl at P1, P4 and P7, respectively.

Intraocular injections were performed by using a glass micropipette inserted at the ora serrata connected to an Hamilton syringe every 72 hours from P1.

At P25-P26, P-ERG recordings were made and retinal acuity was determined for each animal as previously described.

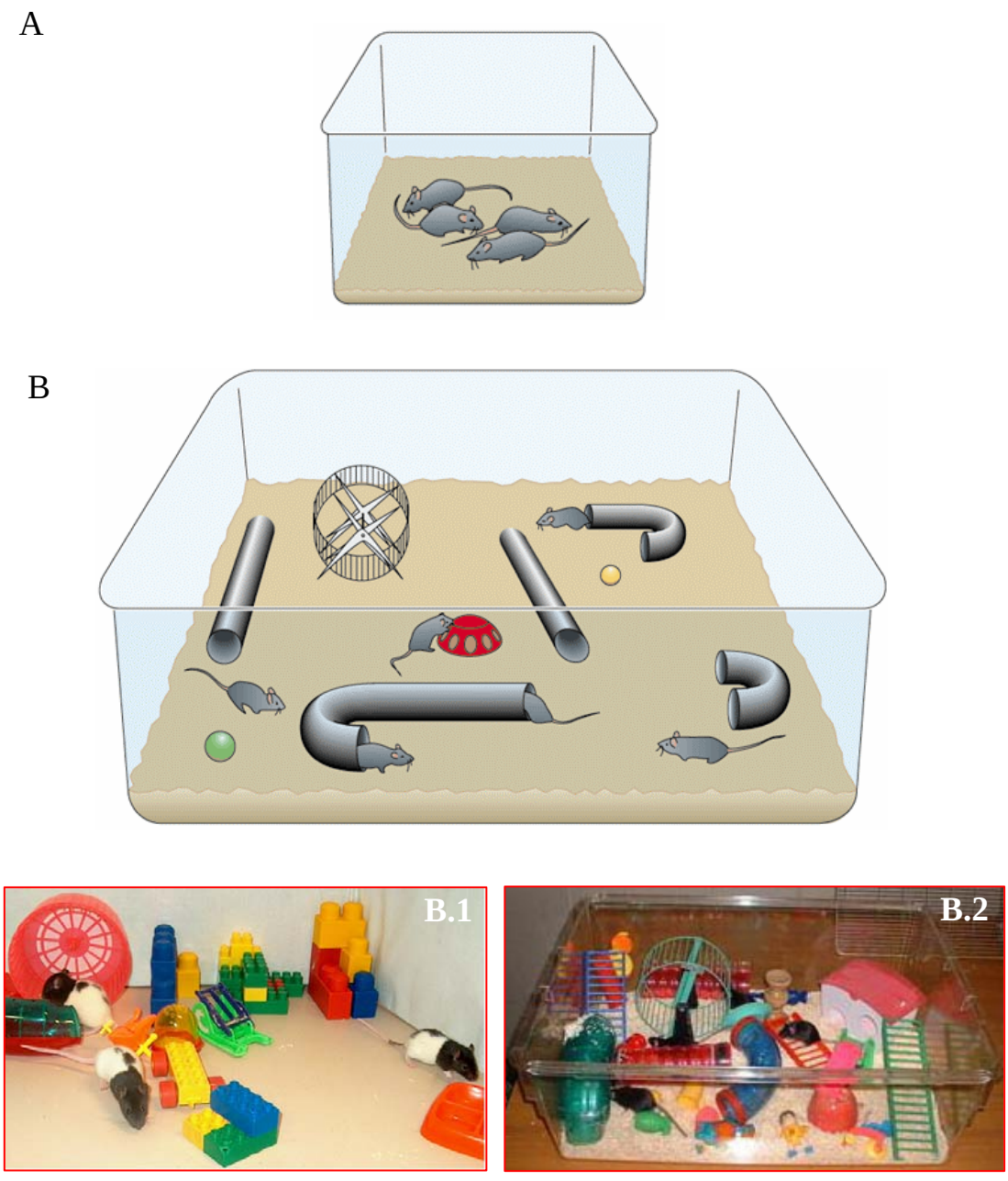


Figure 1: **Different experimental rearing conditions in Rodents (rats and mice)**

(A) Non-enriched environment: animals housed as groups of 2-4 animals in regular size cages without any stimulus object. (B) Enriched environment: enrichment consists of social interactions (6-12 animals in big cages), stimulation of exploratory behaviour with different objects and almost a running wheel for exercise. Exemplificative pictures of our enrichment in rats (B.1) and mice (B.2).

Immunohistochemistry

Mice (or rats) were anesthetized with chloral hydrate (0.2 ml/10g) and perfused transcardially with PBS followed by fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4). Eyes were kindly removed, postfixed in 4% paraformaldehyde and cryoprotected in 30% sucrose. Serial 25 μm thick retinal coronal sections were obtained by using a cryostat.

For analysis of RGC stratification, after a blocking step with 0.3% Triton X-100, mice retinal sections were incubated overnight at 4°C either in goat anti-choline acetyltransferase (ChAT) polyclonal antibody (1:200, Chemicon) or in rabbit anti-GFP polyclonal antibody (1:500, Molecular Probes). The first antibody was detected by incubating sections with Alexa Fluor 568 donkey anti-goat IgG (1:400, Molecular Probes), the second one revealed with biotinylated donkey anti-rabbit (1:200, Vector Lab) followed by extravidin-FITC (1:300, Sigma Aldrich).

For BDNF immunostaining, after a blocking step, sections were incubated overnight in chicken polyclonal anti-BDNF antibody (1:400, Promega), then exposed to the biotinylated donkey anti-chicken IgG (1:200, Promega) followed by extravidin CY3 in mice (1:500, Sigma Aldrich; N= in standard mice, N= in EE mice) or by fluorescein-conjugated extravidin in rats (1:300, Sigma; N=4 animals for each of the three experimental group: P7, P10, P15). Immunostaining was performed for enriched and control retinal sections in parallel within the same experimental set.

Analysis of dendritic RGC arborizations

Images were collected using an Olympus Optical confocal microscope with an UPlanApo 20X objective (N.A.= 0.7). Settings for laser intensity, gain, offset and pinhole size were optimised initially and held constant through each experimental session. For each animal, the entire serial order of coronal sections of the retina was acquired, and for each section, confocal series of 1 μm step size were obtained throughout the whole section thickness (25μm); these confocal series were then averaged and visualized on a single focal plane by Fluoview software. All images of the GFP RGC dendrites patterning were examined visually in blind at the end of each acquisition and each acquired RGC was assigned to its class according its pattern of stratification in different sublaminae of the inner plexiform layer (IPL) according to a protocol similar to that described in Bodnarenko and Chalupa (1993).

Analysis of BDNF expression in the retina

Images of retinal sections were acquired at 20X magnification using a Zeiss HR Axiocam videocamera connected to a Zeiss Axiophot microscope and digitalised by Axiovision software for rats or using an Olympus Optical confocal microscope with an UPlanApo 20X objective (N.A.= 0.7) for mice retinas. To compare different specimens, the time of exposure was optimized at the start and then held constant throughout image acquisition. Then, the collected images of the retina were imported to the image analysis system MetaMorph and used to evaluate pixel intensity of cellular immunofluorescence. All image analyses were done blind. The profile of cells into RGC layer was outlined and pixel intensity was measured within this area.

BDNF immunoreactivity levels were calculated as the ratio between the pixel intensity of RGC profiles and the background level, measured in the outer nuclear layer (ONL). Values obtained from at least 8-10 retinal fields were used to calculate the average pixel intensity value per animal.

Electrophysiological assessment of retinal and cortical acuity in rats

A total of 117 rats [animals non-enriched (non-EE): N=46; enriched (EE): N=48; rats with a forced eye-opening at P10 (EO-P10): N=5; enriched and dark reared between P10 and P14 (EE(DR_P10-14)): N=5; enriched until P10 (EEP10): N=6; enriched treated with BDNF antisense oligonucleotides (EE-AS): N= 4; enriched treated with BDNF sense (EE-S): N=3] was used for electrophysiology. Rats were anesthetized with an intraperitoneal injection of 20% urethane (0,7 ml/hg; Sigma, St. Louis, MO) and mounted on a stereotaxic apparatus allowing full viewing of the visual stimulus. Additional doses of urethane (0.03-0.05 ml/hg) were used to keep anaesthesia level stable throughout the experiment.

During electrophysiology, the body temperature of rats was monitored with a rectal probe and maintained at 37.0°C with a heating pad. Visual stimuli were horizontal sinusoidal gratings of different spatial frequency and contrast generated by a VSG2/2 card (Cambridge Research System, Cheshire, UK) and presented on a computer display (mean luminance=10 candles/meter square; area, 24X26 cm) placed 20 cm in front of the animal. In order to analyze the contribution of the different phases of EE to the development of retinal acuity, one enriched mother with its offspring born in EE was transferred at P10 in a standard environment.

Recordings were always made in blind in relation to the animal's rearing condition to avoid subjective judgements of the experimenter.

- *Pattern electroretinogram (P-ERG).* P-ERG was recorded as in Berardi et al., 1990 and Domenici et al., 1991. The stereotaxic apparatus was oriented with an angle of about 40° as respect to the position of the screen; P-ERG electrodes were small silver rings positioned on the corneal surface by means of a microelectrode drive, so as to avoid occlusion of the pupil. Visual stimuli were sinusoidal gratings alternated in phase with a fixed temporal frequency of 4 Hz. Steady-state recorded signals were filtered (0.1-100 Hz) and amplified in a conventional manner, computer averaged and analysed; 15 packets of 20 sums each (300 events) were averaged for each stimulus spatial frequency, changing ramdomly the spatial frequency from one record to another. For each spatial frequency, the amplitude of the P-ERG signal was taken as the amplitude of the second harmonic in the averaged signal, calculated by a Fast Fourier Transform; the P-ERG amplitude decreases with increasing spatial frequency (Beradi et al., 1990; Rossi et al., 2001). The noise level was estimated by measuring the amplitude of the second harmonic in records were the stimulus was a blank field. Retinal acuity was taken as the highest spatial frequency still evoking a response above noise level.

- *Visual Evoked Potentials (VEPs).* VEPs were recorded as in Di Cristo et al., 2001. Briefly, a large portion of the skull overlying the binocular visual cortex was drilled and removed taking away the dura. A glass micropipette (2-2,5 MΩ) was inserted into the binocular primary visual cortex in correspondence of the vertical meridian representation (Oc1B). Electrical signals were amplified, bandpass filtered (0.1-120 Hz), and averaged (at least sixty events in blocks of ten events each) in synchrony with the stimulus contrast reversal. Transient VEPs in response to abrupt contrast reversal (0.5-1 Hz) were evaluated in the time domain by measuring the peak-to-baseline amplitude and peak latency of the major component. VEPs in response to a blank field were also frequently recorded to have an estimate of the noise. For each animal, VEP amplitude was plotted as a function of log spatial frequency and visual acuity was determined by linearly extrapolating VEP amplitude to 0 V.

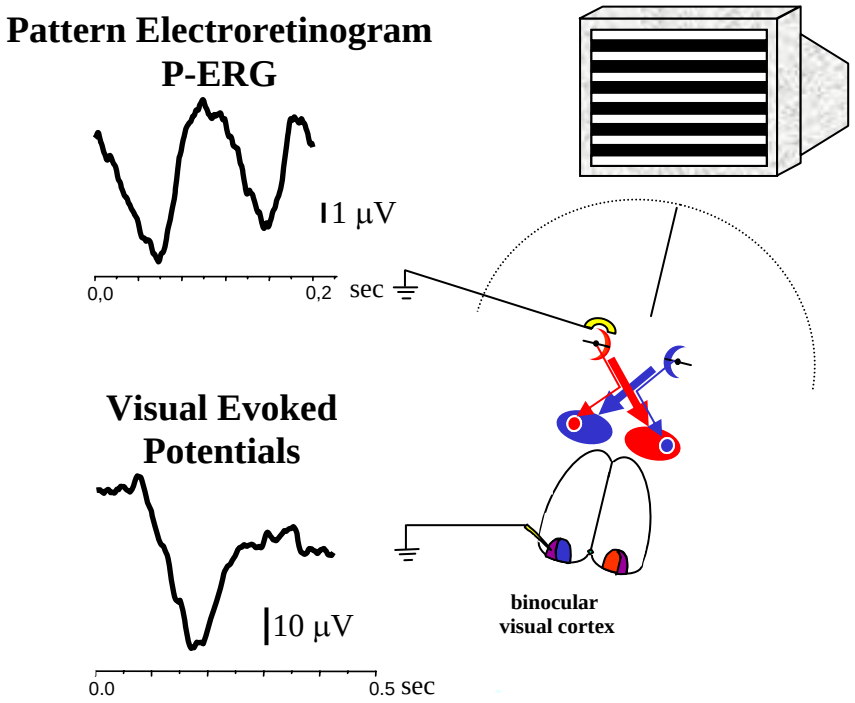


Figure 2: **Schematic representation of P-ERG and VEP recordings**

Schematic representation of the recording electrode position (corneal surface for P-ERG and binocular visual cortex for VEPs) and the examples of the characteristic waveform of the recorded signal for P-ERG and VEP. Note the different amplitude of the signal as evidenced by the scale bar. Steady-state P-ERG responses 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 to valuate retinal and cortical acuity were horizontal sinusoidal gratings of different spatial frequency and contrast.

RESULTS

PART I: RGC developmental stratification is influenced by environmental enrichment

Segregation of RGC dendrite stratification

We analysed the segregation of RGC dendrites in bistratified and monostratified processes into the sublaminae a and b of the inner plexiform layer at different ages after birth. We used a transgenic line of mice expressing plasma-membrane marker green fluorescent protein (m-GFP) under control of Thy-1 promoter (De Paola et al., 2003) on a sample of RGCs. GFP consistently labels dendrites, somata and axons of the RGCs, as shown in Fig. 3A.

We visualized and quantified the stratification patterns of RGC dendrites in retinal coronal sections by using an antibody selectively directed against a specific marker of cholinergic neurons, the choline acetyltransferase (ChAT). ChAt immmunoreactivity identifies two distinct bands inside the IPL (Fig. 3B), as expected by the fact the patterning of retinal cholinergic amacrine cells projections defines the a and b sublaminae very early in retinal development (Feller et al., 1996; Galli-Resta et al., 2000). It is thus possible to clearly detect RGCs as bistratified or monostratified in the two sublaminae of the IPL (Fig. 3C, D, E) in sections immunostained simultaneously with anti-ChAT (red) and anti-GFP to enhance GFP constitutive expression (green). While bistratified RGCs present a double-layered segregated arborisation respect to the two anti-ChAT labelled bands, there are RGCs with dendrites restricted to the ChAT positive band inside sublamina b (correspondent electrophysiologically to ON-center ganglion cells) and others with dendrites around the outermost ChAT positive band in sublamina a (functionally classified as OFF-center ganglion cells).

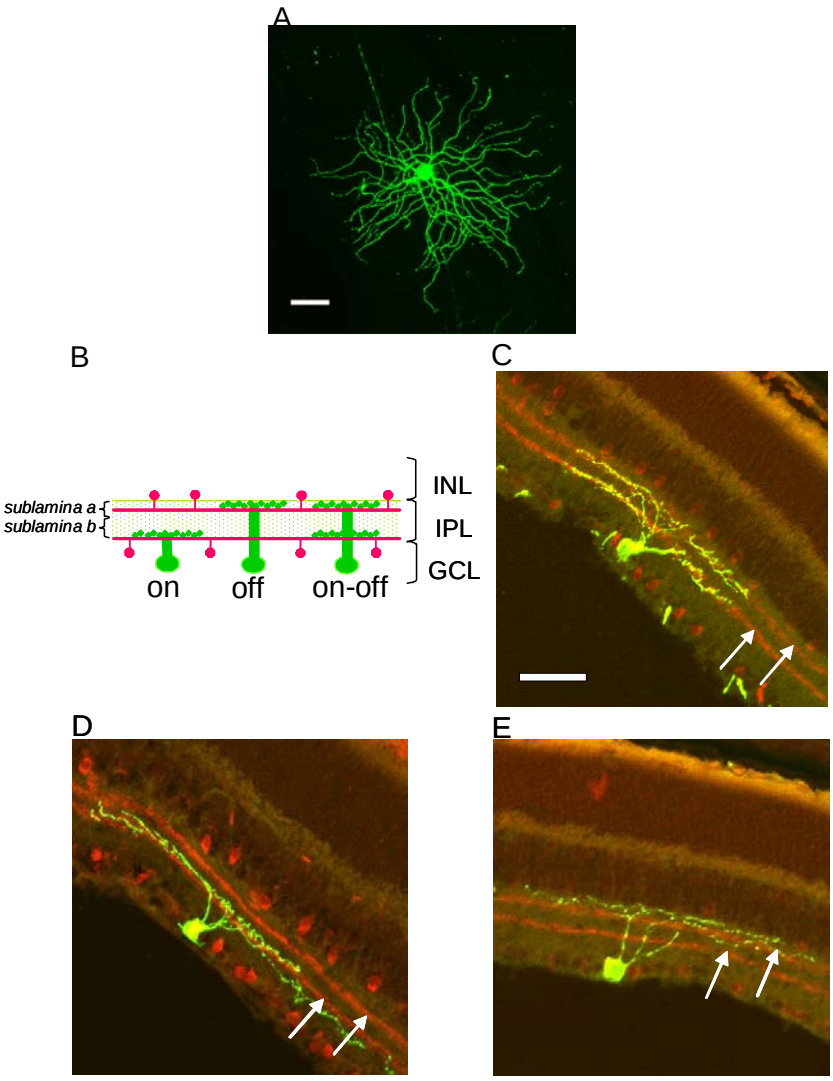


Figure 3: **Stratification of RGC dendritic pattern in Thy-1 mGFP P30 mice.**

(A) Confocal image of a retinal ganglion cell from whole mount retina of mGFP mouse. GFP, which expression is enhanced with a specific immunostaining completely labels RGC somata, dendrites and axons. (B) Schematic diagram illustrating the patterning of amacrine cell (red) and RGC (green) projections. C, D, E. Examples of RGC confocal images (green) taken from 25 µm transverse retinal sections from P30 mGFP mice. Choline acetyltransferase (ChAT) positive cell bodies are respectively in the ganglion cell layer (GCL) and in the inner nuclear layer (INL), while their projections form two clearly visible bands (arrows) that run along the sublamina *a* and *b* of the IPL. Bistratified ganglion cells present a double-layered segregated arborization with respect to the two anti-ChAT labeled bands (C), while monostratified ganglion cells have their dendrites proximal to the cell body and restricted to the ChAT positive band within sublamina *b* (D) or distal to the cell body and restricted to the outermost ChAT positive band in sublamina *a* (E) [A, C, D, E: scale bar= 50 µm].

RGCs stratify during postnatal development

An age-dependent decline of bistratified RGCs has been observed in cat (Maslim and Stone, 1988; Bodnarenko et al., 1995), ferret (Bodnarenko et al., 1999), primate (Kirby and Steineke, 1991) and mouse (Tian and Copenaghen, 2003), as schematically illustrated in Fig. 4A. We observed a comparable developmental decline of bistratified RGCs in our GFP mice reared in standard conditions. Our analysis revealed that in P10 non-EE mice 65.8% of RGCs were bistratified, while at P16 and at P30 this percentage decrease to 53.8% and 30.8%, respectively. In Fig. 4B, I show the percentage of monostratified and bistratified RGCs at the different developmental ages.

EE from birth counteracts dark rearing effects promoting RGC dendritic maturation.

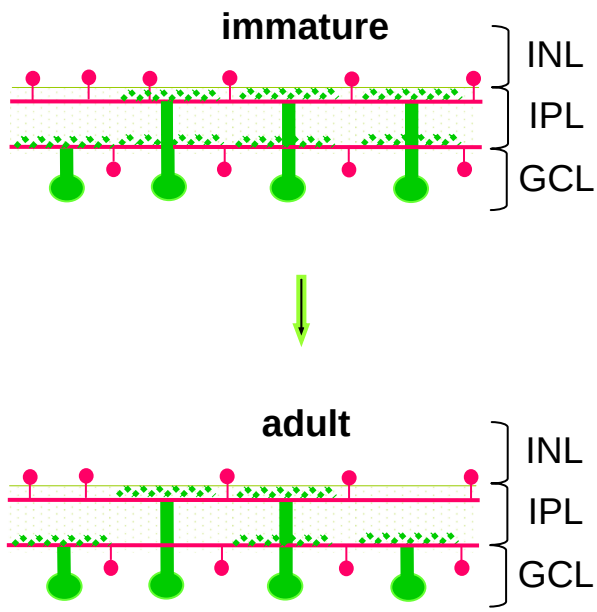
Recent studies demonstrated that DR affects RGC dendrite stratification by blocking RGC developmental remodelling (Tian and Copenaghen, 2003). Since EE prevents DR effects on visual cortical maturation (Bartoletti et al., 2004), we first examined whether DR effects on RGC stratification can be counteracted by EE.

Non-EE or EE mice were dark reared from birth (DR mice or EE-DR mice, respectively) and the percentage of bistratified RGCs was analysed at P30 (as esemplified in Fig. 5).

DR delays the developmental decrease in the percentage of bistratified RGCs seen in DR mice, as expected (56.1% in DR mice versus 30.8% in normal, non-enriched mice at P30, Fig. 6); however, EE-DR animals have a percentage of bistratified RGCs which is not different from that of normal non-enriched mice at the same age (EE-DR 30.5% versus non-EE 30.8 %, Fig. 6).

These results demonstrate that EE from birth strongly prevents DR effects on the developmental remodelling of RGC dendrites.

A



B

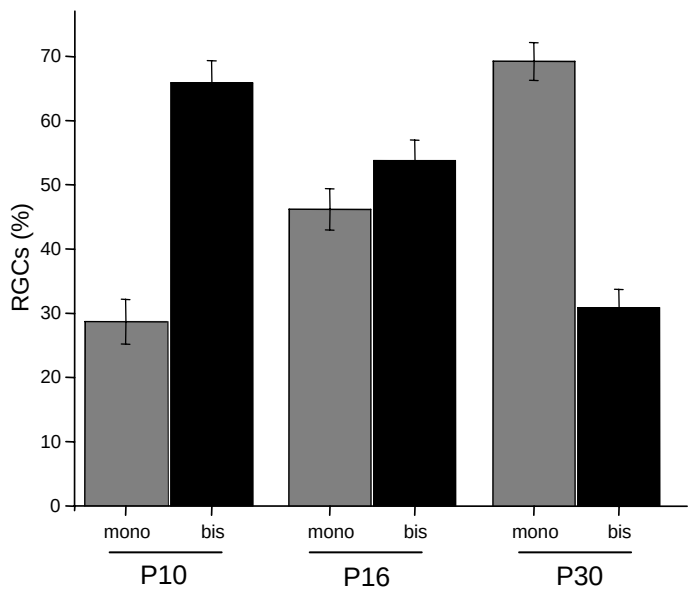


Figure 4: **RGCs stratification during postnatal development in standard Thy-1 mGFPmice.**

(A) Schematic representation illustrating the passage from immature to adult state during development in the level of stratification of RGCs (amacrine cell in red, RGCs in green). (B) Percentages of monostratified and bistratified RGCs during development in non-EE mice between P10 and P30 (P10, N=4; P16, N=4; P30, N=5).

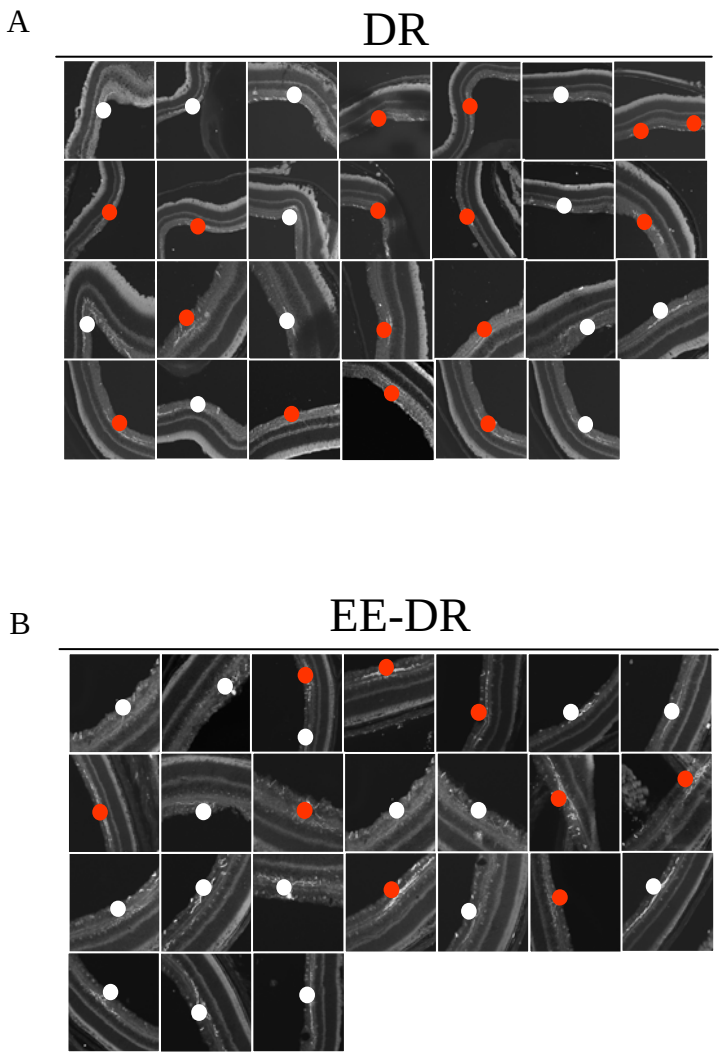


Figure 5: **EE from birth counteracts DR effects promoting RGC dendrite maturation.**

Representative examples of all retinal sections acquired to valuate the presence of bistratified or monostratified RGCs in the retina of DR (A) and EE-DR mice (B). In each section, a circle is superimposed on the position of a GFP labelled ganglion cell: red circles represent bistratified RGCs, while white circles monostratified RGCs. It is evident the preponderance of bistratified RGCs in the retina of the DR animals.

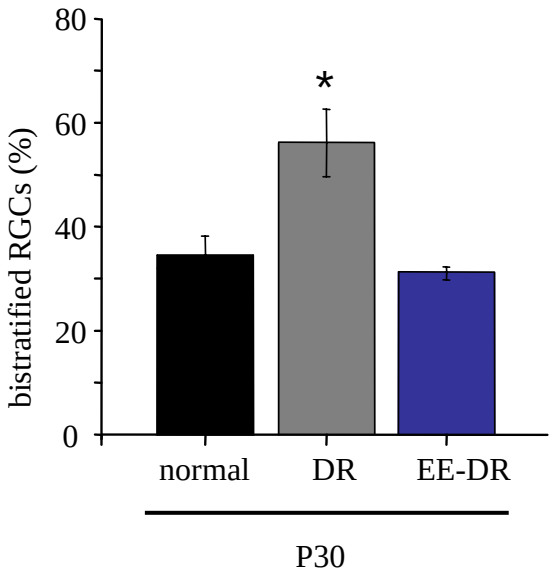


Figure 6: **EE from birth counteracts DR effects promoting RGC dendrite maturation.**

The average percentage of bistratified RGCs in normal (black), DR (grey), and EE-DR mice (blue) at P30. The percentage of bistratified RGCs is 30.8% ± 3.8% in normal mice; DR blocks RGCs stratification process (bistratified cells 56.1% ± 6.5% at P30, N=6 mice, 65/121 cells), while EE in DR animals reduces the percentage of bistratified RGCs to that of P30 non-EE mice (EE-DR mice: bistratified cells 31.1% ± 1.3%, N=3, 29/95 cells). One-Way ANOVA shows a statistically significant difference in the various rearing groups (p= 0.002) between non-EE and DR, and EE and DR mice. The bars indicate SEM. EE from birth prevents DR effects on the developmental remodeling of RGC dendrites.

EE early in life affects the maturational refinement of RGC dendrites

To better characterize the influence of EE on RGC circuitry development, we analyzed the development of RGC dendrite stratification in EE and in non-EE mice reared in a normal environment (light-dark standard 12:12 cycle).

We found that the decrease in the percentage of bistratified RGCs occurs much earlier in EE than in non-EE mice. In P10 EE mice the percentage of RGC stratification decreases remarkably with respect to that of non-EE mice (from 65.8% to 43.9%, Fig. 7) and by P16 the incidence of bistratified RGCs does not differ from that of non-EE P30 mice (30.8% versus 32.9%) indicating that the developmental segregation of RGC dendrite stratification is already completed. We did not observe any differences in the thickness of the IPL between EE and non-EE mice (at P10, EE: 27.3 μm ± 0.7; non-EE: 28.5 ± 0.8; at P16, EE: 28.5 ± 1.1; non-EE: 26.3 ± 1.2). These data indicate that the changes in RGC dendrite segregation induced by EE reflect a true variation in RGC circuitry rather than alterations in retinal architecture.

Thus, EE influences the maturational remodelling of RGC dendrites by accelerating the segregation of RGC arborizations.

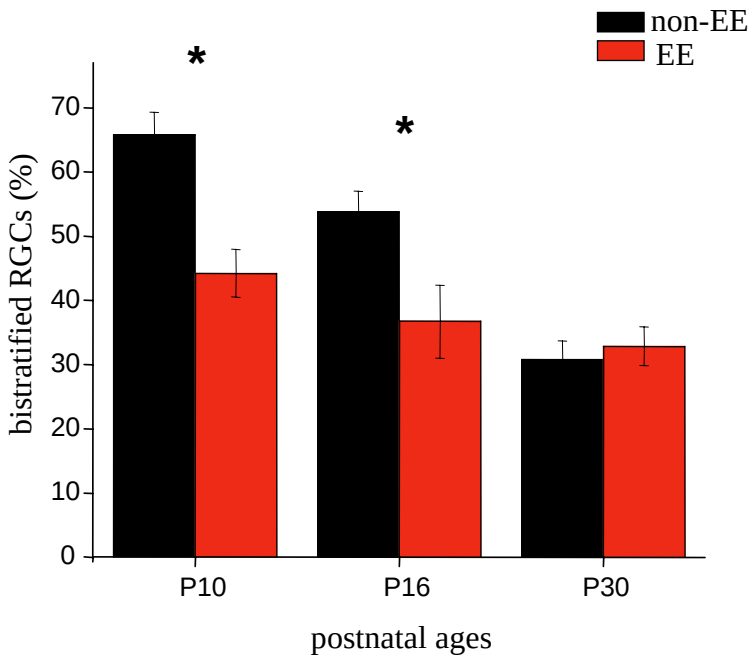


Figure 7: **EE early in life affects the maturational refinement of RGC dendrites.**

The mean percentage of bistratified RGCs in non-EE (black) and EE mice (red) at P10 (non-EE:65.8% ± 3.5%; EE: 44.2 % ± 3.7%, N=5,47/107 cells), P16 (non-EE: 53.8% ± 3.2%; EE: 36.7% ± 5.7%, N=5, 66/193 cells) and P30 (non-EE: 30.8% ± 2.9%; EE: 32.9% ± 3%, N=3, 44/138 cells). Two-Way ANOVA shows a significant effect of age ($p=0.006$) and environmental housing condition ($p<0.001$). Post-hoc Tukey test reveals a significant difference between EE and non-EE at P10 and P16 (*). The bars indicate SEM. EE accelerates the process of the segregation of RGC arborizations.

The acceleration of RGC dendrite segregation induced by EE is dependent on the enhanced levels of BDNF

BDNF expression in the brain is increased by EE in adults (Ickes et al., 2000; Pham et al., 2002) and in developing animals (Cancedda et al., 2004). BDNF is an important factor in RGC development, regulating both their morphological and functional maturation (Cohen-Cory et al., 2004).

We therefore asked whether BDNF could be involved in the effects produced by EE on the developmental stratification of RGC dendrites. Since EE effects on RGC segregation are already evident at P10, we analyzed whether this accelerated developmental process was preceded by an enhanced expression of BDNF in the RGC layer. At P8 we found higher levels of BDNF protein by immunoistochemistry in the retina of EE mice compared to that of non-EE mice as shown in Fig. 8. To investigate directly whether this BDNF increment was implicated in the effects of EE, we decreased BDNF expression in the eyes of EE mice by injecting antisense oligonucleotides against BDNF, with the same protocol employed to suppress BDNF protein levels in the retina (Menna et al., 2003; Mandolesi et al., 2005).
Antisense oligonucleotides against BDNF were injected intraocularly from P6 to P12, and at P16 we analysed the percentage of bistratified RGCs. Injections of sense oligonucleotides were used as controls. We found that the treatment with BDNF antisense oligos blocked the accelerated stratification observed in EE retinas (51% ± 4.2% of bistratified cells at P16 in EE antisense-treated mice, Fig. 9). By contrast, in BDNF sense treated EE mice the percentage of bistratified RGCs was not statistically different from that of EE untreated eyes (One Way Anova, $p=0.029$). Thus, these results suggest that the effects of EE on RGC dendrite patterning is dependent on retinal BDNF.

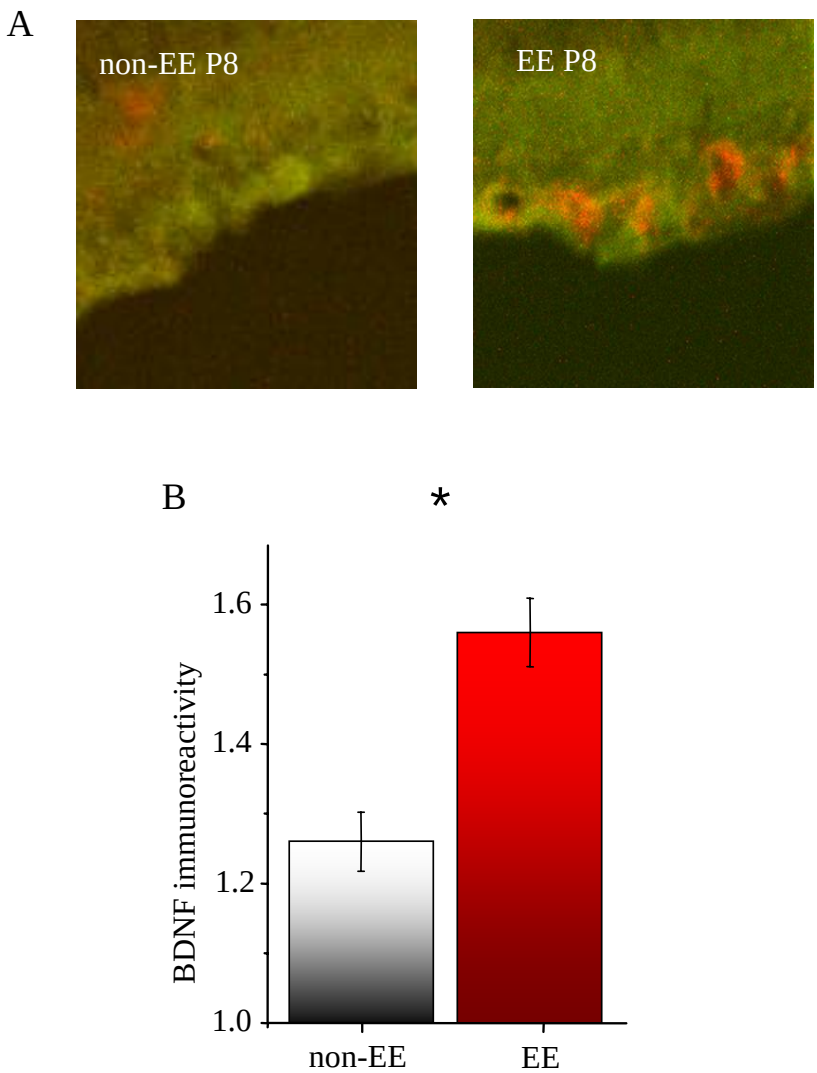


Figure 8: **BDNF mediates the effects of EE on RGC segregation.**

(A) Coronal sections through the retina of P8 mice show that BDNF immunoreactivity is low in RGCs of non-EE mice in comparison with that of EE mice. The scale bar represents 50 µm. (B) Quantification of the BDNF immunofluorescence level in non-EE and EE retinas. Normalized pixel intensity for BDNF immunofluorescence is 1.26 ± 0.04 in non-EE mice (grey) and 1.56 ± 0.05 in EE mice (red). T-test shows a statistical difference (*) between the two groups ($p<0.001$). The bars indicate SEM.

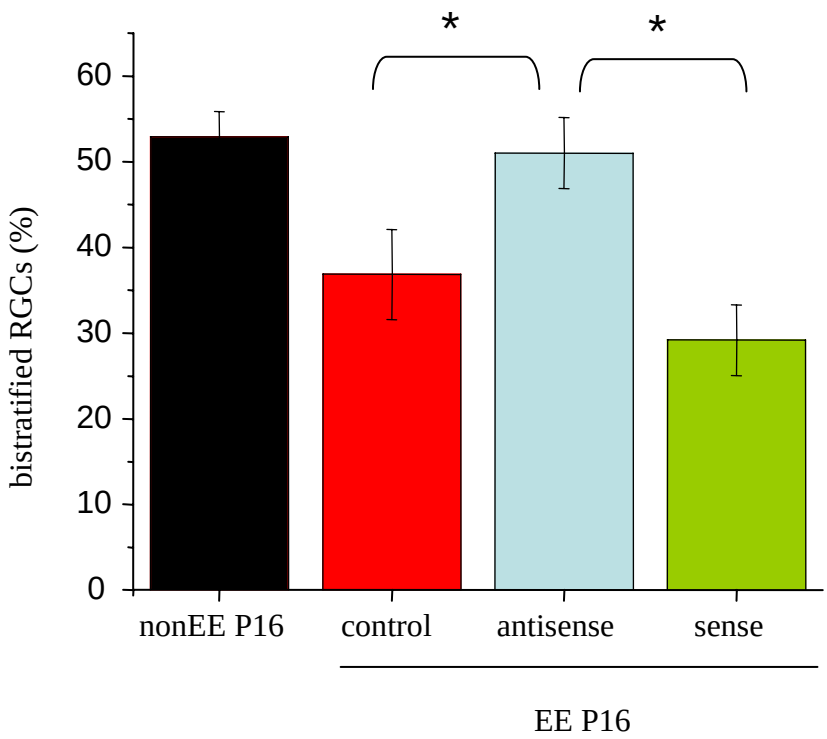


Figure 9: **BDNF mediates the effects of EE on RGC segregation**

EE mice were injected with BDNF oligos at P6, P9, P12 and the percentage of bistratified RGCs was analyzed at P16. The blockade of BDNF expression blocks the effects of EE on RGC stratification. In particular, in the retinas injected with BDNF antisense oligos the percentage of bistratified cells is similar to that of non-EE mice at the same age (51% ± 4.2% versus 52.9% ± 2.9**%**), whereas the control treatment with sense BDNF had no effect on the accelerated development produced by EE (29.2% ± 4.1%). One Way ANOVA indicate a statistical difference between control EE and antisense treated animals (p=0.029). The bars indicate SEM.

PART II: Retinal functional development is affected by environmental enrichment

Development of retinal acuity is accelerated by EE

To test the sensitivity of the retina to the effects of experience, it was important to know if EE also affects RGC functional development.

In observations made at P16, we have found that the disappearance of opacities in the eye optics was complete in EE rats, but not in non-EE rats. P-ERG signals are bigger in EE in respect to non-EE rats as we can see in Fig. 10. However, to avoid confounding effects due to this problem, we have begun a systematic study of P-ERG responses starting from P19-20 (Fig. 11).

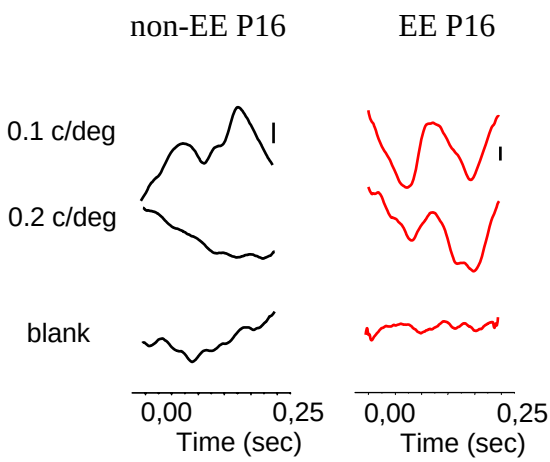


Figure 10: **In EE rats P-ERG responses are already presents at P16.**

Examples of records in non-EE (black) and EE (red) rats at P16. EE rats still present a response at 0,2 c/deg, while non-EE rats do not. Calibration bars= 1 μV.

Retinal acuity in adult rats is 0.87 c/deg ± 0.02 (Domenici et al., 1991). At P19-20, retinal acuity is around 0.5 c/deg in non-EE rats and rapidly increases with age; the adult value is reached at P44-45: at this age, P-ERG acuity is 0.84 c/deg ± 0.02 and does not differ from the value obtained in P60 rats (0.83 ± 0.03 c/deg, N=4).

In EE animals, P-ERG acuity at P19-20 does not differ from that in non-EE animals; however, as evident in Fig.12A, EE clearly accelerates P-ERG acuity developmental time course. Starting from P25-26 up to P34-35, retinal acuity is significantly higher in EE than in non-EE rats and becomes no longer significant from the final adult value in EE rats at P34-35 (P60 EE rats, 0.89 ± 0.02 c/deg, N=5). Thus, the final acuity level is reached almost 10 days before in EE than in non-EE rats. From P44-45 onward P-ERG acuity does not differ between EE rats and non-EE rats, indicating that EE affects the developmental time course of retinal acuity but not its final level (Two Way ANOVA, housing per age, p=<0.001, post hoc Tukey's test). The acceleration in the development of retinal acuity produced by EE is particularly evident normalizing mean retinal acuity for each age group to the respective mean final value (Fig. 12B).

Thus, retinal development is sensitive to the experience provided by an enriched environment.

We then performed an analysis of cortical acuity development in EE and non-EE rats by means of visual evoked potentials (VEPs) as in Cancedda et al., 2004. We found that environmental enrichment strongly accelerates cortical development, with VEP acuity in EE rats overtaking that of non-EE rats at P25-26, as for P-ERG acuity development (Fig. 13A, B).

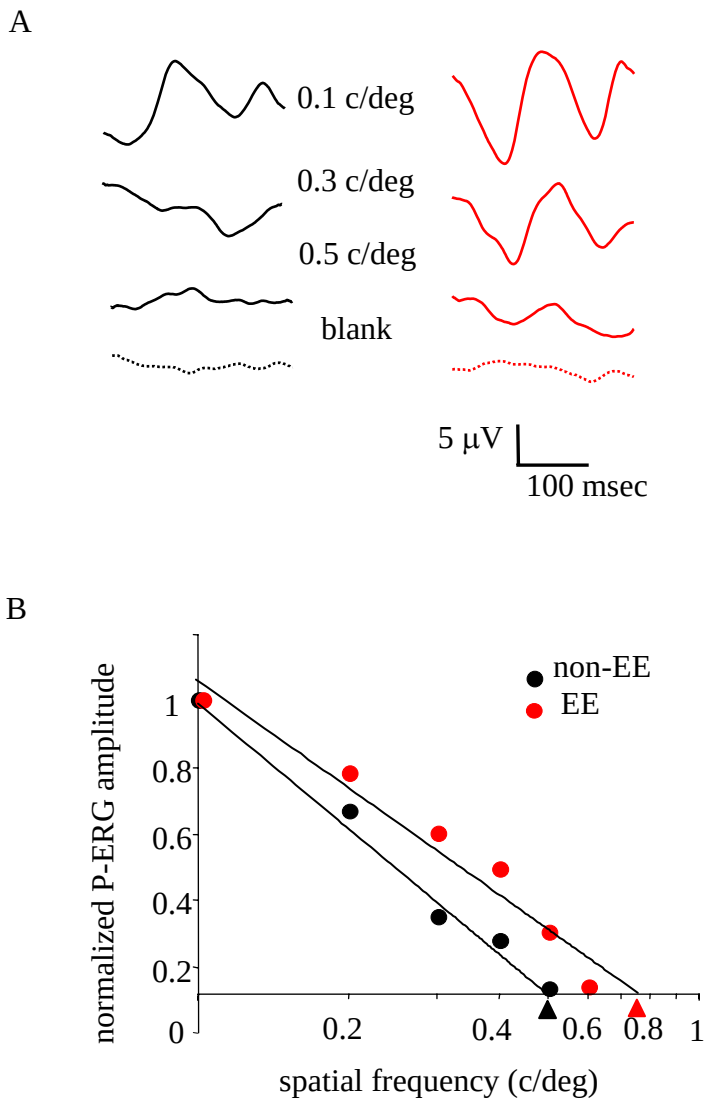


Figure 11: **Development of retinal responses is sensitive to environmental enrichment**

(A) Examples of steady state Pattern Electroretinogram (P-ERG) signals recorded at P25 in response to visual stimulation with gratings of three different spatial frequencies in one non-EE (black traces) and one EE (red traces) rat. The gratings were sinusoidally modulated at a temporal frequency of 4 Hz (period 250 msec) and the principal component of the P-ERG response is on a temporal frequency twice the temporal frequency of the stimulus (second harmonic, two peaks and two troughs of the P-ERG response within one stimulus cycle). P-ERG recorded in response to a blank field is reported to show the noise level. It is evident that a response to a pattern of 0.5 c/deg is still present in the EE but not in the non-EE rat. (B) Examples of retinal acuity estimate for one EE and one non-EE rat at P25. Acuity (arrow) estimated by steady-state P-ERG was calculated by extrapolating the linear regression through normalized data to noise level.

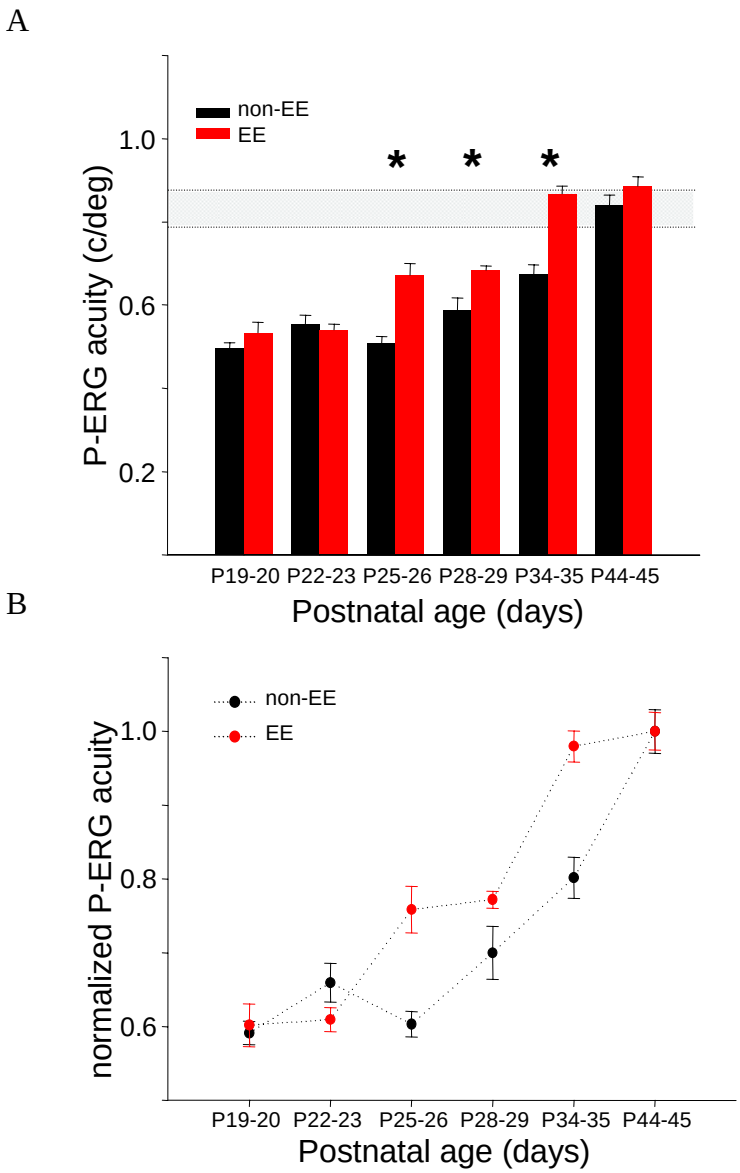


Figure 12: **Retinal development is sensitive to EE**

(A) P-ERG assessment of retinal acuity in non-EE (black) and EE (red) rats during postnatal development. Acuity of animals is plotted as groups of age. The bars indicate SEM. Shaded rectangle indicates the range of retinal acuity in non-EE adult (P60) rats. Two Way ANOVA shows a significant effect of age and environmental housing condition ($p<0.001$) and a significant interaction between age and environmental housing condition ($p<0.001$). *Post-hoc* Tukey test reveals a significant difference (*) from P25 to P34-35 between EE and non-EE groups ($p<0.05$). [non-EE rats, P19-20: N=3, P22-23: N=5, P25-26: N=3, P28-29: N=5, P34-35 N=3, P44-45: N=4; EE rats, P19-20 N=6, P22-23 N=4, P25-26: N=5, P28-29: N=5, P34-35: N=5, P44-45: N=5.] (B) P-ERG acuity normalized to the acuity value at P44-45 is plotted as a function of age for each experimental group, to show the leftward shift of the curve for EE animals, which illustrates the acceleration of visual acuity development produced by EE.

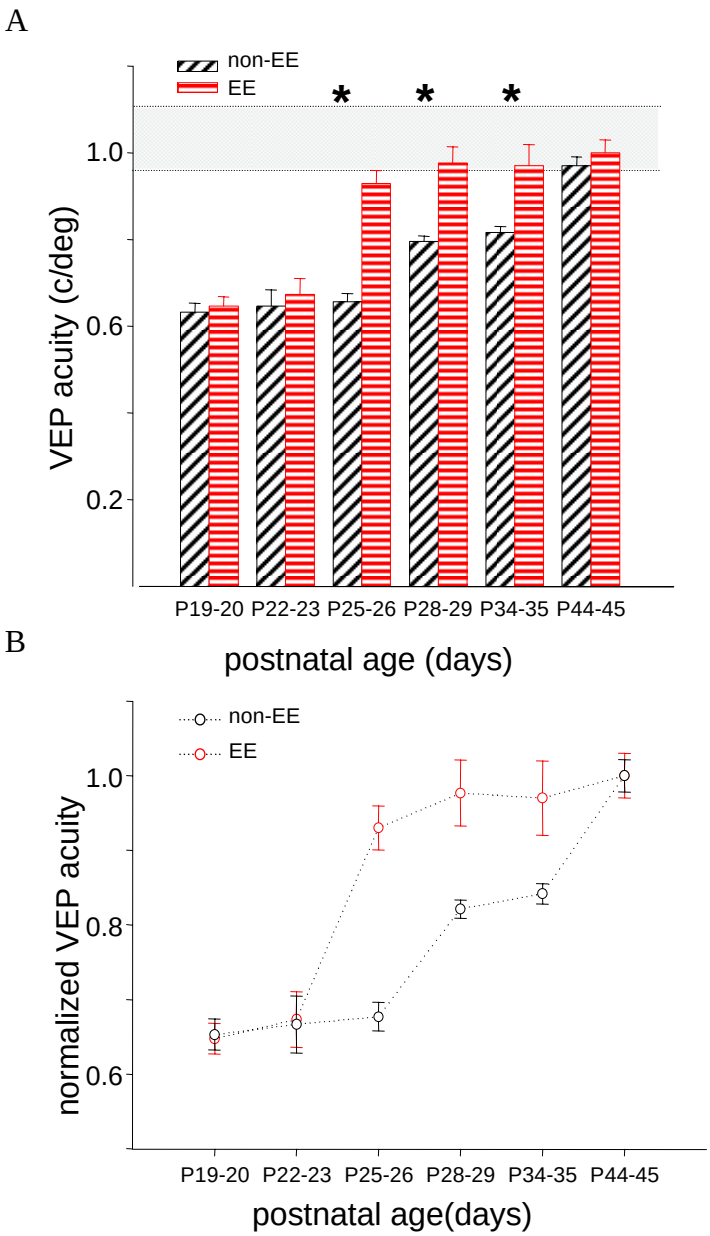


Figure 13: **Cortical development is accelerated in EE rats**

(A) Environmental enrichment influences development of cortical acuity. Visual Evoked potentials (VEP) assessment of visual acuity in non-EE (black stripes) and EE (red stripes) rats during postnatal development. Cortical acuity of animals is plotted as groups of age. The bars indicate SEM. Shaded rectangle indicates the range of cortical acuity in non-EE adult (P60) rats. Two Way ANOVA shows a significant effect of age and environmental housing condition ($p<0.001$) and a significant interaction between age and environmental housing condition ($p<0.05$). *Post-hoc* Tukey test reveals a significant difference (*) from P25 to P34-35 between non-EE and EE groups ($p<0.05$). [non-EE rats, P19-20: N=3, P22-23: N=3, P25-26: N=3, P28-29: N=3, P34-35 N=3, P44-45: N=3; EE rats, P19-20 N=4, P22-23 N=3, P25-26: N=4, P28-29: N=3, P34-35: N=3, P44-45: N=3.]. (B) VEP acuity normalized to the acuity value at P44-45 is plotted as a function of age for each experimental group to show the leftward shift of the curve for EE animals.

Precocious eye opening is not responsible for the effects of EE on retinal acuity development

EE rats open their eyes two days before non-EE rats. We asked whether the precocious eye opening observed in EE rats and presented in Fig.14 gives a contribution to the accelerated retinal development. To answer this question, we made two different experiments.

In the first one, we made a forced eye opening at P10 in non-EE rats (eye opening at P10, EO-P10) and recorded retinal acuity at P25-26; we found that retinal acuity is not affected by this procedure (Fig. 15: non-EE: 0.51 c/deg ± 0.01; EO-P10: 0.56 c/deg ± 0.02, difference not significant). Thus, a precocious visual experience is not sufficient to mimic the increase in acuity seen in EE animals at P25-26.

In the second experiment, we kept animals enriched from birth in a dark room from P10 to P14 [EE(DR_P10-14) rats] in order to abolish their advantage in visual experience due to the precocious eye opening; we have found that at P25-26 the acuity of this experimental group (0.65 c/deg ± 0.03) does not differ from that of EE group (Fig. 13B). Thus, precocious eye opening is not necessary for the accelerated maturation of retinal acuity produced by an enriched environment.

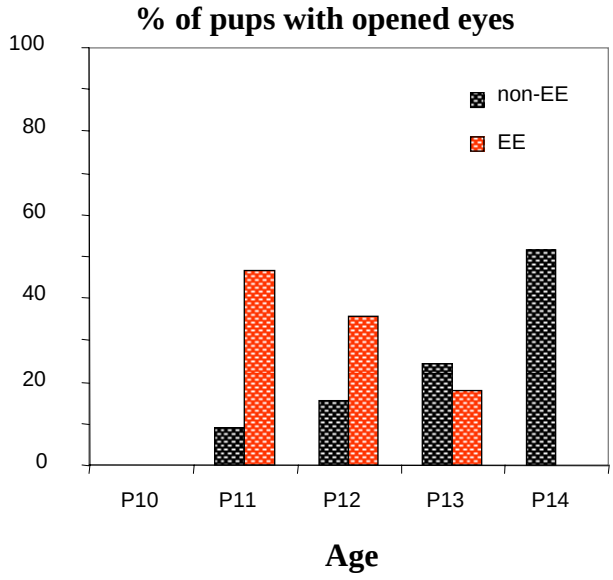


Figure 14: **Precocious eye opening is observed in EE rats.**

As shown by the percentage of EE and non-EE pups which opened their eyes at the age indicated on the abscissa, there is a clear difference between the two groups (Mann–Whitney rank sum test; $p<0.001$), with the median age at eye opening being P12 for EE and P14 for non-EE rats.

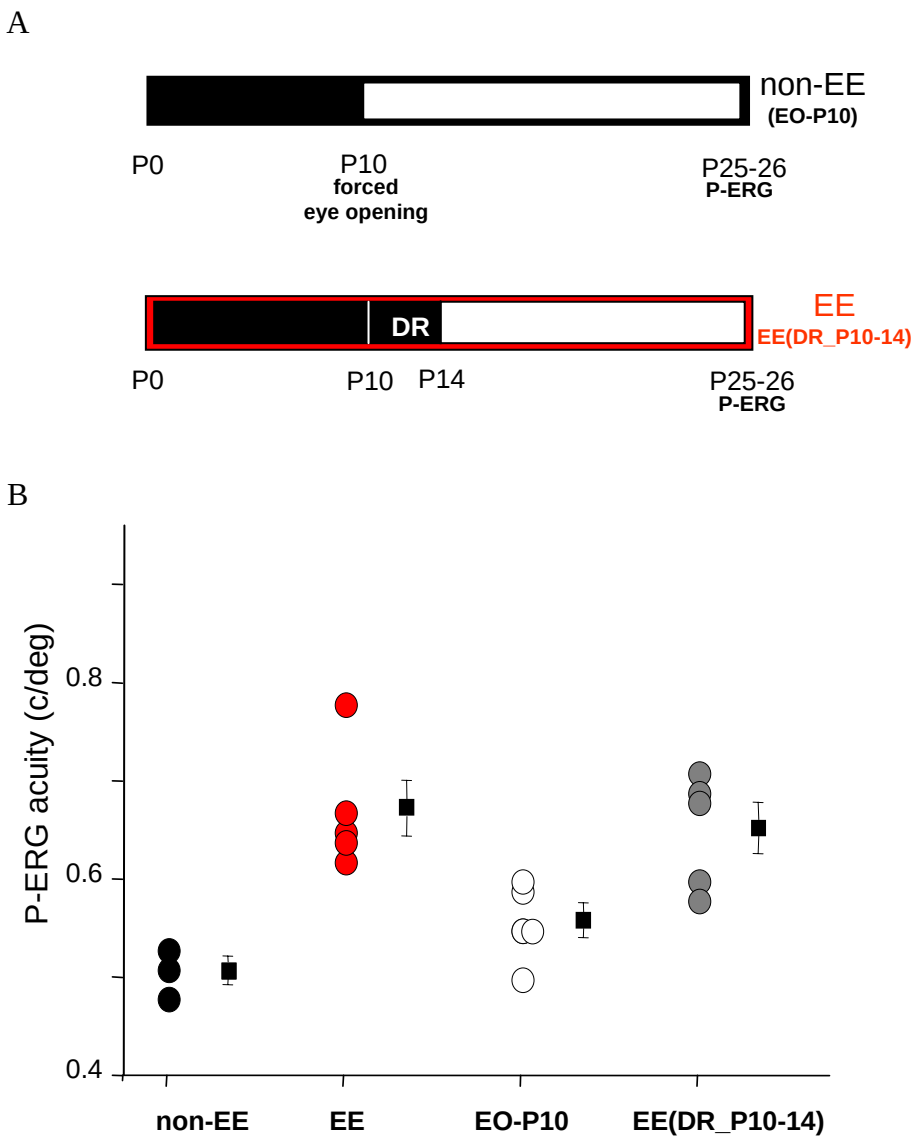


Figure 15: **Precocious eye opening is not responsible for EE effects**

(A) Schematic protocol of the two experiments performed to evaluate the role of precocious eye opening in the accelerated retinal acuity development found in EE rats. (B) Retinal acuity at P25-26 for EE, non-EE, non-EE rats with a forced eye-opening at P10 (EO-P10) and EE animals dark reared from P10 to P14 in order to abolish the visual experience advantage due to the precocious eye opening in EE [EE(DR_P10-14)]. There is a significant difference between non-EE (N=3) and EE rats (N=5), while EE(DR_P10-14) (N=5) do not differ from EE rats and EO-P10 do not differ from non-EE rats [One Way ANOVA ($p<0,001$); *Post-hoc* Tukey test , $p<0,05$].

Accelerated retinal development in EE animals is induced during early phase of enrichment

Our EE animals are enriched from birth until the adulthood. To understand whether the effects of EE on retinal development stemmed from events caused by EE at an early or at a late postnatal age, we reared offsprings in EE until P10 and then transferred them to a standard environment at P10 (EEuntilP10). P-ERG recordings at P25-26 revealed that at this age EEuntilP10 rats had the same retinal acuity of P25-26 EE animals (see Fig. 16; 0.64 c\deg ± 0.03; One Way ANOVA, P=0.017), showing that ten days of EE are sufficient to induce retinal functional maturation seen in EE animals. This suggests that factors influenced by EE and important to trigger rat retinal acuity development are affected during the first ten days of life.

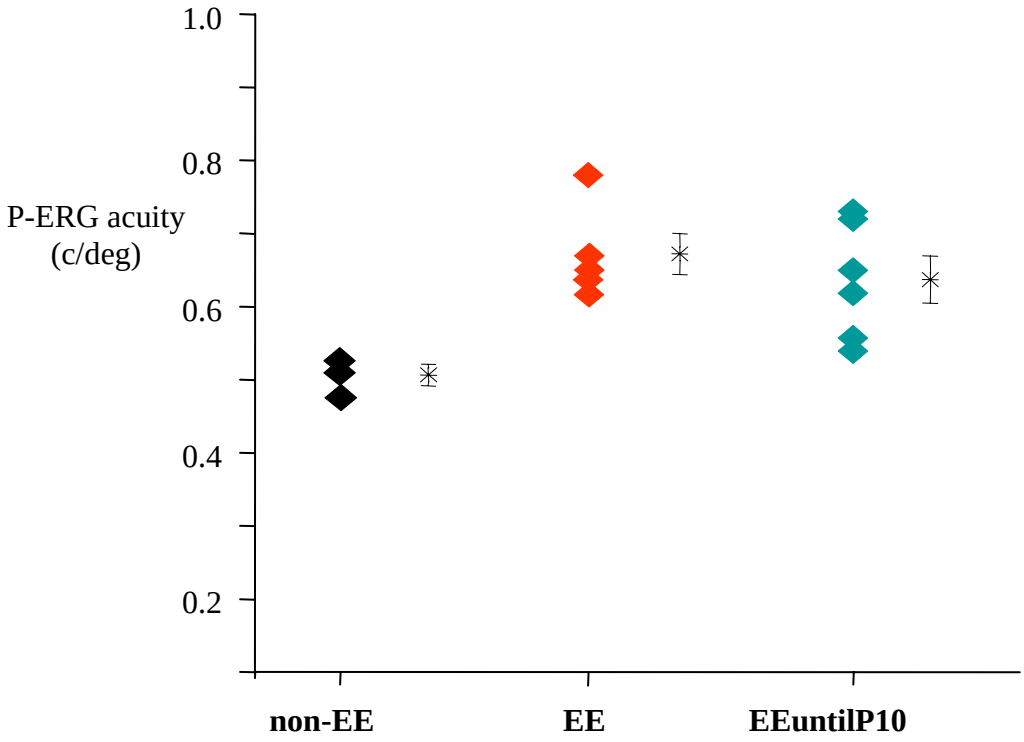


Figure 16: **Early phase of EE is sufficient to induce accelerated retinal development.**

Animals enriched until P10 (EEuntilP10, N=6) show the same retinal acuity of rats enriched until P25 (EE rats) and recorded at P25. Retinal acuity of non-EE rats is reported for comparison. Retinal acuity in EEuntilP10 rats does not differ from that in EE rats (One Way ANOVA, $p<0.05$); both EE and EEuntilP10 rats differ from non-EE rats (N=3, One Way ANOVA, $p=0.017$), suggesting that ten days of enrichment are sufficient to induce EE effects on retinal functional development.

Development of BDNF protein level in RGC layer is affected by EE

We have investigated whether EE affected BDNF protein expression in the developing rat retina, as it did for mice retina.

In normal rats, BDNF protein level is very low before P14 (Seki et al., 2003; see Fig. 17); in EE animals, BDNF immunoreactivity is detectable in the RGC layer already at P7 and at P10 is significantly higher than in non EE rats (Two Way ANOVA, housing per age, post hoc Tukey's test, Fig. 18).

Thus, EE causes a precocious BDNF development in the RGC layer.

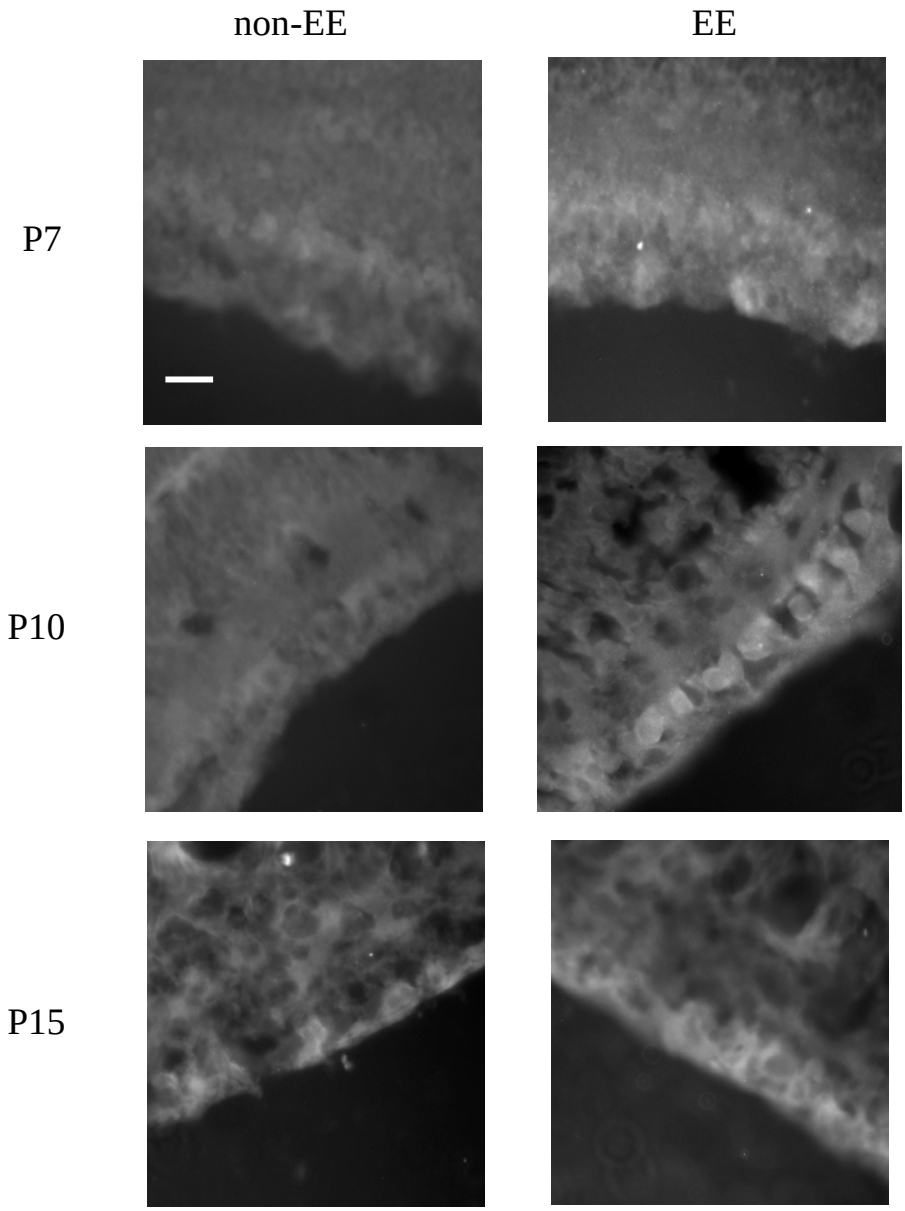


Figure 17: **BDNF immunoreactivity in non-EE and EE rats retinas**

Micrographs of EE and non-EE retinal sections immunostained for BDNF at different ages. BDNF immunolabeled cells are detectable at the level of RGC layer starting from P7 in EE, but not in non-EE retinas. Scale bar is 20 μm.

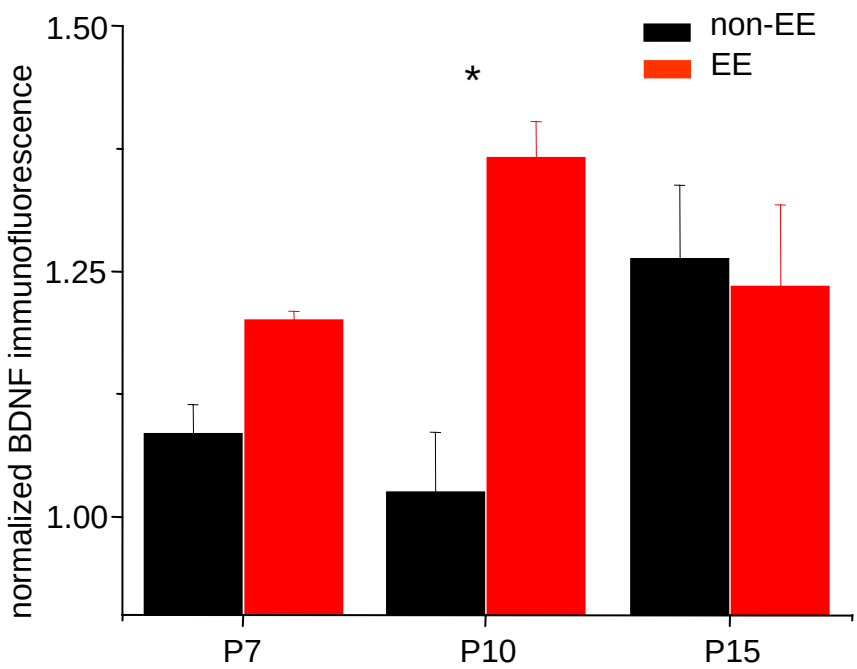


Figure 18: **BDNF is precociously expressed in the RGCs of EE rats**

Quantitative analysis of BDNF immunofluorescence intensity normalized to background level in the RGC layer of non-EE (black) and EE rats (red); the two groups differ significantly at P10 ($p<0.001$), while at P15 BDNF protein level is equal in the two groups ($p=0.73$) and at P7 the difference does not reach the significance level ($p=0.08$) (Two Way ANOVA age per housing, post hoc Tukey's test).

BDNF mediates EE effects on retinal functional development

To test the contribution of the precocious expression of BDNF in the maturation of retinal acuity provided by EE, we reduced BDNF expression during the time window of its enhanced expression with the same protocol previously employed to suppress BDNF protein levels in the rat retina (Menna et al., 2003; Mandolesi et al., 2005) and used before to analyze the role of BDNF in RGC developmental stratification.

We treated a group of EE animals by means of intraocular injections of BDNF antisense oligonuclotide (EE-AS, N=4) and another group by means of BDNF sense oligonucleotide injections as controls (EE-S, N=3) at P6 and P9; we then recorded treated rats at P25-26. We found that retinal acuity in EE-S rats was similar to that of EE rats (0.62 c/deg ± 0.003), while acuity in EE-AS animals did not differ from that of non-EE rats at the same age (0.49 c/deg ± 0.02; One Way ANOVA, P=< 0.001; Fig. 19). It is important to underline that we were able to make an internal comparison in the development of the retinal acuity in three of the EE antisense treated rats, in which one eye was treated with BDNF antisense oligo and the other left untreated. In these animals we have recorded both eyes, the antisense-treated and the untreated; in all three animals, the visual acuity of the treated eye was lower than in the untreated eye (EE-AS rat 1: 0.53 *versus* 0.64 c/deg; EE-AS rat 2: 0.5 c/deg *versus* 0.73 c/deg; EE-AS rat 3: 0.48 *versus* 0.65 c/deg; see Fig. 20).

Thus, the reduction of retinal BDNF blocks the effects of EE on the RGC functional maturation analyzed by means of P-ERG.

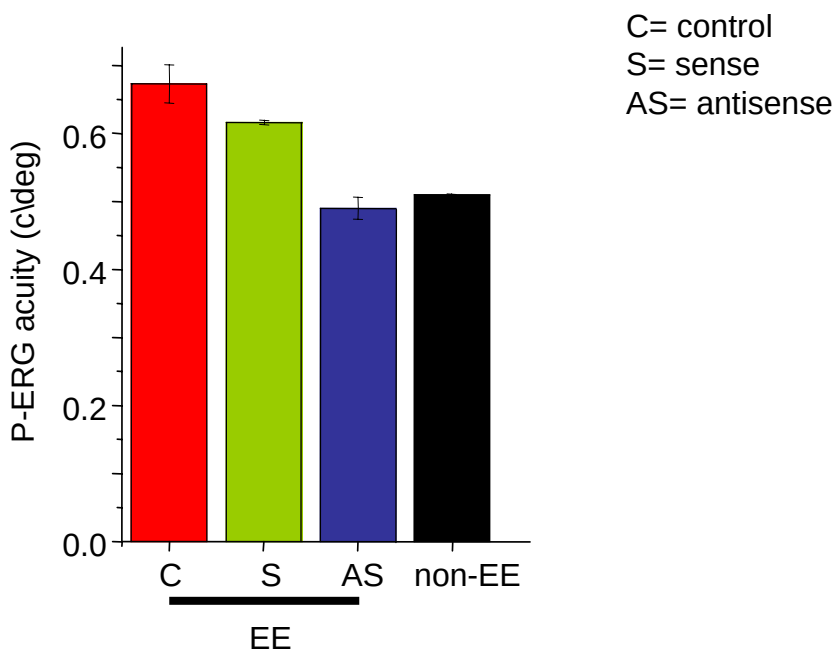


Figure19: **BDNF mediates EE effects on retinal acuity development**

Injections of BDNF antisense oligo block the accelerated maturation of retinal acuity seen in EE animals. Mean retinal acuity in EE, EE treated intraocularly with BDNF sense oligo (EE-S), EE treated with antisense oligo (EE-AS) and non-EE rats. Retinal acuity of EE-AS rats differs from that of EE animals but not from that of non-EE animals, while retinal acuity in EE-S rats differs from that of non EE and EE-AS rats but not from that in EE rats (One Way ANOVA, $p<0.001$, post hoc Tukey's test, $p<0.05$).

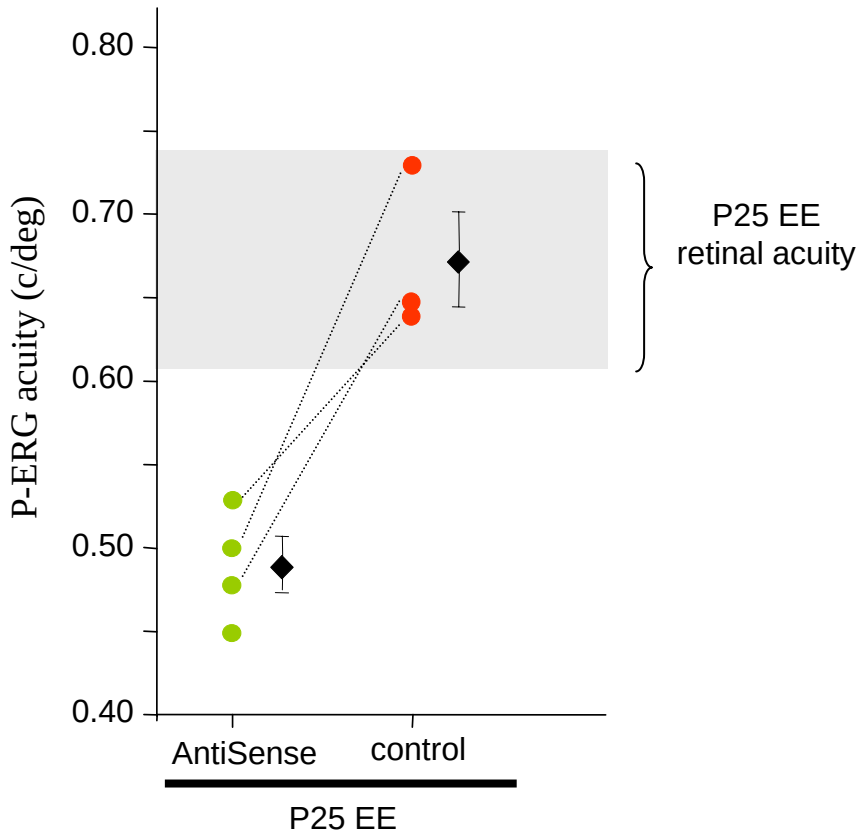


Figure 20: **Examples of P-ERG acuity in the same animal for the antisense treated eye and the untreated eye**

In three EE animals retinal acuity of the BDNF antisense treated eye and of the fellow, untreated eye, is reported; acuity of the BDNF antisense treated eye is significantly lower than that for the fellow eye (paired t-test, p=0.039).

IGF-1 is capable to enhance retinal acuity in standard reared rats

Finally, we tested the role of IGF-I in retinal functional development.

Indeed, in our laboratory it has been recently found that IGF-I levels of expression, higher in EE pregnant rats, are also enhanced in the retina of their offsprings at very early age. In particular, it has been shown that IGF-I is involved in the timing of retinal cell death affected by prenatal enrichment (Sale et al., 2004). On the other hand, it was already known that IGF-I receptors are present in the retina (Rodrigues et al, 1988; Waldbillig et al., 1988) and are expressed in a developmental manner (Frade et al.,1996; Lee et al., 1992).

Here, we were interested to assess whether IGF-I could be a factor capable of controlling retinal functional development and to instruct eventually the development of the entire visual system. According this line of research, we injected rats at P1, P4 and P7 with IGF-I in one eye and with saline in the other eye.

Our results show that IGF-I strongly affects P-ERG acuity development assessed at P25, the age at which we see a jump in acuity produced by EE (0.68 c/deg ± 0.02 in IGF-I treated eye (N=5) in comparison with 0.5 c/deg ± 0.02 in saline-treated eye (N=3)). Retinal acuity at P25 in the eye injected with IGF-I is higher than that of saline-treated eye and comparable to that reached in EE animals (Fig. 21).

Thus, IGF-I is a crucial factor in mediating the EE effects on retinal maturation and IGF-I is capable to mimic the effects of a complex experience on retinal functional development.

Nevertheless, VEP recordings show that the cortex of animals bilaterally injected with IGF-I is not affected by the treatment with this growth factor (Fig. 22; 0.7 c/deg ± 0.02); cortical acuity of IGF-I-treated rats is similar to that of saline-treated rats (0.67 c/deg ± 0.005) and we suggest that retinal functional changes produced by IGF-I effects on RGC circuitry are not sufficient to produce the cortical maturation seen in enriched condition.

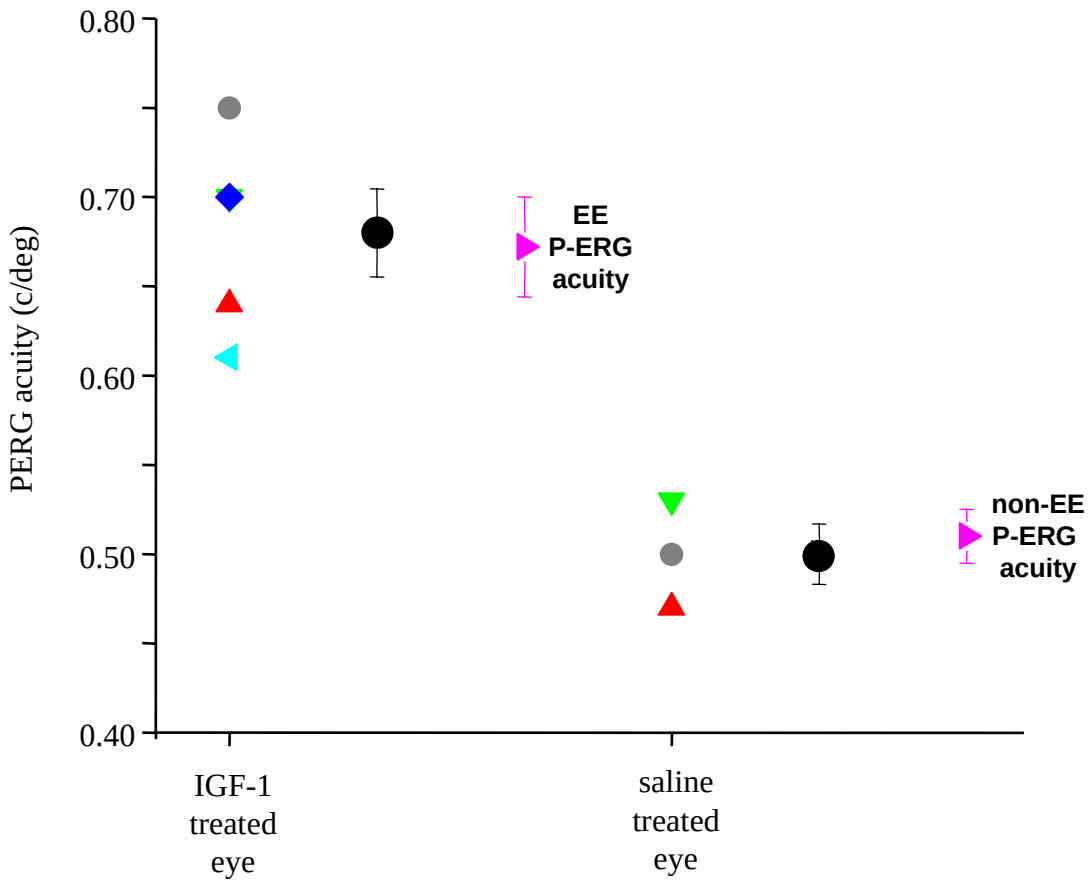


Figure 21: **IGF-I intraocular injections affect retinal acuity development in standard reared rats**

Our results show a strong effect of IGF-I in P-ERG acuity development assessed at P25, the age at which we see a jump in acuity produced by EE (0.68 c/deg ± 0.02 in IGF-I treated eye (N=5) in comparison with 0.5 c/deg ± 0.02 in saline-treated eye (N=3)). Retinal acuity at P25 in the eye injected with IGF-I is higher than that of saline-treated eye and comparable to that reached in EE animals. One Way ANOVA reveals a significant difference between the IGF-I treated eye and the saline injected eye (P= 0.002; post-hoc Tukey test: P<0.05)

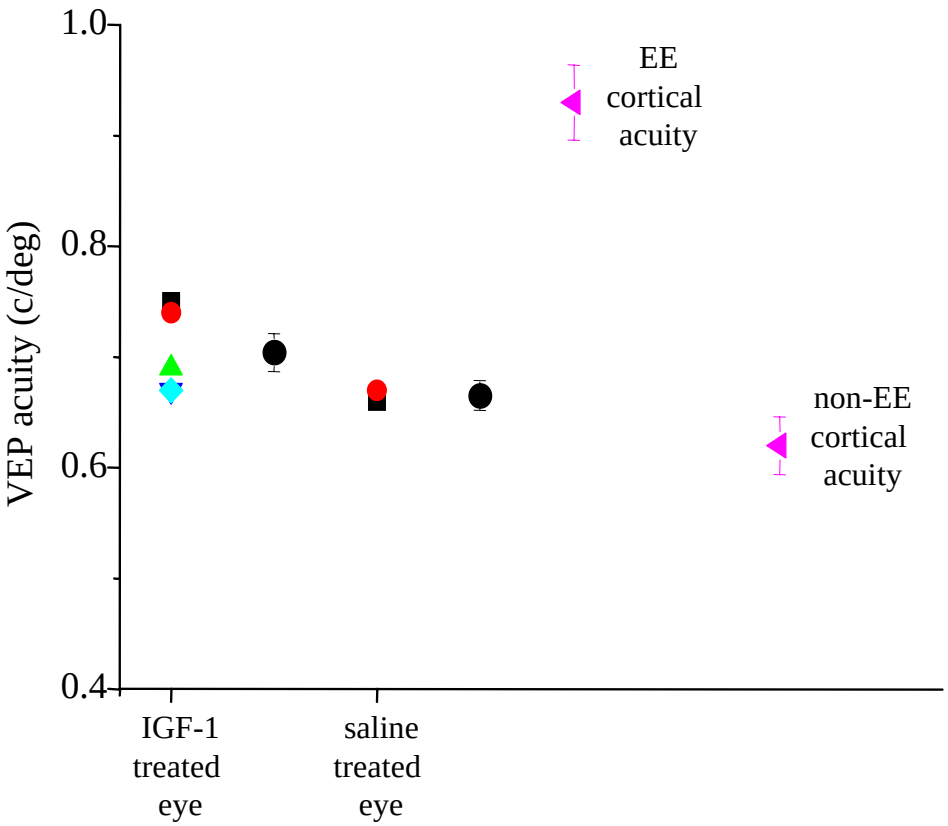


Figure 22: **IGF-I injections in the eye does not influence cortical development**

VEP recordings show that the cortex of animals bilaterally injected with IGF-I is not affected by this growth factor (IGF-I: 0.7 c/deg ± 0.02); cortical acuity of IGF-I-treated rats is similar to that of saline-treated rats (control: 0.67 c/deg ± 0.005). One Way ANOVA reveals a not significant difference between the cortical acuity recorded in IGF-I-treated rats and in saline-treated rats, respectively p=0.061).

DISCUSSION

In the present work, I addressed a new issue: the sensitivity of the retina to experience.

In the recent past, we have shown that EE affects development and plasticity of the visual cortex both accelerating its functional and molecular maturation and counteracting DR effects (Cancedda et al., 2004; Bartoletti et al., 2004).

My question at the beginning of this thesis was: is retina as plastic as other nervous structures as cortex or hippocampus? Until few years ago, it was classically known that retinal development was quite independent of environmental influence; more recently, two papers of Tian and Copenaghen (2001, 2003) clearly showed that retinal development is responsive to DR and is delayed by light deprivation.

To answer this question, I investigated the problem of experience-dependent retinal development by using EE as a paradigm to investigate retinal plasticity.

I have found that retina is capable of responding to a paradigm of an enriched complex sensory-motor stimulation as that provided by EE both at anatomical and functional level. In particular, I have analyzed in Rodents the developmental remodelling of the RGC dendritic arborizations and the development of retinal acuity. I observed that EE accelerates both the anatomical development of RGCs and the maturation of P-ERG acuity. Given the relationship between P-ERG and RGC functional state, this suggest that EE accelerates the maturation of RGC properties on which P-ERG signal depends (Maffei and Fiorentini, 1981; Maffei and Fiorentini, 1982). Both these effects depend on retinal BDNF. Its levels are enhanced in the retinas of EE animals and its blockade counteracts EE effects on the accelerated maturation of the retina.

The novelty of this work is both to show that retinal development is sensitive to EE and to establish a clear role for BDNF in the control of retinal development under the influence of a complex experience.

It is convenient to discuss more in detail the distinct effects of EE on retinal development.

Retinal ganglion cell developmental stratification is influenced by environmental enrichment

I exploited EE as a tool to investigate whether environmental experience could affect the developmental transition of retinal ganglion cell dendrites from the initial bistratified to the final monostratified pattern and whether this revealed a role for factors that can be important for this remodelling process.

During development, the stratification pattern of RGCs undergoes an extensive remodelling. Initially, dendritic arborizations extend in both sublaminae of the IPL (bistratified pattern) and subsequently restrict themselves either to the a or to the b sublamina. This extensive morphological rearrangement is crucial for the emergence of the ON and OFF pathways; indeed, in the adult retina, ON center ganglion cells arborize in the b sublamina, while OFF center cells arborize in the a sublamina. The factors regulating this process are not known, although activity has been demonstrated to play a role (Bodnarenko et al., 1995) and recently, dark rearing has been found to prevent it (Tian e Copenhagen, 2003).

I show for the first time that retinal structural development is responsive to the complex sensory-motor stimulation experience provided by EE. Indeed, not only the development of the retinal circuitry is strongly accelerated in EE mice, but EE promotes the maturation of the RGC dendritic stratification in dark-reared animals. This is the first case in which the environment is reported to act on the development of RGC dendritic segregation, cardinal feature of retinal system development.

Developmental cell death has been suggested to be a factor involved in the mechanisms regulating dendritic segregation since the extent of the dendritic arborization is highly regulated by the local density of RGCs (Perry and Maffei, 1988). However, we have recently shown that EE determine a marked acceleration in the time-course of naturally occurring cell death but does not affect the number of RGCs (Sale et al., 2004). This suggests that the changes in the process of RGC dendrite stratification induced by EE can not be attribute to differences in RGC density in the retina of EE mice.

Afferent input of bipolar cells has been demonstrated to play a critical role in the morphological rearrangement of RGC dendrites. The bipolar terminals are stratified even before ribbon synapse appear in the IPL (Miller et al., 1999; Gunham-Agar et al., 2000) and even in the absence of RGCs (Gunham-Agar et al., 2000) suggesting that bipolar cells stratified their axon terminals in the IPL responding to molecular cues. The stratification

process of RGC dendrites starts at a time when bipolar cells form the first synaptic contacts with RGCs. In addition, injections of APB, a group III metabotropic glutamate receptor agonist, that hyperpolarizes both ON cone and rod bipolar cells preventing their release of glutamate, blocks the dendritic stratification (Bodnarenko et al., 1993 and 1995).

Several observations proposed that also cholinergic amacrine cells may play a role in this process. The finding that processes of these cells stratify into sublaminae a or b very early in the developing retina (Bansal et al., 2000; Stacy et al., 2003 and 2005) suggests that they provide cues for RGC dendrites in the IPL. In addition the ON-OFF stratification in RGCs is altered in mice lacking the β2 nicotinic receptor subunit (Bansal et al., 2000). Recently, it has been shown that a moderate rearranging of the spatial organization of the two cholinergic bands occurs following visual deprivation (Zhang et al., 2005). This finding supports a possible effect of dark-rearing induced delay of postnatal development of cholinergic amacrine cells on ganglion cell development since the maturational decline of ON-OFF ganglion cells was retarded by visual deprivation (Tian and Copenaghen, 2003).

It is known that the neurotrophic factor BDNF influences the complexity of dendritic arborizations; in particular, retinal BDNF has been shown to promote the retraction of these processes in Xenopus tadpoles (Lom and Cohen-Cory, 1999). However as yet, the involvement of this neurotrophin in the remodeling of RGC dendrites stratification was not been demonstrated. My results indicate that BDNF is involved in the anatomical development of RGCs promoted by EE. EE precociously increased BDNF in the retina and blocking retinal BDNF expression by means of antisense oligonucleotides prevented EE from accelerating this developmental segregation. Thus, retinal BDNF is required for EE effects on remodeling of RGC dendrites.

It is important to underline that the expression of this neurotrophin is affected by EE within the first days of postnatal life when pups are still immobile and dependent on the mother. Indeed, we have found that BDNF protein level is enhanced very precociously (around P8) in the retina of enriched mice. This result is in agreement with our previous study indicating that BDNF protein level in the cortex is enhanced at very early age (P7) in the visual cortex of enriched mice (Cancedda et al., 2004). A possible explanation is suggested by results showing that high levels of maternal care enhance BDNF mRNA expression in rat hippocampus (Liu et al., 1997 and 2000); indeed, we have recently shown that EE animals are subjected to higher levels of maternal care (Sale et al., 2004). Thus, enhanced levels of maternal care induced by EE could justify our results for BDNF expression in the retina.

I can speculate about how changes in BDNF expression could regulate the RGC remodelling process. It is known that mRNA for BDNF and its functional receptor, TrkB, are present in the RGCs (Perez and Caminos, 1995; Vecino et al., 2002) and that BDNF controls RGC survival during the period of natural cell death (Isenmann et al., 2003).
In the retina cell specific TrkB labelling was not reported for bipolar cells and cholinergic amacrine cells; in addition, it has been shown that the morphology of the plexus of cholinergic projections is unresponsive to BDNF delivered from P8 to P14 (Cellerino and Kolher, 1997). On the contrary the dopaminergic amacrine cells express TrkB receptor and BDNF controls the development of the retinal dopaminergic network (Cellerino et al., 1998). As yet, a direct evidence of the involvement of these cells in the stratification pattern of RGCs is unknown even if they have a neuromodulatory effects on RGCs via amacrine intermediaries, particularly the AII cells. Interestingly, the projections of dopaminergic cells have been shown innervate the IPL sublaminae with a temporal order overlapping the time period at which glutamatergic and cholinergic systems begin to mature. The dopaminergic fibers start to innervate the sublamina a at the beginning of second postnatal week and progressively grow to innervate both the sublaminae until the end of the third postnatal week when this process is completed. (Cellerino et al., 1998; Witkosky et al., 2004). Finally, it has been also observed an action of dopamine (DA) on the acetylcholine (Ach) release in the retina (Witkosky et al., 2004) suggesting a functional role of dopamine on the cholinergic system. All these evidences suggest the possibility of a role of dopaminergic circuitry on RGC developmental stratification. It would be interesting to validate this speculation made on the basis of literature, observing a differential maturation of dopaminergic network following EE.

Sensitivity of retinal functional development to environmental enrichment

My results show that retinal acuity development is sensitive to the experience provided by the environment.

The effects of EE on retinal acuity development do not seem to depend upon precocious eye opening, suggesting that molecular non visual components activated by enrichment might contribute to the acceleration of retinal development. There are several molecules important for nervous system development and plasticity that are modified by EE in adult animals (Cotman and Berchtold, 2002; Gomez-Pinilla et al., 2002). The results that ten days of enrichment are sufficient to induce an accelerated retinal functional maturation

equal to that observed after a period of enrichment prolonged until P45 suggest that EE acts on molecular factors the expression of which is influenced precociously, when pups are still immobile and largely dependent on the mother. As already shown, BDNF seems to be such a factor.

The results obtained by injecting BDNF antisense oligos in the eye of EE rats clearly show that BDNF is a key molecule in the retinal functional development driven by EE since its reduction in the retina during the time window of its enhanced expression blocks the precocious functional development seen in EE animals.

Thus, we have provided for the first time a direct demonstration of BDNF involvement in the functional development of retinal circuitry in vivo.

As a possible mediator of BDNF control on visual acuity development, we propose the action of BDNF on dopaminergic amacrine cells. Dopaminergic amacrine cells express TrkB (Cellerino et al., 1997) and their development is accelerated by BDNF intraocular injections from P8 to P14 (Cellerino et al., 1998). Undoubtedly, as reviewed in Witkovskj (2004), dopamine has a role as a chemical messenger for light adaptation in the retina. This neurotransmitter is released by a unique set of amacrine cells and activates D1 and D2 dopamine receptors distributed throughout the retina. Multiple dopamine-dependent physiological mechanisms result in an increased signal flow through cone circuits and a diminution of signal flow through rod circuits. Dopamine also has multiple trophic roles in retinal function related to circadian rhythmicity, cell survival and eye growth. In a reciprocal way, the health of the dopaminergic neurons depends on their receiving light-driven synaptic inputs. Dopamine neurons appear early in development, become functional in advance of the animal's onset of vision and begin to die in aging animals. Dopaminergic amacrine cells are interesting from our point of view because they have been shown to contribute to the spatial organization of the receptive fields of RGCs (Jensen, 1986; Witkovskj, 2004). Moreover, it has been suggested that retinal dopamine level affects visual acuity development: children with phenylketonuria who experienced very high phenylalanine levels in the first postnatal days, and who should therefore have particularly low levels of dopamine in the retina, have lower than normal visual acuity (Munakata et al., 2004). Finally, a reduction in retinal dopamine, as occurs in Parkinsonian patients, results in reduced visual contrast sensitivity (Peppe et al., 1998).

It is conceivable that an accelerated development of amacrine dopaminergic cells promoted by the higher retinal BDNF levels in enriched animals could contribute to the accelerated retinal acuity development observed in EE rats. I would like to test this hypothesis by

labelling amacrine dopaminergic cells with tyrosine hydroxylase antibody (Cellerino et al., 1997). Indeed, in this last work it is clear that at P14 in the retina of standard rats dopaminergic cells are not stratified, while this process is already advanced in BDNF treated retinas at the same age.

My study put in evidence a contribute of EE to the development both of the retina and cortex. However, we think that cortical effects are only partially accountable for by the effects induced in the retina. The higher levels of factors as BDNF occurring in EE can promote the faster maturation of the retina and the visual cortex in parallel, without excluding an interacting effect. My data about the effect of IGF-I intraocular injections make us to suggest that a precocious retinal development is not sufficient to drive the acceleration in the development of the visual cortex.

In conclusion, my results are the first evidence that an increased stimulation, such as that provided by EE, can affect the development of retinal visual responses accelerating the maturation of its functional properties by means of factors precociously activated in the retina and that the effects of EE require retinal BDNF action.

A possible link between RGC anatomical development and P-ERG acuity maturation.

The earlier time-course of RGC dendritic refinement induced by EE could be determinant for an accelerated maturation of retinal functional circuits. Indeed, the functional segregation of ON and OFF pathways has an anatomical correspondence in the stratification of RGC dendrites in different sublaminae of the IPL (Famiglietti and Kolb, 1976).

It is not clear if there is a correspondence between development of dendritic arborization and maturation of receptive field size and consequently of retinal acuity. An old work by Rusoff and Dubin (1978) has reported the non-correlation between the dendritic field and receptive field development in kittens from 3-weeks-old until adult age. It was known that cat receptive fields are larger than adult ones (Rusoff and Dubin, 1977) and this is principally caused by neuronal immaturity rather than by cloudy optics. Moreover, the same authors have observed that during early development in cats, there are large receptive fields and small RGC dendritic arborizations, which become larger and more complex during development. The authors conclude that other alternative mechanisms could explain the bigger receptive field size of the brisk-X receptive fields centers such as either the

extrasynaptic input of the center type or the lack of surround-type synapses on RGCs (Rusoff and Dubin, 1978).

I think that the relationship between the development of the RGC circuitry in parallel with other retinal circuitries, as for example that formed by amacrine dopaminergic cells, could in part explain our anatomical and physiological results. Indeed, as aforementioned, retinal dopaminergic cells accelerate their development under increased levels of BDNF (Cellerino et al., 1997) and it has been demonstrated that this retinal population is involved in the spatial organization of the RGC receptive fields (Jensen, 1986).

Eye opening is a trigger or a consequence of EE effects?

Two recent reports annotated by Gandhi (2005) have underlined the importance of eye opening in the functional development of visual cortex and superior colliculus.

Maffei and coworkers (2004) kept one eye closed in young rats while allowing the other eye to open naturally. Because one hemisphere's monocular V1 receives visual input from the open eye and the other from the closed eye, this manipulation provides a nice internal control. Taking slices of each hemisphere and assaying them in a medium that enhanced excitability, the authors recorded the spontaneous discharge of cells in the input layer. Excitatory pyramidal cells that had experienced visual stimulation through the open eye were 20 times less spontaneously active than their counterparts that had not. The authors went on to show that cell type–specific changes in local synaptic connectivity within the input layer were driving the spontaneous discharge. Layer IV in the closed eye's V1 had stronger feedback excitation and weaker inhibition than in the open eye's V1. Such circuit changes, like those found in cultures (Turrigiano and Nelson, 2004), were interpreted to act homeostatically to create similar amounts of activity on the two sides in vivo. The authors interpret these cortical changes in the context of experiments on monocular visual deprivation (Hubel and Wiesel, 1970). But an alternative explanation proposed by Stryker seems even more likely (Gandhi et al., 2005). Could it be that eye opening rather than visual deprivation of the closed eye caused the change in layer IV's local wiring? On the other hand, in Constantine-Pathon laboratory, it has been found that PSD-95 relocates to cortical and collicular dendrites within hours of eye opening (Yoshii et al., 2003) and that eye opening rapidly induces synaptic potentiation and refinement (Lu et al., 2004) .

Indeed, while the importance of the timing of eye opening for visual system development is well established, the mechanisms underlying visual function refinement after eye opening remain unclear. In particular, the effects produced by precocious eye opening may be

mediated by factors other than a change in activity. For instance, BDNF protein levels are increased in the retina upon eye opening (Seki et al., 2003).

My results suggest that molecular factors activated by EE, such as BDNF or IGF-I, rather than precocious eye opening, are determinant for EE effects on retinal development. Indeed, the anatomical remodeling of RGCs in EE condition is already accelerated at P10 (when eyes are still closed), while in the P-ERG experiment, a protocol of forced eye opening in standard rats is ineffective on retinal acuity development and DR between P10 and P14 in EE rats in order to eliminate their anticipated visual experience cannot prevent EE effects.

It was already known that neurotrophins have a major role in the control of visual cortical plasticity during a critical period early in life (reviewed in Berardi et al., 2003). In particular, BDNF exerts profound influences on the development of the visual system; indeed, BDNF overexpressing mice exhibit a pronounced acceleration in both the development of visual acuity and the time course of ocular dominance and synaptic plasticity (Huang et al., 1999). Changes very similar to these, which have been obtained through genetic engineering techniques, can also be induced by EE, as naturalistic condition of increased environmental complexity. In the work of Cancedda et al., 2004, it has been found that EE also during early postnatal development affects BDNF expression in the visual cortex, inducing very precocious changes at P7. Since this enhancement in BDNF level was accompanied by increased expression of GABA biosynthetic enzymes GAD65/67, whose levels were higher in enriched pups at early ages (P7-P15), it has been suggested that one important mediator of environmental-dependent BDNF action at visual cortical level could be the intracortical inhibition. In this respect, it would be interesting to investigate the effects of early EE on the visual system development of BDNF overexpressing mice; if the enrichment procedure would not further increase the effect of accelerated visual system development displayed by these mice (Huang et al., 1999), this would strongly point out to overlapping mechanisms.

A role for BDNF and the related inhibitory system maturation in mediating the influence of enrichment on visual cortical development is further suggested by recent results obtained in this laboratory showing that EE prevents typical DR effects on rat visual cortical development, leading to normal visual acuity development and closure of the critical period for MD: GAD65 expression, decreased in DR rats, was normal in the enriched condition (Bartoletti et al., 2004). A very similar effect of rescue of the developmental blockade caused by DR is observed in mice engineered to overexpress BDNF in the forebrain

(Gianfranceschi et al., 2003). It appears therefore that the effects elicited by EE on visual cortical development and plasticity can be reproduced by artificial increase of BDNF trough increased intracortical inhibition levels.

EE, maternal care and retinal development

Both in mice and in rats, EE acts very early in retinal development, when pups are still immobile and completely dependent on mother and eventual helper females (Sale et al., 2004). In this time window, mother can be considered the most important source of sensory experience for the developing pups (Hofer, 1984; Liu et al., 2000). EE animals receive a continuous physical contact due to the presence of adult females in the nest and also experience the highest levels of licking, provided from both the dam and the filler females. Possibly, a continuous tactile stimulation can strongly influence pup development, providing a source for the earliest changes we observed in EE, as the precocious eye opening. Indeed, it's known that artificial manipulations and tactile stimulation in pups can influence eye opening in Rodents (Barnett and Burn, 1967; Smart et al., 1990). Then, increased levels of licking are accompanied by longer times spent by the mothers out of the nest. Our study allow us to propose that raising pups from birth in a enriched environment may result in a kind of "handling" effect, a protocol eliciting increased care following maternal separation. In this sense, eye opening could be just a consequence of the mechanical stimulation induced in offsprings by the effects of the higher levels of maternal care.

The hypothesis that different level of maternal care in the enriched condition could induce the precocious visual system development and in particular could affect retinal development is supported by the results that ten days of enrichment are sufficient to induce retinal functional development as an enrichment prolonged until P45. I can think that molecular factors and especially BDNF, activated by EE in the retina, are crucial in the first days of enrichment and after that, the molecular cascades already primed continue to function. Yet, it would be interesting to see if mothers, coming from an enriched environment, are still 'enriched' in their level of maternal care once transferred with their pups in standard cages.

Maternal behaviour can control factors important in visual system development, such as growth factors or molecules present in maternal milk. One of these factors could be insuline-like growth factor-I (IGF-I), which is considered a strong modulator of fetal and neonatal somatic and organ growth (Olanrewaju et al., 1996). IGF-I expression is sensitive

to EE (Sale et al., 2004) and it increases electrical activity in neurons bearing IGF-I receptors, promoting the expression of BDNF (Carro et al., 2000; Nunez et al., 2003). IGF-I and its receptor are expressed in the retina by RGCs (Burren et al., 1996). Indeed, increased levels of IGF-I have been found in the retina of pups born in EE to enriched mothers (Sale et al., 2004).

CONCLUSIONS

To understand the effects of experience on neural circuitry development is an important issue in neurobiology and the search for the underlying mechanisms a particularly hot one (Fagiolini et al., 2004; Frenkel et al., 2004; Chattopadhyaya et al., 2004).

Retinal development has been always considered unresponsive to visual experience and only recently DR has been shown to affect ON and OFF RGC development in mice (Tian and Copenhagen, 2002).

Our results show that retinal development is sensitive to EE, challenging the notion that retinal development is largely experience-independent. Indeed, we show for the first time that retinal anatomical and functional development is sensitive to the experience provided by the environment. Also, this is the first time that BDNF is shown to be involved in experience-dependent control of the retina.

We think that these findings are important for neurobiology, since they deal with the crucial problem of the influence of the environment on the development of sensory systems and of its molecular effectors. In addition, a role for BDNF on retinal development has repeatedly been postulated, in particular on RGCs, but a direct demonstration of its involvement in the anatomical and functional development of retinal circuitry in vivo has never been provided.

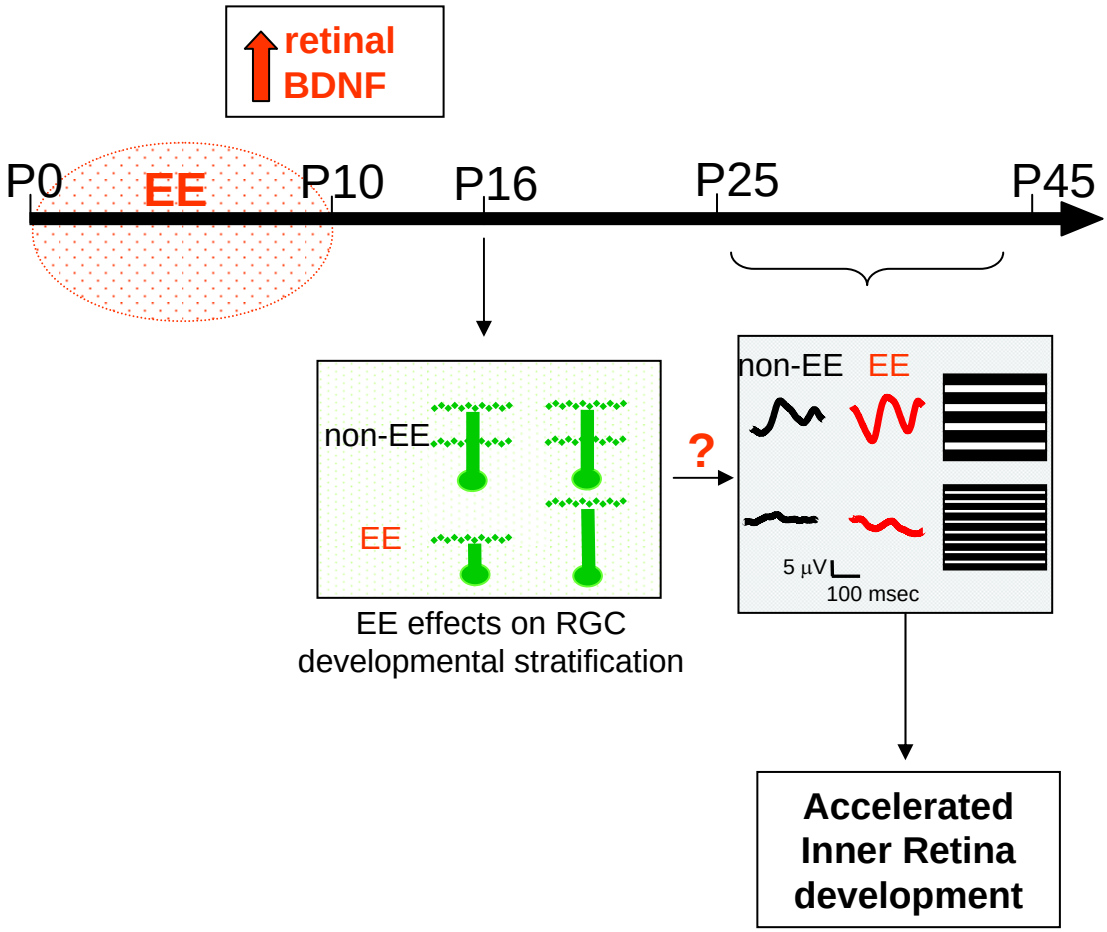


Figure 23: **Schematic diagram of EE developmental effects on the retina**
In the proposed model, EE (red shaded oval) increases retinal BDNF at an early postnatal age (around P10); the increase in BDNF triggers an accelerated inner retina development, both at anatomical and functional level as assessed by immunoistochemistry and by Pattern ERG. Waveforms in the inset represent the P-ERG in response to gratings of low (upper row) and high (bottom row) spatial frequency in a P25 enriched (EE) and non-enriched (non-EE) rat.

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