SCUOLA NORMALE SUPERIORE

Pisa

CLASSE DI SCIENZE MATEMATICHE, FISICHE E NATURALI

CORSO DI PERFEZIONAMENTO IN NEUROBIOLOGIA

Triennio 2004-2006

Tesi di Perfezionamento

INSULIN-LIKE GROWTH FACTOR 1 (IGF-1) MEDIATES THE EFFECTS OF ENRICHED ENVIRONMENT ON VISUAL SYSTEM DEVELOPMENT

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INTRODUCTION

All behavioural functions of the brain – the perception of sensory input, the control of motor and emotional output and the cognitive functions such as learning and memory – are performed by groups of neurons interconnected by means of their axons. During development, axons grow along precise pathways, selecting the appropriate targets and forming specific synaptic connections within the target. This process is initially controlled by genetically determined molecular programs but its final completion requires the individual interaction with the environment. A common feature of many brain areas is the ability to change dynamically in response to experience, a property that is referred to as experience-dependent neuronal plasticity. The mammalian visual cortex represents a classic model for the study of plastic changes driven by sensory experience. Experience-dependent modifications can be elicited in the visual cortex throughout the life but properties of neurons are most susceptible to alteration in the visual input, during an early period of postnatal development, called "critical period" (Sherman and Spear, 1982; Berardi et al., 2000).

Classic works (Rosenzweig, 1966; see for review Rosenzweig and Bennet, 1996; Benefiel et al., 2005), show that physiology, biochemistry and morphology of the nervous system are affected by a complex sensory-motor stimulation, called environmental enrichment (EE), used as experimental paradigm to test the influence of experience on the brain. EE affects the brain both at functional level, enhancing cognitive functions, particularly learning and memory (Rampon et al., 2000a; van Praag et al., 1999; van Praag et al., 2000; Tang et al., 2001) and at an anatomical level, promoting structural changes such as increment in hippocampal neurogenesis (Kempermann et al., 1997 and 2002), dendritic arborization (Greenough et al., 1973) and synaptic density in cerebral cortex, hippocampus and cerebellum (Rampon et al., 2000a; Kolb and Gibbs, 1993).

A few recent works from our laboratory have shown that development of visual system, both at cortical and retinal level, is sensitive to the experience provided by environment. EE has been shown to induce precocious acceleration of visual cortex development (Cancedda et al., 2004) and to prevent dark rearing effects on visual acuity maturation and critical period closure (Bartoletti et al., 2004). The same effect of acceleration has been observed also on retina development (Landi et al., 2007). At the functional level, EE animals show an acceleration of

visual cortical and retinal acuity development (Cancedda et al., 2004; Landi et al., 2007a). At molecular level, we observe a precocious and increased BDNF expression in retinal ganglion cell layer (Landi et al., 2007a), while in the visual cortex we observe an increment of GAD 65/67 protein at postnatal day 7 (P7) and P15 and a precocious peak of BDNF expression at P7 (Cancedda et al., 2004). The molecular mechanisms triggering EE effects on visual system development and plasticity are still unclear.

The Rodent visual system

In the Mammalian visual system, visual information is processed in the retina and sent to different structures of central nervous system (CNS) trough retinal ganglion cells (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 differ from species to species. In Rodents, the great majority of RGCs project to superior colliculus (SC) and the pretectal nuclei, with about 30% of them sending collaterals to dorsal-lateral geniculate nucleus (dLGN) of the thalamus (Dreher et al., 1985). RGCs axons from each eye project to both side of the brain; however inside the dLGN cells are monocular. In higher mammals retinal axon terminals are segregated into alternate eye-specific layers. Each layer is strictly monocular (Hickey and Guillery, 1974). In rodents the majority of afferents to the SC and dLGN arise from the contralateral eye and only 5% of optic axons projects ipsilaterally; there is not a proper lamination of the dLGN; however ipsilateral and contralateral retinal fibers are segregated in a patchy fashion**,** 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 lateral geniculate body is the most important subcortical station projecting to the visual cortex via thalamo-cortical connections that terminate in layer IV of the primary visual cortex. In primates and carnivores, afferents by dLGN segregates by eye within cortical layer IV, in alternate, equal-sized stripes called ocular dominance column (Hubel and Wiesel, 1963; Shatz and Stryker, 1978). Layer 4 neurons relay their information to layers 2-3 neurons and in turn these communicate with layers 5-6 neurons. These eye-specific stripes form the anatomical basis for the functionally defined columnar organisation of ocular dominance that spans all cortical layers. The major difference between the rat visual cortex and that of cat and other mammals is the lack of anatomical ocular dominance columns. However Thurlow and Copper (1988) found hints of a

patchy organisation of ipsilateral and contralateral inputs in the visual cortex of the rats, using a functional mapping by means of deoxyglucose. Recently, this issue has been confirmed trough electrophysiological techniques (Caleo at al., 1999a).

The retina

Architecture of mammalian retina

Anatomical and physiological studies have provided a detailed description of the point-to point connectivity of the visual system where neighboring relations in the retina are conserved in their central projections. The primary visual cortex has been shown to contain a retinotopic map of the visual word in which each point is represented by neurons by specific receptive field that encodes basic visual features.

The retina is part of the CNS and its synaptic organization is similar to that of other central neural structures. It presents a complicate pattern of connections and includes many parallel, anatomically equipotent microcircuits rearranged in anatomical layers. Mammalian retina contains a great number of different neuronal types, approximately 75, each with a different function. 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

The photoreceptors mosaic is optimized to cover the full range of environmental light intensities. This design specification requires two types of detector with different sensitivities, the rod and the cone. In 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). 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 (Nawy et al., 1991). 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 two major classes of bipolar axons segregate at different levels of the IPL, dividing it into OFF and ON laminae and whitin a laminae each type of bipolar axon occupied a defined stratum. 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). A series of experiments show 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). The output of each cone is tapped by several bipolar cell types to provide many parallel channels. 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).

Horizontal cells

Horizontal cells are a small portion of the retina's interneurons, generally less than 5% of cells of the inner nuclear layer (Jeon et al., 1998). Rodents have one type of horizontal cell, while in most mammals, there are two morphologically distinct types of horizontal cells. All rods and cones

receive feedback from horizontal cells which 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 'center–surround' organization, in which a ganglion cell is excited or inhibited by stimuli falling in its receptive field centre, whereas stimulation of the surrounding region has an opposite effect.

Amacrine cells

RGCs 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). Amacrine cells also make inhibitory synapses on the axon terminals of bipolar cells, thus controlling their output to ganglion cells. Amacrine cells have dedicated functions since they carry out specific tasks concerned with shaping and control of ganglion cell responses. 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.

Retinal ganglion cells

RGCs process and transfer 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 variable; their somata and dendritic field vary in size, and they exhibit strikingly varied dendritic architecture (Wassle and Boycott, 1991; Rodieck, 1993) 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, 1993; Dacey, 1999). In Primate retina, RGCs fall into two functional classes, M (for magno or large) or parasol cells and P (for parvo or small) or midget cells. Each class includes both on-center and off-center cells. M cells have large receptive fields (reflecting 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 colour. 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. 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. 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 diversity of RCGs 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 et al., 2000).

<u>*Development of mammalian retina*</u>

Vision in mammals is very poor at birth and develops over a relatively long period (weeks, months, year according to the species) in parallel with anatomical an functional maturation of the visual system. Like other regions of the CNS, the retina is derived from the neural tube and each eye originates as on outgrowth on either side of the neural tube. 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). Extrinsic signals that have been implicated in cell fate determination include neurotrophic factor, such NGF and ciliar neurotrophic factor (CNTF), as well as other factors such as insulin-like growth factor (IGF) and thyroid hormone (reviewed in Harris, 1997). 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 migrate to one of three cell layers in the retina to occupy positions characteristic of their phenotype. Upon becoming postmitotic, retinal cells became polarized and dendrites and axons grow out appropriately, undergoing an extensive process of outgrowth. For each type of inner retinal neuron, such as amacrine cells, process of the same kind ramify in one or a few distinct sublaminae, forming a continuous plexus across the retinal surface.

Synapse formation in the retina has been assessed by electron microscopy (for a review see Robinson, 1991). Synaptogenesis in both plexiform layer begins before eye opening, occurring first in the inner plexiform layer. Ultrastructural studies (Olney, 1968; Fisher, 1979; Blanks et al., 1974) suggest that synaptogenesis between the major neuronal classes of the vertebrate retina occurs in three major steps. Amacrine cells form synapses between themselves and with retinal ganglion cells extending neurites into the inner plexiform layer (IPL) (Maslim and Stone, 1986 and 1988, Nishimura and Rakic, 1987) and originating the earliest functional circuits in the IPL of the developing retina .

Synapses in the outer plexiform layer (OPL) are first formed between horizontal cells and photoreceptors (McArdle et al., 1977). The vertical networks in the inner and outer retina are later interconnected when bipolar cells are born and connections with ganglion cells are established. Bipolar cells, rod photoreceptors and Muller cells are generated throughout the second phase. Bipolar cells appear to be contacted first by cones. Rod bipolar cell differentiation occur later, and synapses from rods are established after those of cones. Synaptogenesis in the OPL continues after eye opening, raising the possibility that the properties of OPL may be more susceptible to sculpting by visual OPL mat be more susceptible to sculpting by visual stimulation (Robinson, 1991).

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, such as acetylcholine (Ach) and $\gamma-$aminobutyric acid (GABA) 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).

Development of Retino-Geniculo pathway

In the adult visual system, information from the two eyes is kept separate thought the early stages of visual processing. In the LGN, RGCs axons from the two eye project to separate eye-specific layer and all LGN cells respond only to visual stimulation from one eye. This adult pattern of segregate inputs from the two eyes is not present in early development, indeed axons from the two eyes are completely overlapping throughout a large portion of the LGN in both carnivores and primates (Linden et al., 1981; Rakic, 1976; Shatz, 1983). During postnatal development in carnivores, adult-like specific segregation of afferents gradually appears as axonal branches in inappropriate locations are pruned and branches in appropriate locations grow and elaborate (Sretavan and Shatz, 1986). Evidence that the segregation of retinal axons into eye-specific layer might be occurring trough a competitive process came from experiments in which one eye was removed from an animal early in development. When the axonal projection from the remaining eye was labeled later in development or in adulthood, axons were found to occupy early the entire LGN (Chalupa and Williams, 1984; Rakic, 1981) suggesting that interaction between afferents and LGN cells are necessary for normal eye-specific segregation in LGN. While the process of segregation of retinal afferents into LGN layers does not depend clearly on visual experience because it occurs in utero in most species and before eye opening in others, it has be found to on spontaneous activity of RGCs. Galli and Maffei. (1988) demonstrated that RGCs are spontaneously active in utero. The pharmacological blockade of this activity it has been shown to prevent the segregation of reticulo-geniculate afferents (Penn et al., 1988; Shatz and Striker, 1988), producing a LGN where afferents form the two eyes remain overlapping. However axonal growth is not prevented by the activity blockade (Sretavan et al., 1988), nor the axons ability to interact with postsynaptic targets is completely abolished (Sretavan et al., 1988). These data suggest that during development a competitive, activity dependent process is responsible fro driving eye-specific segregation in the LGN. Moreover, the following series of experiment demonstrated that the more active eye “win” on the less active eye. If the spontaneous activity of one eye is completely blocked, the LGN area occupied from silenced eye’s ganglion cells is reduced, while area occupied by the normal eye is expanded (Penn et al.,

1988). On the contrary, if the amount of spontaneous activity in one eye is increased, that eye's axons gain territories in the LGN (Stellwagen and Shatz, 2002). If spontaneous activity is blocked during development, in a time immediately after the process of retinogeniculate segregation is completed, the axons desegregate and the projections from the two retinas become overlapping in LGN (Chapman, 2000).

Development of thalamocortical pathways

A series of experiments have shown that patterned visual experience is not necessary for the development of either ocular dominance or orientation preference between the two eyes. For instance, in new born monkeys, after in utero development, and therefore, in the absence of stimulation of the retina, the left and right inputs to the cortex were found to be well segregated into ocular dominance columns (Horton and Hocking, 1996; Rakic, 1976). This led to the suggestion that spontaneous activity, possibly originating in the periodic spontaneous waves of excitation in the immature retina, which are present even before eye-opening, drove segregation (Galli and Maffei, 1988; Maffei and Galli-Resta, 1990; Meister et al., 1991; Mooney et al., 1993; Wong et al., 1993). The existence of spontaneous waves of activity is coming out also in other part of the visual system. Multielectrode recordings in the LGN of awake behaving ferrets, before eye opening, revealed patterns of spontaneous activity that emerge from interactions between retina, thalamus and cortex (Weliky and Katz, 1999; Weliky, 2000).

However another series of studied have suggested that the development of ocular dominance columns can proceed relatively normal in the absence of the retina. Cytochrome oxidase-rich blobs in layer 2/3 in the monkey relate to the ocular dominance columns (Hendrickson, 1985; Hendry and Yoshioka, 1994) and have been shown to develop in the absence of the retina (Dehay et al., 1989; Kennedy et al., 1990; Kuljis and Rakic, 1990) and to show a normal periodicity (Kennedy et al., 1990). More direct evidence for the formation of ocular dominance columns in the absence of the retina has recently been obtained from studies on neonatal enucleated ferrets in which tracer injection in the LGN revealed alternating stripes of label in the striate cortex showing that the ocular dominance columns formations has not been affected (Crowley and Katz, 1999). Subsequently, Crowley and Katz. (2000) have show that the ocular dominance patches are already present in the ferret visual cortex during the initial period

of synaptogenesis in layer 4 (around P15-P18) before the critical period and the period in which geniculostriate axons had been thought to undergo retraction. Katz and colleagues suggest that axons arising from each LGN are molecularly distinct, and that interaction with corresponding cortical cues determines the termination pattern of each sets of axons. Although these results do not exclude a role for sensory periphery in the central development, they suggest that molecular cues play a major role in the formations of ocular dominance columns. The effects of visual experience and deprivation on the development of orientation columns has been a disputed issue (Blakemore and Cooper, 1970; Hirsch and Spinelli, 1970; Stryker and Sherk, 1975) recently reexamined using optical imaging of orientation column during development in normal and deprived animals. This work show that orientation selectivity develops independently of visual experience, but neuronal activity is required to fine-tune and maintain the orientation maps in to adulthood (Crair et al., 1998; Sengpiel et al., 1999; White et al,. 2001; Wiesel and Hubel, 1974).

The emerging consensus on the development of the functional architecture of the cortex is that the construction of both orientation and ocular dominance columns is largely activity independent, but the fine-tuning and maintenance of columns during the critical period depends on activity.

Refinement of connections during development

Even if the relative contribute of intrinsic, genetically, sensory dependent factors on the pattern of neural connections is still unclear, there is a wide consensus about the essential role of sensory driven neural activity in shaping the pattern of developing systems in late phase of development. Visual experience is necessary to achieve the typical electrophysiological properties of adult visual cortex. Hubel and Wiesel had demonstrated that the adjacent territories of area 17 receive input alternatively from the right and left eye. These ocular dominance columns had been explored both by single-unit recording in the cortex, as well as by injections of anterograde tracers in the eye, following transynaptic transport of the tracer to area 17 (Hubel and Wiesel, 1962, 1977).

To investigate the role of the sensory periphery, they carried out similar experiments in normal kittens, as well as kittens having one eye, closed throughout the development. Physiological changes due to monocular deprivation are accompanied by profound changes the anatomical organization of the geniculocortical axons in layer 4. While in normal primates and

cats, the thalamic inputs deriving from the two eyes reach the cortical layer 4 into alternating, equally seized stripes eye, trans-synaptic labeling has shown that, following monocular deprivation, there is an expansion of column receiving inputs from the open eye and the reduction of the cortical territory of deprived eyes to shrunken, broken stripes with its territory invaded by inputs representing the open eye (Hubel and Wiesel, 1977; LeVay et al., 1980). These experiments emphasizes the role of sensory experience in shaping neuronal connections and gave rise to the concept that, during development, the geniculate afferents conveying responses to the right and left initially are extensive and, therefore, overlap. Because binocular deprivation allowed segregation of the two sets of inputs while segregation was prevented by blockage of activity (Stryker and Harris, 1986), it was though that the formation of ocular dominance columns depend on competitive interactions between inputs from both eyes during the critical period. Guillery (1972) supported this hypothesis closing one eye and destroying a small group of retinal ganglion cell in the open eye. As a consequence of this manipulation, the effects on monocular deprivation were present everywhere in the cortex except within the small region receiving input from the lesioned area and the corresponding region of the closed eye. The definitive demonstration that competition between afferent fibers is at the base of the formation of ocular dominance columns derived from the experiment of Costantin-Paton and Law (1978). They proposed that if development of ocular dominance column depends on the competition between afferent fibers, it may be possible to induce the formation of segregated columns where they are normally absent by establishing competition between the two sets of axons. They transplanted a third eye into a region of the frog head near one of the normal eyes during early development. In frogs the retinal ganglion cells from each eye project only to the contralateral side of the brain, thus the afferent fibers do not compete for the same target cells. In the transplanted frog, ganglion cells axons from the extra eye extended to the contralateral optic tectum. Axon terminals from the normal and the transplanted eyes segregated generating a pattern of regular alternating columns. These results suggest than an activity-based competitive process between two sets of afferent neurons for the same population of target neurons is sufficient to segregate the terminals of the presynaptic cells into distinct territories.

The visual cortex

Physiological properties of cortical neurons

Early electrophysiological studies on the visual system of adult rats found that cortical neurons have well defined functional properties (Parnavelas et al., 1981; Maffei et al., 1992) and are distributed in distinct classes of ocular dominance, with high proportion of binocular cells, comparable to that in cats and monkeys (Maffei et al., 1992; Berardi et al., 1993). Fagiolini et al. (1994) performed a fundamental study on postnatal development of functional properties of rat visual cortical neurons. The physiological properties of visual cortical neurons of the rat are immature at postnatal day 17 (P17), three days later than opening of the eyes (P14) and develop gradually during the first month of postnatal life. Visual cortical responses are sluggish and variable at P17, in particularly they present habituation, that is the tendency of cell response to diminish after several stimulation. Neuron responsiveness, evaluated in term amplitude of modulation of cell discharge in response to an optimal visual stimulus (peak response divided spontaneous discharge), increases progressively with age over the third postnatal week: the sluggishness and tendency to habituation disappeared by P23. Ocular dominance distribution does not change significantly through development, indeed the vast majority of visual cortical neurons are binocular and preferentially driven by the contralateral eye. The major component of age dependent changes in ocular dominance distribution is the increase of monocular, contralaterally driven cells.

Receptive fields in adult rats are small and well defined, but this is not the case in younger animals; indeed at P17 receptive fields are very large, extending through almost the whole binocular hemifield. At P19-21 receptive field size is around 34 degrees (deg), and it reaches the value of 10 deg or less in the adult. The progressive decrease in receptive field size is consistent with the time course of visual acuity development. Visual acuity increased from 0,5 c/deg to 0,9 c/deg whiting the first month of life and then reaches the value of 1 c/deg in the adult at P40-45 (Fagiolini at al., 1994).

Critical periods of development

The visual cortex of mammals is immature at birth, both anatomically and physiologically, and develops gradually during the first weeks or months of postnatal life (Hubel and Wiesel, 1963; Sherman and Spear, 1982; Fagiolini et al., 1994, Gordon and Stryker, 1996). Psychologist D.O. Hebb postulated more than a half century ago that experience modifies cortical connections (Hebb, 1947, 1949). Subsequent evidences indicated that Hebb was correct and that neural connections change in response to experience. Cortical circuits are extremely sensitive to manipulation of the sensory environment, in particular during short periods of early postnatal development called "critical period" (for review see Berardi et al., 2000).

The concept of critical period was introduced by Hubel and Wiesel in 1960s. They described for the first time the existence of a columnar organization in the cat primary visual cortex. By electrophysiological recordings Hubel and Wiesel shown that the two eyes differentially activate cortical neurons and that the cells with similar preference for one eye were grouped in columns called dominance ocular columns (Hubel and Wiesel, 1963). They also reported the great finding that occluding one eye early in development (an experimental treatment called monocular deprivation, MD) led to reduction in the number of cortical cells responding to that eye, with a strong increment in the number of neurons activated by the open eye (Hubel and Wiesel, 1963). Since MD treatment is ineffective in the adult life, this period of enhanced plasticity during early development is a clear example of critical period. During critical period, experience modifications, such as visual deprivation, produce permanent and extensive modifications of cortical organization. If during this period of heightened plasticity, one eye is deprived of patterned vision, as in the case of following unilateral congenital cataract or experimental lid suturing (MD), there is an irreversible reduction of visually driven activity in the visual cortex through the deprived eye, which is reflected in a strongly shift in the ocular preference of binocular neurons towards non deprived eye in all mammals tested (Berardi et al., 2000). This dramatic plastic modification occurred when MD is performed during the temporal window of critical period 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). Critical periods are known to occur also during human development including visual function development (Levi, 2005) but also language acquisition (Doupe and Khul, 1999) and maternal attachment

establishment (Leiderman, 1981). Following monocular deprivation treatment, visual acuity and contrast sensitivity of the deprived eye develops poorly originating the phenomenon of amblyopia, which is permanent if the vision is not restored before critical period closure (reviewed in Odom, 1983). The great majority of MD effects have been interpreted as the outcome of an activity driven competition process that follow Hebbian rules. Hebb's principle states that if an electrical activity in a set of afferent fibers is temporally correlated with the activity of postsynaptic neurons (neurons with fire together with together), then afferents will be allowed to maintain and even expand the connections with it. Critical periods are time windows in which brain circuits that perform a given function are particularly receptive to acquiring certain kinds of information or even need the instructive signal for their continued normal development. Visual experience acts by modulating the level and the patterning of neuronal activity within the visual pathway. What remain uncertain is whether the role of neural activity in development is instructive or permissive: it is not clear if patterns of neural activity affects directly the development of neural connections or whether it is simply the presence of neural activity that move other developmental cues, such as molecular factor, to guide appropriate neural connection refinement (for a review, see Crair, 1999).

Plastic and adaptive response to sensory stimulation are present also in the adult but much reduced respect to development: monocular deprivation in the adults produces no effects and the recovery from amblyopia is very limited after the closure of critical period. Classical studies shows that critical period closure is prevented by rearing animals in complete darkness from birth (dark rearing, DR) (Mower et al., 1991; Fagiolini et al., 1994; Gianfranceschi et al., 2003) suggesting that patterned visual experience is crucial for critical period closure. However a recent work (Bartoletti et., 2004) has shown that it is possible to prevent dark rearing effects in the rat visual cortex by varying just the environmental conditions.

Visual cortical plasticity: determinants of critical period

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 unclear, though intensively studied. The first step of neural plasticity which are changes in synaptic efficacy, that no require protein synthesis, are followed by long-lasting changes in

neuronal circuitry that require gene expression and protein synthesis. The molecular basis of plasticity in the visual cortex are summarized in Berardi et al. (2003).

NMDA receptors

NMDA function is thought to be crucial for mechanisms of synaptic plasticity that follow Hebbian rules and rely on NMDA-dependent modification in synaptic efficacy, likely the first modification induced by experience in the visual cortical circuits. NMDA receptors, are synaptic receptors with the 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 synapse. Involvement of NMDA receptors in developmental plasticity of the visual system has been initially suggested by Bear et al., 1990, where block of NMDARs in the visual cortex blocks MD effect.

Recently, the use of NMDAR antagonists (Daw et al., 1999) or antisense oligonucleotide (Roberts et al., 1998) has overcome the problem that pharmacologically NMDARs blocking significantly affects visually driven activity, showing that is possible to block the effects of monocular deprivation without affects visual response (Roberts et al., 1998) and confirming NMDAR involvement in visual cortical plasticity. Two properties of NMDARs that make them candidates to be molecular determinants of critical period are that the characteristics of NMDAR mediated synaptic transmission are developmentally regulated, and that their expression is modified by electrical activity (Livingston and Mooney, 1997; Catalano et al., 1997). 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 that parallels that of critical period and functional visual cortical development. Expression of the 2A subunit correlates with the progressive shortening of NMDA current. Dark rearing, which delays critical period closure and impairs development of function properties of the visual cortex and of visual acuity, delays the developmental shortening of NMDA-receptor currents and of subunit 2A expression, suggest 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 mice with deletion of the NMDA-receptor 2A subunit, the sensitivity to monocular deprivation is restricted to the normal critical period, suggesting that the expression of 2A subunit is not essential to delineate the time course of the critical period of

ocular dominant plasticity (Fagiolini et al., 2003) and might be related to other features of visual cortical plasticity.

Neurotrophins

The family of mammalian neurotrophins comprises nerve growth factor (NGF) and tree closely related factors: brain-derived neurotrophic factor (BDNF), neurotrophins-3 (NT-3) and neurotrophic factor-4 (NT-4). These molecules exert their action on target cells through the binding of two classes of transmembrane receptor: a low affinity receptor (p75), which is bound by all neurotrophins, and high-affinity receptors of the Trk family of receptor tyrosine kinases (TrkA, TrkB, TrkC). Whereas NGF interacts specifically with TrkA, BDNF and NT-4 bind to TrkB, NT-3 has been reported to bind to TrkA and TrkB in addition to its interaction with TrkC. The neurotrophins have been originally characterized from their effects on proliferation, differentiation, and survival of neurons during nervous system development (Lewin and Barde, 1996). More recently, several observations suggest that neurotrophins have additional functions related to activity-dependent plasticity of the brain, particularly of neocortex and hippocampus (Thoenen, 1995; Bonhoeffer, 1996; McAllister et al., 1999). Many insights into the roles played by neurotrophins in synaptic plasticity have come from studies of the mammalian visual cortex (Berardi and Maffei, 1999). For instance, it was shown that exogenous supply of neurotrophins counteracts the effect of MD and DR (Maffei et al., 1992; Riddle et al., 1995; Pizzorusso et al., 2000) and prevents the formation of ocular dominance column (Cabelli et al., 1995). Other studies, which followed the opposite course of antagonizing the action of endogenous neurotrophins, have shown that neurotrophins are important for normal visual cortical development and plasticity (Cabelli et al., 1997; Berardi et al., 1994). There is a clear evidence that neurotrophins control the duration of the critical period; in fact, they are the first molecules for which a causal relation has been established between their action and the duration of critical period in mammals. The first evidence come from the finding that block of endogenous NGF trough the use of antibodies prolongs the duration of critical period, an effect similar to dark rearing (Berardi et a., 1994). More recently, in a study using transgenic mice overexpressing BDNF in the visual cortex, Huang et al., 1999; have found that BDNF overexpression accelerates both the visual acuity development and early closure of the critical period. These effects are accompanied by a precocious development of inhibition and by an early closure of synaptic

plasticity that is usually enabled by LTP in the visual cortex. These findings suggest that BDNF controls the time course of the critical period by accelerating the maturation of GABAergic inhibition.

Inhibitory circuitry

Recently it has become clear that inhibition has an important role in sculpting the pattern of electrical activity and two sets of experiments pointed out its role in visual cortex plasticity during critical period. The first performed by Hensch and colleagues demonstrated that inhibitory interactions are necessary for the manifestation of experience-dependent plasticity. They shown that experience-dependent plasticity is prevented in transgenic (Tg) mice lacking the 65kDa isoform of the GABA synthesizing enzyme GAD (GAD65) and that it can be rescued if GABAergic transmission is enhanced in the visual cortex by means of benzodiazepines (Hensch et al., 1998). The second set of experiments highlighted the role of inhibition as a determinant of critical period. Accelerated development of GABA-mediated inhibition, obtained in mice with overexpression of BDNF, results in an early opening and closure of the critical period (Huang et al., 1999). In according to this finding, the precocious increased of intracortical inhibition by early diazepam administration to the visual cortex accelerates opening of the critical period (Fagiolini and Hensch, 2000). The development of inhibition lags behind that of excitation and this mismatch between inhibition and excitation could determine a time window, the critical period, during which cortical circuitry are particular sensitive to sensory experience. According to these researches, it is becoming clear that excitatory and inhibitory circuit elements reach an optimal balance one in life during which plasticity may occur.
Premature enhancement of intracortical inhibition triggers a precocious onset of the critical period for OD plasticity (Fagiolini and Hensch 2000; Iwai et al., 2003)

Intracellular signalling

Three kinases have been resulted necessary for shift of ocular dominance during MD: c-AMP dependent protein kinase (PKA), extracellular-signal-regulated kinase (ERK) and α Ca^{2+}/calmodulin-dependent protein kinase II (α CaMKII) (Taha et al., 2002; Di Cristo et al., 2001). Each kinase is activated by specific extracellular signal and after visual activation, the

possible targets of these three kinases could be both at cytoplasm and nucleus level. At cytoplasm level, the activated PKA, ERK and α CaMKII could phosphorylate substrates crucial for synaptic transmission, neuronal excitability and morphological stabilization (reviewed in Berardi et al., 2003). Because the PKA, ERK and αCaMKII pathways vary in the signal integration that lead to their activation and in their downstream targets, it's surprising that interfering with the activation of any of these pathways produces the suppression of ocular dominance shift after MD. This could be due to extensive overlap and cross talk of these pathways, so that the blockade of a single kinase reverberates on the entire network (Berardi et al., 2003).

Regulation of gene expression

The pattern of kinase activation has to be translated into a pattern of gene expression, trough the activation of transcription factors. Several transcription factors, such as early-growth-response 1 (erg1/zif 268), are regulated by visual activity (Caleo et al., 1999; Kaczmarek et and Chaudhuri, 1997), but the condition of being visual-activity-dependent does not imply that the factor is necessary for ocular-dominance-plasticity. For instance, mice knock out for erg1/zif 268 exhibit a normal response to MD (Mataga et al., 2001). An important indication to identify the transcription factors necessary for visual cortical plasticity is offered by the finding that the activation of CREB is necessary for ocular-dominance-plasticity (Mower et al., 2002, Liao et al., 2002; Pham et al., 1999). To phosphorylate CREB, activated kinase must translocate to the nucleus, where they start the expression of genes under c-AMP-response-element (CRE) promoter, with the consequent production of gene transcripts essential fro establishment and maintenance of plastic changes (Silva et al., 1998). Both PKA and ERK are well-known activators of CREB (Impey et al., 1996; Mayr and Montminy, 2001), although the ability of αCaMKII to translocate in to the nucleus and directly activate CREB is far less certain (Wu and McMurray, 2001; Matthews et al., 1994; Deisseroth et al., 2002). *In vivo* studies allowed to clarify the pathway responsible for CRE-mediated gene expression activated by visual stimulation. Recently it has been shown that patterned vision is powerful activator of ERK in neurons of the visual cortex and visually induced ERK activation relies, at least partially, on the cAMP-PKA system. Pharmacological block of ERK phosphorylation completely suppress CRE-mediated gene expression after visual stimulation (Cancedda et al., 2002). These results are strong indicators that ERK is the final effector linking extracellular signals with gene expression

in the visual system during the critical period. Our present knowledge about the plasticity-related signalling can be designed with the following scheme: NMDA coincidence detection activates αCaMKII, possibly helped by the co-occuring activation of PKA and the consequent inhibition of the αCaMKII phosphatase, protein phosphatase 1 (PP1) (Bhalla and Iyengar, 1999; Brown et al., 2000). Activated αCaMKII acts on local targets, such as AMPA receptors (Benke et al., 1998; Esteban et al., 2003), contributing to further depolarization. ERK detects the simultaneous and stabilized activation of αCaMKII and PKA, 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 (Berardi et al., 2003). A recent study (Majdan and Shatz, 2006) conducted with microarray technique, reported that critical period in mice has a specific pattern of gene regulation. Four days of MD regulated one set of genes during the critical periods, and different sets before and after. Moreover between the signalling pathways visually experience regulated, they still found the mitogen-activate-protein (MAP) kinase pathway: it was downregulated after MD at every age studied, suggesting that visual deprivation lead to a sustained, rather than transient, downregulation of MAP pathway. These results expands on previous studies reporting that visual stimulation enhances MAP kinase activity (Cancedda et al., 2003; Di Cristo et al., 2001) and that MEK1/2 is necessary for OD shifts induced by MD during the critical period (Di Cristo et al., 2001). Majdan and Shatz, (2006) findings further confirm that a dynamic interplay between experience and gene expression drives activity-dependent circuit maturation and imply also that although gene regulation in response to visual deprivation occurs throughout the life for some genes, the critical period is fundamentally distinct from other developmental time windows in term of its molecular signature.

Recently, Putignano et al. (2007) suggested that intracellular pathways that control gene expression in the visual cortex and are activated by visual experience, could modulate the critical period closure. They found that that visual experience activates intracellular signalling pathways which differ in juvenile and adult animal; indeed in the adult they observe a developmental downregulation of a few signalling pathways which are highly activated during development. Results obtained by Putignano and colleagues suggest that reduction of plasticity occurring in the adult visual cortex could be induced by the reduced activation of molecular targets responsible for plasticity process in the young animal.

In particular, they demonstrated that during the critical period ERK is strongly activated by visual experience and its activation is required for the molecular cascades that involve MSK,

CREB and histone H3 and H4. Putignano and colleagues observe that, in the adult, ERK and MSK are still fully responsive to visual stimulation, while there is a downregulation of the effects of visual stimulation on CREB phosphorylation, H3 phosphoacetylation, and H4 acetylation. The hypothesis that a reduction in experience-dependent regulation of CREB mediated gene transcription could be involved in regulating the closure of critical period is also supported by the observation that MD in the adult mice produce a form of plasticity can be rendered persistent by the expression of a constitutively active CREB mutant (Pham et al., 2004). Moreover, the hypothesis the critical period closure is also associated with a decrease in the ability of visual experience to drive changes in histone phosphorylation and acetylation is supported by the result that pharmacologically increasing histone acetylation in adult animals restores MD-dependent plasticity to the visual cortex (Putignano et al., 2007).

Extracellular environment

Recent findings indicate that degradation 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 MD (Mataga et al., 2002). The first investigations on the role of tPA in visual cortical plasticity indicated that its pharmacological inhibition attenuates the OD shift induced by MD (Mataga et al., 1996) and prevents the effects of reverse suture (a form of plasticity in which the deprived eye is reopened while the contralater, previously open is monocular deprived). During development, this procedure is able to revert the effects of the previous MD, but reverse suture resulted ineffective in kittens treated with tPA activity inhibitors (Muller and Griesinger, 1998). The implications of these studies have been deepened analyzing tPA-knockout mice. These mice displayed an impaired ocular-dominance shift that could be rescued by exogenous tPA (Mataga et al., 2002). tPA has a wide spectrum of possible molecular targets, including extracellular-matrix proteins (Wu et al., 2000), growth factors (Yuan et al., 2002), membrane receptors (Nicole et al., 2001), cell adhesion molecules (Endo et al., 1999), but the available information is not sufficient to distinguish which of these tPA actions are necessary for cortical plasticity. tPA has been recently implicated in the regulation in dendritic spine dynamism after brief MD in two works (Oray et al., 2004; Mataga et al., 2004). Oray et al., 2004 demonstrated

that application of tPA on visual cortical slices induces a dramatic increase in spine motility. If tPA is applied to visual cortical slices obtained from MD animals, they found that the effect of tPA was not additive to the effect elicited by MD, suggesting that tPA is a mediator of MD action on spine motility. Mataga et al. (2004) demonstrated that tPA activity is necessary for MD-changes induced in spine density. Counting the number of spines on dendrites of layer III pyramids, they found that the decrease in spine density caused by 4 days of MD is not present in tPA knockout mice and this effect could be rescued by exogenous tPA.

Recent data (Pizzorusso et al., 2002 and 2006), however, suggest that at least part of the inhibitory action of the extracellular environment could reside in components of the extracellular matrix, the chondrointin-sulfate proteoglycans (CSPGs), a class of glycoproteins that are the major components of the extracellular matrix of the CNS. CSPGs are abundantly expressed in CNS where they are used mainly to create barriers. It has been shown that in the developing CNS, barriers between the two sides of the brain contain large amount of CSPGs (Faissner and Steundler, 1995). CSPGs are inhibitory for axonal sprouting and the are upregulated in the CNS after injury with the effect of blocking axon regeneration (Bradbury et al., 2002).

In the adult CSPGs are condensed in perineuronal nets (PNNs), which completely ensheath neuronal cell bodies and dendrites. PNNs are mainly formed by a family of CSPGs, lecticans and by other CSPGs, such as phosphocan. This specialized form of ECM (called perineuronal network) constitutes aggregates around subpopulation of neurons in the form of network-like aggregates. In the visual cortex the process of condensation of CSPGs into PNNs begins during late development and is completed after the end of critical period (Pizzorusso et al., 2002; Bruckner at al., 2000; Hockfield et al., 1990). Dark rearing, which is known to prolong the critical period for ocular-dominance plasticity (Berardi et al., 2000), also prevents PNN formation (Pizzorusso et al., 2002). The correlation between CSPGs maturation and critical period closure (Sur et al., 1988) suggest that CSPGs could hinder ocular-dominance plasticity in the adult visual cortex (Hockfield et al., 1990). A direct demonstration of this theory comes from the recent analysis of the effects of degradation of CS-GAG chains *in vivo* with the enzyme chondroitinase ABC which destabilizes PNNs and cause their disappearance from the adult visual cortex (Pizzorusso et al., 2002 and 2006). Removal of CSPGs, after this treatment, was able to reactivate ocular-dominance plasticity in MD adult rats, suggesting that developmental maturation of PNNs could contribute to the progressive reduction of plasticity that occurs in the visual cortex at the end of critical periods. A more recent work (Pizzorusso et a., 2006), showed

that degradation of CSPGs by chondroitinase ABC combined with reverse lid-suturing produces a complete recovery of ocular dominance and visual acuity, in adult rats. In this case a functional recovery is accompanied by significant recovery of dendritic spine density; infact, reopening the formerly promotes the restores spine density to normal values in adult animals treated with chondroitinase ABC.

Effects of the environment on nervous system and behaviour: enriched environment

Complex genetic and epigenetic programs generate the mammalian brain and ensure that cells and structural areas are in place by birth. Several studies demonstrated that sensory, cognitive and motor stimulation through the interaction with the environment has a key role in refining the neuronal circuitry required for normal brain function, both during development than in adulthood.

It is difficult to establish the different contribution of genes or environment in shaping nervous system, and in the second half of the last century a large number of studies has focussed on the so-called “nature versus nurture” debate. The central question of this debate was “what is the contribution of genes to overt and covert behaviour, and what is the contribution of environment on the same behaviours?” (for review, see Krubitzer and Kahn, 2003). This debate was solved by the Nobel Price Konrad Lorenz which introduced for the first time the concept of “innate predisposition to learn” (Lorenz, 1961). “Innate” and “learned” constitute the two ways trough which information are available to the organism, registered to the nervous system and resulting in an adaptive behaviour (Lorenz, 1961; Rescorla, 1988). Since species are different physiologically and morphologically and these difference are subjected to the genetic laws of selection and heredity, the potentiality to acquire new information trough learning is also regulated and programmed under specie-specific constraints, so, genetic inheritance during phylogenesis and learning and memory during ontogenesis are intermingled in the construction of the individual personality.

In 1940s Hebb was the first to introduce the idea of the “enriched environment” like an experimental concept. He described that rats that he took home as family pets, and that he reared

in an extremely more complexes environment than a laboratory rodent's setting, showed behavioural improvements in learning task. Subsequently the finding that a more complex and stimulating rearing environment enhanced performance on complex learning tasks was repeated by Hebb's students and by others (Bingham and Griffiths, 1952; Forgays and Read, 1962).

The most direct approach to investigate the effect of experience on nervous system is the manipulation of environmental stimulation pattern in which animals are reared. In this ambit, in the 1960s, two experimental approaches originated to investigate the effects of experience on the brain. On one side, Hubel and Wiesel started to investigate the effects of visual deprivation on the anatomy and physiology of the visual cortex during development (Wiesel and Hubel, 1965; Hubel and Wiesel, 1970). On the other side, Rosenzweig and colleagues introduced enriched environment as an experimental paradigm used to analyze the effects of experience on nervous system, showing that the morphology, biochemistry and physiology of brain can be deeply affected by the quality and the intensity of environmental stimulation (Rosenzweig et al., 1962, Rosenzweig, 1966; Rosenzweig and Bennett, 1969).

Enriched environment: definition and peculiarity of the experimental protocol

Enriched environment was defined for the first time by Rosenzweig et al., 1978 as " a combination of complex inanimate and social stimulation". Environmental enrichment refers to housing conditions, either home cages or exploratory chambers, that facilitate enhanced sensory, cognitive and motor stimulation relative to standard housing conditions. In our experimental paradigm, enrichment include also increased social stimulation through the larger numbers of animals per cage. In particular, enriched animals are reared in groups of numerous individuals (6-12 individuals is the most common situation) and in large dimension cages, with three floors containing running wheels to improve physical activity, and differently shaped objects (tunnels, shelters, stairs) that were completely substituted with others once a week. The enriched environment rearing is compared with standard rearing condition in which animals are reared in groups of at most tree animals in small dimensions cage without particular objects besides water and food *ad libitum*, and impoverishment rearing condition in which animal is reared alone in a cage identical to the standard one.

The environment produced in the enriched condition stimulates animals at varies levels. First of all, presence of running wheels allow animals to perform voluntary physical activity providing a strong stimulation of motor cortex and enhancing motor activity.

On the other and, the environmental complexity, with different objects provides a range of opportunities for a complex multi-sensorial stimulation at a visual, somatosensory and cognitive levels. An important aspect of enriched rearing is the environmental complexity and novelty, obtained by changing the objects and the position of objects which might provide additional cognitive stimulation with respect to the formation of spatial maps (for a review see Rosenzweig, 1966; Rosenzweig and Bennett, 1969; Nithianantharajah et al., 2006).

It is interesting to note, that most of the effects elicited in animals reared in EE, are common to animals reared in standard cage but submitted to voluntary physical exercise for the presence of a running wheel or for a treadmill running (for a review see Cotman and Berchtold, 2002). Physical exercise increases neurotrophins 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) and increases important survival factors levels such as insulin-like growth factor1 (IGF-1) (Carro et al., 2000; Schwart et al., 1996) which considered the mediator of the effects of exercise (Carro et al., 2000) and of EE (Koopmans et al., 2006) and a key molecule related to functional and anatomical plasticity in the brain (for review see Torres-Alemann, 1999 and 2000, Carro and Torres-Aleman, 2005; Aberg et al., 2006). Furthermore physical exercise improves cognitive functions in rats and aging humans (Fordyce and Farrar, 1991; Kramer et al., 1999, Churchill et al., 2002; van Praag et al., 1999a and 2005), attenuates motor deficit (Klintsova et al., 1998), increases neurogenesis (van Praag et al., 1999 a, b), increases angiogenesis (Black et al., 1990; Isaacs et al., 1992) and is neuroprotective ameliorating neurological impairment in different neurodegenerative processes (Arkin et al., 1999; Petajan and White, 1999; Larsen et al., 2000; Mattson et al., 2000; Carro et al., 2000 and 2001).

Since all the effects produced by physical exercise are elicited also by rearing in enriched environment, it is rightful to suppose that physical exercise and EE affect common pathways and common endpoints in the nervous system, including neurogenesis, anatomical changes, production of growth factor and survival factors such as IGF-1 and neurotrophins. For this reason, it is extremely difficult to distinguish the single contribution of physical exercise to the effects produced by EE. Enrichment including exercise, is more effective than exercise alone in improving memory functions (Berstein,1973), while neurogenesis in the adult is more strongly

increase by exercise than by enriched environment. Moreover, a recent work (Stranahan et al., 2006) showed that isolation housing prevents the positive effects of running on adult hippocampal neurogenesis and in the presence of additional stress suppresses the generation of new neurons, likely because individually housed runners had higher levels of corticosterone. Thus, all these results suggest that physical exercise and all the other components of EE (increased exploration, social interactions and novelty) are important and act in synergistic way to evoke EE effects.

Environmental enrichment effects on adult brain and behaviour

Behavioural changes

Hebb's observation about the improvement in behavioural tasks of rats reared freely in his home for some weeks compared to those reared in laboratory (Hebb, 1949), pioneered a large number of studies which showed that the experience of a complex environment can induce the improvement of animals performances in tasks involving superior cognitive function, mostly learning and memory (for a review see Rampon and Tsien, 2000).

EE effects are especially evident in hippocampal-dependent tasks involving spatial memory, such as the Morris water maze task. In this task, animals have to swim in a circular pool filled with opaque water, learning to reach a submerged platform which they can't perceive, but has to be deduced from a spatial map based on the position of visual stimuli external to the pool (D'Hooge and De Deyn, 2001). EE improves the execution of Morris water maze task enhancing spatial learning and memory (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) independently on the gender and age of tested animals, or reducing the cognitive decline of spatial memory typically associated with aging (for a review see Winocur, 1998). This latter effect it has been linked to general signs of "cellular health" in the hippocampus, including increment in the levels of synaptophysin, a glycoprotein present in the membrane of presynaptic vesicles containing neurotransmitters (Saito et al., 1994; Frick and Fernandez, 2003), reduced lipofuscin deposits (Kempermann et al., 2002), indicators of oxidative stress (Terman and Brunk, 1998) and a strong induction of hippocampal neurogenesis (Kempermann et al., 2002). EE

animals have better performances than the standard one, also in nonspatial tasks; in fact enrichment enhances mnemonic performances in three different nonspatial tasks (Rampon et al., 2000b). In particularly, Rampon et al. (2000b) shown that EE animals have enhanced visual recognition memory in a novel object recognition task and retention of memory in contextual fear-conditioning task. Moreover the effects of EE on behavioural task are extended also to tasks independent on the hippocampus, such as the cued fear-conditioning task (Rampon et al., 2000b) in which the animals learn to associate an acoustic stimulus with a mild footshock. Thus, these data suggest that enriched experience has a general impact on various spatial and nonspatial, hippocampal-dependent and –independent tasks.

The other behavioural consequence of enriched experience is the modification of emotionally and stress reaction. The issue about the capability of EE to diminish stress levels has remained controversial for a long time, but the study of Chapillon et al., (1999) on BALB/c mice, a strain usually described as pathologically anxious (Trullas and Skolnick, 1993) which display decreased levels of anxiety if reared in EE, supported the assumption of the EE anxiolitic action. A possible mechanism of action of EE on emotional reactivity could be represented by lowering stress hormone levels such as ACTH and corticosterone. Glucocorticoids are physiological indicators of stress levels (Jost, 1966) and it is interesting to note that Mlynarik et al., (2004) showed that EE prevents the elevation of glucocorticoids elicited by repeated injections of lipopolysaccharide (LPS): treatment with LPS resulted in increased corticosterone levels in non-EE but on EE animals. Furthermore, enriched animals did not display signs of discomfort after LPS treatment, such as suppression of grooming and decrease in body weight, suggesting that the prevention in corticosterone rise was beneficial and likely responsible for the better ability of EE mice to face with stress. This assumption is reinforced by work of Banaroya-Milsthein et al., (2004) that show how a stressing procedure significantly increases serum corticosterone levels in animals reared in standard condition, while does not affect corticosterone levels of EE animals which also display higher natural cells killer cytotoxicity, an effect not abolished by stressing protocol. The study of interaction between behavioural, neural and endocrine function and immunity is a very fascinating topic. Banaroya-Milsthein et al., (2004) confirm the hypothesis of Larsson et al. (2002) that the EE effect on emotional behaviour could be partially due to the action on the hypothalamic-pituitary-adrenocortical (HPA) axis and to a more efficient action of corticosteroids in these animals. At the same time, EE may regulated the activity of immune system in response to distressing situation as confirmed by Kingston and Hoffman-Goetz, (1996)

which found lower splenic proliferative responses to an acute distressing episode relative to responses obtained in non-EE mice. Thus, EE may affect at different levels the communication pathways existing among the immune, nervous and endocrine system with relevant implications in the field of psychoneuroimmunology.

Anatomical changes

Rosenzweig and colleagues demonstrated that the improvements in learning an behavioural tasks observed in EE animals were accompanied with deeply changes at neurochemical and anatomical level in brain. The initial experiments of Rosenzweig et al. (1964) showed that the entire dorsal cortex, including frontal, parietal and occipital cortex of enriched lived rats for 30 days, increased in thickness a weight compared with that of standard reared rats. Since this pioneer observation, many other studies have reported wide anatomical changes elicited by exposure to EE, such as an increment of the size and of the nucleus of nerve cells (Diamond, 1988); increased dendritic arborisation (Holloway, 1996**;** 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 post-synaptic thickening (Diamond et al., 1964) and gliogenesis (Diamond et al., 1966).

It has been found that almost four consecutive days of enrichment in one month old rats, are able to increase cortical thickness in the visual association area (Diamond, 1988) and an increase in total dendritic length and total branches number in the primary visual cortex (Wallace et al., 1992). Longer periods, such as thirty days beginning at P23-25, of enriched living conditions produced long lasting effects persisting even after thirty days of housing individual cages (Camel et al., 1986). The anatomical changes are not limited to the cortical regions, but include other areas such as hippocampus. Indeed, similar effects to that reported for the cerebral cortex, have been found for pyramidal cells of CA1 and CA3 and for dentate granule neurons (Walsh et al., 1969; Walsh and Cummins, 1979; Rosenzweig and Bennet, 1996; Rampon et al., 2000b).

Another anatomical effect of EE has been described on hippocampal neurogenesis. Studies about this problem, have started when it has been shown that some brain structures, such as the olfactory bulb and the hippocampal dentate gyrus, maintain the potentiality of neurogenesis even after sexual maturity (Gueneau et al., 1982; Kuhn et al., 1996) like monkeys

and humans respectively brain (respectively, Gould et al., 1999; Eriksson et al., 1998). Recent studies have revealed that enriched experience produce a significant increases in hippocampal and dentate gyrus neurogenesis (Kempermann et al., 1997; Nilsson et al., 1999). EE increases hippocampal neurogenesis and the integration of these new newly born cells into functional circuits (van Praag et al., 2000; Kempermann et al., 1997; Bruel-Jungerman et al., 2005). It has been suggested that increase in neurogenesis could be mediated by mechanisms involving vascular endothelial factor (VEGF) (During and Cao, 2006) and the recruitment of T cells and the activation of microglia (Ziv et al., 2006). Both enrichment and physical exercise, in particular running, fundamental component of EE for the presence of running wheels, increases neurogenesis (van Praag et al., 1999a, b); however the mechanism by which new neurons are generated seems to differ between the two conditions. Running alone, in standard cages is responsible for both proliferation of neural precursor and of survival of new generated neurons, while enriched living increases number of survival new borne neurons without affecting cell proliferation (van Praag et al., 1999b, van Praag et al., 2005). The capability of EE to interact with these programs of nervous cells goes beyond the action on neurogenesis, because it reduce cell death in the rat hippocampus under both physiological and pathological conditions (Young et al., 1999).

Molecular changes

Many of the anatomical and behavioural effects observed in EE animals, are consistent with enriched experience modulation of neurotransmitter systems and expression of genes involved in synaptic function and cellular plasticity (for review see Rampon and Tsien, 2000; van Praag et al., 2000; Nithianantharajah et al., 2006).

It has been shown that EE affects the functioning of cholinergic, serotoninergic an noradrenergic system increasing acetylcholinesterasi activity (Rosenzweig et al., 1962 and 1967); augmenting mRNA expression levels of serotonin 1A receptor for serotonin (Rasmuson et al., 1995) and increasing beta-adrenoceptor transduction system (Escorihuela et al., 1995; Naka et al., 2002). All these neurotransmitters have been reported to influence learning and plasticity in the adult brain (van Praag et al., 2000) and to regulate the arousal state of the brain (Hobson et al., 1975; Berridge and Waterhouse, 2003).

Enrichment can increase levels and action of neurotrophins, a class of secreted proteins promoting neural development and survival and implicated in structural and functional neural circuits rearrangement both during development and adult life plasticity processes (reviewed in Bonhoeffer, 1996; Berardi and Maffei, 1999; Thoenen, 2000; Berardi et al., 2003; Caleo and Cenni, 2004). In particular, EE increases levels of mRNA for NT-3 and NGF in the visual cortex, hippocampus (Torasdotter et al., 1996 and 1998) and other brain regions (Ickes et al., 2000; Pham et al., 2002) of the early candidate-plasticity gene, the early growth factor induced-A (NGFI-A or Zif/268) throughout the brain (Pinaud et al., 2002).

Moreover, enriched experience also increases the phosphorylation of the transcription factor cyclic-cAMP response element binding protein (CREB; Cancedda et al., 2004), which is known to regulate BDNF expression (Tao et al., 1998), to mediate the plasticity changes required for memory formation (Bailey et al., 1996; Yin and Tully, 1996; Silva et al., 1998) and involved in visual cortical development and plasticity (Berardi et al., 2003).

As previous reported, many of the effects elicited by EE are common to those produced by physical exercise. Sustained levels of physical activity occurring in enriched living condition can increase the production and the brain uptake of IGF-1. IGF-1 is considered the molecular mediator of most of the effects of physical exercise on the brain including increase of BDNF expression and c-fos activation (Carro et al., 2000), increased hippocampal neurogenesis (Trejo et al., 2001), protective effects of exercise and possible of EE against brain insults and on neuronal cell death (Carro et al., 2001, Koopmans et al., 2006) and the enhancement in hippocampal plasticity and in learning and memory (Markowska et al., 1998; Cotman and Berchtold , 2002; Aberg et al., 2006).

EE affects expression levels of a large number of genes grouped in functional class of genes linked to neuronal structure, synaptic plasticity and transmission, neuronal excitability, neuroprotection and learning and memory capacity (Rampon et al., 2000a; Keyvani et al., 2004). The expression of several synaptic proteins, such as the presynaptic vesicle protein, synaptophysin and postsynaptic density protein (PSD-95) are affected by enriched experience (Frick and Fernandez, 2003; Nithianantharajah et al., 2004).

Development of conditional gene knockout techniques offered a valuable way to study the molecular mediators of EE effects on the brain. A conditional knock out mice in which the NMDA (N-methyl-D-aspartate) receptor for the excitatory neurotransmitter glutamate was deleted in the CA1 sub-region of hippocampus (CA1-KO mice) was used to study the role of

NMDA receptor in functional and anatomical changes elicited by EE (Rampon et al., 2000b). These CA1-KO mice, lacking of NMDA current and LTP/LTD resulted profoundly impaired in spatial and non spatial memory tasks (Tsien et al., 1996; McHugh et al., 1996; Rampon et al., 2000b). The learning deficits exhibited by CA1-KO mice in tree hippocampus-dependent behavioural tasks (novel objects recognition, social transmission of food preference and contextual-fear conditioning) are largely or completed healed after two months of daily training in the EE (Rampon et al., 2000b). A first attempt to clarify the mechanisms by which EE restore memory capacity of CA1-KO mice proposed that EE increased the density of nonperforated synapses, the most abundant kind of synapses in cortex and hippocampus (Eichenbaum and Harris, 2000). On the other and, Rampon et al. (2000b) proposed the compensation of EE could be due an enhancement in the connectivity outside the functionally deleted region of the hippocampus, likely the neocortex. The existence of overlapping mechanisms between EE and the NMDA receptor function was suggested also by Tang et al. (2001) which studied transgenic (Tg) mice whit an enhanced NMDA receptor function in the forebrain showing an enhanced learning and memory ability in hippocampal-dependent task. Tang and colleagues found that while EE improved performances of wild type mice in the same behavioural tasks, it was no able to further increase performances of Tg mice, likely for a saturation toward EE effects. Also the expression of AMPA (α-amino-3-hydroxy-5-methyl-4-isoazole propionic acid) receptors subunits, involving in glutamatergic signalling (Tand et al., 2001; Naka et al., 2005) are affected by EE. The alterations on NMDA and AMPA receptors subunits confirm the increased elicited by EE in synaptic strength, including long term potentiation (LTP), a specific form of synaptic plasticity (Green and Greenough, 1986, Foster et al., 1996; Artola et al., 2006).

Environmental enrichment effects on visual system development

Despite the large numbers of data showing the effects of the complex sensory-motor stimulation provided by enriched living condition in the adult brain, just recently have began to be investigated the effects of EE during development. Analysing the Rodent visual system development as a paradigmatic model of nervous system development, a series of experiments performed in our laboratory showed that EE strongly affects visual cortex and retinal development and plasticity (Cancedda et al., 2004; Sale et al., 2004; Landi et al., 2007) and

prevents dark rearing effects on visual acuity development and critical period closure (Bartoletti et al, 2004).

Effects of environmental enrichment on visual cortex development

Cancedda et al. (2004) demonstrated that rearing mice in EE from birth produces a strong acceleration of visual system development at behavioural, electrophysiological and molecular level. Surprisingly these EE mice showed a precocious eye-opening, a precocious developmental decline of the white-matter long term potentiation (WT-LTP), *in vitro* parameter of visual cortical maturation (Kirkwood et al., 1995) and an accelerated maturation of visual acuity development tested both electrophysiologically with VEPs and behaviourally with Prusky task (Prusky et al., 2000). In particular, at P25 VEPs acuity of EE mice was higher than that of non-EE mice, and in EE animals visual acuity development was accelerated significantly by 6 days compared with non-EE mice.

Some of the molecular changes found in EE mice were observed at very early age (P7-P15), when pups spend all their time in the nest: enriched pups shown an increment of GAD 65/67 protein at P7 and P15 and a precocious peak of BDNF expression at P7. Using Tg mice that carry the *LacZ* reporter gene under the control of CRE promoter (Impey et al., 1996), Cancedda et al. (2004) demonstrated that EE is able to affect also the cAMP/CREB pathway, crucial hub in the development and plasticity of visual system (Impey et al., 1996; Pham et al., 1999a; Barth et al., 2000; Mower et al., 2002; Cancedda et al., 2003). EE mice from birth shown an acceleration of the developmentally regulated CRE-mediated gene expression with a precocious peak at P20, and treatment of non-EE mice with rolipram, a specific inhibitor of the high-affinity phosphodiesterase type IV that activate cAMP system, resulting in an increased phosphorylation of the transcription factor CREB (Tohda et al., 1996; Kato et al., 1998; Nakagawa et al., 2002) partially mimics EE effects on CREB pathway and on visual system development.

Bartoletti et al. (2004) demonstrated that post-weaning EE (starting from P18) prevents the effects of dark rearing (DR) on the closure of critical period for MD and on the Chondroitin Sulphate Proteoglycans (CSPG) developmental organization into perineuronal nets (PNNs) in the visual cortex. While a week of MD is effective in dark reared rats also after critical period closure (Cynader and Mitchell, 1980; Mower, 1991; Fagiolini et al., 1994), this same protocol of

sensorial alteration is not effective in dark reared enriched rats (Bartoletti et al., 2004) as in adult rats with a normal visual experience. Thus enriched living condition promoted the consolidation of developing visual connections, allowing a normal critical period closure in dark reared rats. Dark rearing also inhibit the maturation of intracortical inhibitory system maturation (Benevento et al., 1995) and prevents the developmental organization into PNNs of CSPGs (Pizzorusso et al., 2002 and 2006), two determinant events for the of critical period closure (Berardi et al., 2003). Bartoletti and colleagues. (2004) found that EE significantly reduces the effects of dark rearing on the development of PNNs and of GABAergic system. These findings suggests that molecules not exclusively under the control of visual experience, responding to EE, may contribute to visual cortical development. A particularly good candidate is IGF-1 the brain levels of which are increased by physical exercise (Carro et al., 2000). IGF-1 receptors are present in the occipital cortex (Frolich et al., 1998) and therefore it could influence the expression of molecules relevant for visual cortical plasticity such as NGF and BDNF. Moreover the demonstration that IGF-1 is involved in experience-dependent plasticity of the visual cortex arise from the recent work of Tropea et al. (2006), showing that monocular deprivation (MD) affects IGF-1 pathway increasing the expression of IGF-1 binding protein 5 (IGFBP-5) and exogenous application of IGF-1 prevents the physiological effects of MD on ocular dominance plasticity examined in vivo.

Effects of environmental enrichment on retinal development

A more recent finding (Landi et al., 2007) was the demonstration that also retina development, commonly assumed to be independent from sensory inputs, is affected by experience provided by EE both at electrophysiological and molecular level. Landi and colleagues showed that EE from birth elicits a strong acceleration of retinal acuity development such as starting from P25-26 up to P34, retinal acuity is significantly higher in EE than non-EE rats and the final acuity level is reached almost 10 days before in EE than in non-EE rats. The same effect is obtained exposing animals to EE just for the first ten days of life. Effects produced by EE on retinal acuity development seem to be due to the precocious increment of BDNF protein levels in the retinal ganglion cell (RGC) layer of EE rats in which at P10 BDNF immunoreactivity is higher than in non-EE rats. This hypothesis has been confirmed by the observation that reduction of BDNF levels in EE rats by means of BDNF antisense oligonucleotides intraocular injections during the time window of its enhanced expression,

prevents the precocious development of retinal acuity (Landi et al., 2007). Since the complete effect of EE from birth on retinal acuity maturation is reproduced also by enrichment up to P10, before eye opening, the acceleration in visual acuity elicited by EE cannot be dependent on vision.

Results of Landi et al. (2007) are in line with previous findings that show that it is possible to modulate the outcome of visual deprivation by varying the environmental conditions (Bartoletti et al., 2004) or the availability of BDNF to cortical neurons (Gianfranceschi et al., 2003). These data confirm, according to Bartoletti et al. (2004), that developmental factors that are not under exclusively visual experience but are modified by EE (Cotman and Berchtold., 2002; Gomez-Pinilla et al., 2002), such as IGF-1, may contribute to visual system development.

Experiments done recently in our laboratory, have support the hypothesis of IGF-1 role in mediating EE effects. It has been seen that maternal enrichment during pregnancy affects retinal development of the fetus, influencing the dynamics of neuronal cell death.. This effect seem under the control of IGF-1 levels of which higher in enriched pregnant rats and their milk, are increased also in the retina of their pups. Blockade of IGF-1 action obtained by the infusion of an IGF-1 receptor antagonist in EE females prevents the effects of maternal enrichment on retinal development, while infusion of recombinant IGF-1 to standard-reared females, mimics the effects of EE on the fetus (Sale A., Cenni MC., Ciucci F., Putignano E., Chierzi S., Maffei L. unpublished data).

Maternal care and early environmental enriched

The very precocious effects (between P7 and P15) elicited by EE in the expression of GAD65/67 and BDNF (Cancedda et al., 2004), and in the development of retinal acuity (Landi et al., 2007) are unlikely elicited by the direct interaction of pups with the richness of the environment, but they are supposed to derive from the higher levels of licking behaviour provided by adult females to their pups in the enriched condition (Cancedda et al., 2004; Sale et al., 2004). Detailed analysis of maternal behaviour shown that EE pups receive higher levels of maternal care respect to standard one (Cancedda et al., 2004; Sale et al., 2004). In rodents maternal care occur at intervals of time, alternate to period with the mother is absent from the nest and pups remain lonely: the time spent from pups in the nest without mother is much shorter in EE animals than in non-EE pups (Cancedda et al., 2004). In the first two weeks of life, rodent

spend all their time in the nest, do not interact with environment and their most important sources of sensory experience is maternal influence (Hofer et al., 1984; Ronca et al., 1993; Liu et al., 2000). Licking and physical contact given by mother and by filler females constitutes a tactile stimulation that could facilitated the precocious eye-opening seen in EE pups (Cancedda et al., 2004).

The hypothesis that higher levels of maternal care can accelerate visual system development is confirmed by results showing that alterations in maternal care can affect BDNF levels and neural development of pups (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).

This finding is extremely important because BDNF and neurotrophins in general, have a major role in the control of visual cortical development and plasticity during a critical period early in life (for a review see Berardi et al., 2003). Liu et al. (2000) demonstrated that the offspring of mothers that supplied higher levels of maternal care showed higher levels of NMDA receptor subunit and of BDNF mRNA in the hippocampus, enhanced hippocampal synaptogenesis and spatial learning and memory. Early work by Levine (1957) has shown that stimulation of neonatal rodents affects their endocrine and behavioral responses later in life. Long term consequences of maternal alterations on behavioral and neuroendocrine responses have been reported also by following works (reviewed in Cirulli et al., 2003). Maternal care influences several process regulating pups development such as the synthesis of ornithine decarboxilase, an enzyme essential for cell growth and development, DNA synthesis, neuroendocrine secretion, and the response to growth hormone, prolactin and insulin (Kuhn and Schanberg, 1998; Schanberg et al., 2003). Thus all these data confirm that different levels of in maternal care in different rearing condition, could act like an indirect mediator to elicit the more precocious effects of enrichment. Maternal behaviour, can affect other factor important for visual system development, such as growth factors present in maternal milk, as for example IGF-1, that are involved in regulation of the development. In particular, the finding that pups injected with IGF-1 from P3 until P15 exhibit precocious eye opening (Philips et al., 1988), suggest that increased levels of maternal care could be directly involved in the eye opening acceleration found in EE pups, affecting IGF-1 production or expression levels.

Insulin like growth factor 1 (IGF-1)

Historical perspective

Classically, IGF-1 has been implicated in prenatal and postnatal events in CNS development such as the control of cell proliferation, gliogenesis, neurogenesis, neuron survival, differentiation, synaptogenesis, myelination (D'Ercole et al., 1996 and 2002; O'Kusky et al., 2000; Aberg et al., 2006). Recently, IGF-1 has been shown to be neuroprotective against the effects of lesions in the adult CNS and to mediate both the neuroprotective effects of physical exercise and possibly of EE on neuronal death (Carro et al., 2001; Koopmans et al., 2006) and the enhancement caused by exercise in hippocampal plasticity and in learning and memory (Markowska et al., 1998; Cotman and Berchtold, 2002; Aberg et al., 2006). Running induces uptake of IGF-1 by specific groups of neurons and IGF-1 enhances neuronal electrical activity (Carro et al., 2000). It has been suggested that the increase in IGF-1 caused by EE and exercise determines an increase in BDNF (Cotman and Berchtold, 2002). This hypothesis has been confirmed by Carro et al. (2000) showing that IGF-1 administration produces increment of BDNF expression in cortex and hippocampus. More recently, it has been demonstrated (Ding et al., 2006) that IGF-1 affects BDNF system to mediate exercise effects on cognitive processes and synaptic plasticity and that IGF-1 enhance the activity of BDNF on cerebrocortical neurons (McCusker et al., 2006). All these data support the hypothesis that IGF-1 could be a mediator of the EE effects on visual system development. For these reason we have chosen to investigate the role of IGF-1 on visual cortex and retina development and to deepen its action on BDNF expression. Indeed, all results demonstrating a causal relationship between IGF-1 and BDNF concern with the adult brain, but it is not known whether this holds true also for the developing brain.

In 1957, Salmon and Daughaday reported a serum factor that mediated the cartilage sulfation and longitudinal bone growth activity of somatotrophic hormone (growth hormone, GH) (Rinderknecht and Humbel, 1976a; Daughaday et al., 1972). This factor was termed "sulfation factor" and was produced by hepatic cells after exposure to GH (Salmon and Daughaday, 1957). In conjunction, Dulak and Termin were investigating the cell proliferative factors in serum, and termed one such activity Multiplication-Stimulating Activity (MSA) (Dulak and Termin, 1973).

These circulating factors, which also showed insulin-like activity not suppressible by anti-insulin antibodies (non suppressible insulin-like activity, NSILA I/II), were later found to have similar biochemical structure to β chain of insulin (Rinderknecht and Humbel, 1976b; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). It was apparent that this activity represented a similar group of substances with a wider biological activity than first suspected. Daughaday, 1972 proposed that NSILA I and II were two forms of an insulin-like hormone with predominant effects on cell and tissue growth (Rinderknecht and Humbel, 1976b). These two small molecular mass peptides (NSILA I and II), were later renamed as somatomedins (mediator of growth-hormone actions) replacing the "non suppressible insulin-like activity" and sulfaction factor terminology. Further investigation revealed that these factors mediated the actions of pituitary derived GH, giving birth to the Somatomedin Hypotesis (Daughaday, 1972).

Two mammalian somatomedins were identified by protein sequence and cDNA data (Rinderknecht and Humbel, 1976b; Humbel, 1990; Hintz and Rinderknecht, 1980; Jansen et al., 1985; Jansen et al., 1983; de Pagter et al., 1989) and their structural homology with proinsulin led to their current designation of insulin-like growth factors I and II (IGF-I and IGF-II).

In 1980's Sara et al., 1993; identified a brain specific variant of IGF-1, des(1-3) or "truncated" IGF-1, which lacks the first tree aminoacids and is more potent than intact IGF-1 in various cell culture system (Sara et al., 1993; Giacobini et al., 1990; Russo et al., 1994), probably due to its lower affinity for IGFBP binding proteins (Oh et al., 1993). These findings suggested brain synthesis of IGF-1 or its truncated form. Some *in situ* hybridisation studies (Bondy et al., 1990; Bondy et al., 1992) demonstrated that IGF-1 and IGF-1 receptor mRNA is synthesised in the rat brain in specific regions, such as olfactory bulb, hippocampus and cerebellulm (Werther et al., 1990). In addition IGF-carrier proteins, later named as insulin-like growth factor binding proteins (IGFBPs), were also found expressed in similar regions of the brain (Pons et al., 1991; Bondy and Lee, 1993; Lee et al., 1993, Russo et al., 1994). The finding of IGF-1 mRNA co-localisation with IGF-1 receptors and the presence of IGFBPs, suggested a pacrine or autocrine role for IGF-1, potentially modulated by IGFBPs, in developing brain (Werther et al., 1990; D'Ercole et al., 1996; Leventhal et al., 1999; Werther et al., 1998). The presence of IGF-1 (D'Ercole et al., 1984) and IGF-1R mRNA (Lund et al., 1986) in multiple tissue has necessitated the revision of the original "somatomedin hypotesis" to include both autocrine and paracrine actions of IGF-1 in addition to its classical endocrine aspects. The somatomedin hypothesis has been studied employing the specific IGF-1 deficient mice (LID), that have reduced circulating IGF-1 and

elevated GH (Yakar et al., 1999; Sjogren et al., 1999) and the acid-labile subunit knockout ALSKO mice (Ueki et al., 2000) that have reduced circulating IGF-1 and IGFBP-3, but normal GH levels. The LID mice showed normal postnatal growth and development (Ueki et al., 2000; Haluzik et al., 2003), while the ALSKO mice, despite the 65% reduction in circulating IGF-1, demonstrated only 10% reduction in body weight. When LID mice were crossed with the ALSKO mice, the LID- ALSKO mice (very low IGF-I and very high GH levels) showed postnatal growth retardation and osteopenia, suggesting that IGF-1 is important for post-natal growth and development (Haluzik et al., 2003) exerting its functions in and endocrine and paracrine fashion (Russo et al., 2005). Growing evidence suggest that IGF-1 may have a neutrophic role.

The IGF-1 peptide: structure and synthesis

IGF-1 is a growth promoting peptide, member of a superfamily of related insulin-hormones that includes insulin and relaxin in the vertebrates (Rinderknecht and Humbel., 1978a; Rinderknecht and Humbel., 1978b; Isaacs et al., 1978); however, insulin and IGFs are the most closely related in terms of primary sequence and biological activity. IGF-1 is a major growth factor whereas insulin predominantly regulates glucose uptake and cellular metabolism. They consist of A, B, C and D domains. Large parts of the sequence within the A and B domains are homologous to the α and β of the human pro-insulin. This sequence homology is 43% for IGF-I. No sequence homology exist between the C domains of IGF-1 and C region of human proinsulin (Russo et al., 2005). The C domain of IGF-1 is not removed during pro-hormone processing, thus the mature IGF-1 peptide is a single chain polypeptides (Zapf et al., 1986; Daughaday et al., 1989). The gene encoding IGF-1 is highly conserved, such that 57 of 70 residues of the mature protein are identical among mammals, birds and amphibians (Zapf et al., 1986; Shimatsu et al., 1987; Kajimoto et al., 1987; Perfetti et al., 1994; Koval et al., 1994; Chan et al., 1990).

Expression of the IGF-1 gene is affected at many levels including gene transcription, splicing, translation and secretion (Russo et al., 2005). IGF-1 expression is also influenced by hormonal (GH) (Salmon et al., 1957; Bichell et al., 1992; Gronowski et al., 1995; Gluckman, 1994; Benbassat et al., 1999; Meton et al., 1999), nutritional (Thissen et al., 1994; Rabkin, 1997; Muaku et al., 1996), tissue-specific and developmental factors (D'Ercole et al., 1996, D'Ercole,

1987; Sara et al., 1994; Milner and Hill., 1989; Rappolee and Hill, 1991). IGF-1 is synthesized mainly by liver (Haselbacher et al., 1980) and its synthesis is regulated by ipophisary GH (Daugaday and Rotwein, 1989; Clemmons and Underwood, 1991). However IGF-1 is synthesized also locally in many other tissues, among them the nervous system: these findings suggest that IGF-1 could have both autocrine and paracrine tissue-specific role during development (Behringer et al., 1990).

The IGF-1 receptor and its functions

The biological effects of IGF-1 are triggered by specific binding to the α subunit of a membrane-bound tyrosin-kinase receptor that is called IGF type I receptor (IGF-1R) (Schlessinger and Ullrich, 1992; White and Khan, 1994). The insulin-like growth factor 1 receptor is synthesized as a single polypeptide precursor that is cleaved in α and β subunits. The mature receptor is an $\alpha_2\beta_2$ heterotetramer. The glycosylated α subunits are entirely extracellular and bind its ligand.

The β subunit of the receptor is a transmembrane polypeptide which contains a highly conserved tyrosinekinase catalytic domain. The first step of the pathway transducing the IGF-1 stimulus is binding to IGF-1 tyrosine receptor. Binding of the ligand induces a rapid autophosphorilation of the tyrosines in the β subunits and the activated receptor signals to the cytoplasm and nucleus by phosphorylation or recruitment, or both, of several endogenous substrates, member of the insulin receptor substrate family (Izumi et al., 1987; Shemer et al., 1987). The best known is insulin-receptor substrate 1 (IRS-1) (White and Khan, 1994), which then serves as a docking protein by binding the numerous SH2 domain containing proteins. These include the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) and the guanine nucleotide exchange factor Grb2/Sos (Myers et al., 1992; Backer et al., 1993; Skolnik et al., 1993a,b).

IGF-1R is known to mediate the activation of a variety of overlapping pathways promoting cell proliferation, cell differentiation, cell survival and metabolic events. Among them, the main downstream signalling cascade activated by IGF-1R phosphorylation is the phosphatidylinositol 3-kinase (PI3K) cascade, while a second signalling pathway activated by IGF-1 is the the mitogen-activated protein kinase (MAPK) pathway. Both PI3K and MAPK pathways are known

to be involved in IGF-1 actions on severall cell types (van der Geer et al., 1994; Guan, 1994; Chakravarthy et al., 2000; Prisco et al., 1999; Baserga et al., 1999; Melmed et al., 1996).

Activation of PI3K and formation of its lipid products in vivo lead to activation of Akt/PKB and $p70^{S6}$ kinase, two downstream serine-treonine kinase involved in cell survival and protein synthesis pathways, respectively (Brunet et al., 2001; Cheatham et al., 1994; Datta et al., 1996). PI3K leads to the phosphorilation of protein kinase B/Akt (Summers and Birnbaum, 1997) regulating synthesis and translocation of glucose transporters (GLUTs), in particular GLUT4 (Cheng et al., 2000), from intracellular pool to plasmatic membrane, enhancing in this way glucose entry into cells (Khon et al., 1996; Summers and Birnbaum, 1997). Cheng et al., 2000; demonstrated that in *Igf1*-null mice brains, both GLUT4 immunoreactivity and hexokinase I activity, responsible for neuronal glucose utilization, are reduced: this finding suggest a role of IGF-1 to augment glucose utilisation during brain development.

Another target of Akt protein in IGF-1 signalling, is the glycogen sinthasis kinase 3β (GSK3β): IGF-1 stimulates the GSK3β phosphorylation in cultured of human neurons (Hong and Lee; 1997) preventing the inhibition of glycogen synthasis and of transcription factor (eIF2B), promoting glycogen and protein synthesis (Summers et al., 1999). Cheng et al., 2000 supports the role of IGF-1 in GSK3β activation because ser^9-phospgo- GSK3β is abundant in IGF-1 expressing neurons in WT brains, but is rarely found in theses same neurons in *Igf1-/-* brains. The presence of phospho- GSK3β is associated with abundant glycogen stores, consisting with the hypothesis that IGF-1 promotes glucose uptake and storage as glycogen in developing projection neurons. Neuronal glycogen synthesis is abundant during postnatal development and it is spatiotemporally correlated with a peak of IGF-1 expression (Borke and Nau, 1984; Bondy, 1991).

A substrate of GSK3β is also tau, a protein associated to microtubular filaments and involved in their stabilization. *In vitro* studies, demonstrated that IGF-1 by means of GSK3β, enhances tau phosphorylation (Hong and Lee, 1997; Lesort and Jhonson, 2000), leading to cytoskeleton reorganization necessary for neurite growth and develompent. Iperphosphorylated tau proteins leads to formation of intracellular neurofibrillar clusters, responsible for neuronal degeneration (Cheng et al., 2004). It has been demonstrated that tau phosphorylation is enhanced in *Igf-1 -/-* mice respectt to WT (Bondy and Cheng, 2004). The finding that IGF-1 modulates thau

phosphorylation, suggest the GSK3β inhibition induced by IGF-1 is central mechanism of IGF-1 actions on central nervous system.

Finally protein kinase Akt causes phosphorylation of Bcl-2 proteins family, involved in apoptosis regulation; in particular IGF-1 stimulates antiapoptotic protein and in inhibit apoptotic one.

Coupling of Grb2 to IGF-IR appears to occur through IRS-1 and Shc and results in a sequential activation of Ras, the protein kinase Raf and the mitogen activated protein kinase (MEK1), which then activates MAPKs [also called extracellular signal-regulated protein kinases (ERKs)] (Kyriakis et al., 1992; Wood et al., 1992; Moodie et al., 1993). Activation of the Ras/MAPK pathway is known to mediate signalling leading to proliferation in many cell types (Blenis et al., 1993; Coolican et al., 1997; Reiss et al., 1998) and to activate other kinases and protein mediating genic expression. One well-studied target of MAPK is the transcription factor cAMP-response element (CRE)-binding protein (CREB; Shaywitz and Greenberg, 1999). CREB is a transcription factor with a fundamental role in development and plasticity of the visual system either at a cortical level (Pham et al., 1999; Cancedda et al., 2003) and at geniculate level (Pham et al., 2001) and its expression is affected by EE (Cancedda et al., 2004). Several findings demonstrated that IGF-1 activates CREB in different cell types. Fernandez et al., 2005 shows that treatment of pituitary cells with IGF-1 promotes a rapid phosphorylation of CREB through the activation of MAPK signalling pathway (Ras-Raf1-MEK). Palacios et al., 2005 demonstrated that IGF-1 activates CREB and CREB activation is necessary for IGF-1 to induce myelin basic protein (MBP) in oligodendrocytes.

The IGFBPs

IGF-1 biological activity is regulated by a family of high affinity IGF-binding proteins (IGFBPs) of which six have been characterized to date (IGFBP-1 through IGFBP-6). IGFBPs regulate IGF-1 pharmacokinetics, both in circulation and at the tissue level, in several ways, including regulation of IGF-1 transport in plasma and control of its diffusion between intra- and extravascular space; prolongation of IGF-1 half-life in circulation; modulation of IGF-1 binding to its receptor (Jones and Clemmons; 1995; Rajaram et al., 1997; Bach et al., 1995; Russo et al., 1997; Firth and Baxter; 2002; Monzavi and Cohen, 2002). They might serve to transport IGF-1

from CSF to cells, present ligand that are synthesized in one cell to receptors in neighbouring cells, sequester IGF-1 in the extracellular matrix or protect it from enzymatic degradation.

IGFBPs biological activity is regulated by post-translation modifications such as glycosylation and phosphorylation and by proteases (BP-Pr) that cleave them, generating fragments whit reduced or no binding affinity for the IGF-1(Jones and Clemmons; 1995; Firth and Baxter; 2002).

It is hypothesised that IGFBPs, in addition to stabilizing and regulating levels of diffusible IGF-1, might regulate IGF-1 cellular response by facilitating IGF-1 receptor targeting or modulating IGF-1 bioavailability in the pericellular space (Jones and Clemmons; 1995; Firth and Baxter; 2002). In the extracellular cell matrix or at the cell surface, IGFBPs can either inhibit or enhance the presentation of IGF-1 to its receptor. The neuroanatomical distribution of IGF-1 and IGFBPs overlap partially. Colocalization of IGFBPs and IGF-1 has been reported to occur during brain development and has been suggested to be a mechanism for modulating IGF-1 actions (Leventhal et al., 1999; Walter et al., 1999). For instance the effects of IGF-1 in development are most modulated by the IGFBPs. Three IGFBPs, 2, 4 and 5, which appear to be the predominant binding proteins, are expressed very early in embryonic genesis and show developmental regulation in rodents CNS (de Pablo et al., 1995). Expression of IGFBP-2 simultaneously with IGF-1 has been reported in neuronal structures (Lee et al., 1993) and IGFBP-5 has been shown to be more selectively coexpressed with IGF-1 or in the vicinity of IGF-1 producing neurons (Bondy and Lee; 1993). IGFBPs selective and regulated expression suggest that they participate in the autocrine-paracrine network of IGF-1 signalling within CNS.

IGF-1 expression in the central nervous system

Increasing evidences strongly supports a role for IGF-1 in central nervous system (CNS) development by promoting neural cell proliferation, survival, and differentiation (for a review see D'Ercole at al., 1996; Folli et al., 1996; Anlar et al., 1999; D'Erole et al., 2002). For example, *in situ* hybridization studies, have detected IGF-1 messenger RNA (mRNA) in the subventricular zone, hippocampus, retina and cerebellum at developmental times when glial and neuronal precursor undergo cell division (Bondy, 1991; Lee et al., 1992; Bartlett et al., 1991; Bartlett at al., 1992; Bondy and Lee, 1993). Transient IGF-1 mRNA expression occurs in developing retina,

hippocampus, cerebellum, cerebral cortex, and several sensory thalamic and brain stem nuclei during periods of neuronal growth and synaptogenesis (Bondy, 1991; Lee et al., 1992; Bartlett et al., 1991; Bartlett at al., 1992) In the olfactory bulb and hypothalamus, structure that remain plastic into adulthood, IGF-1 mRNA expression persist throughout life (Lee et al., 1992; Bartlett at al., 1992).

Although the principal source of circulating IGF-1 is the liver (Yakar et al., 1999); IGF-1 is produced by many cell types trough the body, including the brain (D'Ercole et al., 1996).

The expression pattern of IGF system proteins during brain growth suggests highly regulated and developmentally timed IGF actions on specific neural cell populations. IGF-I expression is predominantly in neurons and, in many brain regions, peaks in a fashion temporally coincident with periods in development when neuron progenitor proliferation and/or neuritic outgrowth occurs (D'Ercole et al., 1996). The first studies about IGF-1 pattern expression in CNS (Rotwein et al., 1988; Werther et al., 1990; Bondy et al., 1993) during different periods of embrional and postnatal development have been shown that IGF-1 is highly expressed in rat brain from late fetal E16-E20, but diminish rapidly with a plateau at P1. Both neurons and glia synthesize IGF-1 mRNA (Rotwein et al., 1988). IGF-1 pattern expression is regulated during development with the highest levels of gene expression coinciding with early phases of neuronal growth.

Following studies (Bartlett et al., 1991; Bondy, 1991; Bondy and Lee, 1993) analyzed IGF-1 expression during the maturation of different groups of functionally related sensory and cerebellar projections neurons, more precisely IGF-1 is found expressed in neuronal cells with large some and complex dendritic formation (Bondy and Lee, 1993; Cheng et al., 2003). IGF-1 mRNA is abundant within the developing cerebellar Purkinje cells and in the major cerebellar relay centres. Similarly IGF-1 is localized in the synaptic stations the developing olfactory, auditory, visual and somatosensory system with a transient high level of IGF-1 expression during a relatively late phase in their development, at a time of maturation of dendrites and synapse formation. IGF-1 gene expression is found to be time–locked to periods of dendritic maturation and synaptogenesis suggesting that IGF-1 may have a role in the shaping of system-specific synaptic connections or myelinization. For instance, the development of rat cerebellar cortex takes place in the first postnatal week and during this period, there is an intense IGF-1 gene expression in the principal cerebellar cortical neurons, the Purkinje cells. Large Purkinje cells bodies are recognizable in the cerebellar anlage as early as E18, but Purkinje mRNA IGF-1

expression is not detected until the day of birth and is maximal between P4 and P10 (Table 1). By P28, when the development of cerebellar cortex is largely complete, Purkinje cell mRNA IGF-1 detection is significantly diminished compared with P10 (Table 1).

IGF-1 gene expression is also very marked during the maturation of the components of the cerebellar relay system (Bondy, 1991; Aguado et al., 1992, Torres-Alemann et al., 1994).

In the hippocampus, IGF-1 expression is high during the two first postnatal weeks, whereas it remains for a long time in dentate gyrus, where is not restricted to developmental phases.

IGF-1 gene expression is very intense in the maturing trigeminal somatosensory relay system. The highest level of somatosensory IGF-1 mRNA are found during the first three postnatal weeks in thalamic nuclei, but is also transiently localized in projection neurons of the principal trigeminal nucleus and trigeminal ganglion neurons (Bondy, 1991).

The onset of IGF-1 gene expression in the olfactory cortex occurs between P4 and P7 and continues unabated throughout adult life, as in the olfactory bulb (Bondy, 1991). IGF-1 gene expression is detected in the olfactory bulb in the late phases of embrional life whereas it's present in the piriform cortex just in the first postnatal days (Bondy, 1991).

System	Component	Period[a]
Cerebellar	Purkinje cell	D4–D28
	Inferior olive	E20–D21
	Deep cerebellar nuclei	E20–D21
	Red nucleus	D4–D28
	Ventrolateral nucleus	D4–D21
Somatosensory	Spinal trigeminal nucleus	E20–D21
	Ventrobasilar nucleus	D4–D21
	Gelatinosa nucleus	E20–D14
	Intralaminar nucleus	E18–
	Anterior pretectal nucleus	E16–D28
	Dorsal column nucleus[b]	D0–
Olfactory	Mitral cell	E16–
	Tufted cell	E18–
	Piriform cortex[c]	D7–
Visual	Retinal ganglion cells	E20–D10
	Superior colliculus	D0–
	Lateral geniculate nucleus	D7–D28
	Lateral posterior nucleus	D7–D28
	Anterior pretectal nucleus	E16–D28
Auditory-vestibular	Medial vestibular nucleus	E20–D21
	Superior vestibular nucleus	E20–D21
	Dorsal cochlear nucleus	E20–D14
	Ventral cochlear nucleus	D0–D14
	Superior olive	E20–
	Lateral lemniscal nucleus	E20–D28
	Medial geniculate nucleus	D4–D21
	Inferior colliculus	D0–D28
	Interstitial nucleus	E20–D14
Other	Nucleus basalis	E20–D14
	Diagonal band nucleus	E20–D21
	Lateral septal nucleus	E20–D21
	Medial habenula	E20–D14
	Ventral tegmental nucleus	E20–D14
	Dorsal tegmental nucleus	E20–D14
	Entopeduncular nucleus	D0–D21
	Parafascicular nucleus	E16–D7
	Retroflexus nucleus	E16–D7
	Parabrachial nucleus	E20–D7
	Substantia nigra, pars lateralis	D0–D21
	Hippocampus[d]	E20–D28
	Neocortex[e]	D0–D28

Table 1: **Timing of peak IGF-1 expression in specific neuroanatomical loci of the developing rat CNS** (taken form Bondy, 1991).

Bondy, 1991 analyzed IGF-1 gene expression also throughout the stations of the visual system. The IGF-1 signal over the developing neural retina is confined to the postmitotic, postmigratory ganglion cells layer. IGF-1 mRNA is present in the major projection centres, the lateral geniculate nuclei and the superior colliculus. The lateral dorsal and anterior pretectal nuclei of the thalamus also contain IGF-1 mRNA.

IGF-1 mRNA is abundant in the developing auditory and vestibular relays systems, including the cochlear (dorsal and ventral) and medial and superior vestibular nuclei from E20 through D14 (Bondy, 1991).

Other structures expressing IGF-1 during development are listed in the table 1 of following page. These *in situ* hibrydization studies have been demonstrated that IGF-1 gene expression shows a peak of expression just for a brief time-window, differing in the varies regions, during the CNS maturation.

In most neurons IGF-1 transcription decreases significantly postnatally, a decrease that correlates with the degree of cell maturation and which reach very low level in the adult (Rotwein et al., 1988; Andersson et al., 1988). In rat adult brain it has been shown an high level of IGF-1 gene expression in hippocampus, olfactory bulb and cerebellum (Rotwein et al., 1988; Werther et al., 1990; Bondy et al., 1993).

IGF-1 receptor and IGFBPs expression in CNS

Several studies have addressed the expression and distribution of IGF-1R in the brain. The first characterization was performed with ligand binding experiments and subsequently with mRNA and protein expression patterns. IGF-1R is expressed in neural stem cells (Aberg et al., 2003), but also appear to be present mostly in neurons, in glial cells throughout the brain, with the highest density in the cerebral cortex and the striatum (Werther et al., 1990; Chung et al., 2002). IGF-1R is highly abundant in the choroid plexus as shown in both ligand binding experiments (Araujo et al., 1989) and from IGF-1 R mRNA experiments (Aguado et al., 1993).

IGF-1R is widely expressed in the CNS from early stages of embryogenesis, and its ligand also show similar “temporal-spatial” pattern of expression. It is evident that the components of IGF-1 system are crucial modulators of the processes activated during brain development.

By means of *in situ* hybridization (Bondy et al., 1990), it has been demonstrated that IGF-1R mRNA gene expression, is highly expressed in rat brain from E14. Immunoblot studies showed that IGF-1R expression is highest at E15 to E20 (Baron-Van Evercooren et al., 1991). Two feature are remarkable for IGF-1R expression pattern: first, a stable pattern of IGF-1R expression in all neural cell types appears to be distributed largely according to cell density in each region (de Pablo and de la Rosa, 1995); and second, during differentiation, specific sites of neurons show higher levels of IGF-1R in conjunction with local expression of IGF-1 (Bondy et al., 1992).

In the adult brain, the IGF-1R expression pattern overlaps with that of IGF-1. IGF-1R expression is associated to structures that remain plastic into adulthood, such as the dentate gyrus of the hippocampus, olfactory bulb, hypothalamic areas and choroid plexus (Sonntag et al., 1999; Stenvers et al., 1996; Walter et al., 1999; Eriksson et al., 1998).

IGFBP-2 (Beilharz et al., 1998), IGFBP-4 (Stenvers et al., 1994) and IGFBP-5 (Stenvers et al., 1994) appear to be the predominant binding protein expressed in the brain, although low expression of IGFBP-1 (Walter et al., 1999), IGFBP-3 (Beilharz et al., 1998), and IGFBP-6 (Walter et al., 1999). The neuroanatomical distribution of IGF-1 and IGFBPs overlap partially, with a clear association with neuronal elements (de Pablo and de la Rosa, 1995). IGFBP-2 expression starts precociously during rat embryogenesis, at least at E7 (Wood et al., 1992); during postnatal life IGFPB-2 expression is confined to astroglia (Lee et al., 1993). During development, IGF-1 and IGFPB-2 expression sites are temporal and spatial correlated, in particular in the cerebellum, retina and sensory systems (Wood et al., 1990; Wood et al., 1992; Lee et al., 1992; Lee et al., 1993), suggesting that IGFBP-2 interacting with IGF-1 could affect IGF-1 physiology, incrementing its concentrations at the target cells or modulating its effects.

IGFBP-4 expression is higher in late phases of development (Brar and Chernausek, 1993; Stenvers et al., 1994): at E20 IGFBP-4 expression is present in basal ganglia neurons, in the precocious postnatal period hippocampus starts to express IGFBP-4 and in the adult brain IGFBP-4 is expressed also in neurons of layers II and IV of cerebral cortex, in the olfactory bulb and in amigdala.

Expression of IGFBP-5 has been shown to be more selectively coexpressed with IGF-1 or in the vicinity of IGF-1 binding neurons (Bondy and Lee, 1993). For instance, IGFBP-5 and IGF-1 are coexpressed in sensory system neurons, such us that of olfactory bulb, geniculate and vestibular nuclei (Bondy and Lee; 1993).

IGF-1 actions on CNS

IGF-1 is an anabolic peptides that promotes growth and development of CNS (D'Ercole et al., 1996; 2002; Folli et al., 1996).

In this paragraph I will illustrate several works giving experimental evidences that IGF-1 exert pleiotropic, fundamental actions in the developing and adult CNS.

Transgenic mice models for the study of in vivo IGF-1 role on CNS

Manipulation of the murine genome, either by insertion of transgenes or by disruption of native genes (so-called gene knockout, accomplished by homologous recombination), and study of the resultant mutant mice have proved invaluable to our understanding of insulin-like growth factor 1 physiology.

Accumulating evidence indicates a major role for IGF-1 in CNS development (for reviews see D'Ercole et al., 1996; Folli et al., 1996; Anlar et al., 1999). IGF-1 stimulates increase in neurons and oligodendrocyte numbers by mechanism that involve both the stimulation of proliferation and the promotion of survival. IGF-1 also influences neuron and oligodendrocyte differentiation and function: it stimulates neuritic outgrowth, synaptogenesis and myelination (D'Ercole et al., 2002).

To directly evaluate *in vivo* IGF-1 action, several studies focussed on transgenic (Tg) mice models with genetic alterations of IGF-1, IGF-1 receptor and IGFBPs expression.

Several studies on Tg mice show that IGF-1 regulates brain growth. Tg mice overexpressing IGF-1 have increased brain weight and size compared with their wild type (wt) littermates (Behringer et al., 1990; Mathews et al., 1988; Carson et al., 1993; Ye et al., 1995; Ye et al., 1996) because of an apparent increase in neuron number (Behringer et al., 1990) and increases in both total brain myelin (Carson et al., 1990) and regional density of myelinated axons (Ye et al., 1995) . Conversely, mice carrying IGF-1 genes disrupted by homologous recombination (Beck et al., 1995), and those with ectopic expression of IGF-1 (Ye et al., 1995a; Ye et al., 1995b; D'Ercole et al., 1994), an inhibitor of IGF-1 actions, have smaller brain than

their counterparts. Changes in brain size in theses Tg mouse line are due in part to the effects of IGF-1 on oligodendrocyte survival and function (Carson et al., 1993; Beck et al., 1995; Ye et al., 1995a; Ye et al., 1995b), but there is also evidence of alterations in neurons number (Behringer et al., 1990, Mathews et al., 1988; Beck et al., 1995).The transient early postnatal expression of IGF-1 in sensory projection neurons suggest that IGF-1 may be especially important to neuronal growth and synaptogenesis in developing sensory system (Bondy et al; 1991).

The following table illustrates transgenic mice with different mutations of IGF-1 proteic system with correlate alterations of development and/or cerebral phenotype.

Protein	Description of Mutant Line	Abbreviation in Text	Brain Phenotype
IGF-I	m Metallothionein-I (mMT-I) driven hIGF-IA cDNA	mMT-I/IGF-I	Increased postnatal brain growth
	m IGF-II 5' flanking region	mIGF-II/IGF-I	Increased postnatal brain growth, especially the cerebellum
	Gene ablation by homologous recombination:	IGF-I KO	
	Heterozygous ablation	IGF-I $^{+/-}$	No abnormality
	Homozygous ablation	IGF-I $^{-/-}$	Brain growth retardation
	Partial gene ablation by homologous recombination	Midi-IGF-I	Brain growth retardation
IGF1R	Gene ablation by homologous recombination:	IGF1R KO	
	Heterozygous ablation	IGF1R $^{+/-}$	No abnormality
	Homozygous ablation	IGF1R $^{-/-}$	Brain growth retardation
IGFBP-1	mMT-I promoter driven hIGFBP-1 cDNA	mMT-I/hIGFBP-1	Postnatal brain growth retardation
	m Phospoglycerate Kinase-1 promoter driven rIGFBP-1 gene	mPGK/rIGFBP-1	Brain growth retardation from fetal life
	hα-1 antitrypsin promoter driven hIGFBP-1 cDNA	α-1AT/hIGFBP-1	Brain abnormalities
IGFBP-2	CMV promoter driven mIGFBP-2 cDNA	CMV/IGFBP-2	Brain growth retardation

Table 2: IGF-1 system Protein Mutant Mouse Lines with known alterations in brain phenotype. (taken from D'Ercole et al., 2002)

IGF-1 overexpressing mice were among the first transgenic Tg mice generated (Behinger et al., 1990; Mathews at al., 1998). The initial line of transgenic mice was created using a transgene driven by the mouse metallothionein (mMT-1) promoter and encoding a human IGF-1 cDNA. The mice in this and subsequent lines generated express IGF-1 in multiple tissue beginning at birth (Mathews at al., 1998; Ye et al., 1995a). Depending upon the level of IGF-1 transgene expression in the tissues among these lines of mice, postnatal somatic overgrowth begins at 3-4 weeks of age and produce a moderate increase in weight (30%) by early adulthood. The IGF-1 Tg mice shown a disproportionate growth of some organs, most markedly the brain

(Ye et al., 1995a). In different MT-I/IGF-1 Tg lines brain weights are increased from 25% to 85% and mice with overgrowth of brain often do not survive post weaning (D'Ercole et al., 2002). Mice with largest brain weights usually do not exhibit somatic overgrowth; rather they have modestly reduced adult body weight (about 15%) and similarly reduced serum IGF-1 levels. D'Ercole et al., 2002 speculate that increased IGF-1 expression in the pituitary results in decreased GH secretion and consequent decreased expression of the native IGF-1 gene in somatic tissue, that is not fully compensated by transgene IGF-1 expression.

Many other transgenic mice with an organ specific IGF-1 overexpression have been created using different types of promoters (for a review see D'Ercole et al., 1996). For instance it has been produced a line of IGF-1 Tg mice overexpressing IGF-1 only in CNS (Ye et al., 1996). : even if the transgene is widely expressed in the brain, its expression is markedly increased in the cerebellum causing a near doubling of this structure weight by adulthood. The cerebral IGF-1 overexpression increases neurons number (Behringer et al., 1990), size of neurons cellular body, extension of neuritic ramifications (Gutierrez-Ospina et al., 1996) and myelin content (Ye et al., 1995a,b).

Generation and study of transgenic mice with IGF-1 and IGF-1R null mutations have provided direct evidences on the central role of IGF-1 in somatic growth and of its receptor in mediating IGF-1 actions (Efstratiadis, et al., 1998). These studies gave the evidence that IGF-1 is necessary for normal *in utero* and postnatal growth and that IGF1R mediates all IGF-1 growth promoting actions.

Mice that are homozygous for the disrupted IGF-1 gene, i.e., knockout mice (IGF-1$^{-/-}$ or IGF-1 KO mice) exhibit marked *in utero* and postnatal growth retardation, and depending upon the genetic background, have a reduced survival post birth (<5%). In particular, at birth there is a strong reduction in the mass of skeletal muscle, bone and organs and, in addition, delayed ossification and impaired development of lungs and epidermis (Liu et al., 1993; Baker et al., 1993; Powell-Braxton et al., 1993). Disruption of a single IGF-1 gene has little impact. Mice without IGF-1 expression have birth weights that are ~60% of normal, and continued poor postnatal growth such that they weight about ~25% of normal as adults (Baker et al., 1993).

Homozygous IGF1R null mutants (IGF1R KO mice) exhibit more profound *in utero* growth retardation than IGF1 KO mice. They reach only ~45% of normal size by the end of gestation, and do not survive the perinatal period.

The brains of IGF-1 KO and IGF1R KO mice are small relative to controls, although they are not as growth retarded as body weight, and the brain phenotype in each appears to be identical (Liu et al., 1993, Baker et al., 1993). At birth the striking histological feature of these mice is their increase in neuronal density (Liu et al., 1993).

Deficits in specific population of neurons such as granular cells in dentate gyrus of hippocampus and parvaibumin-conteining neurons in the striatum and hippocampus, have been identified in another line of adult IGF-1 KO mice (Beck et al., 1995). The present study established a reduction in brain weight (38%) distributed evenly over all major brain areas of these homozygous IGF-1^{-}/$^{-}$ analyzed at 2 months of age. Most evident was the strong reduction in size of the two major white matter structures of the forebrain, the anterior commissure and the corpus callosum, which were reduced by approximately 70% compared with wild-type littermates. It has been observed also a reduction in the number of oligodendrocytes and in the CNS myelination because the density of myelinated axons within the anterior commissure and corpus callosum was decreased by about 35% in IGF-1^{-}/$^{-}$ mice, while the density of un myelinated and total axons were increased by 40%-61% and 28%-33% respectively. Decreases proportional to the 38% loss of brain weight were found for the numbers of calbindin-and calretinin-containg neurons and for the volume occupied by the striatum and hippocampal CA1-4 cell body. Numbers of cortical and hippocampal parvalbumin immunopositive neurons were also reduced approximately 30%, but in the dorsal striatum, they were decreased by 52%. (Beck et al., 1995).

Lines of transgenic mice with altered in IGFBPs expression has been produced (for a review see Schneider et al., 2000). Using different types of promoter, several scientific group produced IGFBP-1 Tg mice which is an inhibitor of IGF-1 action when present in molar excess and each line exhibits a somatic and brain growth retardtaion (Murphy et al., 1993; D'Ercole et al., 1994; Ye et al., 1995; Gay et al., 1997). The time when somatic and brain growth retardation occurs appears to depend upon the developmental time when the transgene promoter is activated. For example, brain growth retardation is apparent earlier and more severe when the transgene is expressed during fetal life, as in mPGK/rIGFFBP-1 Tg mice (Murphy et al., 1993). IGFB-1 does not appear to be normally expressed in the brain. Both of the above note transgenes drive IGFBP-1 expression in the brain (as well as in other tissue), and therefore, IGFBP-1 expression is ectopic and likely acts in brain to block IGF-1 stimulated growth. Circulating IGFBP-1, however,

also may affect the brain, because a line of IGFBP-1 Tg with no brain transgene expression may also exhibit CNS abnormalities (Doublier et al., 2000).

Tg mice overexpressing IGFBP-2, -3, -4 and -5 also have been generated. IGFBP-2 Tg mice with brain transgene expression are characterized by a modest reduction in brain weight at 5 weeks of age (Hoeflic et al., 2001). No changes in brain growth or phenotype has been reported in IGFBP-3 Tg mice, made with a mMT-I-driven transgene (Murphy et al., 1995). IGFBP-4 Tg mice have been generated, but they have been created with promoters that do not drive CNS expression, and as expected no alteration in brain size were reported (Wang et al., 1998). IGFBP-5 overexpression from early gestation also appears to inhibit somatic growth including that of the brain (Salih et al., 2004). When it has been used the MT-I promoter to generate Tg mice with postnatal brain IGFBP-5 overexpression, it has been noted a possible minimal increase in brain weight, consistent with the known capacity of IGFBP-5 to augment IGF actions (unpublished form D'Ercole et al., 2002).

Mice with null mutations in IGFBPs genes have not been found to show anomalous brain phenotypic traits (Pintar et al., 1995; Pintar et al., 1996): presumably, the absence of a single IGFBP protein is compensated by others IGFBPs.

IGF-1 effects on cell differentiation

Neurons

IGF-1 enhances the differentiations of specific sets of neural populations in several regions of CNS, among them, cerebellum, cerebral cortex, hippocampus, hypothalamus, retina and striatum (for a review see Varela-Nieto et al., 2003). In vivo and in vitro studies demonstrated that IGF-1 play significant role in cholinergic and GABAergic differentiation of the neurons in the rodent septum and hippocampus (Konishi et al., 1994; Kelsh et al., 2001).

Konishi et al., 1994, demonstrated that IGF-1 stimulates choline acetyltransferase activity and is a potent trophic factor for central cholinergic neurons and could potentially play a significant role in the deifferentiation, maintenance and regeneration of these neurons.

In the developing rat hippocampus, fast GABAergic transmission is depolarizing early in development and became hyperpolarizing and strictly inhibitory only by the end of the first postnatal week (Cherubini et al., 1991). Hyperpolarizing inhibition requires Cl^- outward transport that is accomplished by KCC2, a K^+/ Cl^- cotransporter. Kelsh et al., 2001 showed that cultured hippocampal neurons initially contain an active form of the KCC2 protein, which becomes activated during subsequent maturation of the neurons and they also demonstrated that this process is accelerated by transient stimulation of IGF-1 receptors.

Glia

Fewer data are available on the in vivo capacity of IGF-1 to induce glial differentiation, but in vitro studies using primary antibody suggest that it also promotes differentiation and maturation of glial precursors (for a review see Varela-Nieto et al., 2003).

IGF-1 action on oligodendrocytes and myelination have been well studied in mice with mutations in IGF-1 protein system. There are few studies on the other type of glial cells such as astocytes or microglia which seems to be affected by IGF-1 expression just in the response to injury. In fact, even if astrocytes and microglia develop normally in mice with either reduced IGF-1 availability (IGF-1 KO and IGFBP-1 Tg mice) or with IGF-1 overexpression (Beck et al., 1995; Ni et al., 1997) their response to injury is altered by IGF-1 expression. For instance, inhibition of IGF-1 action by means of ectopic expression of IGFBP-1 reduce astrocite response to injury (Ni et al., 1997).

It has been shown that multiple growth factors and hormones tightly controlled myelination (Legrand et al., 1980; Eccleston and Silberg, 1985; Van der Pal et al., 1988; Besnard et al., 1989). Increasing evidences suggest that IGF-1 could play an important role in myelination process, promoting proliferation and maturation and stimulating directly myelination.

Studies on IGF-1 Tg mice demonstrated that IGF-1 overexpression produces an increase in the number of oligodendrocytes and of their precursors (Ye et al., 1995a; Mason et al. 2000, Ye et al., 2000). Conversely, mutant mice with reduced IGF-1 availability, show a reduced oligodendrocytes and their precursors number (Beck et al., 1995; Ye et al., 1995; Ni et al., 1997).

In vitro studies confirmed that IGF-1 promotes proliferation of oligodendrocytes precursors and survival of oligodendrocytes and of their precursors (McMorris et al., 1986; McMorris and Dubois-Dalcq, 1988; McMorris et al., 1993; Ye and D'Ercole, 1999). IGF-1

stimulates also oligodendrocytes maturation (Ye et al., 1995a; Ye et al., 2000) and promotes their survival after injury (Barres et al., 1993; Mason et al., 2000). Further confirmations comes from Mason et al., (2000) which showed that treatment with cuprizone, a copper chelator and neurotoxicant causing oligodendrocytes and myelin damage, produced massive apoptosis of oligodendrocytes lineage cells in the cerebral cortex followed by demyelination. If the same injury was induced in IGF-1 Tg mice, demyelination occurred but oligodendrocytes apoptosis was, and the surviving oligodendrocytes retained the ability to reinitiate axon myelination (Mason et al., 2000).

Finally, studies on Tg mice have strongly demonstrated that IGF-1 stimulates myelin synthesis (Carson et al., 1993; Beck et al., 1995; Ye et al., 1995; Ni et al., 1997; Ye et al., 2000). In particular, Carson et al., 1993 demonstrated that myelin content in Tg mice is increased four fold; while IGF-1 KO mice and/or availability (IGFBP-1 Tg mice) exhibit evidence of decreased myelination (Beck et al., 1995; Ye et al., 1995; Ni et al., 1997).

IGF-1 promotes re-myelination process after injury. Mason et al., (2000) showed that in contrast to failure of re-myelination in cuprizone-treated WT mice, surviving oligodendtrcytes re-myelinate axons rapidly in cuprizone–treated IGF-1 Tg mice. These results are confirmed also by amelioration induced by IGF-1 overexpression in brain, on under-nutrition induced hypomyelination (Ye et al., 2000).

IGF-1 effects on neurogenesis, apoptosis and synaptogenesis

Neuogenesis and apoptosis

Experimental data reported up to now about IGF-1 expression and studies *in vitro* and *in vivo* on Tg mice lines show that IGF-1 promotes CNS development, enhancing synaptic connections and neurons number and promoting extension of single neurons neuritic arborisation. Moreover the increment in neurons number seems to be derived either from an increase in neuronal proliferation either from an inhibition of apoptosis during the phase of development characterized by massive event of neuronal death.

Morphometric and stereological analyses of the developing brain in IGF-1 Tg overexpressing mice have reported substantial increases in the total number of neurons in the

cerebral cortex (Gutierrez-Ospina et al., 1996), cerebellar cortex (Ye et al., 1996), dentate gyrus of hippocampus (O'Kusky et al., 2000) and selected brainstem nuclei (Dentremont., 1991). By contrast, in IGF-1 null mutants (Beck et al., 1995; Camarero et al., 2001) and in IGFBP-1 Tg mice, in whom IGF actions are inhibited (Gutierrez-Ospina et al., 1996; Ni et al., 1997) significant decrease in neurons number have been reported in the cerebral cortex, hippocampus, dentate gyrus, stratum and cochlear nucleus.

IGF-1 has been shown to promote the proliferation and differentiations of neurons *in vitro* (DiCicco-Bloom and Black, 1998; McMorris and Dubois-Dalcq, 1998; Torres-Aleman et al., 1990; Drago et al., 1991; Werther et al., 1993; Zackenfels et al., 1995; Arsenijevic and Weiss, 1998; for a review see D'Ercole et al., 1996) and *in vivo* Ye et al., 1996; Aberg et al., 2000). In contrast, IGF-1 has well documented anti-apoptotic effects in cells for several tissues *in vitro* (Le Roith et al., 1997) and *in vivo* (Bozyczko-Coyne et al., 1993; Hughes et al., 1993; Mathews and Feldman, 1996; Dudek et al., 1997; Blair et al.,1999).

Morphometric studies have been performed in Tg mice carrying transgenes that are expressed postnatally, only after neuron precursor proliferation has occurred in most CNS regions. Neurons in layer IV of the cerebral cortex in mouse are generated during prenatal development (Hicks and D'Amato, 1968), while apoptotic neurons death occurs predominantly from birth to P10 (Spreafico et al., 1995; Verney et al., 2000). Morphometric analysis of the somatosensory cortex in MT-I/IGF-1 mice revealed a 24% increase in the total numbers of neurons in somatosensory barrels in cortical layer IV by P90. Given that the transgene in MT-I/IGF-1 mice is first expressed after birth (Ye et al., 1995), it would appear that increased neuron number in these Tg adults results from a decreased apoptosis during the regressive phase of neurogenesis.

In a subsequently study (Chrysis et al., 2001) , morphometric analysis of apoptotic cells in the cerebellum, detected by terminal deoxynucleotidyl transferase-mediated UTP nick end labelling (TUNEL), revealed a 47% decrease in Tg mice when compared to controls. Activities of procaspase-3 and caspase-3 were also decreased in Tg mice, accompanied by an increased expression of the antiapoptotic Bcl genes, Bcl-x_L and Bcl-2. In another study (Baker et al.,1999) Bcl-2 was found to be increased in immunihistochemical studies of the cerebellum in Tg mice. Theses results provide direct evidences that elevated IGF-1 acts to inhibit apoptosis during early postnatal development.

During normal development, the final number of neurons to reach the maturity in a given region of the brain is determined by the combine effects of neurons proliferation and naturally occurring neuron death. An unique opportunity to investigate the *in vivo* role of IGF-1 in controlling the final number of neurons and the apoptotic process during postnatal development, was provided by the Tg mice used in O'Kusky et al., 2000. The line of Tg mice used in this study to investigated effects of IGF-1 on neurogenesis in the hippocampal dentate gyrus, overexpress IGF-1 exclusively in the brain during postnatal development: expression of the transgene begin at approximately the time of birth, increases the peak levels at 20-30 days of age, and then, remains constant throughout the life (Ye et al., 1996; Dentremont et al., 1999). In this line of Tg mice O'Kusky et al., 2000 had been observed that increased expression of IGF-1 produces both an increment in cell proliferation both a reduction in apoptosis rate. Such study added to the opposite observations effected from Ni et al., 1997 in dentate gyrus of Tg mice overexpressing IGFBP-1, suggest that IGF-1 produces more neurons in the dentate gyrus by both increasing the rate of neuronal proliferation and decreasing the rate of cell death in this cerebral region, in which neurogenesis is prolonged until adult life.

IGF-1 expression in rodent brain, begins from embrional life (from E14 or before; Bach et al., 1991); it has been expressed also in neural stem cells of adult brain (Brooker et al., 2000). Moreover several regions of rodent CNS show peak of IGF-1 expression from E16 to E21 (Bondy et al., 1991). All these observations suggest an action of IGF-1 also during embrional development of CNS.

In fact, a recently work (Popken et al., 2004) pointed out that IGF-1 plays an important roll in the development of nervous system also more precociously than massive neuronal death phase. Popken and colleagues produced a Tg mice overexpressing IGF-1 prenatally under the control of regulatory sequences from the nestin gene, a cytoskeleton protein, expressed in neural progenitors (Hockfield and McKay, 1985) with expression levels rapidly decreasing as soon as cells differentiate versus a neuronal or glial fate (Zimmermann et al., 1994; Dahlstrand et al., 1995; Matsuda et al., 1996). In this Tg mice embryos at E16, the volume of the cortical plate was significantly increased by 52% and total cell number was increased by 54%. At 12 days of age, Popken et al., 2004 observed significant increases in regional tissue volumes of cerebral cortex (29%), subcortical white matter (52%), caudate-putamen (37%), hippocampus (49%), dentate gyrus (71%) and habenular complex (48%). During embrional and postnatal age, the numerical density of cells did not differ significantly between Tg and control mice brain, meanwhile the

total number of cell is significantly greater in Tg mice brain. IGF-1 embrional expression in these mice starts during the developmental proliferative phase of neurogenesis and before the beginning of apoptosis process. S-phase labelling with 5-bromo-2'-deoxyuridine revealed a 13-15% increase in the proportion of labeled neuroepithelial cells in Tg embryos at E14 suggesting that IGF-1 overexpression accelerates mitosis process. At the same time, the numerical density of apoptotic cells in the cerebral cortex, labeled by antibodies against active caspase-3, was reduced by 26% in Tg mice at P7, showing a significantly reduction of neurons death.

Such results demonstrated that IGF-1 promotes brain development both stimulating proliferation of neural cells in the embryonic CNS and inhibiting their apoptosis during early postnatal life: likely, stimulation of embryonic cells proliferations occur by a mechanism involving reduction of cell cycle length (Popken et al., 2004).

Given that the transgene in nestin/IGF-1 Tg embryos is expressed as early E13 (Popken et al., 2004), these mice provide an unique opportunity to study *in vivo* the role of IGF-1 in controlling cell cycle kinetics in the embryonic brain. Hodge et al., 2004 observed that increased IGF-1 expression in nestin/IGF-1 Tg mice, reduces cell cycle length and augments neural progenitor cells reentry in to the cell cycle during an otherwise normal duration of cortical neurogenesis. In nestin/IGF-1 Tg mice, the proportion of cells reentry in cell cycle is significantly increased and it has been demonstrated that IGF-1 acts specifically to reduce G_1 phase length during cortical neurogenesis, without affecting the length of the G_2, M, and S phases of the cell cycle. Together, these data suggest that cells are retained in the cell cycle during cortical neurogenesis in Tg embryos. As more cells reenter the cell cycle in Tg embryos, the progenitor population expands and the potential for the production of greater numbers of neurons is increased (Hodge et al., 2004).

Morphometric analysis of MT-1/IGF-1cerebral cortex (in particular, somatosensory area), in spite of increment in the total neurons number, showed a significantly reduction in the density of cell bodies (cellule for unit volume): this observation suggest that the volume occupied by neuritic processes of neurons separating single cellular bodies, is greatly increased and it is perfectly concordant with *in vitro* studies demonstrating that IGF-1 promotes differentiation of some neurons populations (Aizeman and DeVellis, 1987; Nataf and Monier, 1992; Fernyhough et al., 1993; Zackenfels et al., 1995; Brooker et al., 2000). Further confirmation arise from similar morphometric analysis in somatosensory cortex of IGFBP-1 Tg mice which showed a decrement in the total number of neurons, respect to an increment of cellular body density (Gutierrz-Ospina

et al., 1996): the reduction in cortical volume of these Tg mice is caused by both a reduction in the neurons number and in neuropil volume.

Synaptogenesis

The effects of elevated IGF-1 expression on synaptogenesis have been investigated using stereological analysis by the light and electron microscope in the hypoglossal nucleus in IGF-1 Tg mice (O'Kusky et al., 2000 and 2003). The hypoglossal nucleus is unlike most other CNS regions that it has been studied in IGF-1 Tg mice because increased volume is not accompanied by an increase in neuron number; so that changes in neuron number would not confound the interpretation of changes in synapse number. The absence of an increase in the number of hypoglossal nucleus neurons is in these Tg mice most likely was due to the fact the proliferation and apoptotic cell death have already occurred before overexpression of IGF-1. Surprisingly, O'Kusky et al., (2003) observed a significant decreases of neuronal density in IGF-1 Tg mice indicating a greater separation of neuronal cell bodies and an increased volume of neuropil. This result is in accordance with Gutierrez-Ospina et al., (1996) which on other lines of Tg mice with increased expression or inhibited actions of IGF-1, found an increase or decrease, respectively, in the volume of neuropil in somatosensory barrels. These findings were further on consistent with multiple in *vitro studies* showing that IGF-1 promotes neuritic outhgrowth form cortical neurons (Aizeman and De Vellis; 1987), hypothalamic neurons (Torres-Alemann et al., 1990), sensory dorsal root neurons (Zackenfels et al., 1995), and motor neurons (Caroni and Grandes, 1990). In organotypic cultured slices of rat somatosensory cortex, IGF-1 stimulates dendritic growth by increasing the number of branching points and the total extent of dendritic segments on cortical neurons (Niblock et al., 2000). These results are confirmed also by the findings that IGF-1 increases the expression of proteins involved in the growth of axons and dendrites, such as α- and β-tubulin, neurofilament proteins, and growth-associated protein 43 in developing neurons (Fernyhough et al., 1989; Wang et al., 1992).

Although O'Kusky et al., 2003 observed that the density of synapses did not change in IGF-1 Tg mice, there were an increases in total synapse number (52%), the synapse to neuron-ratio (51%), and the total cumulative length of myelinated axons within the hypoglossal nucleus (81%). They found that IGF-1 overesxpression promoted the progressive phase of synaptogenesis

by 46% at P21, but it did not alter synapse elimination during the regressive phase after P21. In studies of neuromuscular synapses, the transient peak in muscle IGF-1 gene expression during normal postnatal development has been reported to parallel the progressive and regressive phase of neuromuscular synaptogenesis (reviewed in Ishii et al., 1993). These results indicate that the increased *in vivo* expression of IGF-1 during postnatal development, augments the progressive phase of synaptogenesis, although it does not prevent synapse elimination during the regressive phase, but likely decreased IGF-1 facilitates synapse elimination.

Immunihistochemical studies using IGF-1$^{-/-}$ null mutants have reported abnormal synaptophysin expression in the organ of Corti of the null mutants at P20 (Camarero et al., 2001). The pattern of immunoreactivity in the cell bodies of cochlear ganglion neurons and sensory hair cells in IGF-1$^{-/-}$ mice at P20 more closely resembled controls at P5, indicating the persistence of an immature pattern of synapses distribution in the absence of IGF-1 (D'Ercole et al., 2000).

IGF-1 actions on adult CNS

While IGF-1 role as molecular mediator of CNS development is well established, its involvement in functions of adult brain is less clear. However several data suggest that IGF-1 could be involved in the neuronal plasticity also in the adult life.

In particular IGF-1 affect synaptic efficacy and plasticity, modifying the number and structure of synapses (Cardona-Gomez et al., 2000; Fernandez-Galaz et al., 1999), affecting neurotrophins release and post-synaptic receptors expression or regulating neuron firing and evoked field potentials (for a review see Torres-Alemann, 1999).

In fact IGF-1 administration produces wide neuronal c-fos expression (Carro et al., 2000), increment of BDNF expression in cortex and hippocampus (Carro et al., 2000), stimulation of hippocampal neurogenesis (Aberg et al., 2000; Trejo et al., 2001; Anderson et al., 2002), modulation of NMDA and GABA receptors expression (Ramsey et al., 2004) and an increment in neuronal activity in neurons accumulating IGF-1 (Aberg et al., 2000).

Modulation of neuronal excitability by IGF-1 it has been described, but it has no well understood yet. IGF-1 modulates ionic currents trough L-and N-type calcium channels (Chik et al., 1997; Blair et al., 1997) and modulates the activity of neurotransmitters receptors (Wan et al., 1997; Man et al., 2000; Gonzales del la Vega et al., 2001, Ramsey et al., 2004). Analysis of

cellular mechanisms, mediating the stimulatory effects of IGF-1 on neurons of sensory ganglia in spinal cord, demonstrated that IGF-1 modulates their intrinsic electrophysiological properties and facilitates their synaptic answers (Nunez et al., 2003). In the hippocampus, des-IGF-1 has been shown to increase the field excitatory postsynaptic potentials (fEPSP) slope, appearing to be mediated through a postsynaptic mechanism involving alpha-amino-3-hydroxyl-5-methyl-4-isoxazolepropionate (AMPA), but not NMDA receptors (Ramsey et al., 2005).

Furthermore, IGF-1 can regulate synapse activity by modulating neurotransmitter release and uptake, receptor endocytosis, and signal transduction of several neurotransmitters including glutamate, γ-aminobutyric acid (GABA), noradrenaline, and taurine (for a review see Varela-Nieto et al., 2003). There is a plenty of evidence that IGF-1 affect several of the major neurotransmitters differently in several brain regions, including the monoamine (serotonin, noradrenalin=norepinephrine) system, the gluatamatergic system, the dopaminergic system and the cholinergic system. Many of these effects act directly via neurons and can be observed after only a short exposure.

Interestingly, it has been shown (Bitar et al., 2000) that IGF-1 enhances release of norepinephrine in lumbar motoneurons augmenting the lumbospinal noradrenergic system, while a recent report (Khawaja et al., 2004) has shown that chronic administration of antidepressants venlafaxine and fluoxetine (inhibitors of monoamine reuptake) increased the protein levels of IGF-1 in the hippocampus. In addition, Hoshaw et al., (2005) found that acute administration of IGF-1 has the potential to produce antidepressant behaviour effects in a well-established model of antidepressant activity: the rat forced swim test (FST). In particular the specific increase in swimming behaviour seen after IGF-1 administration, might suggest that antidepressant effects of IGF-1 in FST are mediated by serotonin-dependent mechanisms. In fact, an increase in swimming behaviour in the FST test has been shown to be associated with activation of the serotonergic system since serotonergic compounds, such as the selective serotonin reuptake inhibitors and serotonin receptor antagonist (Detke et al., 1995).

IGF-1 also contributes to synaptic plasticity and the neural mechanism necessary for learning and memory affecting both the glutamatergic and dopaminergic system (for a review see Sonntag et al., 2000). The glutamatergic NMDA receptors are associated with learning and memory (Michaelis, 1998). Analysis of NMDA receptor subtypes indicated that NMDAR1 did not change with age; however subtypes NMDAR2A, 2B and 2C decrease with age in the hippocampus. In particular, NMDAR2A and 2B levels were reversed by administration of IGF-1

for 28 days (Sonntag et al., 2000). Similarly, dopamine D_2 receptors GTPase protein induced activity declines with age and IGF-1 administration increased it (Thornton et al., 1998).

Finally it appears that also cholinergic system has functional link with IGF-1. IGF-1 affects potassium evoked acetylcholine (Ach) release in tissue slices of adult hippocampus and cerebral cortex. In slices from the adult hippocampus, IGF-1 decreases the release of Ach (Araujo et al., 1989). In slices of the adult cerebral cortex, however, IGF-1 appears to increase the release of Ach at a defined concentration window (Nilsson-Håkansson et al., 1993).

IGF-1 supplies also a trophic support to CNS neurons, maintaining in this way their functionality (for a review see Torres-Alemann, 2000). A decrement of this trophic support, may produce the onset of serious neurological diseases. In fact, in several neurodegenerative diseases, both in human and animals, are been observed alterations in serum and cerebral levels of IGF-1 (Busiguina et al., 2000; Trejo et al., 2004). The alteration of IGF-1 levels may be the cause of neuronal damage or it can develop secondarily: both of these two possibilities can occur in relation to the specific pathology. For instance, alterations in IGF-1 synthesis , caused by liver damages, diabetes or conditions, produce decreases in IGF-1 levels causing neurological damage. On the other and, the reduced IGF-1 neuronal sensibility, caused by inflammation (Ventres et al., 1999), excitotoxicity (Garcia-Galloway et al., 2003) or extracellular amiloyd accumulation (Jain et al., 1998; Gasparini et al., 2002; Carro and Torres-Alemann, 2004) could contribute to neuronal death processes.

Thus we can conclude that the alterations in IGF-1 signalling is a common feature of many neurodegenerative diseases. Determining the precise alteration site, it could be possible to discover new useful therapeutic targets. At this aim, it has been recently proposed IGF-1 like the possible therapeutic agents for cerebellar ataxies (Fernandez et al., 2005): IGF-1 treatment seem effective in animal models an clinical trials suggest to further test IGF-1, because it could be the treatment ameliorating the progression of pathology.

IGF-1 mediates the effects of physical exercise on the brain

The Latin aphorism “mens sana in corpore sano” and the evidence accumulating over many decades, well illustrates the beneficial action of physical activity in maintaining and improving neural functions in humans (Kramer et al., 1999) and animals (Fordyce and Farrar, 1991).

Exercise is associated with a sensation of well being and this subjective state has been objective quantified with psychometric, cardiovascular, and neurophysiological data**.** Exercise has been shown to reduce the cognitive decline during ageing (Laurin et al., 2001), to help recover functional loss after CNS damage (Mattson, 2000) and to promote neurogenesis in the adult hippocampus (van Praag et al., 1999). Recent studies links physical activity to diverse indicators of neural function showing the capacity of exercise to induce the increment of specific trophic factors in select brain regions, early response genes, or hippocampal neurogenesis (Neeper et al., 1995; Iwamoto et al., 1996; Gomez-Pinilla et al., 1997). However the mechanism underlying these changes are not yet known (Molteni et al., 2002).

Several works, both in human and in rodents, suggest that IGF-I could be the key molecule modulating brain response to physical exercise. Physical exercise leads to increased serum levels of GH and subsequent, stimulating the growth hormone-IGF-I axis, increased IGF-I levels in serum and also in the brain (Eliakim et al., 2000; Schwarz et al., 1996; Wallace et al., 1999, Carro et al., 2000). Carro et al., 2000 has been shown that in rats, 1 hr of treadmill running induce profuse labelling of different brain areas with IGF-I (cortex, hippocampus, striatum, septum, thalamus, hypothalamus, cerebellum and several brain stem nuclei), whereas non exercised control animals show no brain IGF-1 labeling. The most interesting finding is that IGF-1 administration is able to mimic the effects of physical exercise: both exercise and IGF-1 administration elicits the same pattern of increased c-Fos staining throughout central nervous system and a similar increase in hippocampal expression of BDNF mRNA (Carro et al., 2000). Moreover Carro et al, 2000, give the first evidence that IGF-1 (by means of intracaroid injection) directly modulates neuronal activity *in vivo*. In particular, it has been demonstrated that IGF-1 accumulating neurons such as those of cerebellar cortex and dorsal column nuclei (DCN), had increased sensitivity to afferent stimulation, together with an increased spontaneous firing rate. To determine whether entrance of IGF-1 in the brain is a critical intermediary of exercise actions on the brain, Carro et al., 2000 blocked the uptake of IGF-1 by brain cells before animals were subjected to 1 hr of treadmill running. Chronic administration in to the CSF of anti-IGF-1 antibody plus an IGF-1 receptor antagonist (JB-1) resulted in the blockage of IGF-1 entrance into the brain after exercise and also in blockage of exercise-induced c-Fos staining of brain cells. This paper suggests a new physiological role for IGF-1 through a novel mechanism that includes passage of circulating IGF-1 into the brain, its accumulation by specific group of neurons, stimulation of neuronal expression of c-Fos and BDNF, and long lasting changes in neuronal

activity. The presence of IGF-I receptors in choroid plexus epithelialium (Marks et al., 1991) as well as in endothelial cells of brain capillaries (Frank et al., 1986) supports the possibility that IGF-I of serum enters in to the brain by either crossing the blood-brain barrier interfaces or the blood CSF. Experimental evidences obtained by Carro et al., 2000; suggest that the blood-CSF pathway is the major route used by serum IGF-1 to access the brain. A recent work (Carro et al., 2005) indicates that megalin/low-density lipoprotein receptor-related protrein-2 (LRP2), a multicargo transporter expressed in choroid plexus epithelium at the brain barrier, is involved in transport of serum IGF-1 from the bloodstream in to the CSF and mediated in regulation of Aβ clearance by serum IGF-1. Successive studies from the same research group, reinforce the evidence that circulating IGF-1 mediates the effects of exercise on the brain. Results obtained by Trejo et al. (2001) suggest that circulating IGF-1 is necessary for the observed increase in the number of $BrdU^+$ hippocampal neurons produced by exercise. They found that subcutaneous IGF-1 administration mimics in sedentary rats the increment in the number of $BrdU^+$ hippocampal granule cells , instead infusion of a blocking anti-IGF-1 completely prevents the effects of exercise on neurogenesi.

IGF-1 has been shown to be neuroprotective against the effects of lesions in the adult CNS and to mediate both the neuroprotective effects of physical exercise and possibly of EE on neuronal death (Carro et al., 2001; Koopmans et al., 2006), the enhancement caused by exercise in hippocampal plasticity and in learning and memory (Markowska et al., 1998; Cotman and Berchtold, 2002; Aberg et al., 2006) and the increased brain vessel growth (Lopez-Lopez et al., 2004).

Altogether, exercise affects the CNS via multiple mechanism: both serum IGF-I and possibly locally IGF-I appears to be involved in these processes. Molecular mechanisms by means of IGF-I is able to mediate the effects of exercise on the brain, are not completely known yet, but they should involve a variety of processes supporting an appropriate neural function, ranging from those aimed to fulfill basic metabolic demands to those direct to maintain neuronal plasticity (Torres-Alemann, 2000). Several of the neuroprotective mechanism elicited by IGF-I include modulation of cell-death and enhancement of survival plasticity-promoting molecules in degenerating neurons, such as PSA-NCAM and GAP-43 (Fernandez et al., 1999).

A very interesting point is the complex interplay between IGF-I and BDNF. Physical activity (Cotman and Berchold, 2002) and cognitive stimulation (Young et al., 1999) increases BDNF expression in several brain regions of rodents suggesting a role for BDNF signaling in the

brain multiple benefits of exercise stimulation. It has been suggested that the increase in IGF-1 caused by EE and exercise determines an increase in BDNF (Cotman and Berchtold, 2002). Recently it has been demonstrated (Ding et al., 2006) that IGF-1 affects BDNF system to mediate exercise effects on cognitive processes and synaptic plasticity and that IGF-I enhances the biological activity of BDNF in cerebrocortical neurons (McCusker et al., 2006). These evidences suggest that IGF-I and BDNF may act synergistically in regulating responses of CNS to physical exercise stimulation.

IGF-I play a fundamental role in controlling glucose metabolism and an additional mechanism involved in IGF-I mediated exercise neuroprotection is likely related to enhanced neuronal glucose metabolism (Carro et al., 2001). IGF-I enhances glucose use by neurons through upregulation of glucose transporters and modulation of glycolytic enzymes (Cheng et al., 2000) and stimulated brain glucose metabolism in brain injured animals in a way indistinguishable of exercise (Carro et al., 2001).

Other homeostatic processes involved in IGF-I-mediated exercise effects may include increased angiogenesis and improved handling of oxygen by neurons (Carro et al., 2001). Although the normal adult brain do not show angiogenesis except in response to specific types of insults, exercise stimulates angiogenesis in the adult brain (Black et al., 1990) and IGF-I is involved in angiogenesis in the brain and other tissues (Sonntag et al., 1997; Dunn, 2000). Oxygen availability is also compromised in neurodegenerative conditions involving vascular derangements, and IGF-I is known to induce expression of HIF-1 (Zelzer et al., 1998), a transcription factor central in the cell response to hypoxia. Additional mechanisms by which IGF-I mediates effects of exercise on CNS, could be modulation by IGF-I of neuronal excitability through modulation of membrane ion channels, glutamate receptors or synapse size (Torres-Alemann, 2001).

Therapeutic potential of IGF-1 in neurodegenerative disorders

IGF-1 is considered a good candidate as a neuroprotective treatment in different neurodegenerative diseases, ranging from those of high prevalence, such as Alzheimer disease (AD) or amyotropic lateral sclerosis (ASL) (Lang et al., 2001; Torres-Aleman and Fernandez, 1998) to the less frequent illness such as cerebellar ataxia (Chen et al., 2003; de Pablo et al., 1995; Moll et al., 1993; Peretz et al., 2001).

Alzheimer disease (AD)

AD is the most common type of senile dementia in the elderly. AD is characterized by the presence of amyloid plaques, neurofibrillary tangles and the loss of neurons in defined region of the brain (Hardy, 1997; Wisniewski et al., 1997; Selkoe et al., 1999; Selkoe et al., 2001; Clark and Karlawish, 2003). Preliminary studies in Alzheimer patients reported either high (late-onset AD) or low (familial AD) serum IGF-1 levels (Mustafa et al., 1999; Tham et al., 1993).

Carro and Torres-Aleman, 2004, consider the disrupted IGF-1 signaling the primary event in the late –onset AD and a necessary effector of mutations in familial forms of the disease. IGF-1 increases Aβ clearance from the brain by enhancing transport of Aβ carriers proteins into the brain through the choroid plexus (Carro et al., 2002) and inhibits glycogen synthetase kinase (GSK)-3β, a kinase involved in hyperphosphorylation of tau, the major component of neurofibrillary tangles (Quevedo et al., 2000; Clark and Karlawish, 2003).

Carro and Torres-Aleman hypothesized a dual role for IGF-1 in the development of AD. First, IGF-1 levels decreased in serum during aging, will impair IGF-1 input to choroid plexus and brain vessels. Consequently, Aβ clearance facilitated by IGF-1, which increases transport of β amyloid carriers proteins into the brain trough the choroid plexus (Carro et al., 2002) will be reduced and the brain will accumulate Aβ. Since Aβ compete with IGF-1 receptors, an accumulation of amyloid could reduce IGF-1 signaling to the brain.

Disrupted brain IGF-1 input will originate disturbance at cellular level including lower neuronal resistance to Aβ toxicity, increased susceptibility to other cell death-triggering signals, hyperphosphorylation of tau, insulin resistance and at the tissue level brain accumulation of Aβ (Carro and Torres-Aleman, 2004). Liver-specific deletion of *igf-1* gene prematurely increases cerebral levels of Aβ (Carro et al., 2002). IGF-1 administration to aged rats or to mice over-expressing mutant amyloid decreases the level of Aβ burden in the brain parenchyma (Carro et al., 2002; Carro and Torres-Aleman, 2004).

The primary objective to reach in translating use of IGF-1 from laboratory animals to clinic is the effective delivery of IGF-1 to the brain in sufficient concentrations to influence neuronal functions (Dore et al., 2000; Carrascosa et al., 2004; Gasparini and Xu, 2003).

ASL and cerebellar ataxia

Moto neuron (MN) diseases is a group of neurodegenerative disorders that selectively affect upper and/or lower moto neurons leading to a muscle atrophy and weakness. Amyotropiclateral sclerosis accounts for approximately 80% of all cases of MN diseases (Feldman, 2004).

Accumulating data support the therapeutic use of IGF-1 in the treatment of ASL. IGF-1 prevents apoptosis in MN (Kaspar et al., 2003; Vincent et al., 2004), glial cells (Leinninger et al., 2002), and muscle cells (Singleton et al., 2001) all cell types affected by ASL (Cleveland et al., 2001). IGF-1 serum levels are decreased in ASL patients (Torres-Aleman et al., 1998) and could contribute to the development if the disease (Torres-Aleman et al., 1998; Wilczak et al., 2003). Two placebo-controlled trials of IGF-1 in ASL have produced mixed results. The North American ASL /IGF-1 Study Group found that patients receiving IGF-1 daily for 9 months had slower disease progression and reported a better quality of life than placebo-treated controls (Lai et al., 1997). However, the European ASL /IGF-1 Study Group showed no benfit to IGF-1 therapy (Borasio et al., 1998). Although the sc adminstration of IGF-1 to ALS patients was of uncertain clinical benefits (Lai et al., 1997; Ackerman et al., 1999) the data obtained were sufficiently promising to support other clinical trials.

Another group of neurodegenerative diseases characterized by motor discoordination for which no effective therapeutic treatment exist are cerebellar ataxias (Harding et al., 1993). With the exception of the rare ataxia telangiectasia (AT) (Shimoata et al., 2000), ataxia is associated with low IGF-1 serum both in humans and animals (Backeljauw et al., 2001; Chen et al., 2003; de Pablo and de la Rosa, 1995; Marks et al., 1991; Torres-Aleman et al., 1996). Since the etiology of ataxias is remarkably varied, it appears unlikely that low serum IGF-1 has an etiopathogenic significance. It is more probable that low IGF-1 serum participates in the subsequent pathological cascade, favouring progression of ataxia (Fernadez et al., 2005).

Systemic efficacy of IGF-1 treatment in animal models of cerebellar ataxia (Chen et al., 2003; de Pablo and de la Rosa, 1995; Moll et al., 1993), together with the observation that ataxic animals and different types of human ataxic patients showed altered IGF-1 levels encourage the use of IGF-1 as a possible therapeutic application in this neurodegenerative disease.

It has to be underlined that, both for AD and other neurodegenerative disorders, the major obstacle in developing an IGF-1 therapy is still the purported relationship between serum IGF-1 and increased cancer risk (Beck et al., 1995).

Aim of the thesis

In this PhD thesis I have investigated the molecular mediators of the effects of EE on visual system development. In particular we focussed our attention on insulin-like growth factor 1 (IGF-1), a trophic factor the levels of which are increased by physical exercise (Carro et al., 2001) which is a fundamental component of enriched living condition. IGF-1 is produced mainly by the liver but it is also able to cross blood brain barrier with the possibility to modulate the expression of molecules important for cortical and retinal plasticity such as Nerve Growth Factor (NGF) and Brain Derived Neurotrophic Factor (BDNF), through the binding to IGF-1 receptors present in the occipital cortex (Frolich et al., 1998) and in the retina (Rodrigues et al., 1988; Waldbilling et al., 1998). Moreover IGF-1, a modulator of foetal and neonatal growth (Philips et al., 1988; Popken et al., 2004), has a central role in building the architecture of the retina (Hernandez-Sanchez et al., 1995) and also in normal development of the cortex (for a review see Aberg et al., 2006).

We here show that IGF-1 levels are affected by EE which increases the number of IGF-1 positive neurons in the visual cortex at P18 an accelerates the developmental time course of IGF-1 labelling . This action on IGF-1 levels seems to be the key event mediating EE effects on visual cortical development. Infact increasing IGF-1 in the visual cortex of non-EE rats by means of osmotic minipumps implanted at P18 mimics the effect of EE on the acceleration of visual acuity development, while blocking IGF-1 action in the visual cortex of EE rats by the infusion of IGF-1 receptor antagonist JB1 from P18 completely blocks EE action on visual acuity development.

EE increases IGF-1 expression in the retina at embryonic day 15 (E15), E18 and P1. Intraocular IGF-1 injections in non-EE rats at P1, P4 and P7, accelerates retinal acuity development reproducing the same effects observed in EE animals by Landi and colleagues (2007).

These results show that IGF-1 is a key factor mediating EE effects on visual cortical and retinal development.

We than show that IGF-1 affects GAD65 immunoreactivity in perisomatic innervation and the condensation of Chondroitin Sulphate Proteoglycans (CSPGs) in perineuronal nets (PNNs) in the visual cortex. This suggests that IGF-1 action in mediating EE effects on visual cortical development could be exerted through the modulation of intracortical inhibitory circuitry and PNN development.

At retinal levels IGF-1 administration in non-EE rats elicits the same increase of BDNF expression in RGCs cell layer observed in EE animals, suggesting that IGF-1 is able to trigger the molecular events responsible for EE effects on retinal development.

MATERIALS AND METHODS

Animal treatment

All experiments were performed on rats in accordance with the Italian Ministry of Public Health guidelines for care and use of laboratory animals.

Long Evans hooded rats lived in an animal house with a temperature of 21 °C, 12h/12h light/dark cycle, and food and water available ad libitum. For both housing conditions, matings were made inside the cage. After birth all the litters were housed with their mother until the date of experiment.

Rearing environments. Enriched environment (EE) consisted of large wire netting cages (60X50X80 cm) with three floors containing several foodhoppers, two running wheels (one bigger for adults, the other for post-weaning pups) to improve physical activity, and differently shaped objects (tunnels, shelters, stairs) that were completely substituted with others once a week. Every cage housed at least 4-5 females and their pups. Cages for standard environment (non-EE) were standard laboratory cages (30X40X20 cm) housing one dam with her pups as established by the Italian law for the care of laboratory animals.

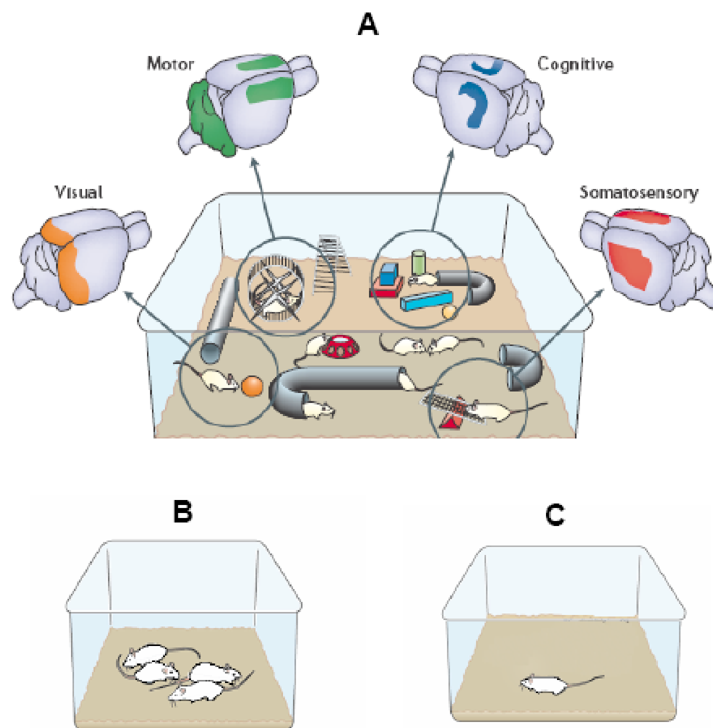


Figure 1: **Living conditions in different experimental groups**.

A. *Enriched environmental condition*. Enriched environment consist of social interaction (6-12 animals in big cages), stimulation of explorative behaviour with different objects and running wheels. Enriched environment rearing promotes neuronal activation, signaling and plasticity throughout various brain regions. Increased sensory stimulation, including increased somatosensory and visual input, activates the somatosensory (red) and visual (orange) cortices. Increased cognitive stimulation (encoding of information relating to spatial maps, objects recognition, novelty and modulation of attention) is likely to activate the hippocampus (blue) and other cortical areas. Enhanced motor activity stimulates areas such as the motor cortex and cerebellum (green) (Nithianantharajah et al., 2006). B. *Standard environmental condition*: The animals are housed in small groups of 2-4 animals in regular size cage without any stimulus objects. C. *Impoverished environmental condition*: The animals are housed individually in regular size cages without any stimulus objects.

IGF-1 and JB1 intracortical administration

Drugs (IGF-1 or JB1) were infused with osmotic minipumps (model 1007D; 0,5µl/h; Alzet, Palo Alto, CA) starting at P18. Minipumps were connected via PVC tubing to a stainless steel 30-gauge cannula implanted 1 mm lateral to lambda of the left visual cortex [44, 45]. IGF-1 (IBT; 1µg/µl) was infused in the visual cortex of non-EE rats (N= 12), while JB1 (Bachem AG; 10

μg/ml) was infused in the visual cortex of EE rats (N= 11), and non-EE rats (N=5). As control, PBS was infused in the visual cortex of non-EE rats (N= 6) and EE rats (N=4). All treatments were made for one week between P18 and P25. We also recorded non-EE animals implanted with JB1 filled minipumps from P18 to P25 (N=4, recordings at P25) and from P21 to P28 (N=4, recordings at P28).

Intraocular injections of IGF-1 or JB1

For the analysis of the role of IGF-1 on retinal acuity development standard rats (N=5) received intraocular injections of IGF-1 (IBT) while EE rats (N=5) received intraocular injections of JB1 (Bachem AG). As control contralateral eye was injected (0.9% NaCl). Injections were performed under ether anesthesia at P1, P4 and P7. IGF-1 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). JB1 concentration was according the increasing size of the eye camera to maintain a final intraocular concentration of the factor equal to 1μg/ ml, demonstrated to be effective for at least three days in vitro (Etgen et al., 2003; Emlinger et al., 1998; Kahlert et al., 2000; Duan et al., 2001). Both for IGF-1 and JB1 the volume injected 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.

Chronic infusions of anti-IGF-I antiserum or IGF to pregnant rats

Timed pregnant rats were reared in either non-EE or EE since the start of gestation. At E10, anti-IGF-1 antiserum or IGF-1 protein were infused to EE and non-EE pregnant rats, respectively. It has been reported that the anti-IGF-1 antibody has <1% cross-reactivity with either insulin or IGF-II, as determined by competition with ^{125}I-IGF-1 (Trejo et al., 2001). Infusions were done through implantation of a subcutaneous osmotic minipump (Alzet; anti-IGF-1 infusion: 20% in saline; IGF-1 protein infusion:1 μg/μl; infusion rate: 0.25 μl/h in both cases) placed in the back of the animal in the scapula (Carro et al., 2000). Qualitative observations

performed during both daytime and during the dark phase of the daily cycle revealed an apparently normal behavior of implanted pregnant rats. In particular, EE pregnant rats were frequently observed to use the running wheel. At E18, pregnant rats were perfused transcardially and their embryos were removed through surgical hysterotomy. The eyes of the embryos were removed, fixed and processed for RGC apoptosis analysis (N=13 animals for EE, N=20 animals for non-EE) and IGF-1 expression levels (N=7 animals for EE, N=5 animals for non-EE), as previously described. Examination of histological brain sections revealed no signs of malformations or gross morphological abnormalities in both anti-IGF-1 and IGF-1 embryos.

Electrophysiological assessment of cortical and retinal acuity

A total of 46 rats were VEPs recorded [non-EE: N=7; EE: N=4; non-EE treated with IGF-1 (IGF-1): N=5; non-EE treated with vehicle (PBS): N=6; EE treated with JB1(EE-JB1): N=5; EE treated with vehicle (EE-PBS): N=4; non-EE treated with JB1: N=8, non-EE intraocular injected with IGF-1: N=4; non-EE intraocular injected with saline, N=3] was used for electrophysiology. A total of 20 rats were PERG recorded [EE: N=5; non-EE: N=5; non-EE intraocular injected with IGF-1(IGF-1) in one eye and with saline (saline) in the contralateral eye: N=5; EE intraocular injected with JB1 (EE-JB1) in one eye and with saline in the other eye (non-EE saline)].
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 anesthesia 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=25 candles/m^2; area, 24X26 cm) placed 20 cm in front of the animal. Recordings were always made in blind in relation to the animal's rearing condition to avoid subjective judgements of the experimenter.
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 (Oc1B**;** Paxinos and Watson, 1986) in correspondence of the vertical meridian

representation and advanced 100 or 450 µm within the cortex. At these depths, VEPs had their maximal amplitude. 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.

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 randomly 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 (Berardi 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.

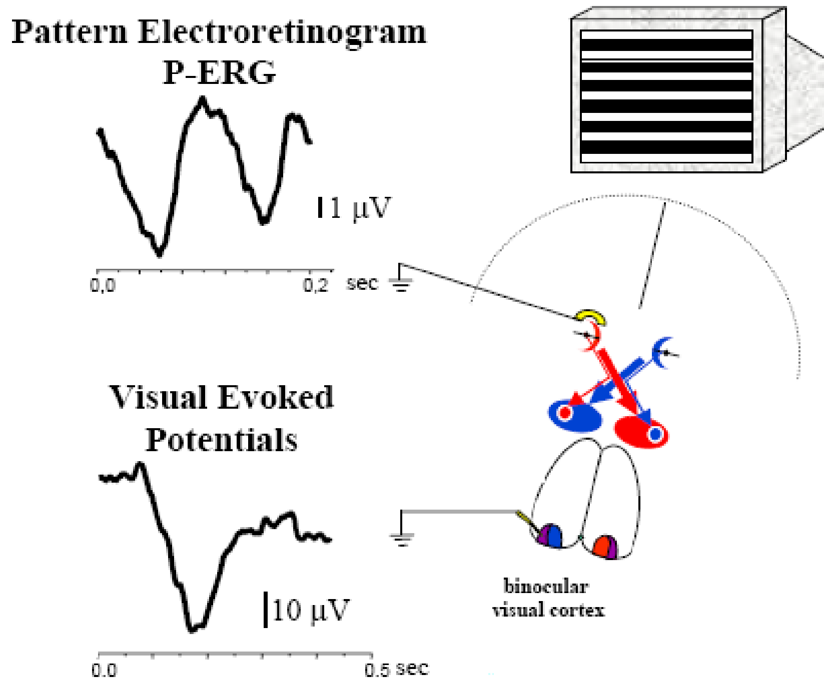


Figure 2: **Schematic representation of P-ERG and VEP recording**

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.

Immunohistochemistry on visual cortical sections

A total of 80 (EE: N= 35; non-EE: N= 45) Long Evans hooded rats aged between P15 and P25 were employed (P15, EE: N= 6, non-EE: N= 5; P18, EE: N= 10, non-EE: N= 12; P21, EE: N= 6, non-EE: N= 6; P25, EE: N= 13; non-EE: N= 22). Animals were deeply anesthetized with chloral hydrate and perfused transcardially with PBS 1X followed by fixative (4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.4; PB). Brains were removed, post-fixed in the same fixative at 4°C,

cryoprotected by immersion in 30% sucrose with 0,01% sodium azide solution in PB at 4°C and frozen by isopentane. 35 μm coronal sections were cut on a microtome and processed for immunohistochemistry. Free floating sections were incubated for 1-2 hours in a blocking solution (containing 10% BSA, 0,3% Triton X-100 in PBS or 3% BSA in PBS for WFA staining) followed by incubation with the appropriate antibodies.

For IGF-1 we used rabbit polyclonal anti-IGF-1 antibody (1:500 in 1% BSA, 0,2% Triton; antibody kindly provided by I. Torres-Aleman) revealed with biotinylated secondary antibody goat anti-rabbit IgG (1:200, Vector Laboratories, Burlingame, CA) followed by fluorescein-conjugated extravidin (1:300, Sigma). For NeuN we used Chemicon, MAB377, (1:500, in 1% BSA, 0,2% Triton) revealed with Alexa 568 (Molecular Probes, 1:400). For GAD67 we used mouse anti GAD67 (Chemicon, MAB5406, 1:1000 in 1% BSA, 0,3% Triton) revealed with Alexa 568 (Molecular Probes, 1:400). For WFA staining sections were incubated overnight at 4°C in a solution of biotinylated Wisteria floribunda lectin (WFA) (1:100, Vector). WFA was stained with 1h incubation in fluorescein-conjugated extravidin (1:300, Sigma). For GAD65 we used monoclonal antibody anti GAD65 (Chemicon, MAB351, 1:500, in 1% BSA, 0,2% Triton) revealed with biotinylated secondary antibody goat anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA) followed by incubation in fluorescein-conjugated extravidin (1:300, Sigma). For IGF1-R we used polyclonal anti-IGF-1R antibody (C-20, sc-713, Santa Cruz, 1:50 in 1% BSA, 0,2% Triton) revealed with Alexa 488 (Molecular Probes, 1:400) for IGF-1R/GAD67 double staining or with Alexa 568 (Molecular Probes, 1:400) for IGF-1R/WFA double staining. Sections were then mounted on slides with Vectashield.

IGF-I immunoreactivity analysis

At all ages, images were acquired with a confocal Olympus microscope at 40 X magnification, (N.A.=0,85 field 353 x 353 μm acquired at 1024x1024 pixels) to analyze the colocalization of antigens and at 20 X (N.A.=0,7 field 707 x 707 μm acquired at 1024x1024 pixels) to analyze the number of IGF-1 and NeuN positive cells in sections double labelled for IGF-1 and NeuN. To compare different specimens, the parameters of acquisition were optimized at the start and then held constant throughout image acquisition. The collected images from Oc1B cortical fields were imported to the image analysis system MetaMorph. For each animal, at least three Oc1B sections were analyzed. At each age, counts were done on the entire thickness of Oc1B. The number of

IGF-1 positive cells was normalized to the number of NeuN positive cells both in EE and non-EE animals. The number of NeuN positive cells was also compared between EE and non-EE rats for each age. To identify whether the IGF-1 positive cells were neurons, the number of double labelled IGF-1 and NeuN positive cells was counted and, for each age and housing condition, was normalized to the total number of IGF-1 positive cells. To identify the proportion of IGF-1 positive cells which are neurons the number of double labelled IGF-1 and NeuN positive cells was counted and normalized to the total number of NeuN positive cells at P18. To identify whether IGF-1 positive cells were excitatory neurons or inhibitory interneurons and to assess whether EE affected the number of IGF-1 positive inhibitory interneurons, double labelling for IGF-1 and GAD 67 was performed at P18 in EE (N=4) and non-EE animals (N=3). For each animal at least 3 Oc1B sections were analyzed; acquisition were done at 40 X magnification, zoom 1 X (N.A.=0,85 field 353 x 353 μm acquired at 1024x1024 pixels). The number of double labelled cells was counted on the entire Oc1B thickness. All image acquisition and analysis were carried out in blind.

WFA quantification

Images were acquired at 20 X (N.A.=0,7, field 707 x 707 μm acquired at 1024x1024 pixels). To compare different specimens, the parameters of acquisition were optimized at the start and then held constant throughout image acquisition. For each animal at least 6 Oc1B sections were analyzed (three for IGF-1 or JB1 treated cortex and three for PBS contralateral cortex). The collected images from Oc1B cortical fields were imported to the image analysis system MetaMorph. Counts were done on the entire thickness of Oc1B (mosaic of three 20X images) and the ratio WFA-positive cells/ NeuN positive cells was calculated. All images acquisition and analysis were carried out in blind.

GAD65 puncta rings quantification

Images were acquired at 60X (N.A.=1,40 field 105 x 105 μm acquired at 512 x 512 pixels). Settings for laser intensity, gain, offset and pinhole were optimized initially and held constant through the study. During image collection, confocal settings were regulated so that the full range of pixel intensities (0-255) was used, with very little saturation at either end of intensity range. For each animal at least six sections (three from IGF-1 or JB1 treated cortex and three

from PBS contralateral cortex) were analyzed. For each section, we imaged six field taken from layer 2/3 of the primary visual cortex. In each field, a stack of ten GAD65 optical sections separated by 1um was collected at the top face of the tissue section. The image within each stack with the highest average pixel intensity was selected for the quantitative analysis of GAD65 immunoreactivity (Silver et al., 2000, Tropea et al., 2001). Perisomatic GAD65 signals ("puncta-ring") from at least three target neurons were outlined for each image and GAD65 signal intensity was calculated (Methamorph). For each neuron, signal intensity were divided by the background labelling in the cell soma. A total sample of 40-50 neurons were analyzed for each cortex. All images acquisition and analysis were carried out in blind.

Double labelling IGF-1 receptor and GAG67 or IGF-1 receptor and WFA

To assess the presence of IGF-1 receptor on inhibitory interneurons and on PNN surrounded neurons in the time window of our treatment, double labelling for IGF-1 receptor and either GAD 67 or WFA was performed in non-EE animals at P18 in (N=4) and at P25 (N=4). For each animal at least 3 Oc1B sections were analyzed; acquisition were done at 40 X magnification, zoom 1 X (N.A.=0,85 field 353 x 353 µm acquired at 1024x1024 pixels). The collected images from Oc1B cortical fields were imported to the image analysis system MetaMorph. For each animal, at least three Oc1B sections were analyzed. At each age, counts were done on the entire thickness of Oc1B. The number of double labelled cells was counted on the entire Oc1B thickness. All image acquisition and analysis were carried out in blind.

IGF-1 immunohistochemistry on retinal sections

For IGF-1 immunostaining, vertical retinal sections (25 µm thick) and cerebellar sections (40 µm thick) were cut using a cryostat and then processed as follows. Sections were permeabilized in 0,3% triton X-100 and incubated in 1:500 rabbit polyclonal anti-IGF-I antibody (kindly provided by Prof. Ignacio Torres-Aleman). Bound antibody was detected by incubating sections with biotinylated goat anti-rabbit IgG (1:200, Vector) followed by fluorescein-conjugated extravidin (1:300, Sigma). The number of animals used for IGF-I analysis in the retinas was: 10 (E15), 7 (E18), 7 (P1), 4 (P10), for EC; 6 (E15), 5 (E18), 7 (P1), 4 (P10), for SC. Immunostaining of enriched and control retinal sections was performed in parallel within the same experimental set. Images of RGC portions were acquired at 20x magnification using a Zeiss

HR Axiocam videocamera connected to a Zeiss Axiophot microscope and digitalized by Axiovision software. To compare different specimens, the time of exposure was optimized at the start and then held constant throughout image acquisition. Collected images were imported to the image analysis system MetaMorph and used to evaluate pixel intensity of cellular immunofluorescence. All image analyses were done blind. IGF-1 pixel intensity was measured within the area contoured by the edges of the RGC layer. Pixel intensity values were divided by background level, measured in the outer nuclear layer. Values obtained from at least 8-10 retinal fields were used to calculate the average pixel intensity value per animal.

Analysis of pyknotic cells

To take the eyes from E15 and E18 embryos, pregnant dams were anesthetized with chloral hydrate, perfused through the hearth with 4% paraformaldehyde in 0,1 phosphate buffer (pH 7,4) and the embryos were removed after surgical hysterotomy. To take the eyes from P1 pups, rats were perfused through the hearth with 4% paraformaldehyde in 0,1 phosphate buffer (pH 7,4). The eyes of E18 and P1 rats were fixed in 4% paraformaldehyde for 24 h. Retinas (E18: N=19 for EE and 10 for non-EE; P1: N=25 for EE and 12 for non-EE; retinas derived from at least two littermates per experimental group) were then dissected from the eyes, flattened on gelatinized slides and fixed with 2,5% glutaraldehyde and then with formalin-ethanol solution (1:9). Whole-mounted retinas were stained with cresyl violet (0,1%). The number of pyknotic profiles was counted following a "blind procedure" in the RGC layer of 60 fields (80x80 mm) per retina on average, uniformly distributed across the retinas. The proportion of retina sampled in this way ranged from 2.1 to 13.8%. Pyknotic cells were counted at 100x magnification using a Zeiss computerized microscope (software, Stereo Investigator, Microbrighfield). Pyknotic cells were identified by the presence of darkly and uniformly stained nuclei, sometimes fragmented. When two or more fragments were seen within a cell diameter distance from each other, they were counted as a single pyknotic cell. Total number of pyknotic cells per retina was estimated by multiplying the average number of cells per field times the ratio of the total area of each retina to field area. For microglial cell number analysis, the *Griffonia simplicifolia* lectin labeling was performed on whole-mount retinas of P1 rats (EC, n = 6; SC, n = 6). Retinas were incubated overnight in B4 isolectin biotinylated (0,025 mg/ml, Sigma). Bound lectin was revealed by ABC kit (Vector) and nickel-enhanced diaminobenzidine (DAB) reaction. Microglial cells were

counted at 100x magnification. The total number of microglial cells per retina was estimated using the same counting procedure described before.

Analysis of natural cell death with the Tunel method

Since the newborn rat ganglion cells are stacked in a pseudostratified fashion and it is difficult to detect the border between the RGC layer and the inner plexiform layer (Perry et al., 1983), we cannot exclude the possibility to have counted pyknotic profiles in both of these layers with the cresyl violet staining procedure. Therefore, we repeated the analysis on natural cell death using the Tunel method, analyzing also levels of apoptosis at E20. In this analysis, counterstaining of retinal vertical sections with the nuclear marker TOTO (see below) allowed us to clearly visualize the position of fragmented nuclei in different retinal layers.

To detect DNA fragmentation in RGC layer dying cells, terminal deoxynucleotidyl transferase mediated dUTPNick.End Labeling (TUNEL) technique was employed, using a commercially available kit (DeadEnd^{TM}Fluorometric Tunel System, Promega). Eyes were immersion-fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded in Tissue-Tek. Retinal sections of 18 μm were cut using a cryostat and collected in serial order through the entire retina. After treatment with proteinase K (20 μg/μl) to dissociate proteins from DNA, sections were incubated (1h at 37 °C) with "the Tunel reaction mixture", containing the TdT enzyme and Nucleotide mix with fluorescein-12-dUTP. Retinal sections (n = 4 SC and EC rats for each age) were then counterstained with TOTO-3 iodide (Molecular Probes) to visualize the different cell layers, and rinsed in PBS. In the negative controls, which never gave any significant staining, the Tunel reaction mixture was omitted. Tunel-positive cells were counted with "a blind procedure" by the use of a 40x objective, in the RGC layer of 10 equally spaced sections per retina. Each retinal section was completely sampled. The total number of cells per retina displaying fragmented nuclei in the RGC layer was calculated by multiplying the average number of labeled cells per section times the total number of retinal sections. The morphological appearance of retinal layers was indistinguishable between EE and non-EE rats.

BDNF immunohistochemistry

A total of 14 (non-EE treated with IGF-1 (IGF-1): N=7; EE treated with JB1 (EE-JB1): N=7) Long Evans hooded rats, aged P10, were employed. Animals were deeply anesthetized with chloral hydrate and perfused transcardially with PBS 1X followed by fixative (4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.4; PB). Eyes were kindly removed, postfixed in 4% paraformaldehyde in 0.1 phosphate buffer (pH=7.4) and cryoprotected in 30% sucrose. Vertical retinal sections (25 µm thick) were cut using a cryostat and then processed as follows. After a blocking step, sections were permeabilized in 0,01% triton X-100 and incubated in 1:400 anti-human BDNF antibody (Promega). Bound antibody was detected by incubating sections with byotinilated rabbit anti-chicken IgG (1:200, Promega) followed by fluorescein-conjugated extravidin (1:300, Sigma). Images of RGC portions were acquired with a confocal Olympus microscope at 20 X magnification (N.A.=0,7 field 707 x 707 µm acquired at 1024x1024 pixels). Settings for laser intensity, gain, offset and pinhole were optimized initially and held constant through the experiment. Then, the collected images of the retina were imported to the image analysis system MetaMorph and used to evaluate pixel intensity of cellular immunofluorescence. For each animal, at least 24 retinal sections (12 of the IGF-1 or JB1 treated eye and 12 for saline treated eye) were analyzed. 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). To compare different experimental sets of himmuhistochemistry the BDNF immunofluorescence value obtained, was further normalized to the value of BDNF intensity estimated in the RGC layer of retinas taken from non-EE rats intraocularly injected with saline.

Determination of IGF-1 concentration in maternal milk

Milk samples were collected from P1 and P10 suckling pups. Pups (P1: N=15 for both EE and non-EE; P10: N=6 for both non-EE and EE groups) were killed between 9 and 10 a.m. through decapitation and the gastric content was quickly removed, weighed and frozen at –80°C until assayed. Milk samples were homogenized with distillate water and centrifuged at 14000 rpm at 4°C for 30 min to separate the whey (infranatant) from the fat (supernatant) and casein (pellet).

The whey milk was acid-ethanol extracted to remove IGF-I binding proteins. The concentration of IGF-I was determined by radio immunoassay (RIA) using a commercial kit specific for rodents (DSL-2900, Diagnostic Systems Laboratories, Webster, TX), with a sensitivity of 21 ng/ml.

RESULTS

PART I: IGF-1 mediates the effects of enriched environment on visual cortical development

EE affects the developmental time course of IGF-1 labelling in the visual cortex

Several works analysed IGF-1 mRNA expression and protein levels in the central nervous system during development (Rotwein et al., 1988; Bondy et al., 1991); however, no data are available on IGF-1 presence in the developing visual cortex. We have therefore assessed IGF-1 levels in the visual cortex between P15 and P25 and then evaluated EE effects on this developmental time course. The period P15-P25, which follows eye opening, marks the beginning of the experience dependency of rat visual cortical development and the opening of the critical period for ocular dominance plasticity (Fagiolini et al., 1994; Berardi et al., 2000).

IGF-1 protein was revealed by means of an immunohistochemical protocol repeatedly used to analyse IGF-1 presence in the central nervous system (Carro et al., 2000; Trejo et al., 2001; Carro et al., 2001). Typical appearance of IGF-1 positive cells is shown in Fig. 3A.

At all ages, the great majority of IGF-1 positive cells are neurons (Fig. 3B) both in non-EE and EE rats (Two Way ANOVA, housing x age, factor housing $p = 0,16$, factor age $p < 0,001$, no significant interaction age x housing). The fraction of IGF-1 and NeuN double labeled cells is: at P15, EE: $0,74 \pm 0,02$ N = 5; non-EE: $0,8 \pm 0,04$ N = 4; at P18, EE: $0,89 \pm 0,03$ N = 4; non-EE: $0,88 \pm 0,02$ N = 5; at P21, EE: $0,78 \pm 0,03$ N = 3; non-EE: $0,79 \pm 0,02$ N = 4; at P25, EE: $0,79 \pm 0,04$ N = 3; non-EE: $0,83 \pm 0,02$ N = 4. We quantified the presence of IGF-1 in the visual cortex at different developmental ages in terms of the number of IGF-1 positive cells normalized to the number of neurons (NeuN positive cells; Fig. 4A).

The normalized number of IGF-1 positive cells increases between P15 and P21 in non-EE rats (Two Way ANOVA, housing (two levels) x age (four levels), factor age significant, $p < 0,001$, normalized number of IGF-1 positive cells at P21 in non-EE rats 0,44 ± 0,02 (N = 6) differs from the value at P15, 0,3 ± 0,02 (N = 5) and P18, 0,37 ± 0,02 (N = 8); *post-hoc* Tukey's test, $p < 0,05$). In EE rats the normalized number of IGF-1 positive cells at P18 (0,44 ± 0,01, N = 10) is higher than at P15 (0,32 ± 0,01 (N = 6)) and is increased with respect to P18 non-EE rats (Fig. 4A and B) (Two Way ANOVA, interaction housing age significant, $p = 0,011$, housing within P18, normalized number of IGF-1 positive cells in P18 EE rats differs from P18 non-EE rats, age within EE, EE P18 differs from EE P15; *post-hoc* Tukey's test, $p < 0,05$). An example of the effect of EE on the number of IGF-1 positive cells at P18 is reported in Fig. 4B. The increase in IGF-1 positive cells caused by EE at P18 is due to the increase in IGF-1 positive neurons: indeed, the number of neurons positive for IGF-1 (cells double labelled for NeuN and IGF-1) normalized to NeuN positive cells is significantly different between EE and non-EE rats at P18 (t-test, $p = 0,015$).

At no age the number of NeuN positive cells is increased by EE with respect to non-EE animals (Two Way ANOVA, age x housing, factor age not significant, $p = 0,345$, factor housing not significant, $p = 0,457$); therefore, the increase in the density of IGF-1 positive neurons caused by EE is due to an increased presence of IGF-1 labeled neuronal cells, not to an increase in the density of neurons.

Thus, EE accelerates the developmental time course of IGF-1 protein levels in the visual cortex.

At P18, a double labelling was also performed for IGF-1 and GAD67 to identify whether the IGF-1 positive cells were inhibitory or excitatory neurons. The density of IGF-1 positive inhibitory interneurons is significantly increased by EE (23% ± 3,8% in non-EE rats and 37% ± 2,6% in EE rats, t-test, $p = 0,03$). Thus, EE affects IGF-1 labelling both for excitatory and inhibitory neurons in the developing visual cortex.

EE is known to affect different brain areas in the adult (van Praag et al., 2000). To determine whether the effects of our enrichment protocol on IGF-1 levels documented for the visual cortex were due to a generalized increase of IGF-1 levels in the brain, we assessed the density of IGF-1 positive cells in S1 of P18 EE and non-EE rats. We found that the increment in IGF-1 positive cells observed in the visual cortex of P18 EE animals is absent in S1 [number of

IGF-1 positive cells/field in EE rats 697 ± 32 (N = 3) and in non-EE rats 654 ± 49 (N = 4), t-test, p= 0,522].

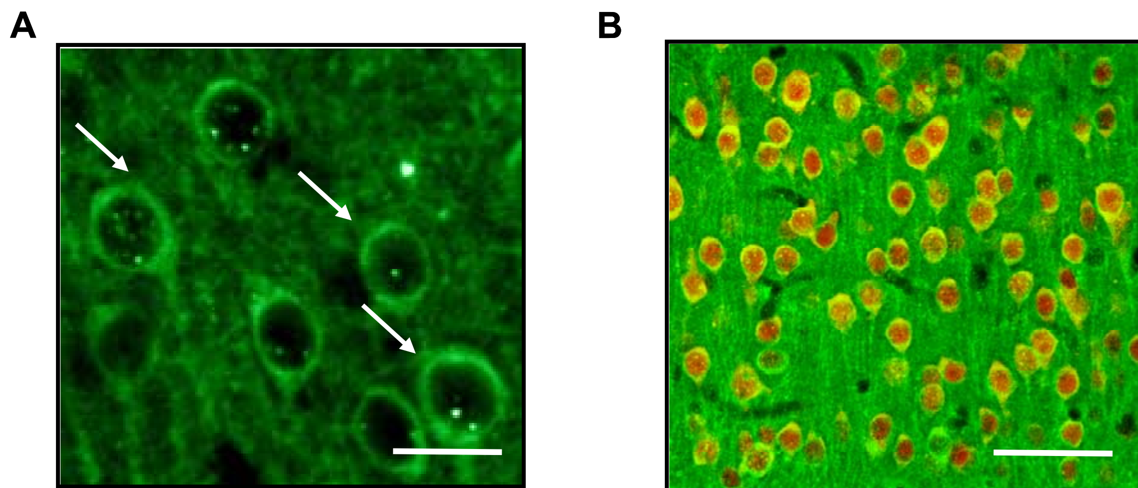


Figure 3: **Immunoreactivity for IGF-1 in the developing visual cortex.**
(**A**) Typical appearance of IGF-1 positive cells in the developing rat binocular visual cortex Oc1B. Age of the animal, P25, calibration bar 25 µm. (**B**) Example showing the preponderance of the neuronal phenotype in IGF-1 positive cells in the developing rat binocular visual cortex Oc1B. Age of the animal P18. Staining for IGF-1 green, staining for NeuN (neuronal marker) red, merged image. Calibration bar: 50 µm.

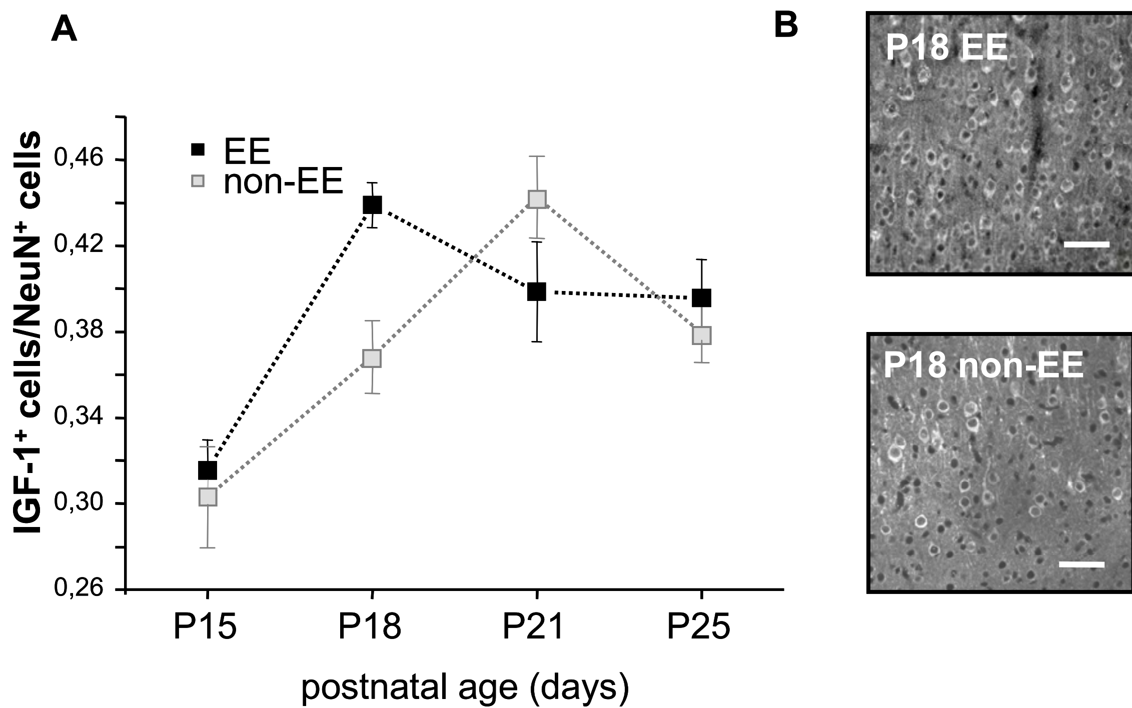


Figure 4: **Immunoreactivity for IGF-1 in the developing visual cortex: effects of enriched environment.**

(**A**) Mean number of IGF-1 positive cells in the visual cortex, normalized to the number of neurons (Neu N positive cells) for each developmental age analysed. Black dots are data from EE rats and light grey dots data from non-EE rats. Vertical bars represent SEM. The number of animals analyzed is: for non-EE rats, N = 5 at P15, N = 8 at P18, N = 6 at P21, N = 6 at P25; for EE rats, N = 6 at P15, N = 10 at P18, N = 6 at P21, N = 7 at P25. The normalized number of IGF-1 positive cells increases between P15 and P21 in non-EE rats (Two Way ANOVA, housing (two levels) x age (four levels), factor age significant, $p < 0,001$; *post-hoc* Tukey's test, $p < 0,05$). In EE rats the normalized number of IGF-1 positive cells increases significantly between P15 and P18; at P18 the normalized number in EE rats is significantly increased with respect to non-EE rats (Two Way ANOVA, housing (two levels) x age (four levels), factor age significant, $p < 0,001$, interaction housing x age significant, $p = 0,011$; *post-hoc* Tukey's test, $p < 0,05$). Vertical bars are SEM. (**B**) Example of IGF-1 labelling from fields taken in the layers II/III of the rat visual cortex of one P18 EE and one P18 non-EE rat. It is evident the increase in IGF-1 positive cells caused by EE. Calibration bar: 50 µm.

IGF-1 administration in the visual cortex accelerates visual acuity development

We assessed whether an increase in IGF-1 levels in the visual cortex of non-EE rats from P18 to P25, achieved by infusing exogenous IGF-1 in the visual cortex, mimics EE effects on visual acuity development. IGF-1 (1µg/µl) was infused by a minipump connected to a cannula implanted 1 mm lateral to lambda (Pizzorusso et al., 1999; Lodovichi et al., 2000; Di Cristo et al., 2001). Visual acuity was assessed by VEP recordings at P25, after a week of IGF-1 treatment (Fig. 5, 6). As control, animals implanted at P18 with PBS filled minipumps and recorded at P25 were used. Two other groups of animals were recorded at P25, animals reared in standard cages (non-EE rats) and animals reared from birth in enriched cages (EE rats), to compare the effects of IGF-1 infusion with those of EE.

The diffusion of IGF-1 to the binocular portion of the primary visual cortex (Oc1B), where VEPs were recorded from, was assessed immunohistochemically at P25 in 4 animals infused with IGF-1 from P18 evaluating the number of IGF-1 positive cells in Oc1B; for comparison, the contralateral cortex, infused with PBS, was used. We found that the density of IGF-1 positive cells (number of IGF-1 positive cells divided by number of neurons (NeuN positive cells) is significantly increased in the IGF-1 treated Oc1B with respect to the contralateral Oc1B (paired t-test, $p < 0,05$) and the number of NeuN positive cells is not increased (paired t-test, $p = 0,884$). A higher IGF-1 labeling was still visually detectable in visual areas more lateral than Oc1B.

We found that IGF-1 accelerates visual acuity development (Fig. 5, 6); indeed visual acuity of P25 IGF-1 treated animals (0,9 ± 0,08 c/deg, N = 5) is significantly higher than non-EE vehicle treated (0,67 ± 0,03 c/deg, N = 6) or untreated animals (0,63 ± 0,01 c/deg, N = 7) (One Way ANOVA, $p < 0,001$; *post-hoc* Tukey's test, $p < 0,01$ for IGF-1 vs vehicle treated rats and vehicle treated vs EE rats; $p < 0,001$ for IGF-1 treated vs non-EE rats and non-EE vs EE rats); the latter two do not differ (One Way ANOVA $p < 0,001$, *post-hoc* Tukey's test $p > 0,05$; Fig. 6B); the effects of IGF-1 treatment are comparable with those produced by EE: visual acuity of P25 IGF-1 treated rats does not differ from that of P25 EE rats (0,93 ± 0,03 c/deg, N = 4, One Way ANOVA $p < 0,001$, *post-hoc* Tukey's test $p > 0,05$; Fig. 6B).

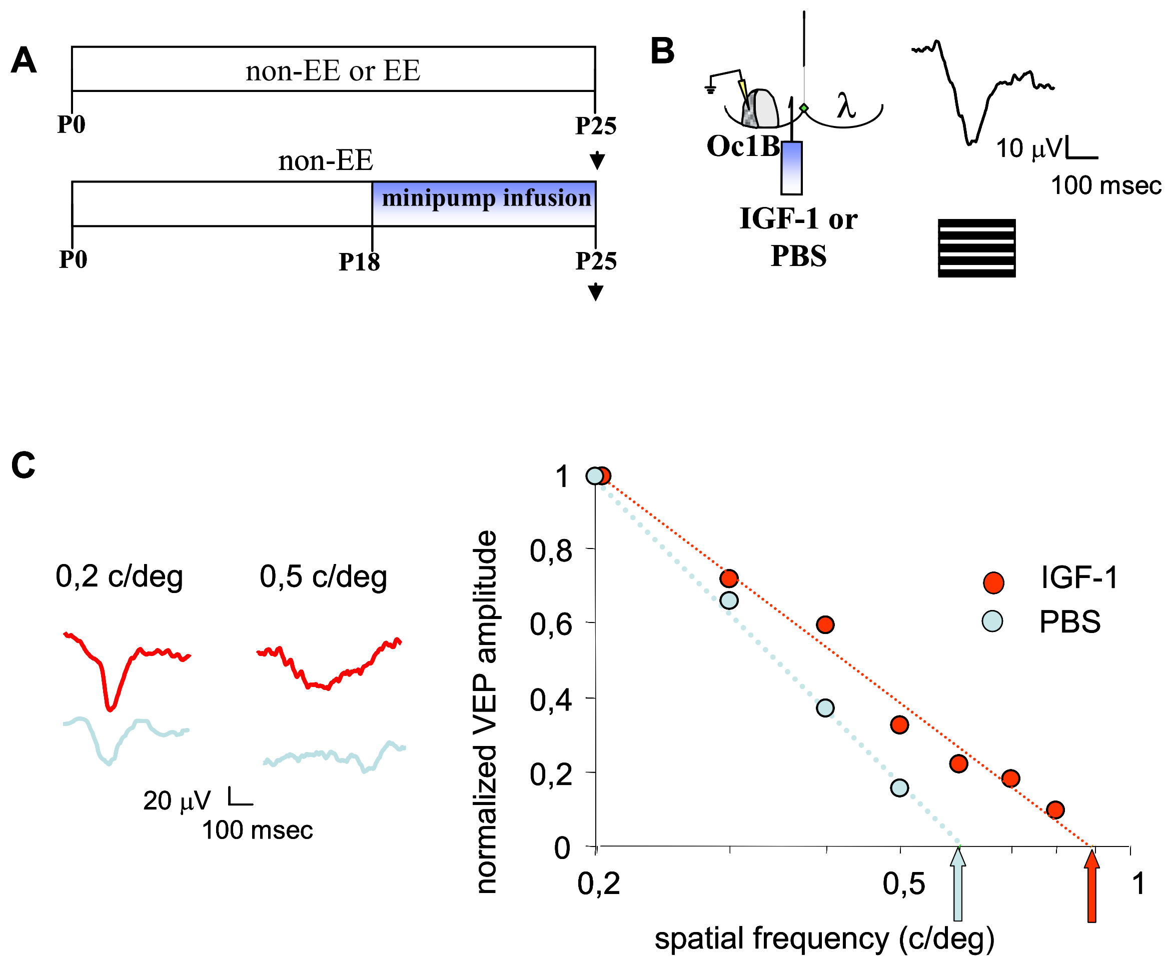


Figure 5: **IGF-1 administration in the visual cortex accelerates visual acuity development**. (A) Experimental protocol. (B) Left, schematic representation of minipump implant and recording site for Visual Evoked Potentials (VEPs). Right, representative waveforms of VEP recorded from Oc1B in response to visual stimulation with gratings sinuosoidally modulated in contrats at 1 Hz. (C) Example of visual acuity estimated in one IGF-1 (red) and one vehicle (light blue) treated animal. Experimental points are VEP amplitudes normalized to the mean amplitude of VEP at 0,2 c/deg; thick lines are linear fits to the data. Estimated visual acuities (arrows) are taken as the extrapolation to 0 level of the fitting line. Waveforms above the graph are the VEP recordings obtained at 0,2 and 0,5 c/deg for the IGF-1 (red) and the vehicle (PBS) treated animal (light blue). It is evident that at the higher spatial frequency response is obtained only in the IGF-1 treated rat.

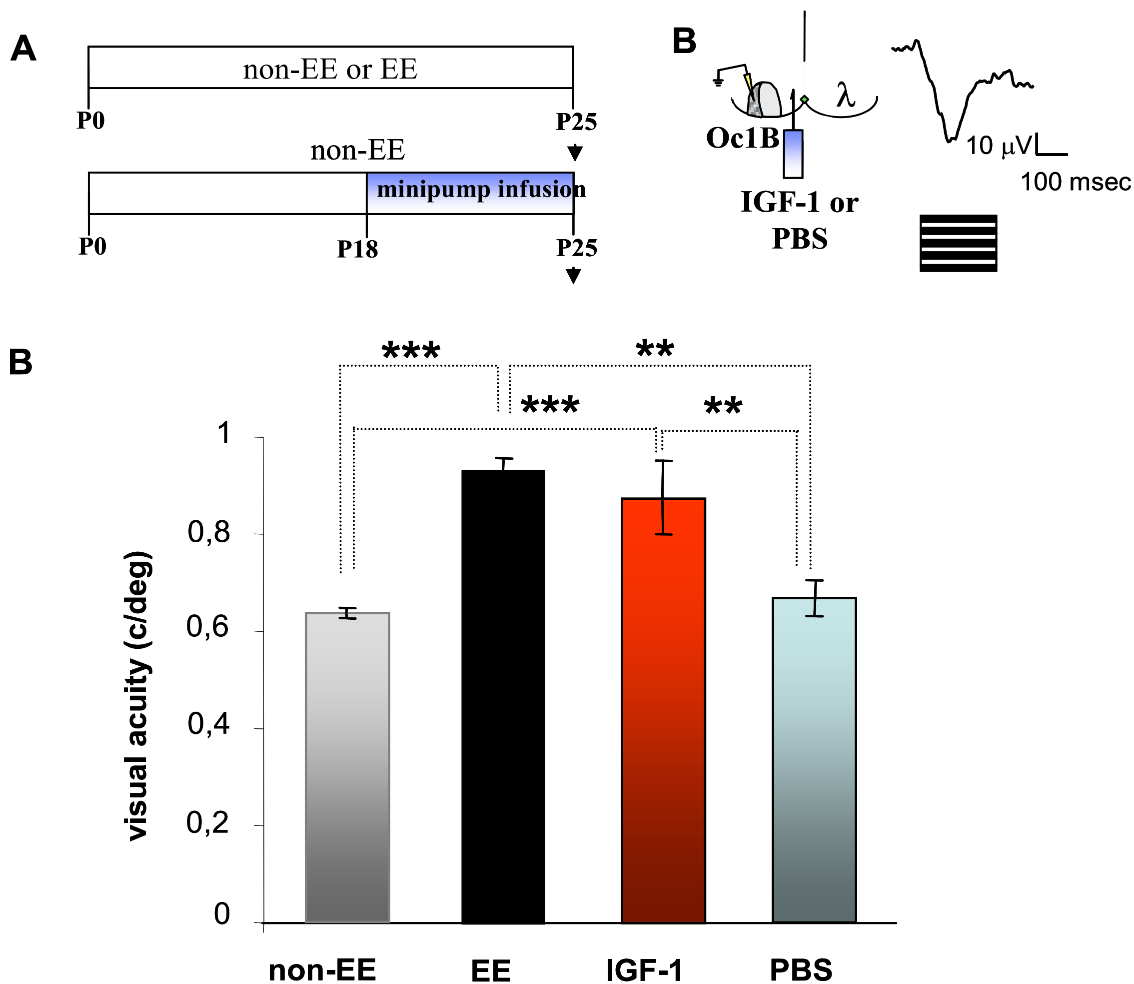


Figure 6: **IGF-1 administration in the visual cortex accelerates visual acuity development.** (A) Experimental protocol. (B) Left, schematic representation of minipump implant and recording site for Visual Evoked Potentials (VEPs). Right, representative waveforms of VEP recorded from Oc1B in response to visual stimulation with gratings sinuosoidally modulated in contrats at 1 Hz.(C) Summary of visual acuity in all groups. Data are mean visual acuity and vertical bars represent SEM. Visual acuity of non-EE IGF-1 treated animals (IGF-1, 0,9 ± 0,08 c/deg, N = 5) is significantly higher than in non-EE vehicle treated animals (PBS, 0,67 ± 0,03 c/deg, N = 6) or in non-EE untreated animals (non-EE 0,63 ± 0,01 c/deg, N = 7); the latter two do not differ (One Way ANOVA, p < 0,001; *post-hoc* Tukey's test, significance level 0,05). The visual acuity in non-EE IGF-1 treated rats do not differ from that in P25 EE rats (EE, 0,93 ± 0,03, N = 4) (One Way ANOVA, *post-hoc* Tukey's test p > 0,05). Asterisks denote significant difference (two asterisks, p < 0,01, three asterisks p < 0,001).

Block of IGF-1 in the visual cortex prevents the acceleration of visual acuity development in enriched animals

To assess if IGF-1 is a crucial factor mediating EE effect on visual acuity development, we performed also the experiment of antagonizing IGF-1 action in EE rats. IGF-1 action was antagonized in the visual cortex from P18 to P25 infusing the IGF-1 receptor antagonist JB1 (Fig. 7, 8) (concentration in the minipump 10 μg/ml, as in Fernandez et al., 1997). JB1 has been repeatedly used to block IGF-1 (Pietrzkowski et al., 1992; Pietrzkowski et al., 1993; Camarero et al., 2003) and in particular in the CNS (Fernandez et al., 1997; Carro et al., 2000). In JB1 treated EE animals we have measured visual acuity at P25 to assess whether antagonizing IGF-1 blocked EE action on visual acuity development. As control, EE animals implanted with PBS containing minipumps were recorded at P25.

To control for possible adverse effects of antagonizing IGF-1 action with JB1 on visual cortical neurons, we have assessed the density of NeuN positive cells at P25 in 5 animals implanted at P18 with a JB1 filled minipump in one cortex and with a PBS filled minipump in the contralateral cortex. We found that neuronal density in the JB1 treated Oc1B (2661 ± 44 $NeuN^{+}cells/mm^{2}$) does not differ from that in the contralateral Oc1B (2602 ± 63 $NeuN^{+}cells/mm^{2}$, paired t-test p = 0,532); neither the density in the JB1 treated nor that in the PBS treated Oc1B differ from that in Oc1B of untreated of P25 rats (2440 ± 91 $NeuN^{+}/mm^{2}$, One Way ANOVA, p = 0,107). The cortical thickness in Oc1B is not affected by JB1 treatment (JB1 treated cortex: 0,915 ± 0,02 mm; PBS treated cortex: 0,87 ± 0,01 mm, paired t-test, p = 0,052). We also recorded non-EE animals implanted with JB1 filled minipumps from P18 to P25 (N = 4, recordings at P25) and from P21 to P28 (N = 4, recordings at P28). We found no difference between visual acuity assessed in these two groups of animals and the visual acuity assessed in non-EE rats of the same age (JB1 treated P25 non-EE, 0,57 ± 0,04 c/deg, untreated P25 non-EE (same data as for Fig. 2) 0,63 ± 0,01 c/deg, p = 0,12, t-test; JB1 treated P28 non-EE rats, 0,75 ± 0,03 c/deg, untreated P28 non-EE-rats (N = 3), 0,79 ± 0,01 c/deg, p = 0,263, t-test). Thus, JB1 treatment, at the concentration employed by us, does not seem to have negative effects on the visual cortex.

JB1 treatment blocks EE effects on visual acuity maturation. As shown in Fig. 8, visual acuity of P25 JB1 treated EE animals (0,55 ± 0,05 c/deg, N = 5) is significantly lower than in P25

EE animals either treated with vehicle (0,81 ± 0,07 c/deg, N = 4) or untreated (0,93 ± 0,03 c/deg, N = 4, same data as for Fig. 6B) and does not differ from the visual acuity of P25 non-EE rats (0,63 ± 0,01c/deg, N = 7, same data as for Fig. 6B) (One Way ANOVA, $p < 0,001$, *post-hoc* Tukey's test, EE untreated *vs* EE JB1 treated rats, $p < 0,001$, EE vehicle treated *vs* EE JB1 treated rats, $p < 0,01$, EE JB1 treated *vs* non-EE rats, $p > 0,05$, EE *vs* EE vehicle treated rats, $p > 0,05$).

Thus, antagonizing IGF-1 action completely prevents EE effects on visual acuity development.

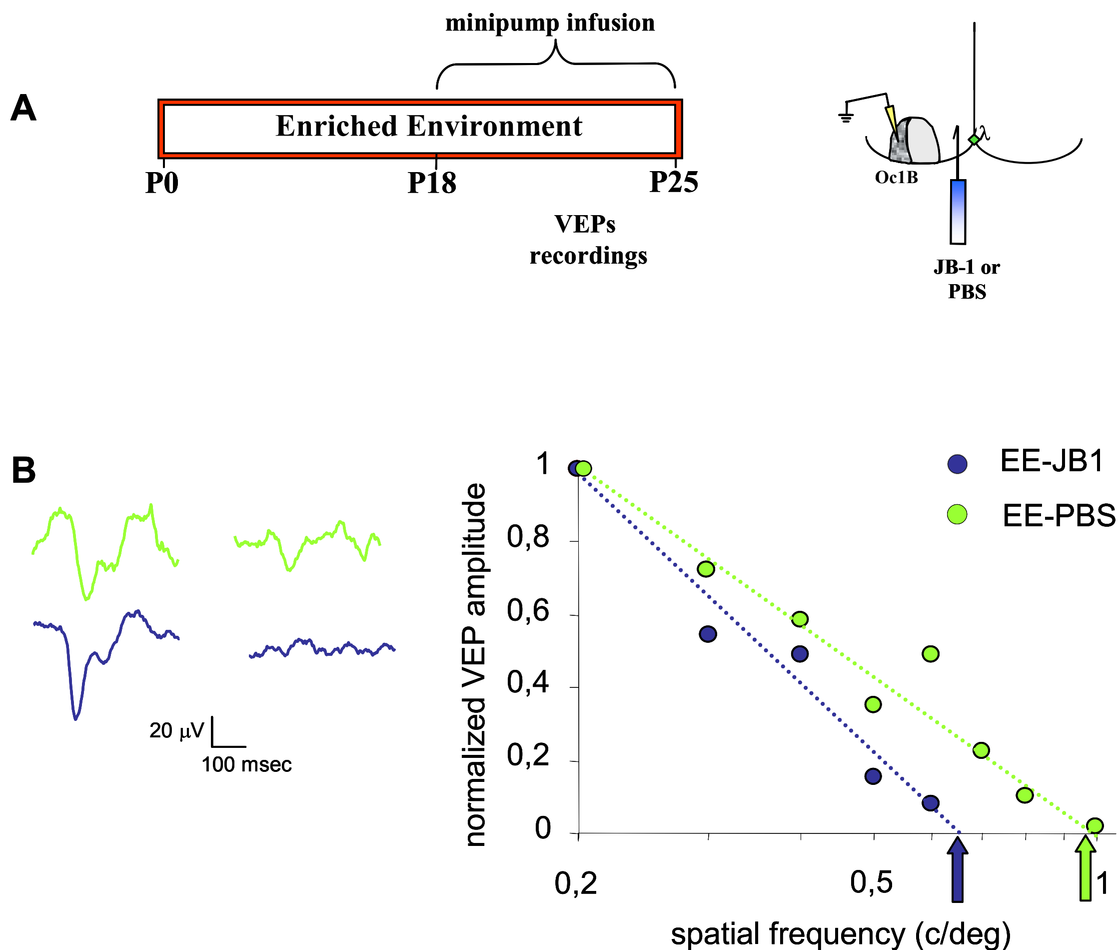


Figure 7: **IGF-1 blockade prevents the acceleration of visual acuity development in enriched animals.**

(A) Experimental protocol and schematic representation of minipump implant and recording site for VEPs. (B) Example of visual acuity estimated in one JB1 treated EE rat (EE-JB1, blue) and one vehicle treated EE animal (EE-PBS, green). Experimental points are normalized VEP amplitudes; thick lines are linear fits to the data. Estimated visual acuities are indicated by arrows. Waveforms above the graph are VEPs recorded in response to visual stimulation with gratings of spatial frequencies 0,2 and 0,5 c/deg for the JB1 treated (blue) and the vehicle treated EE animal (green). It is evident that at the higher spatial frequency a response is obtained only in the vehicle treated EE rat.

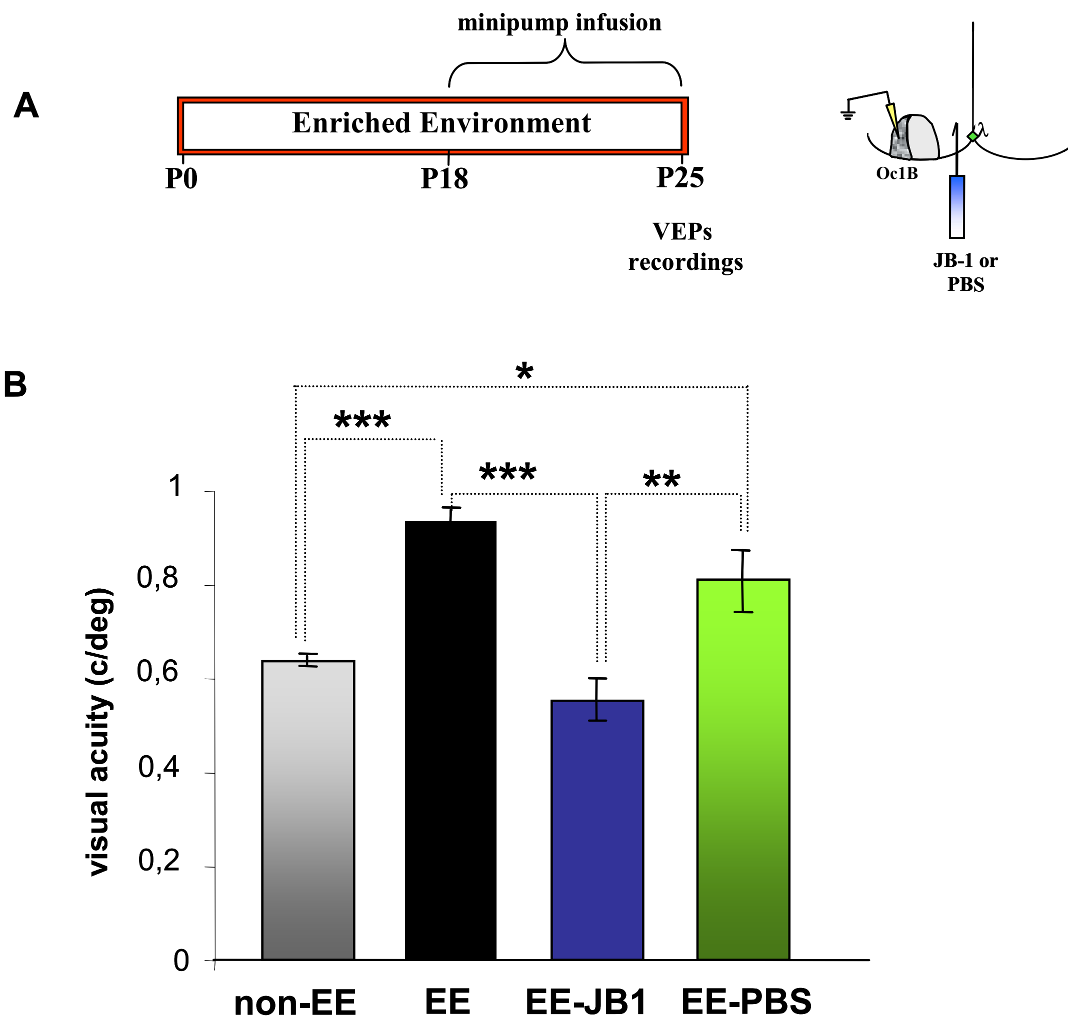


Figure 8: **IGF-1 blockade prevents the acceleration of visual acuity development in enriched animals.**

(A) Experimental protocol performed and schematic representation of minipump implant and recording site for VEPs. (B) Summary of mean visual acuity in all JB1 (0,55 ± 0,05 c/deg, N = 5) and PBS treated (0,81 ± 0,07 c/deg, N = 4) P25 EE animals; data for P25 EE and non-EE rats are replotted from Fig. 6B for comparison. Vertical bars represent SEM. Visual acuity of JB1 treated EE animals is significantly lower than in EE animals either treated with vehicle or untreated and does not differ from the visual acuity of P25 non-EE rats (One Way ANOVA, p < 0,001; *post-hoc* Tukey's test, significance level 0,05). Asterisks denote significant difference (one asterisk, p < 0,05; two asterisks, p < 0,01; three asterisks, p < 0,001).

IGF-1 affects the density of inhibitory synapses and of perineuronal nets in the visual cortex

How could IGF-1 increase mediate EE effects on visual acuity development? One factor which is likely to be relevant for visual acuity development is the intracortical inhibitory tone. The increase of visual acuity is well correlated with a decrease of mean receptive field size of neurons in the primary visual cortex (Fagiolini et al., 1994) and with the postnatal development of intracortical inhibition (Wolff et al., 1984; Huang et al., 1999; Morales et al., 2002) which plays a crucial role in shaping visual cortical receptive fields (Sillito et al., 1975; Hensch et al., 1998). Visual deprivation, which prevents visual acuity development (Fagiolini et al., 1994), also affects the developmental increase of GABAergic inhibition (Benevento et al., 1995; Morales et al., 2002; Gianfranceschi et al., 2003). BDNF overexpressing mice, which exhibit a precocious development of intracortical inhibition, also show an accelerated development of visual acuity (Huang et al., 1999). EE, which accelerates visual acuity development (Cancedda et al., 2004; Landi et al., 2007) and prevents dark rearing effects on visual acuity (Bartoletti et al., 2004), also affects the developmental expression of GAD65/67 (Cancedda et al., 2004) and prevents DR effects on intracortical inhibition development (Bartoletti et al., 2004).

We have therefore investigated whether the development of GABAergic intracortical inhibition was affected by IGF-1 infusion in the visual cortex assessing the presence of perisomatic inhibitory innervation (Huang et al., 1999). Perisomatic innervation mediated by basket interneurons, which constitutes up to 50% of GABAergic interneurons in the visual cortex, is likely a component of the overall developmental maturation of GABAergic innervation in the primary visual cortex and has been previously used to characterize intracortical inhibition development (Huang et al., 1999, Bartoletti et al., 2004, Chattopadhyaya et al., 2004). Huang et al. (1999) found that the development of GABAergic perisomatic inhibition is not completed before the fifth postnatal week. We have quantified the expression of GAD65 in the presynaptic boutons of GABAgergic interneurons around the soma of target neurons (perisomatic puncta rings, (Huang et al., 1999, Bartoletti et al., 2004) at P25, the age of visual acuity assessment, in non-EE animals (N = 7) implanted at P18 with an IGF-1 filled minipump in one cortex and a PBS filled minipump in the contralateral cortex and in EE animals (N = 5) implanted at P18 with JB1 filled minipump in one cortex and a PBS filled minipump in the contralateral cortex. We have

found that GAD65 immunoreactivity in puncta rings was significantly higher in the visual cortex treated with IGF-1 than in the PBS treated cortex (paired t-test, $p < 0,05$, one asterisk) (Fig. 9A, right, light bar), while it was significantly lower in the visual cortex treated with JB1 than in the PBS treated cortex (paired t-test, $p < 0,05$, one asterisk) (Fig. 9A, right, dark bar).

Thus, IGF-1 increase could be a mediator of EE effects on visual acuity development via an effect on inhibitory system development. To assess whether this IGF-1 action on inhibitory interneurons could be direct or indirect, we determined the presence of IGF-1 receptor on GABAergic interneurons both at P18 and at P25, the beginning and the end of our IGF-1 treatment. IGF-1 receptor is abundantly expressed in the visual cortex at both ages (Fig. 11). To quantify its presence on GABAergic interneurons we performed a double stain for IGF-1 receptor and GAD67, one of the GABA biosynthetic enzymes (Fig. 12A). We found that both at P18 and at P25 the great majority of GAD67 positive interneurons (96% at P18, 76% at P25, no significant difference, Mann-Whitney Rank sum test, $p > 0,05$) were also positive for IGF-1 receptor labelling. Thus, a direct effect of IGF-1 on GABAergic interneurons is possible. The maturation of visual acuity is correlated with the developmental decline of plasticity in the visual cortex (Berardi et al., 2000). We have recently shown (Bartoletti et al., 2004) that EE is able to prevent dark rearing effects on the developmental organization into perineuronal nets (PNNs) of Chondroitin Sulphate Proteoglycans (CSPGs), components of the extracellular matrix recently shown to be non permissive factors for visual cortical plasticity (Pizzorusso et al., 2002, 2006). Moreover, EE affects the developmental time course of synaptic plasticity in the visual cortex (Cancedda et al., 2004) and counteracts dark rearing effects on the critical period for ocular dominance plasticity. Tropea et al. 2006 have shown that IGF-1 is involved in ocular dominance plasticity.

We have therefore investigated whether IGF-1 increase in standard animals or IGF-1 blockade in EE animals, around P18 affects PNN development.

The density of PNN surrounded neurons increases from P22 to P70 (Pizzorusso et al., 2002). We examined PNN formation using Wisteria floribunda lectin (WFA) as in (Pizzorusso et al., 2002); the density of PNN surrounded cells has been determined at P25 in non-EE animals ($N = 5$) implanted at P18 with an IGF-1 filled minipump in one cortex and a PBS filled minipump in the contralateral cortex and in EE animals ($N = 6$) implanted at P18 with an IGF-1 filled minipump in one cortex and a PBS filled minipump in the contralateral cortex. We have found that the density of PNN surrounded cells (number of normalized to NeuN positive cells was

significantly higher in the IGF-1 treated cortex than in the contralateral cortex (paired t-test; $p<0,005$ two asterisks) (Fig. 10B, right, leftmost), while it was significantly lower in the visual cortex treated with JB1 than in the PBS treated cortex (paired t-test, $p < 0,05$, one asterisk) (Fig. 10B, right, rightmost). Thus, IGF-1 is able to affect PNN development and, through this effect, could contribute to EE action on visual cortex experience dependent plasticity.

To assess whether this IGF-1 action on PNN surrounded interneurons was direct or indirect, we determined the presence of IGF-1 receptor on PNN surrounded interneurons both at P18 and at P25. We performed a double stain for IGF-1 receptor and WFA, to label PNNs (Fig. 12B). We found that both at P18 and at P25 a large proportion of WFA positive interneurons (64% at P18, 50% at P25, $p = 0,03$, Mann-Whitney Rank sum test) were also positive for IGF-1 receptor labelling. Thus, a direct effect of IGF-1 on PNN surrounded interneurons is possible.

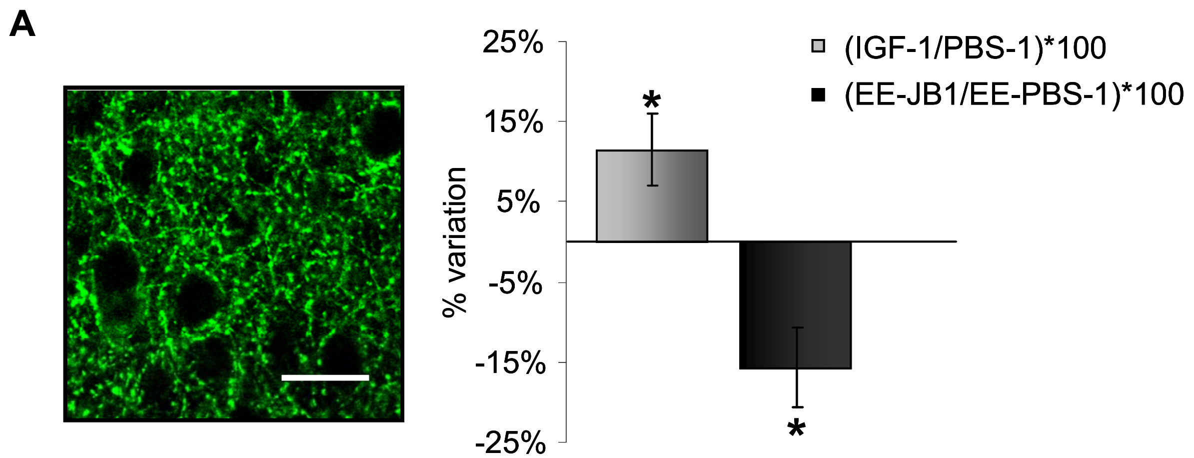
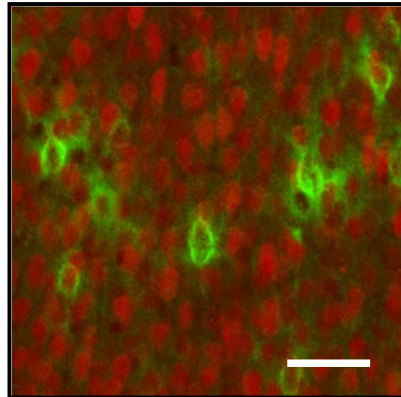


Figure 9: **IGF-1 affects intracortical inhibition in the developing visual cortex.**

(A) Left, representative example of GAD65 immunoreactivity in the rat visual cortex at P25. It is evident the punctate nature of the staining around cell bodies (puncta-ring). To quantify GAD65 immunoreactivity in puncta rings, immunofluorescence in puncta ring was normalized to background signal. Calibration bar 20 μm. Right: light bar: percentage variation of GAD65 puncta ring immunoreactivity between the cortex implanted at P18 with a IGF-1 filled minipump and the cortex implanted with a PBS filled minipump in P25 non-EE animals (N = 7). Percentage variation computed as [GAD65 immunoreactivity in IGF-1 treated/ (GAD65 immunoreactivity in PBS treated cortex –1)]x 100. GAD65 immunoreactivity is significantly higher in the IGF-1 treated than in the PBS treated cortex (paired t-test, $p < 0,05$, one asterisk). Right, dark bar: percentage variation of GAD65 puncta ring immunoreactivity between the cortex implanted at P18 with a JB1 filled minipump and the cortex implanted with a PBS filled minipump in P25 EE animals (N = 5). Percentage variation computed as [GAD65 immunoreactivity in JB1 treated/ (GAD65 immunoreactivity in PBS treated cortex –1)]x 100. GAD65 immunoreactivity is significantly lower in the JB1 treated than in the PBS treated cortex (paired t-test, $p < 0,05$, one asterisk). Vertical bars indicate SEM.

A



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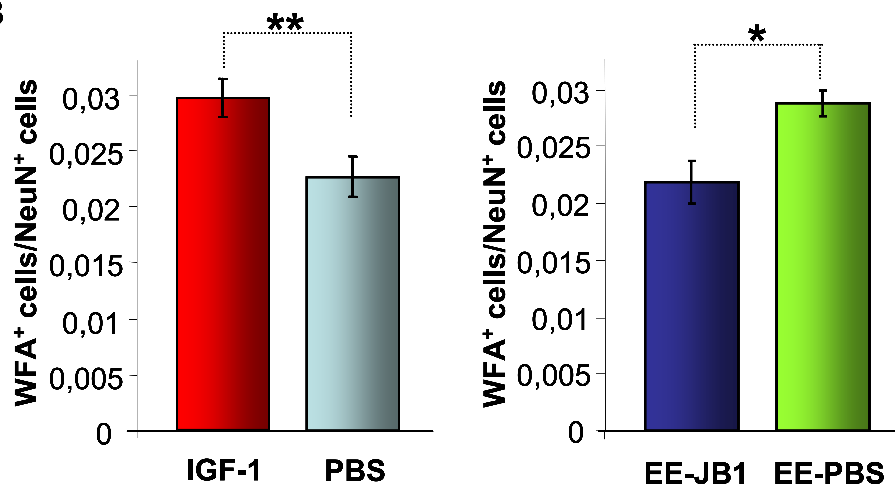


Figure 10: **IGF-1 mediates the increase in the density of perineuronal nets (PNNs) produced by EE in the visual cortex.**

(A) Representative example of WFA staining (green) and NeuN staining (red) merged image in the rat visual cortex at P25. WFA stained PNN completely surround cortical neurons. Calibration bar 50 μm. (B) Left: PNN surrounded cells (WFA positive cells/NeuN positive cells) are more numerous in the visual cortex treated from P18 to P25 with IGF-1 than in the contralateral, PBS treated cortex of non-EE animals (N = 5 animals, paired t-test, $p < 0,01$, two asterisks). Right: PNN surrounded cells (WFA positive cells/NeuN positive cells) are less numerous in the visual cortex treated from P18 to P25 with JB1 than in the contralateral, PBS treated cortex of EE animals (N = 6 animals, paired t-test, $p < 0,05$, one asterisks). Vertical bars represent SEM.

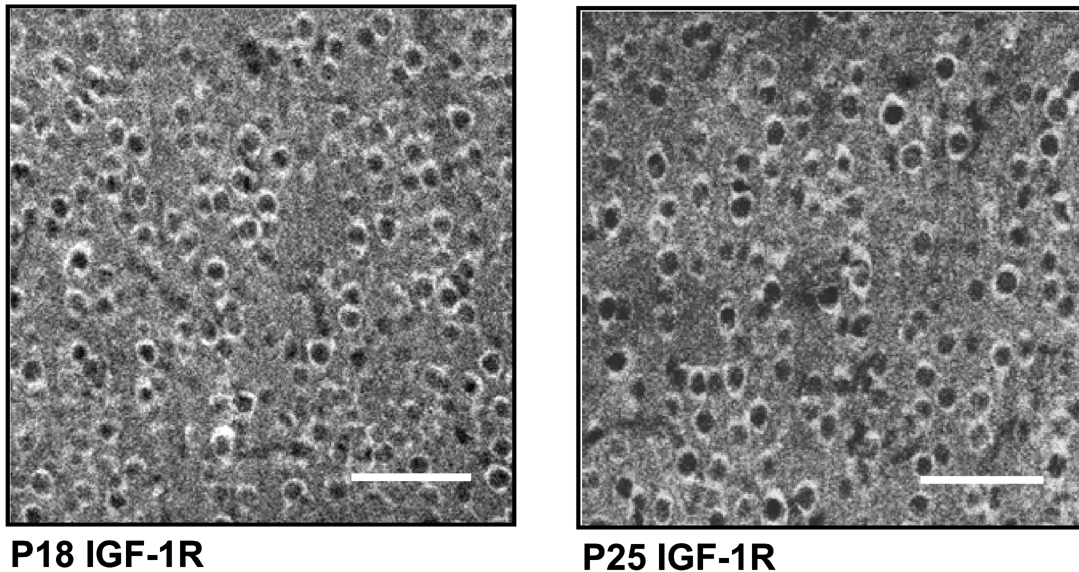
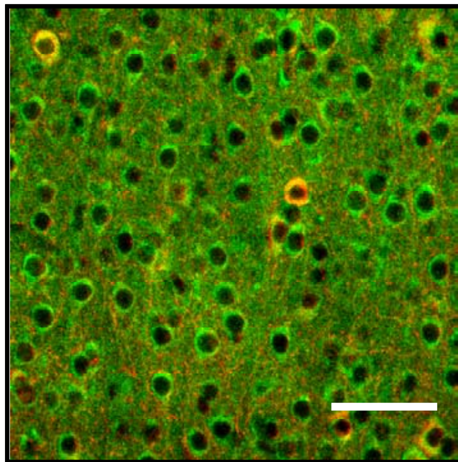
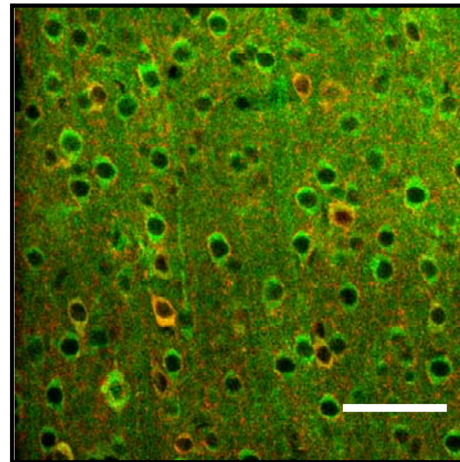


Figure 11: **Immunoreactivity for IGF-1R in the developing visual cortex.**
Representative example of staining for IGF-1 receptor in the visual cortex of a P18 and a P25 rat. Microphotographs from layers II-III. Images were acquired at 20 X (N.A.= 0,7, field 707 x 707 μm acquired at 1024 x 1024 pixels). Calibration bar 50 μm. It is evident that IGF-1 receptor is abundantly expressed in the visual cortex during this developmental period.

A

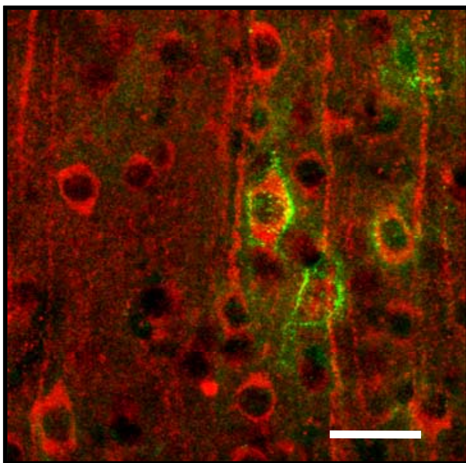


P18 IGF-1R/GAD67

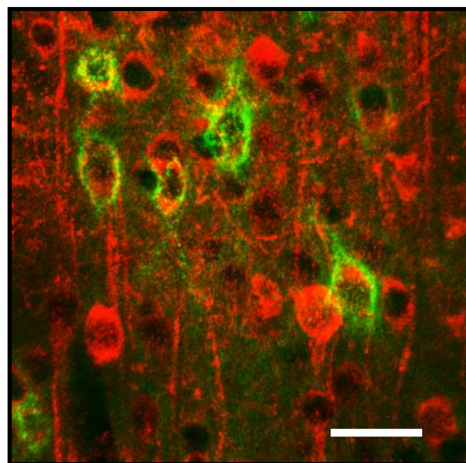


P25 IGF-1R/GAD67

B



P18 IGF-1R/WFA



P25 IGF-1R/WFA

Figure 12: IGF-1R colocalization with PNNs and GAD67 immunopositive neurons.

(A) Left, representative example of GAD67 staining red, IGF-1R staining gree, merged image in the rat visual cortex at P18. Right, representative example of GAD67 staining red, IGF-1R staining green, merged image in the rat visual cortex at P25. At P18 96% of inhibitory interneurons GAD67 immunopositive express also IGF-1R while at P25 the value is 76%. Scale bar: 50 µm. (B) Left, representative example of WFA staining green, IGF-1R staining red, merged image in the rat visual cortex at P18. Right, representative example of WFA staining green, IGF-1R staining red, merged image in the rat visual cortex at P25. At P18 64% of neurons surrounded by PNN express also IGF-1R, while at P25 the value is 47% . Calibration bar 25 µm.

Enrichment up to P12: effects on visual cortex IGF-1 levels and visual acuity development

Cancedda et al., (2004) demonstrated that EE, which causes a precocious increase of visual acuity at P25, has a very precocious effect on BDNF expression in the visual cortex; indeed, EE pups show a significant increase in BDNF protein at P7 and of GAD65/67 at P7 and P15.

However it is not known if these precocious effects of EE can trigger the increase in IGF-1 levels observed in the visual cortex of EE animals at P18 and documented in the previous section. To answer this question we reared rats in EE up to P12, and then moved litter and mother in a standard cage. At P18 we analyzed by immunohistochemistry IGF-1 cortical levels in EE and non-EE animals. We found that in P18 rats exposed to EE up to P12 (N = 4) the density of IGF-1 positive cells (0,397 ± 0,018 IGF-1 positive cells/Neu N positive cells) shows a small increase with respect to non-EE P18 rats (N = 8, mean number of IGF-1 positive cells/Neu N positive cells 0,368 ± 0,017) which, however, is not significant (One Way ANOVA, *post-hoc* Tukey's test, $p > 0,05$) (Fig. 13).

To understand whether in absence of a significant increase of IGF-1 at P18 exposure to EE up to P12 was sufficient to trigger the increase in visual acuity at P25 caused by continuous exposure to EE we reared rats in EE up to P12 and at P25 we assessed their visual acuity by VEP recordings (Fig. 14). We found that visual acuity in P25 rats exposed to EE up to P12 is significantly higher (Fig. 14, 0,73 c/deg ± 0,02, N = 4) with respect to P25 non-EE rats (0,63 c/deg ± 0,01, N = 7, same date as for Fig. 6B); however, it is significantly lower with respect to P25 EE rats (0,93 c/deg ± 0,03, N = 4, same data as for figure 6B) (One Way ANOVA, $p < 0,001$, *post-hoc* Tukey's test, EE up to P12 *vs* non-EE, $p < 0,05$, EE *vs* EE up to P12, $p < 0,05$).

An interesting finding to point out is that the visual acuity of P25 rats enriched up to P12 correspond to the visual acuity of non-EE rats exposed to artificial stimulation mimicking increased maternal cares observed in EE from P1 to P12 (Baldini S., Baroncelli L., Ciucci F., Putignano E., Sale A., Berardi N., Maffei L. unpublished results).

The observation that visual acuity at P25 in animals exposed to EE up to P12 is lower than that of animals exposed to EE up to P25 suggest that effects of early EE exposure on

molecular events in the visual cortex are not enough to induce the increase in visual acuity at P25 found in animals exposed to a continuous EE. Some later occurring events, necessary for the completion of visual acuity development acceleration, are missing in animals EE up to P12.

We think that lack of significant IGF-1 increase at P18 is one of the events missing, although we cannot say it is the only one.

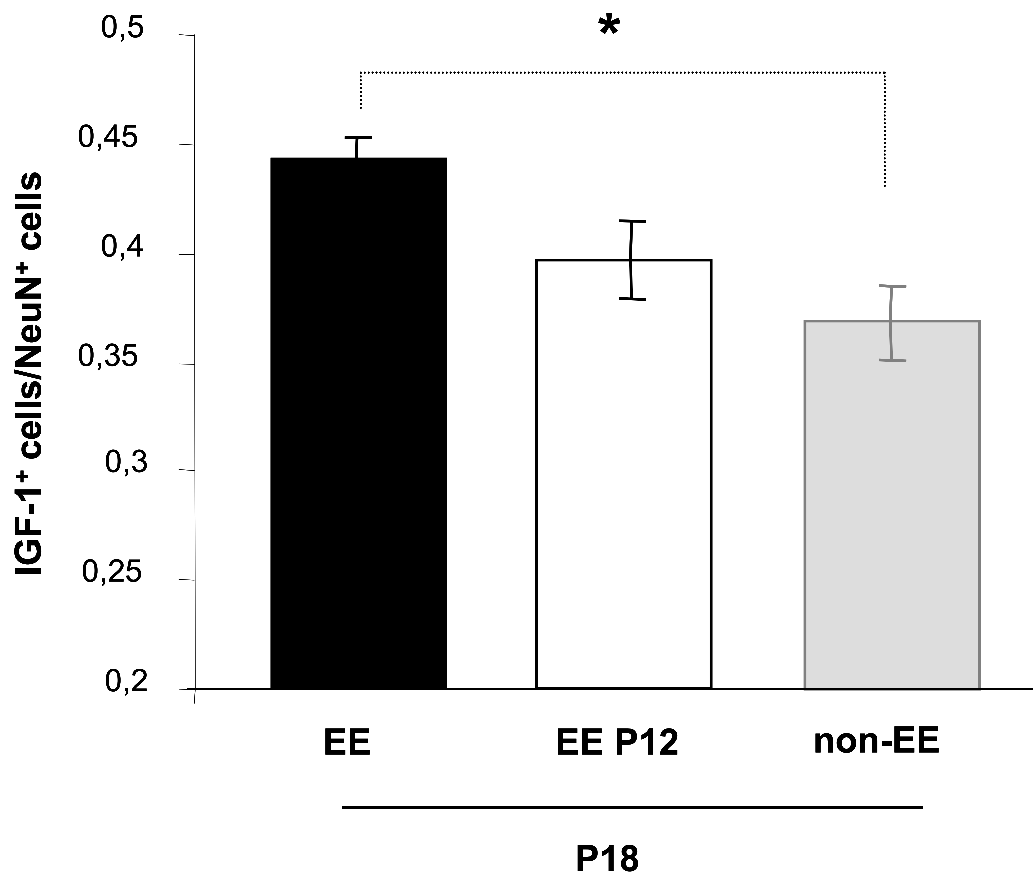


Figure 13: **Enrichment up to P12: effects on visual cortex IGF-1 levels.**

Mean number of IGF-1 immunopositive cells normalized to the number of neurons (NeuN positive cells) in the visual cortex of P18 rats (rats enriched up to P12, EE rats, 0,44 ± 0,01 IGF-1/ Neu N cells, N = 10; rats enriched up to P12 (EE P12), 0,397 ± 0,018 IGF-1/ Neu N cells, N = 4; non-EE rats, 0,368 ± 0,017 IGF-1/ Neu N cells, N = 8). Data for EE rats and non-EE rats are replotted from Fig. 4A. Vertical bars represent SEM. In P18 rats exposed to EE up to P12 the density of IGF-1 positive cells shows a small increase with respect to non-EE P18 rats which, however, is not significant (One Way ANOVA, *post-hoc* Tukey's test, p > 0,05).

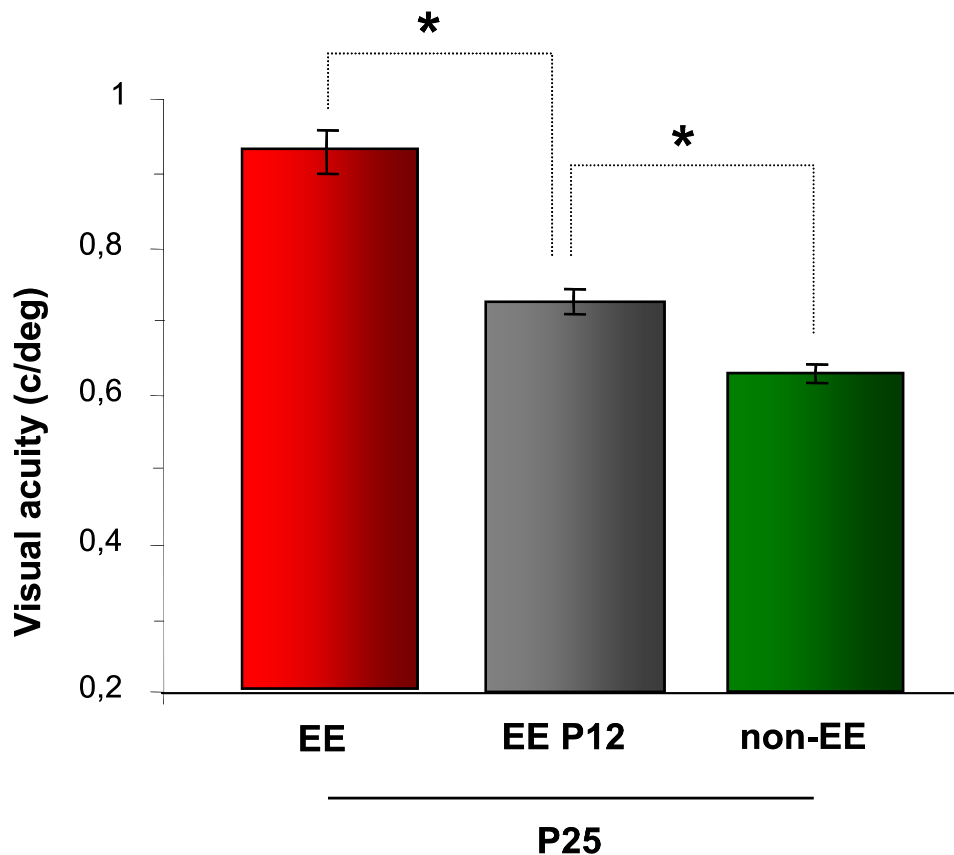


Figure 14: **Enrichment up to P12: Effects on visual acuity development.**

Mean visual acuity at P25 for EE rats (0,93 ± 0,03 c/deg, N = 4), EE P12 rats (0,73 ± 0,02 c/deg, N = 4) and non-EE rats (0,63 ± 0,01 c/deg, N = 7). Vertical bars represent SEM. Visual acuity in P25 rats exposed to EE up to P12 is significantly higher with respect to that of P25 non-EE rats, but is significantly lower with respect to that of P25 EE rats (One Way ANOVA, $p < 0,001$; *post-hoc* Tukey's test, significant level $p < 0,05$). Asterisk denote significant difference ($p < 0,001$).

PART II: IGF-1 mediates the effects of enriched environment on retinal development

Previous works (Cancedda et al., 2004; Bartoletti at al., 2004) demonstrated that EE affects development and plasticity of the visual cortex. Results obtained in this thesis suggest that IGF-1 is a key factor mediating these EE effects on visual cortex development.

Landi et al. (2007) have shown that also retina, considered less plastic than cortex, is sensitive to enriched environment which accelerates its development at a functional and anatomical level. However the visual cortex and the retina show two different responses to enriched living condition. In particular, exposure of rats to EE just for the first 10 postnatal days produces in the retina an accelerated maturation equal to that observed after a period of enrichment until P45 (Landi et al., 2007). On the other hand, a similar early enrichment produces in the visual cortex only a partial effect on visual acuity and on IGF-1 labelling development.

These results suggest that the effects elicited by EE on the retina are principally mediated by molecular factors the expression of which is influenced precociously, likely dependent on the enrichment of the mother; the molecular events triggered by EE to promote visual acuity development are dependent also from the interaction of pups with the richness of the environment.

To clarify if the same molecules which mediate EE effects on visual cortical development are at work also in the retina, we investigated the role of IGF-1 in retinal development.

Prenatal enrichment affects IGF-1 levels in the milk and in the pup retina

The final number of RGCs in the adult is the result of a period of RGCs overproduction, followed by a process of programmed cell death (called "apoptosis"). We demonstrated that at embryonic day 18 (E18) and E20 the number of apoptotic cells was 70% higher in EE with respect to non-EE fetuses, and lower in EE compared to non-EE pups at postnatal day 1 (P1), when the peak of natural cell death is typically seen (Perry et al., 1983; Horsburgh and Sefton, 1987) (Fig. 15). These results demonstrated that maternal enrichment accelerates the temporal dynamics of RGC death in EC animals.

All these effects seems to be mediate by IGF-1; in fact while the blockade of its action in the EE pregnant females prevents the effects of maternal enrichment on RGC programmed cell death, chronic IGF-1 administration to standard-reared females mimics them (Sale A., Cenni MC., Ciucci F., Putignano E., Chierzi S., Maffei L., unpublished data).

These precocious effects of EE on fetal development could be imputed to a different supply of nutrients, including hormones, provided by EE mothers to the fetus during pregnancy, throughout placental exchanges: the enriched living condition of the mothers during pregnancy, with increase physical activity and sensorial social stimulation, could affect the production of factors potentially important for visual system development, such as growth factors crossing the placental barrier or present in maternal milk, that are involved in the regulation of the development.

Since it is known that differences in blood-borne IGF-1 are difficult to detect because of its uptake by the tissues (Carro et al., 2000), we measured IGF-1 levels in the brain and in the milk of EE and non-EE pregnant rats. We found higher levels of IGF-1 in the brain of EE compared to SC pregnant rats. Milk is the sole source of nutrition for the neonatal rat for at least the first 2 weeks postpartum and IGF-1 is known to be present in the rat maternal milk (Donovan et al., 1991; Olanrewaju et al., 1996). We studied whether different levels of IGF-1 were provided to EE and non-EE pups through maternal milk investigating IGF-1 concentration in the gastric content of EE and non-EE suckling pups during the first ten postnatal days, using RIA. We found enhanced levels of IGF-1 in the milk of EE pups at P1, but not at P10 (Fig. 16). This result indicates increased concentration of IGF-1 in EE maternal milk as a possible postnatal source mediating the effects of early EE on the offspring nervous system development.

The increment if IGF-1 detected in the mother was found also in the offspring. In particular, we have shown that IGF-1 protein levels are increased in RGC layer of E15, E18 and P1 of enriched animals (Two-ways ANOVA showed an effect of age ($p < 0{,}001$) and housing condition ($p < 0001$). T -test with Bonferroni correction revealed a statistical difference between EE and non-EE groups at E15 (p=0,009), E18 ($p < 0{,}01$) but not at P1 (p=0,025) and at P10 (p=0,319) (Fig. 17). We found that IGF-1 expression is developmentally regulated in the RGC layer progressively increasing during late embryonic life and decreasing in the postnatal period.

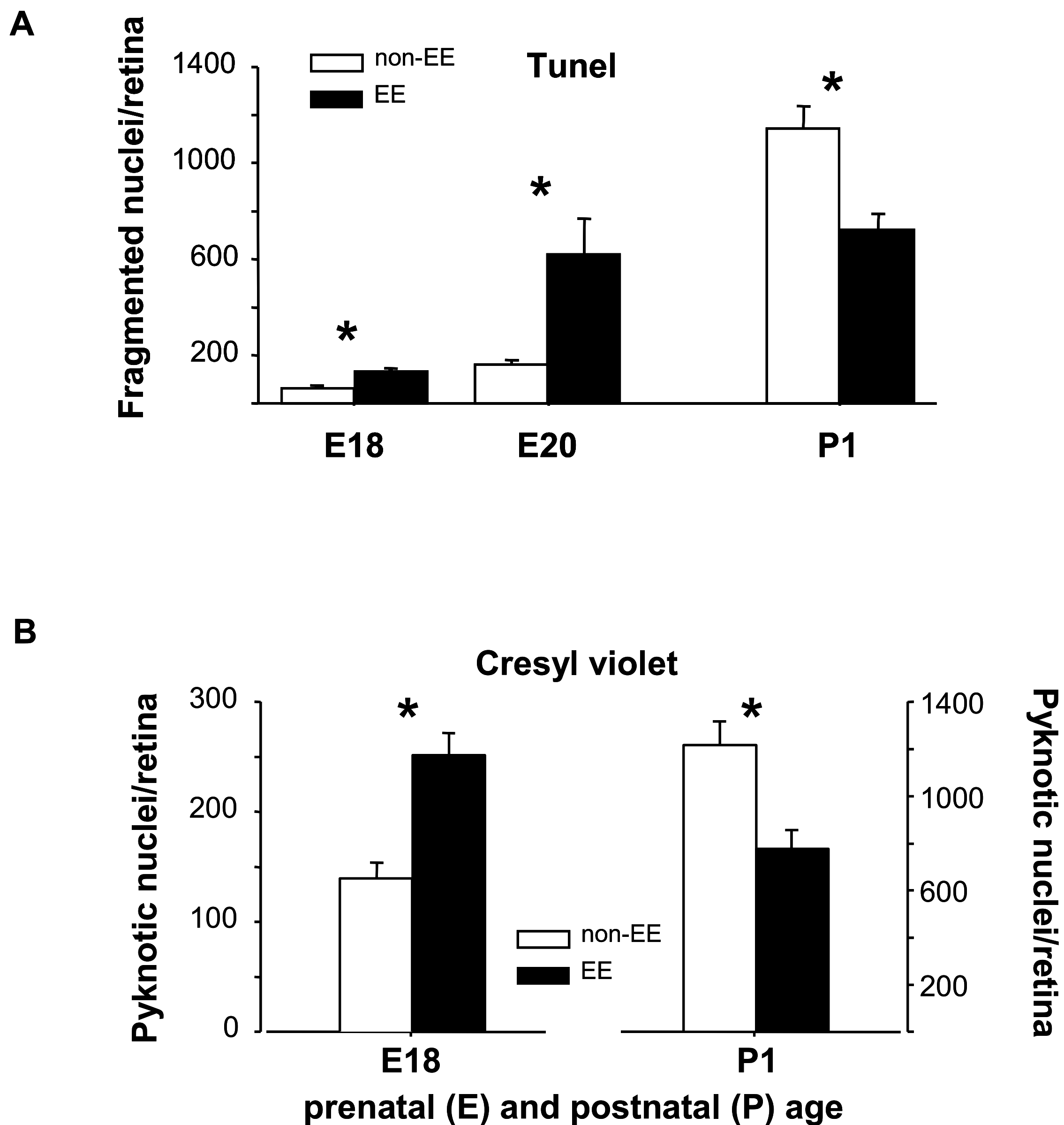


Fig 15: **Accelerated natural cell death in the RGC layer of EE rats.**

RGC layer apoptotic cell number in EE and non-EE rats, analyzed at the indicated ages with the Tunel method (A) and with cresyl violet staining of whole-mount retinas (B). With both methods, two-ways ANOVA showed an effect of age ($p < 0,001$) and housing condition ($p < 0,05$) and a significant age x housing condition interaction ($p < 0,001$). Mann-Whitney rank sum test with Bonferroni correction revealed a difference between EE and non-EE at E18, E20 and P1 ($p < 0,001$) for the Tunel method, and at E18 and P1 ($p = 0,002$) for cresyl violet staining.

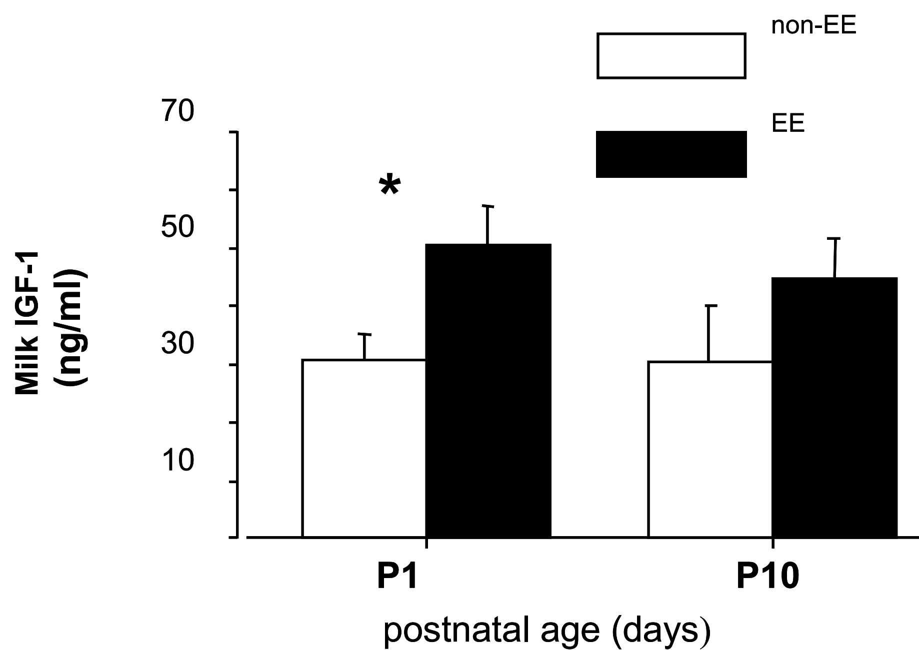


Figure 16: **Increased IGF-1 concentration in maternal milk**.

RIA determination of IGF-1 concentration in the milk of non-EE and EE suckling pups: two-ways ANOVA showed a significant age x housing condition interaction ($p < 0,05$). *post-hoc* Tukey's test revealed a difference at P1 ($p < 0,05$), but not at P10 ($p = 0,258$) between EE and non-EE groups. Bars indicate SEM.

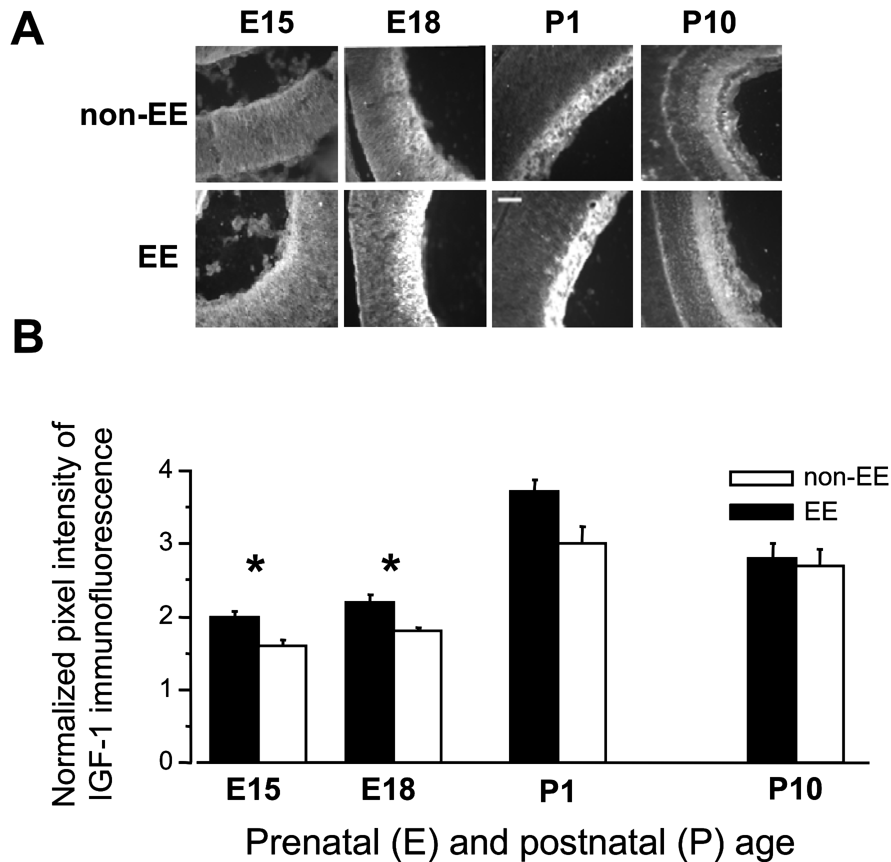


Fig 17: **Enhanced IGF-1 expression in the RGC layer of EE rats.**

(A) Micrographs of EE and non-EE retinal sections immunostained for IGF-1 at different ages. IGF-1 was increased in EE rats at E15, E18 and P1. Scale bar: 50 µm. (B) Quantitative analysis of IGF-1 immunofluorescence intensity in the RGC layer of non-EE and EE rats. Two-ways ANOVA showed an effect of age ($p < 0,001$) and housing condition ($p < 0,001$). T-test with Bonferroni corrections revealed a statistical difference between EE and non-EE groups at E15 ($p = 0,009$), E18 ($p < 0,01$) but not at P1 ($p = 0,025$) and at P10 ($p = 0,319$).

IGF-1 mediates the effects of EE on retinal acuity maturation

IGF-1 levels of expression, higher in EE pregnant rats, are also enhanced in the offspring retina at very early ages. IGF-1 receptors are present in the retina (Rodrigues et al, 1988; Waldbillig et al., 1988) and their expression is regulated in a developmental manner (Frade et al.,1996; Lee et al., 1992).

Here, we were interested to assess whether IGF-1 could be a factor capable of controlling also retinal functional development during precocious postnatal (P1-P7) period 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-1 in one eye and with saline in the other eye. In IGF-1 injected eyes we have measured acuity at P25 with PERG and as control we recorded the saline treated eyes.

Our results show that IGF-1 strongly affects P-ERG acuity development at P25, the age at which we saw an increase in retinal acuity produced by EE (Landi et al., 2007). Indeed retinal acuity of P25 IGF-1 treated eyes (0,68 c/deg ± 0,02, N = 5) is significantly higher than acuity of non-EE saline treated eyes (0,5 c/deg ± 0,02 in saline-treated eye, N = 3) or untreated eyes (0,51 ± 0,008, N = 5) (One Way ANOVA, p < 0,001, *post-hoc* Tukey's test, p < 0,001). The latter two do not differ (One Way ANOVA, p < 0,001, *post-hoc* Tukey's test, p > 0,05). The effects of IGF-1 treatment are comparable with those produced by EE: acuity of IGF-1 treated eyes does not differ from acuity of P25 EE eyes (0,68 ± 0,008, N = 5) (One Way ANOVA, p < 0,001, *post-hoc* Tukey's test, p > 0,05). (Fig 18B, left).

To assess whether IGF-1 is a crucial factor mediating EE effect on retinal acuity development, we antagonized IGF-1 action in EE rats. IGF-1 action was inhibited in the retina injecting intraocularly EE rats at P1, P4 and P7 with JB1, IGF-1 receptor antagonist, in one eye and with saline in the other eye. In JB1 injected animals we have measured retinal acuity at P25 with PERG and as control we recorded from the saline treated eyes. We found that JB1 treatment blocks EE effects on retinal acuity maturation. As shown in 19B, PERG acuity assessed for the JB1 treated eyes of EE rats at P25 (0,5 ± 0,02, N = 5) is significantly lower than the acuity assessed for the eyes either treated with saline (0,61 ± 0,008, N = 5) or untreated (0,68 ± 0,008, N=5, same data as Fig. 18B) of P25 EE rats and does not differ from the PERG acuity for the eyes of P25 non-EE rats (0,51 ± 0,008, N = 5, same data as Fig.18B) (One Way ANOVA, p<

0,001, *post-hoc* Tukey's test, EE untreated eyes *vs* EE JB1 treated eyes, $p<0{,}001$; EE saline treated eyes *vs* EE JB1 treated eyes, $p < 0{,}001$; EE JB1 *vs* non-EE eyes, $p > 0{,}05$; EE *vs* EE saline treated eyes, $p > 0{,}05$).

Thus, all these experiments support the hypothesis of IGF-1 role in mediating EE effects on retinal maturation.

To investigate whether the accelerated maturation of the retina induced by IGF-1 treatment causes an increase in visual acuity assessed at cortical level we recorded P25 non-EE animals intraocularly injected with IGF-1 or saline at P1, P4 and P7. We found that acceleration of retinal development does not cause an acceleration of cortical circuit development. Indeed, VEP recordings show that the cortex of animals injected with IGF-1 in both eyes is not affected by the treatment (Fig. 20): cortical acuity of intraocularly IGF-1 treated rats (0,7 c/deg ± 0.02; N = 5) does not differ from that of saline treated animals (0,67 c/deg ± 0.005; N = 3) and from that of non-EE untreated animals (0,63 c/deg ± 0.01; N = 7), but is significantly lower than that of EE animals (0,93 ± 0,03, N = 4) (One way ANOVA $P<0.001$; post-hoc Tukey test, $P < 0{,}05$). We suggest that retinal functional changes produced by IGF-I effects on RGC circuitry are not sufficient to produce the cortical maturation seen in the enriched condition or in the animals infused with IGF-1 intracortically.

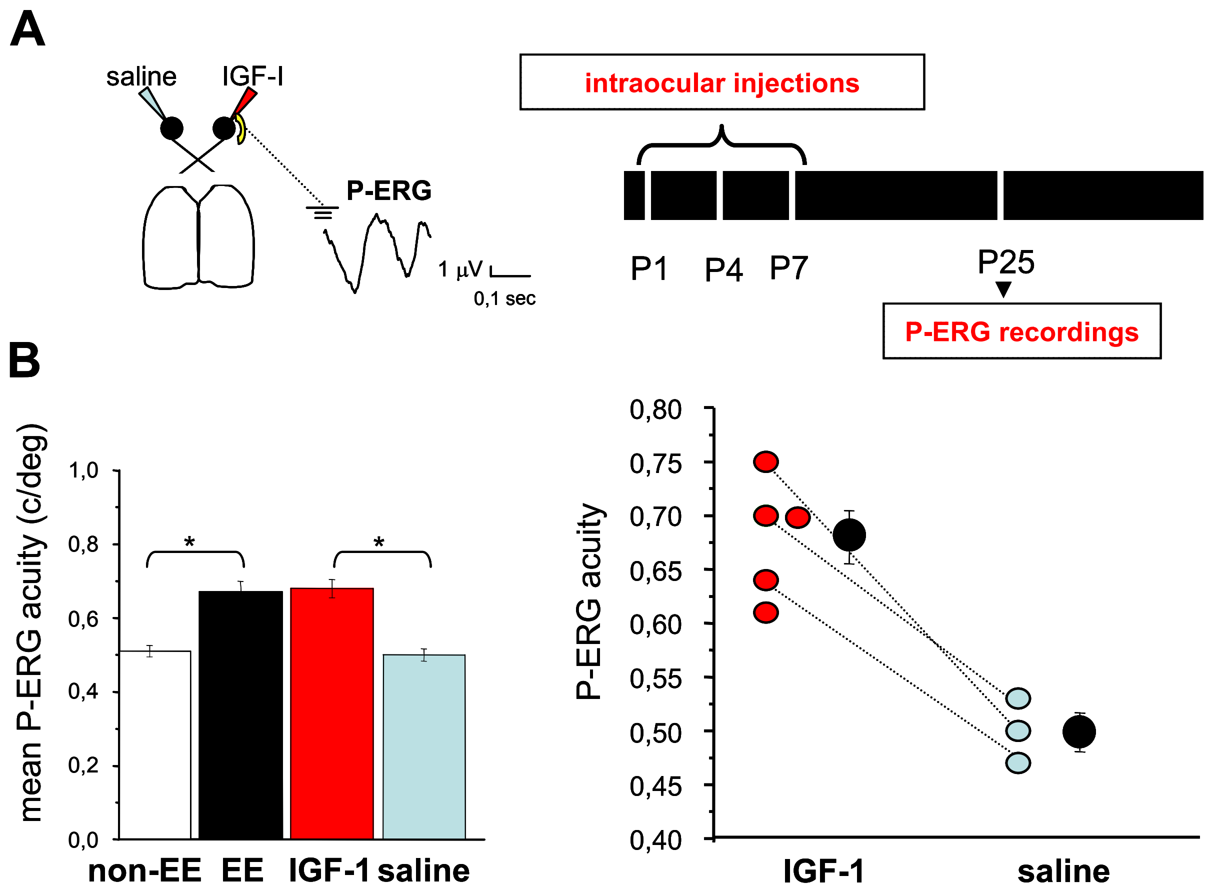


Figure 18: **IGF-1 intraocular injections affect retinal acuity development in standard reared rats.** (**A**) Schematic protocol of the experiment performed to evaluate the effects of IGF-1 intraocular injections on retinal acuity development. **(B)** Effect of IGF-1 treatment on retinal acuity development. Retinal acuity was assessed at P25. Left, mean retinal acuity in all experimental groups. The effect of IGF- 1 is evident: P-ERG acuity in IGF-1 treated eyes (0,68 c/deg ± 0.02, N = 5) is higher than in saline treated eyes (0,5 c/deg ± 0,02, N = 3) or in untreated eyes (non-EE) and does not differ from that in EE animals (One Way ANOVA P = 0,002; *post-hoc* Tukey's test, significance level 0,05). Vertical bars represent SEM. Right: results for the single animals treated with IGF-1 the mean acuity of which has been reported on the left. Retinal acuity of the IGF-1 treated eye (red dots) and of the saline treated eye (blue dots) is reported. The acuity of the IGF-1 tretated eye is joined to that of the fellow eye by a dotted line when it was possible to record both eyes in the same animal. Black, larger symbols represent the mean of each group; vertical bars represent SEM. The acuity of IGF-1 treated eyes is significantly higher than that of saline treated eyes (t-test, P = 0,002).

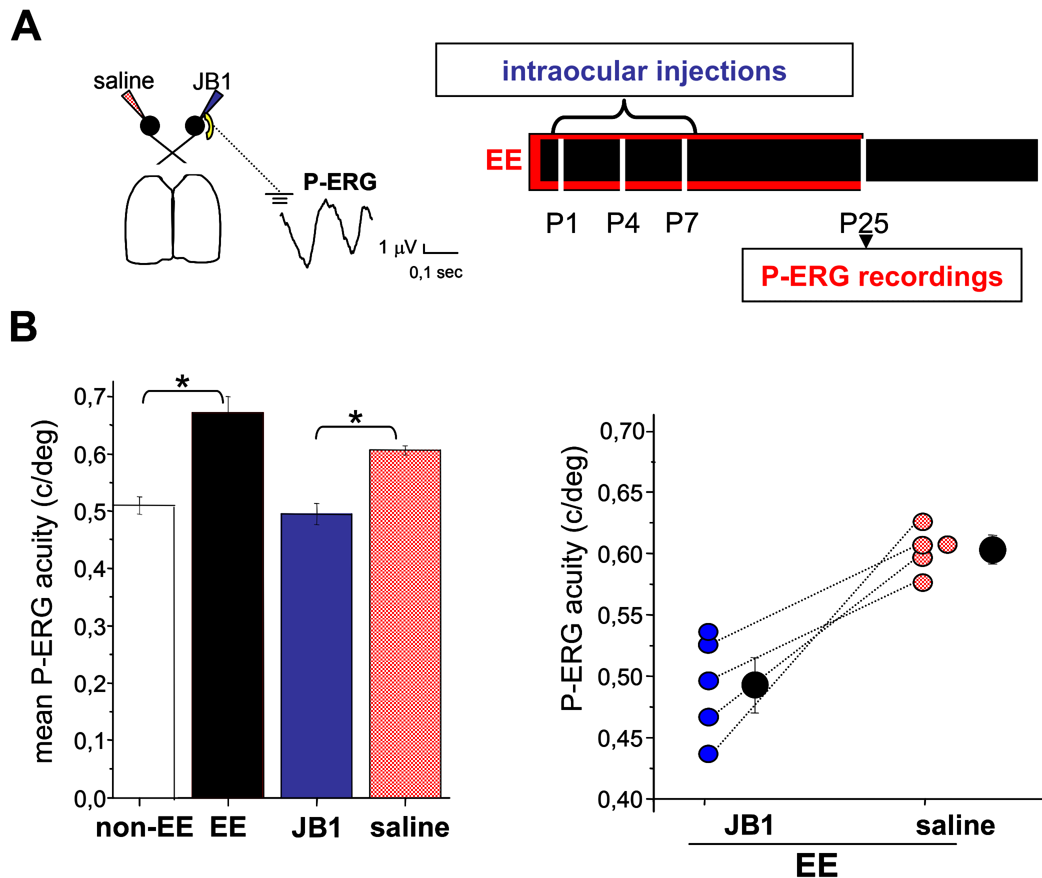


Figure 19: **IGF-1 blockade prevents the acceleration of retinal acuity development in enriched animals**. **(A)** Schematic protocol of the experiment performed to evaluate the effects of IGF-1 blockade by JB1 intraocularly injections on retinal acuity development. **(B)** Effect of JB1 treatment on retinal acuity development. Retinal acuity was assessed at P25. Left, mean retinal acuity in all experimental groups. The effect of JB1 is evident: PERG acuity in JB1 treated eyes (0,5 ± 0,02, N = 5) is lower than in saline treated eyes (0,61 ± 0,008, N = 5) or in eyes of EE animals and does not differ from that in untreated eyes (non-EE). (One Way ANOVA $p < 0,001$; post *hoc-hoc* Tukey, significance level 0,05). Vertical bars represent SEM. Right: results for the single animals treated with JB1 the mean acuity of which has been reported on ther left. Retinal acuity of the JB1 treated eye (dark blue dots) and of the saline treated eye (red dots) is reported. The acuity of the JB1 tretaed eye is joined to that of the fellow eye by a dotted line when it was possible to record both eyes in the same animals. Black, larger symbols represent the mean of each group; verticals bars represent SEM. The acuity of JB1 treated eyes is significantly lower than that of saline treataed eye (paired t-test, P = 0,01).

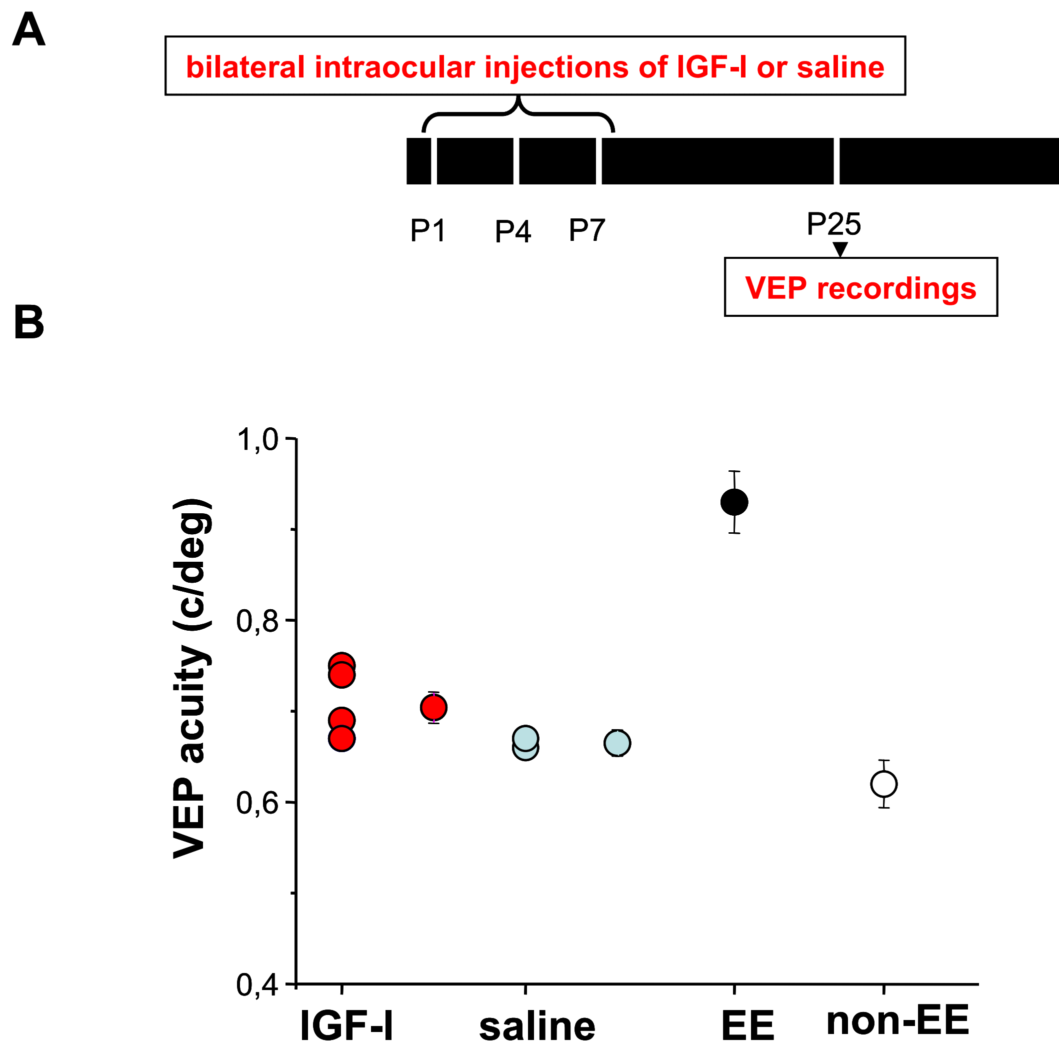


Figure 20**: IGF-1 intraocular injections do not influence visual cortical acuity development**. **(**A) Schematic protocol of the experiment performed to evaluate the role of intraocularly injected IGF-1 on visual cortical acuity development. (**B**) VEP recordings show that IGF-1 treatment does not affect the development of visual cortical acuity assessed at P25, the age at which we see a jump in acuity produced by EE. The acuity of IGF-1 treated animals (0,7 c/deg ± 0,02, N = 5) does not differ from that of saline treated animals (0,67 ± 0,005, N = 3) and from that of non-EE untreated animals (0,63 ± 0,01 c/deg, N = 7), but it is significantly lower than that of EE animals recoreded for comparison (One Way ANOVA P < 0,001; *post-hoc* Tukeytest: P < 0,05).

Intraocular IGF-1 administration mimics EE effects on BDNF protein levels at P10.

Landi et al., 2007 demonstrated that BDNF protein levels are affected by EE in RGC layer of developing retina. While in standard rats BDNF protein levels are very low at P14, EE rats show a precocious BDNF expression: indeed BDNF immunoreactivity is detectable in RGC layer already at P7, and it is significantly higher than in non-EE rats at P10. Moreover, Landi and colleagues (2007) found that reduction of BDNF expression during this time of enhanced expression by intraocular injections of BDNF antisense oligonucleotides, prevents EE effects on the acceleration of retinal acuity development. Since BDNF is known to be required for EE effect on the maturation of RGC functional properties, and IGF-1 mimics this effect, we investigated if IGF-1 affected BDNF expression levels in RGC layer.

EE effects on IGF-1 expression levels are observed between P1 and P10 in RGC layers of EE rats and the expression of BDNF is affected by EE in P10 rat retina (Landi et al., 2007), we therefore intraocularly injected with IGF-1 non-EE animals at P1, P4 and P7.

Non-EE pups were injected with IGF-1 in one eye, and with vehicle (saline) in the other eye for comparison. BDNF protein levels in RGC layer were then analysed at P10 by immunohistochemistry. Our results show that IGF-1 treatment strongly affects BDNF protein expression. In particular, we have found that BDNF immunoreactivity is significant higher in RGC layer of IGF-1 treated eyes ($1{,}103 \pm 0{,}003$, $N = 7$) than of saline treated ones ($1 \pm 0{,}0033$, $N = 7$) (t-test, $p < 0{,}001$) (Fig 21B and C left). Conversely, EE pups were injected with JB1 in one eye, and with vehicle (saline) in the other eye for comparison. BDNF protein levels analysed at P10 in RGC layer were significantly lower in RGC layer of JB1 treated eyes ($1{,}002 \pm 0{,}034$, $N = 7$) respect to saline treated ones ($1{,}104 \pm 0{,}034$, $N = 5$) (t-test, $p < 0{,}001$) (Fig 21B and C right).

Thus, these data show that IGF-1 triggers the enhancement of BDNF expression in RGC layer, mimicking EE effects on retinal development.

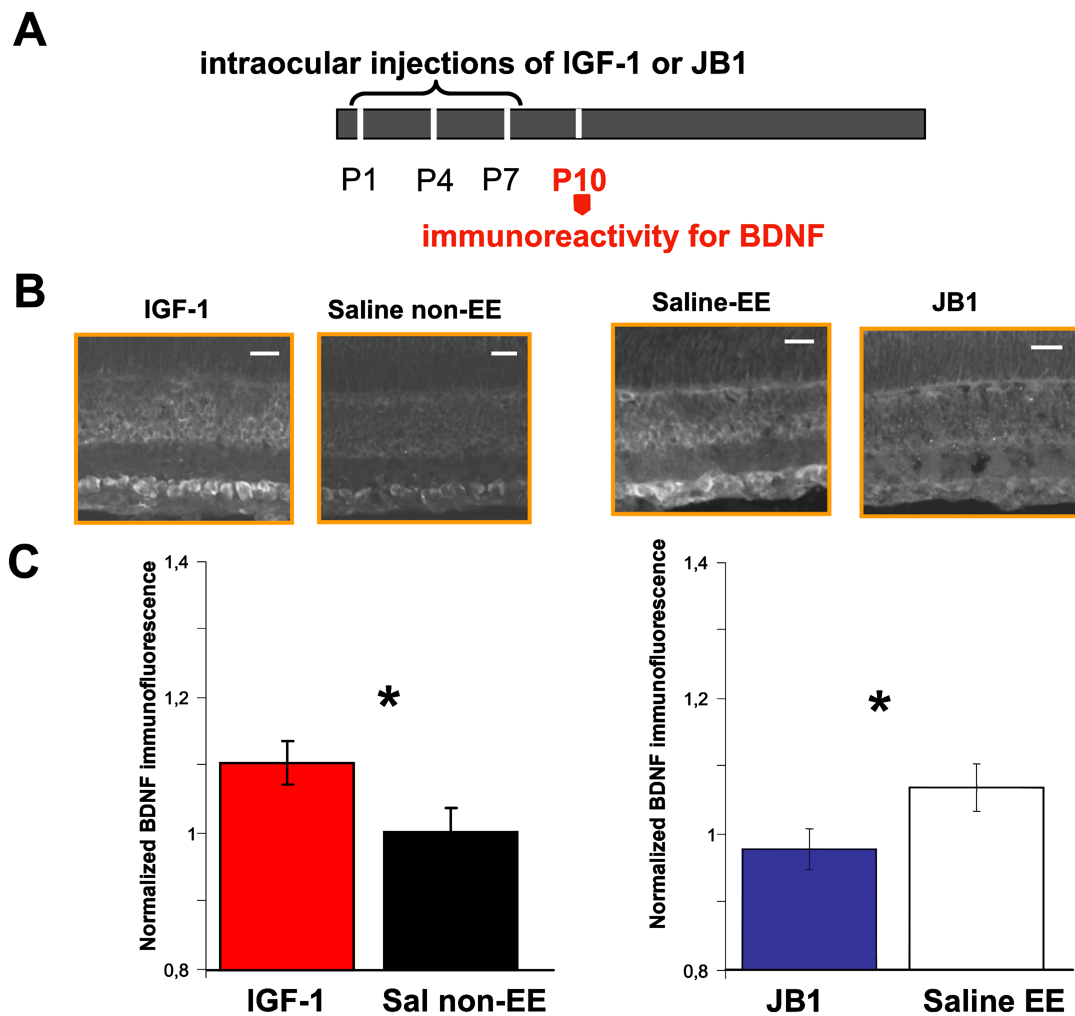


Figure 21: **IGF-1 intraocular injections increase BDNF protein levels in RGC layer at P10.** **(A)** Experimental protocol. **(B)** Left: P10 Retinal sections microghaphs of non-EE rats intraocularly injected with saline or IGF-1. Right: Retinal section micrographs of EE rats intraocularly injected with saline or JB1. In both cases, BDNF immunolabeled cells are detectable at the level of RGC layer. Scale bar: 50 µm. **(C)** Left: Quantitative analysis of mean BDNF immunofluorescence intensity normalized to background level in RGC layer of non-EE rats intraocularly injected with IGF-1 (red) or vehicle (black) and immunostained for BDNF at P10. BDNF immunoreactivity is significantly higher in the eyes of IGF-1 non-EE treated rats (1,103 ± 0,033, N = 7) respect to those of non-EE saline treated rats (1 ± 0,033, N = 7) (Mann-Whitney Rank Sum Test; $p < 0,001$). Vertical bars represent SEM. Right: Quantitative analysis of mean BDNF immunofluorescence intensity normalized to background level in RGC layer of EE rats intraocularly injected with JB1 (blue) or vehiche (pink) and immunostained for BDNF at

P10. BDNF immunoreactivity is significantly lower in the eyes of EE JB1 treated rats (1,002 ± 0,034, N = 7) respect to those of EE saline treated rats (1,104 ± 0,039, N = 5) (Mann-Whitney Rank Sum Test; $p < 0{,}001$). Vertical bars represent SEM.

DISCUSSION

Enriched environment is a powerful experimental paradigm which deeply affects brain plasticity processes, in development and adult life. Important results about the consequences of exposing animals to enriched living conditions during development come recently from our laboratory. It has been demonstrated that EE accelerates visual system development both at cortical and retinal level (Cancedda et al., 2004; Sale et al., 2004; Landi et al., 2007) and prevents dark rearing effects on visual cortex maturation (Bartoletti et al., 2004). At molecular and cellular level, EE enhances levels of intracortical inhibition, affecting GAD67 and 65 expression and prevents dark rearing effects on the developmental organization into perineuronal nets (PNNs) of chondroitin sulphate proteoglycans (CSPGs); promotes an earlier developmental time course of BDNF and of CRE-mediated gene expression (Cancedda et al., 2004; Bartoletti et al., 2004; Landi et al., 2007). However, is still poorly understood how environmental experience exerts its strong effects on visual system development. The topic of this thesis is to gain insight into the molecular mediators triggering the faster maturation of visual cortex and retina under enriched conditions and to investigate if the changes detected in these two different centres of the visual system in response to enriched environment stimulation are dependent on the same molecular mediators. We focused our attention on IGF-1, a molecule increased by physical exercise and EE (Carro et al., 2000) and able to increase the expression of factors, such as BDNF (Cotman and Berchtold, 2002) important for visual cortical and retinal plasticity such as BDNF (Landi et al., 2007).

Our most relevant finding is that IGF-1 mediates the effects of EE on retinal and on visual cortical development.

At retinal level, IGF-1 is the mediator of the acceleration of the pattern of retinal ganglion cell migration and death in the fetus and of the accelerated development of retinal acuity in postnatal rats. Its action may be exerted through retinal BDNF: indeed, IGF-1 induces in non EE rats an increase of BDNF expression at P10, as observed in EE rats (Landi et al., 2007) and block of IGF-1 in EE rats prevents both EE induced retinal BDNF increase and accelerated retinal acuity development.

At cortical level, IGF-1 mediates EE effects on visual acuity development in postnatal rats. IGF-1 action may be exerted through the control on inhibitory circuitry maturation and the development of PNNs.

A totally new finding is the identification of the specific developmental time windows in which EE elicits the molecular changes responsible for its effects on visual cortex and retina maturation.

Early (up to P10) exposure to enrichment, which causes an increase of retinal IGF-1 around P0 and of BDNF around P10, is sufficient to trigger the acceleration of retinal acuity maturation similar to that observed at P25 in EE animals continuously exposed to EE (Landi et al., 2007). On the contrary, early enrichment is sufficient to increase BDNF and GAD65/67 at P7 in the visual cortex, but it produces a small increment in IGF-1 cortical levels at P18 and only partially reproduces the effects of continuous exposure to EE on visual cortical acuity recorded at P25.

IGF-1 mediates the effects of EE on visual cortical development

EE affects the developmental time course of IGF-1 levels in the developing visual cortex

Pioneering work of Carro and colleagues. 2000, demonstrated for the first time that wheel running produces an increment of circulating IGF-1 and of its brain uptake affecting different cerebral areas. This, and following works (Trejo et al., 2001 and Carro et al., 2001) pointed out to IGF-1 as one of the major mediator of the neuroprotective effects produced by physical exercise in the adult brain**.** Running induces uptake of IGF-1 by specific groups of neurons (Carro et al., 2000) and increases IGF-1 expression in the hippocampus (Ding et al., 2006). Recently, IGF-1 has been shown to mediate in the adult the protective effects of EE on neuronal death (Koopmans et al., 2006). Interestingly, it has been demonstrated that also environmental enrichment affects IGF-1 pathway: EE has been shown to up-regulate IGF-1 receptor gene in the adult rat hippocampus and sensorimotor cortex (Keyvani et al., 2004).

To analyze whether EE was able to affect IGF-1 levels in the brain during development we analyzed IGF-1 protein levels in the visual cortex of EE and non-EE animals at different ages.

The presence and development of IGF-1 levels protein in the visual cortex and the possible functional involvement of IGF-1 in experience – dependent plasticity evoked by EE has

not been examined to date. Previous studies analyzed IGF-1 expression pattern at the level of visual system, but only in peripheral and sub cortical structures (Bondy et al., 1991). IGF-1 expression in the developing brain is generally transient and different regions show different time courses of IGF-1 developmental expression; in each system, IGF-1 expression appears during relatively late stages of their development, at a time of maturation of dendrites and synapse formation (Bondy, 1991). We found that IGF-1 protein expression in the visual cortex increases between P15 and P21, a period of active synaptogenesis in all cortical layers (Miller et al., 1986) and which corresponds to the beginning of the critical period for experience-dependent remodelling of visual connections in the rat (Fagiolini et al., 1994). This is consistent also with the role for IGF-1 in experience-dependent visual cortical plasticity suggested by (Tropea et al., 2006) which show an up-regulation of insulin-like growth factor 1 binding protein 5 (IGFBP-5) and a downregulation of IGF-1R after monocular deprivation.

The first indication that IGF-1 might be involved in the effects produced by EE on the developing visual cortex is that IGF-1 expression in the visual cortex is affected by EE. In particular, IGF-1 immunoreactivity at P18 is higher in rats exposed to EE (EE rats) than in non-EE rats.

At all ages, the great majority of IGF-1 positive cells are neurons. The number of NeuN positive cells is not increased by EE; therefore the increase in the density of IGF-1 positive neurons in EE animals is due to an increased presence of IGF-1 labelled neuronal cells, not to an increase in the density of neurons. Also the density of IGF-1 positive inhibitory interneurons in the visual cortex is significantly increased by EE at P18. Thus, we show for the first time that IGF-1 presence in the developing visual cortex is sensitive to the experience provided by EE and that both excitatory neurons and inhibitory interneurons could be potentially affected by IGF-1 increase.

During development, IGF-1 expression in neurons is well documented (Bondy, 1991; Bondy et al., 1992). The effects of EE on IGF-1 protein levels in the visual cortex could therefore be attributed to an increased IGF-1 mRNA expression in the visual cortex. However, we cannot exclude a contribution to the increased IGF-1 immunoreactivity found in P18 EE rats from an increase in circulating IGF-1, a decrease in IGF-1 binding protein (Tropea et al., 2006) or an increase in IGF-1 receptors on visual cortical neurons.

IGF-1 mediates the effects of EE on visual cortical development

To assess if the enhanced IGF-1 levels we observed in the visual cortex of enriched rats are responsible for the acceleration of visual acuity development evoked by EE we performed two mirror experiment. On one hand, we mimicked IGF-1 increase infusing exogenous IGF-1 from P18 to P25 in the visual cortex of non-EE rats; on the other hand, we blocked IGF-1 action, infusing JB-1, IGF-1 receptor antagonist (Pietrzkwoski et al., 1992) during the same time window in the visual cortex of EE rats.

The choice of the age to start IGF-1 treatment in non-EE rats or IGF-1 blockage in EE rats was performed evaluating the pattern of IGF-1 expression in the visual cortex of EE rats which present the higher levels of expression around P18.

During this time window the visual cortex is capable of responding to IGF-1 because IGF-1 receptor is present on visual cortical neurons of standard rats both at P18 and at P25. In particular, we observed an high number of IGF-1 receptor positive cells, comparable to the number of IGF-1 positive cells. This does not differ between P18 and P25. Similar to that of IGF-1, IGF-1R immunostaining appears periplasmatic, but it is present also in the long dendritic processes of pyramidal neurons which cross different visual cortical layers.

Our results show that it is sufficient to increase IGF-1 availability in the visual cortex, in this specific time window, to mimic the strong effect of EE on visual cortical development and that the increment of IGF-1 levels observed in the visual cortex or EE rats at P18 is necessary for the effects of enriched living condition on cortical development to take place.

Possible adverse effects of antagonizing IGF-1 action with JB1 on visual cortical neurons can be excluded because the density of NeuN positive cells is not altered by JB1 infusion. Also the cortical thickness in Oc1B is not affected by JB1 treatment. A further confirmation that JB1 treatment, at the concentration employed by us, does not have negative effects on the visual cortex is our VEPs recordings in non-EE animals implanted with JB1 filled minipumps from P18 to P25 (recordings at P25) and from P21 to P28 (recordings at P28). We found no difference between visual acuity assessed in these two groups of animals and the visual acuity assessed in non-EE rats of the same age.

Visual acuity is a sensitive index of visual cortical development (Huang et al., 1999; Porciatti et al., 1999); thus, our observations suggest that IGF-1 is an important factor in

mediating EE effects on the development of the visual cortex. At the same time, our results show for the first time that IGF-1 is able to accelerate visual acuity development.

Another indication that IGF-1 increase at P18 is necessary for EE to determine its maximal effect on visual acuity maturation comes from the observation that exposure to EE up to P12, which is not sufficient to trigger a significant increase in IGF-1 labeling in the visual cortex at P18, produces only a partial effect on visual acuity development. This results also suggests that the early molecular effects of EE in the visual cortex (Cancedda et al., 2004) are not sufficient to trigger the full increase in the visual acuity of P25 animals caused by continuous exposure to EE up to P25.

A very interesting result confirming our hypothesis that early EE is effective in affecting visual acuity development but is not sufficient to trigger the maximal effects of continuous exposure to EE on visual acuity maturation has been recently obtained in our laboratory. Assuming that the more precocious EE effects are imputed to increased levels of maternal care under EE conditions (Sale et al., 2004), an experiment was performed in our laboratory that reproduced in non EE pups the enhanced levels of maternal care experienced by EE pups and in particular the enhanced levels of tactile stimulation provided by licking. Non-EE pups were subjected to artificial stimulation mimicking maternal licking from P1 to P12 and visual acuity was assessed at P25. The results (Baldini S., Baroncelli L., Ciucci F., Putignano E., Sale A., Berardi N., Maffei L. unpublished results) show that animals artificial stimulated have a visual acuity of 0,74 ± 0,02 c/deg, almost identical to that of animals exposed to EE up to P12 (0,73 ± 0,02).

Increment of IGF-1 at P18 likely derives from the direct interaction of animals with the richness of the environment, but our results show that a trend towards increased IGF-1 levels at P18 are found also in animals exposed to EE up to P12, suggesting that some of the early molecular events caused by EE may “prime” IGF-1 cortical levels. Thus, both the higher levels of maternal care, which can be considered an indirect interaction of the developing pups with the environment, and the direct interaction of the pups with the environment would be necessary to cause a significant increase of IGF-1 and to obtain the maximal acceleration of visual development at cortical level.

IGF-1 affects the density of inhibitory synapses in the visual cortex

What could be the specific targets affecting by IGF-1 to accelerate visual acuity development? One hypothesis stems from the known effects of IGF-1 in increasing neuronal activity (Carro et al., 2000). The enhanced presence of IGF-1 in EE rat visual cortex might act on neurons bearing IGF-1 receptors, determining an increase in spontaneous or evoked neural activity, and in the production of activity-dependent factors, such as neurotrophins (Ickes et al., 2000), or in the activation of activity-dependent pathways, such as ERK/CREB, important for visual cortical development and plasticity (Di Cristo et al., 2001; Berardi et al., 2003). Expression of genes under CREB promoter control has been involved in several paradigm of visual cortex plasticity both in vivo and in vitro, like in several other cerebral region (Impey et al., 1996; Impey et al., 1998a, b; Pham et al., 1999; Barth et al., 2000; Watt and Storm, 2001; Athos et al., 2002; Barrot et al., 2002). Recently it has been suggested that CRE mediated gene expression could be involved in the precocious phases of visual system development (Pham et al., 2001): this study shows that a temporal window exist during thalamus development in which CRE mediated gene expression is particularly intense, corresponding to the period of thalamic connections refinement. Cancedda et al. (2004) found that in non-EE mice the peak of CRE mediated gene expression in the visual cortex is at P25, while in EE mice it is anticipated at P20, which is in good agreement with the increase of IGF-1 immunoreactivity we found at P18 in EE rats. These data, show, that similarly to developmental profile of IGF-1 protein levels, there is a window of CRE-mediated gene expression in the developing visual cortex and this event occurs earlier in EE animals. Cancedda and colleagues (2004) demonstrates also that pharmacological induction of cAMP/CREB by means of rolipram treatments from P7 to P28, reproduces EE on the development of visual acuity. Because it is known that the binding of IGF-1 to its receptor is able to affect also cAMP/CREB pathway (for a review see D'Ercole et al., 1996), our results suggest that IGF-1 action on visual acuity development could be mediated by IGF-1 induction on cAMP/CREB pathway. It would be important to assess whether IGF-1 administration activates the ERK/CREB pathway and in which neuronal types in the visual cortex.

We have shown for the first time that IGF-1 labelling is present in inhibitory interneurons (GAD_{67} positive neurons) in the developing visual cortex and that the density of IGF-1 positive interneurons is increased by EE. This suggests that another, non alternative, explanation for IGF-1 effects on visual acuity development could be an action of IGF-1 on inhibitory interneurons.

Glutamic acid decarboxylase is the rate-limiting enzyme that converts the excitatory neurotransmitter glutamate into the inhibitory neurotransmitter GABA. At least two isoform of GAD are present in the central nervous system coded by separate genes (Erlander et al., 1991). One gene codes for a protein approximately 65 kDa and the other gene codes for a protein approximately 67 kDa in size and both enzyme possesses decarboxylase activity. The observation that IGF-1 affects GAD65 immunoreactivity in puncta-rings demonstrates that inhibitory interneurons respond to IGF-1 with a GAD65 increase in their synaptic terminals, an effect possibly mediated by an increase in BDNF expression, which is known to be caused by IGF-1 in the adult (Carro et al., 2000; Cotman and Berchtold, 2002) and which affects intracortical inhibitory system development in the visual cortex (Huang et al., 1999). The result that GABAergic interneurons in the visual cortex express IGF-1 receptor during development suggest that IGF-1 can also act directly on inhibitory interneuron development. The nature of this action, whether it is directed on neuronal activity or/and the expression of synaptic proteins and GABA biosynthetic enzymes remains to be determined. Since inhibitory interneuron development has been suggested to contribute to visual acuity development (Huang et al., 1999; Bartoletti et al., 2004) we suggest that IGF-1 action on inhibitory interneuron development is a possible mediator of EE effects on visual acuity development, although a contribution from an IGF-1 action on excitatory neuron development cannot be excluded.

IGF-1 action on inhibitory circuitry development is also suggested by the fact that release of GABA neurotransmitter is regulated by IGF-1 (Castro-Alamancos and Torres-Alemann, 1993; Castro-Alamancos et al., 1996; Seto et al., 2002).

A link between IGF-1 pathways and the maturation of inhibitory circuitry is confirmed also by the detailed anatomical analysis of the brain of *Igf1*$^{-}$/$^{-}$ mice at two months of age reported by Beck et al., 1995. They observed that these mice carrying inactive IGF-1 genes showed a reduction in a parvalbumin-containing neurons which were counted in the striatum and in the hippocampus. In the cerebral cortex and in the hippocampus, parvalbumin is expressed by a subset of GABAergic neurons that display, at least in the hippocampus, a rapid firing rate and high metabolic activity (Celio, 1984 and 1990; Bergmann et al., 1991; Solbach and Celio, 1991). The exact transmitter type of striatal parvalbumin immunoreactive neurons is not known, but they are most likely not GABAergic (Celio, 1990). In the hippocampus of *Igf1*$^{-}$/$^{-}$ mice the numbers of parvalbumin-containing neurons were reduced by 32% and 35% in CA1-4 area and the dentate

gyrus, respectively. In the dorsal striatum, the decrease strongly than in the hippocampus, was 52%.

IGF-1 affects the density of perineuronal nets in the visual cortex

We have shown for the first time that IGF-1 increases the density of neurons ensheated by PNNs in the visual cortex of P25 non-EE rats treated with intracortical infusion of IGF-1 from P18 respect to contralateral cortex treated with the vehicle. We also analyzed the density of PNNs in the visual cortex of P25 EE animals treated from P18 with JB1, IGF-1 receptor antagonist, in one cortex and with the vehicle in the contralateral cortex. We observed that EE significantly increase the density of PNNs respect to the standard condition, and this effect of EE on the increase in PPNs number was prevented by IGF-1 action blockade. On the other and, we further confirmate that JB1 treatment in the concentration used by us is not detrimental for the normal development of PNNs, the number of which does not differ from the visual standard animals treated with vehicle or enriched animals treated with JB1.

PNNs are multimolecular assemblies mainly formed by CSPGs, hyaluronan and glycoprotein such as Tenascin-R (for a review see Dityatev and Schachner, 2003). Proteoglycans are formed by a core protein with attached lateral chains of glycosminoglycans (GAGs), negatively charged polysaccharide chains composed of repeating disaccharide units. Results obtained by Thiébot et al., 1997 in rat testis suggest that IGF-1 could stimulate glycosaminoglycan biosynthesis by inducing glycosyltransgerase(s) or it could increase core protein pool glycosylation, or further IGF-1 could also stimulate the expression of core proteins concomitant with those of glycosiltransferase. Silberg et al., 1981 postulated that IGF-1 could stimulate proteoglycan syntheis not only by elongating existing chondroitin sulphate chains but also by increased synthesis of other sugar chains.

Recent studies have demonstrated association of some IGFBPs with the ECM or cell surface via glycoproteins, collagens, integrins (Jones et al., 1993 a and b), and glycosaminoglycans (Arai et al., 1994; Smith et al., 1994). Some growth factors, such as basic fibroblast factor, bind to cell membrane and ECM proteoglycans and this interaction is important for binding to specific high affcinity receptors (Berrou et al, 1995). In contrast IGF-1 do not

substantially bind to proteoglycans or other ECM components. Cell-associated IGFBPs may therefore act as a linker molecules allowing pericellular sequestration of IGF-1. Russo et al., 1997 demonstrated that IGFBP-2 binds to the glycosaminoglycan component of membrane proteoglycans *in vivo*, in the rat brain olfactory bulb. They shown that *in vitro*, IGFBP-2 bound to chondroithin-4-sulphate, chondroithin-6-sulphate, keratan sulfate, heparin and the proteoglycans aggrecan. Both IGFBP-3 and IGFBP-5 bind to membranes and/ or ECM from different cells, and salt and heparin displacement suggest that binding to glycosaminoglycan may be involved (Jones et al., 1993; Arai et al., 1994; Smith et al., 1994; Martin et al., 1992; Hodgkinson et al., 1995). The mechanism of potentiation of IGF-1 activity by IGFBPs is not completely understood; in many situations IGFBPs inhibit IGF-1 activity, but they may also enhance IGF-1 activity. It could postulated that the decrease in binding affinity of IGFBPs bound to proteoglycans for IGF-1, facilitate release of IGF-1 from IGFBPs for binding by receptors enhancing IGF-1 activity. A very interesting question to answer could therefore be whether EE affects also IGFBPs levels and if EE animals show an higher percentage of IGFBPs colocalizing with PNNs.

Performing a double staining IGF-1 receptor and WFA, to label PNN we found that both at P18 and at P25 a large proportion of WFA positive interneurons (64% at P18, 50% at P25) were also positive for IGF-1 receptor labelling; thus, a direct effect of IGF-1 on PNN surrounded interneurons is possible. A mechanism by means of which IGF-1 could affect PNNs density could be also the enhancement of neural activity. Evidences demonstrated that synthesis of CSPGs is activity-dependent.

The action of IGF-1 on PNN and inhibitory interneurons suggests that IGF-1 could be also the mediator of EE effects on visual cortical plasticity shown by Bartoletti et al. (2004). This would be in agreement with the involvement of IGF-1 in ocular dominance plasticity in response to monocular deprivation (Tropea et al., 2006).

It has been described that IGF-1 could mediate the neuroprotective effects of exercise enhancing neuronal glucose metabolism and improving oxygen consumption by neurons (Carro et al., 2001). Indeed, IGF-1 enhances glucose uptake (Bondy and Cheng, 2002) and glucose use by neurons through up regulation of glucose transporters and modulation of glycolitic enzymes (Cheng et al., 2000). On the other hand, IGF-1 could increase neurons oxygen availability inducing the expression of HIF-1 (Zelzer et al., 1998). Enriched living conditions, with enhanced physical movement and multisensory stimulation, is likely accompanied by changes in

respiratory and cardiovascular activity proportional to the increased metabolic demendas of organs, incluing brain. In which way IGF-1 effects on glucose neuronal metabolism could contribute to EE effects on visual cortical development, if they do, is not yet understood. Tropea et al., 2006 hypothesized that IGF-1 enhancement glucose uptake could be involved in preventing the effects of MD reducing deprivation-induced competition between afferents from the two eyes. However, Tropea et al also propose, as we do, that the action of IGF-1 is on cortical plasticity and is mediated by cortical neurotrphins. Whether an increased glucose metabolism could contribute to the eccelerated visual cortical development observed in EE rats or in IGF-1 treated rats remains to be ascertained.

Early EE, IGF-1 and retinal development

Landi et al. (2007) demonstrated that EE from birth exerts a strong influence on the postnatal development of the retina, causing a marked acceleration in retinal acuity maturation, an effect dependent upon the increase in BDNF levels in the RGC layer of EE pups at P10. We show here that exposure of the mother to EE during pregnancy accelerates the development of the retina in the offspring. The acceleration is evident already at prenatal ages. In particular, our results show that enrichment of the mother during pregnancy accelerates the physiological time course of apoptotic process, anticipating at E18 the peak of natural cell death observed at P1 in the offspring of non-EE mothers. The changes we observe in the rate of RGC apoptosis were paralleled by an increased IGF-1 immunostaining in the retinas of EE pups at E15 and E18. These experiments suggested that the EE effects we found on retinal apoptotic process could be dependent on IGF-1. Recent results (Sale A., Cenni MC., Ciucci F., Putignano E., Chierzi S., Maffei L., unpublished data), report that neutralization of IGF-1 abolished the action of maternal enrichment on RGC death. Furthermore, chronic IGF-1 infusions during late pregnancy were sufficient to induce in non-EE animals an IGF-1 increase in their RGC layer and increased levels of pyknosis, thus mimicking the changes elicited by EE on RGC layer development. This result demonstrated that IGF-1 is involved in mediating EE effects on the acceleration of fetus retinal maturation. IGF-1 receptors are present in the retina (Rodrigues et al., 1998; Waldbillig et al., 1998), particularly in RGC layer (Burren et al., 1996) and their expression is developmentally regulated (Hernandez-Sanches et al., 1995; Lee et al., 1992). The influence of IGF-1 on cell cycle kinetics has been recently demonstrated by Hodge et al. (2004), who showed that it

accelerates cell cycle during embryonic development. If this effect, which has been documented for cortical neurogenesis, is present also in the retina, it would contribute to accelerate the time course of RGC death in the enriched condition, where we found increased levels of IGF-1. Therefore, it is possible that alterations in the expression of IGF-I may affect naturally-occurring developmental cell death in the RGC layer.

The increased level of IGF-1 we found in the retina of EE embryos could be elicited by enhanced transfer of maternal substrates to the foetus occurring in the enriched condition. This could derive from maternal exercise during pregnancy, which is known to increase the foetal brain/body weight ratio (Houghton et al., 2000). Enhancement in glucose and placental lactogens received by the foetus results in a direct stimulation of IGF production (Owens, 1991; Anthony et al., 1995; Fowden et al., 1998; Javaid et al., 2004). We found that increased levels of IGF-1 at P1 was present in RGC layer of EE pups. At this age, we found higher levels of IGF-1 in the gastric content of suckling EE pups. Even if we did not assess IGF-1 levels in the milk directly taken from the mother, it is known that the concentration of IGF-1 in maternal milk is reflected in the concentration of the peptide gastric content of the pups (Olanrewaju et al., 1996). Luminal IGF-1 supplied in the milk may act by modulating gastrointestinal proliferation and differentiation (Donovan et al., 1991; Philipps et al., 2000). On the other hand, it has been shown that IGF-1 administration in the fetal gut increases gastrointestinal development in fetal sheep (Kimble et al., 1999), thus enhanced level of IGF-1 observed in the milk of EE pups could accelerate gastrointestinal tract development.

We suggest that an indirect source for the difference we found in IGF-1 expression between EE and non-EE rats in the retina at P1 could be the increased intestine growth which allow in EE pups an enhanced nutrition, that it is known to enhance IGF-1 production (Clemmons and Underwood, 1991; Thissen et al., 1994).

IGF-1 mediates the effects of EE on retinal acuity development

The acceleration of retinal acuity development produced by EE is not dependent on vision since EE is effective in causing it before P10, when pup eyes are still closed (Landi et al., 2007). Molecular changes triggering the acceleration of retinal maturation are likely influenced by EE precociously, when pups are still immobile and dependent on the mother.

Our results show that IGF-1 is one of these factors. Enrichment during pregnancy affect IGF-1 levels in pups retina, cerebellum and in maternal milk. Many studies demonstrate that tactile interactions between rats pups and mothers modulated pup physiology an neuroendocrine function at various stages of development. Short-term periods of separation of preweanling rat pups (<20 days old) from their mother, triggers a coordinates pattern of neuroendrocrine response that includes (1) a decrease in ornithine decarboxylase (ODC) basal levels in major organs, (2) a suppression of tissue ODC responsiveness to administered prolactin (PRL) and growth hormone (GH), (3) an inhibition of GH secretion (Butler et al., 1978; Kuhn et al., 1979; Evoniuk et al., 1979; Schanberg et al., 1984; Pauk et al., 1986; Wang et al., 1996) and (4) decrease in DNA synthesis (Greer et al., 1991). Because GH which is affected by different levels of maternal care, regulates IGF-1 synthesis we hypothesized that IGF-1 could be a mechanism by means of EE elicits also its earlier effects on visual system development.

Our results showing that early IGF-1 injections in eyes of non-EE pups mimic EE effects either increasing BDNF levels in RGCs layer and accelerating retinal acuity development, suggest that IGF-1 could be a key factor that EE acts upon to prime P-ERG acuity development.

This hypothesis is further confirmed by the observation that the reverse experiment (IGF-1 action blockade by intraocular injections of JB1, IGF-1 receptor antagonist) prevents EE effects on retinal development. Thus, we have provided for the first time a direct demonstration of IGF-1 involvement in the functional development of retinal circuitry *in vivo*.

Another early molecular change induced by EE at retinal level is the BDNF increment observed at P10 in EE animals; this BDNF increase has been demonstrated to be necessary for the effects of EE on retinal acuity maturation (Landi et al., 2007). Also for the effects of prenatal exposure to EE on RGC developmental pattern of cell death could involve BDNF. Exercise during pregnancy increases hippocampal expression of BDNF in the neonatal rat (Parnpiansil et al., 2003) and it has been proposed that placenta and amniotic fluid are a source of neurotrophic factors for the developing foetus (Uchida et al., 2000). BDNF exerts a strong influence on retinal development by directly regulating natural cell death of RGCs, and it is thought to be the target-derived trophic factor for which the ganglion cells compete during development (reviewed in von Bartheld, 1998 and Cellerino et al., 2000). Results showing that an effect on RGC-death rate similar to that we found in EE animals have been reported in mice with null mutation of BDNF

(Pollock et al., 2003) suggest that different levels of BDNF in the retina of EE rats are implicated in the developmental changes in RGC death dynamics displayed by enriched rats.

Because pheripherical administration of IGF-1 induces BDNF mRNA in the brain (Carro et al., 2000), BDNF is potentially a down-stream target that could mediate some of the effects of IGF-1. Our results suggest indeed that IGF-1, in its role of mediator of EE effects on retinal acuity maturation acts by increasing BDNF levels at P10 in RGC layer of EE pups.

In the adult, BDNF expression is known to be increased both by environmental enrichment and physical exercise (Carro et al., 2000; Cotman and Berchtold, 2002; Adlard et al., 2004; Klintsova et al., 2004) and that this increase is mediated by IGF-1. Recently it has been also demonstrated (Ding et al., 2006) that IGF-1 affects BDNF system to mediate exercise effects on cognitive processes and synaptic plasticity. However a direct relationship between IGF-1 and BDNF during development, such as we have now demonstrated, had never been demonstrated before.

As a possible target of IGF-1/BDNF control on visual acuity development, we propose the action of IGF-1, by means of BDNF, on dopaminergic amacrine cells. 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). 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). It would be extremely interesting to study if also intraocular IGF-1 injections from P1 to P7 affect the development of dopaminergic amacrine cells.

Retina and visual cortex developmental interactions in EE

Landi et al., 2007 demonstrated that enriched environment effect on retinal maturation starts very early: exposing pups to EE only for the first 10 days of life is sufficient to reproduce

the acceleration of retinal acuity development observed in animals enriched up to the day of P-ERG recordings.

The effect of early EE is extremely different between retina and cortex. Exposure to EE up to P12 is not sufficient to trigger a significant increase in IGF-1 labelling in the visual cortex and produces only a partial effect on visual acuity development. This suggests that the early molecular effects produced by EE in the visual cortex (Cancedda et al., 2004, Sale et al., 2004) are not sufficient to trigger the full increase in the visual acuity of P25 animals caused by continuous exposure to EE up to P25. In addition, our results show that the visual cortex of animals intraocularly injected with IGF-1 and showing an accelerated maturation of retinal acuity, does not show an increased visual acuity. This results suggest that the faster maturation of retinal development, triggered by early EE or early IGF-1 intraocular injections, is not sufficient to drive the acceleration of the visual cortex maturation observed in continuous enriched condition or in animals intracortically infused with IGF-1 from P18. To obtain the maximal effects of EE exposure on cortical visual acuity and, ultimately, on visual behaviour, both the early component of EE, likely mediated by enhanced maternal care, and the later components, likely mediated by direct pup interaction with the environment, are necessary.

We demonstrated that IGF-1 is the key molecule in mediating both the effects of early EE at retinal level and of postweaning EE at cortical level. In the retina, a temporal cascade of molecular events has been delineated, IGF-1 triggering BDNF increase and the later developmental events. At cortical level, it remains to be ascertained whether the early BDNF increase documented at P7 (Cancedda et al., 2004) is subsequent to the early IGF-1 increase in maternal milk or whether IGF-1 acts only at later developmental ages on visual cortical neurons.

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ACKNOWLEDGEMENTS

Desidero ringraziare tutti coloro che, in varia misura, hanno contribuito alla realizzazione di questo percorso di ricerca e di vita.

Rivolgo il mio più profondo ringraziamento al Professor Lamberto Maffei, la cui guida preziosa è stata per me il punto di riferimento costante nello studio della Neurobiologia. Sono infinitamente grata alla Professoressa Nicoletta Berardi che, con la sua professionalità e disponibilià, mi ha seguito passo dopo passo in tutto il corso del dottorato. E' difficile esprimere tutto l'affetto e la stima che provo per la Dottoressa Elena Putignano, il cui sostegno e la cui amicizia sono stati fondamentali sia nei momenti sereni che nei momenti difficili che si sono presentati. Rivolgo un ringraziamento particolare alla Dottoressa Silvia Landi il cui aiuto e la cui determinazione mi hanno spronato ha perseguire i miei obiettivi. Ringrazio di tutto cuore la Dottoressa Maria Cristina Cenni che mi ha fornito un aiuto prezioso rispondendo sempre con disponibilità alle mie richieste. Ringrazio il Dottor Alessandro Viegi ed il Dottor Tommaso Pizzorusso con cui ho avuto la fortuna di lavorare apprendendo nozioni fondamentali. Ringrazio il Dottor Matteo Caleo, il Dottor Gian Michele Ratto ed il Dottor Yuri Bozzi che mi hanno dato importanti consigli. Desidero esprimere la mia più profonda gratitudine a Giulio Cappagli e a Carlo Orsini che mi hanno aiutato a risolvere qualsiasi problema informatico e meccanico che si sia presentato. Ringrazio tutti i miei colleghi ed amici del laboratorio in particolare Marco, Laura, Alessandro, Dario, Matilde, Fernando, Sara, Flavia, Maria, Francesco per la loro amicizia ed il loro sostegno.

Infine desidero esprimere tutto il mio affetto a Barbara, Francesco, Tommaso ed ai miei genitori a cui questa tesi è dedicata.