SCUOLA NORMALE SUPERIORE

Pisa

CLASSE DI SCIENZE MATEMATICHE, FISICHE E NATURALI

CORSO DI PERFEZIONAMENTO IN NEUROBIOLOGIA

2005-2008

PhD Thesis

MASSAGE ACCELERATES BRAIN DEVELOPMENT AND THE MATURATION OF VISUAL FUNCTION

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INTRODUCTION

CHAPTER 1

THE RELATIONSHIP BETWEEN BRAIN AND BEHAVIOUR: A NATURE-NURTURE INTERPLAY

Over the past several decades, significant advances have been made in our understanding of the basic stages and mechanisms of mammalian brain development. This large body of work provides a picture of brain development as the product of a complex series of dynamic and adaptive processes operating within a highly constrained, genetically organized but constantly changing context (Morange, 2001). The view of brain development that has emerged from the developmental neurobiology literature presents challenges and opportunities to understand the fundamental process that underlie the different aspects of behaviour and the neural systems that mediate them. Our current view about brain and behaviour has risen over the last century from convergence of several experimental disciplines, including anatomy, embryology, physiology, pharmacology and psychology.

A lasting influence came from the studies of Charles Darwin, who set the stage for the use of animals as models of human actions and behaviour by publishing his observation on the continuity of species in evolution. This new approach gave rise to ethology, the study of animal behaviour in the natural environment, and later to experimental psychology, the study of human and animal behaviour under controlled conditions. A large number of studies was focused on the genetic and environmental determinants of behaviour within the debate "nature versus nurture". The old but always fascinating question addressed in this debate was " is the human mind a *"tabula rasa"*, a

blank slate upon which experience leaves its mark or are its proprieties innate, brain shaping being driven by a sequence of coded instructions which are already written in its structure?".

In the second half of the last century, the debate between Behaviourists and Ethologists was very informative regarding nature-nurture debate. In North America, a new psychological current called Behaviourism emerged proposing a highly objective approach wherein the observable behaviour was the focus of its experimental research, leaving out all the internal process. Behaviourism considered the human mind as "black box". Cardinal points of Behaviourism were that the basic processes of learning were common to all vertebrate as well as all behaviours must be learned rejecting the concept of "innate". This approach, albeit incomplete and reductive, led to a considerable amount of experimental research on learning processes, that was essential for advancing in the knowledge of the organization of behaviour (Skinner, 1938; Munn, 1950). In turn, in Europe, the Ethological school emerged in part as reaction against the subjective interpretations of behaviour and in part against an unorthodox interpretations of Darwinian evolution. The study of animal behaviour allowed to detect the properties of invariance characterising the behaviour of individuals belonging to the same species. These ethological investigation revealed the existence of highly stereotyped behaviours, perfectly executed the first time of their appearance, in the total absence of previous learning or training. This led to the concept of 'instinctive behaviour' and to the now widely accepted idea that some behavioural traits can be inherited. However, this new approach was limited by the frequent tendency exhibited by the Ethologists to ignore those behaviours not classifiable as genetically programmed.

A fundamental contribution to the solution of this debate came from the ethologist Konrad Lorenz. He introduced for the first time the concept of "innate predisposition to learn" (Lorenz, 1961), focusing on the remarkable evidence that all learning processes

have to occur within the physiological limits and constraints of each species. A predisposition to respond and to learn from some stimulus rather than others (Hinde and Hinde, 1973) occurs also in human infants (Johnson and Morton, 1991). The behaviour is modified by learning allowing a better adaptation to environment where an organism lives and grows and every learning process is fitted with the natural necessities of every single species. In this synthesis 'innate' and 'learned' are not antithetic, but constitute the two main ways through which information about the environment are available to the organism and registered in the nervous system circuits, finally resulting in an adaptive behaviour (Lorenz, 1961; Rescorla, 1988).

A straightforward example of a learning process which is genetically determined to occur at a biologically optimum time is the 'filial imprinting' (for reviews, see Bolhuis and Honey, 1998; Horn, 1998; Horn, 2004). Filial imprinting was originally studied in precocial birds, such as ducklings or domestic chicks, which learn to recognise and follow their mother (or an inanimate mother-surrogate) very quickly after hatching, subsequently following and preferring her (or the surrogate) to another adult female or object (Lorenz, 1937; Slucking, 1972; Horn, 1985). Since the reaction of mother-following is completely innate, while the knowledge of which particular object has to be followed is acquired through the early postnatal experience, the filial imprinting is a paradigmatic example of an innate component, the predisposition to follow a large, moving object, with a learned component, which object has to be followed, the interaction of which give rise to the observed behaviour. By now, we know that during development experience is crucial to ensure that the cerebral circuits, initially built on the basis of genetically coded instructions, mature appropriately, thus assuring a normal development of brain functions and of behaviour.

1.1 Role of Experience in neuronal development and Critical Periods.

The effects of experience on the brain are strong during restricted time windows in development named "sensitive periods", during which neural circuits display a heightened sensitivity to external stimuli. "Critical periods" are a special class of sensitive periods wherein an appropriated experience is absolutely required, at fixed development periods, for the normal development of a pathway or a set of connections inducing irreversible changes in brain function (for review, see Berardi et al., 2000; Knudsen, 2004). How early experience transforms the nervous systems initially crude wiring plan into the exquisitely precise patterns of connectivity that are required to mediate adaptive behaviour is a question of great interest for the neuroscience. Understanding the mechanisms involved in the development and plasticity of neural connections has also an important clinical relevance. It may potentially allow reactivation of neural circuit plasticity in the adult brain which is normally less plastic than the juvenile brain, with obvious sequences for brain repair. In addition, it might provide insights on the factors underling deviations from the typical developmental plan, and hence on the aetiology of developmental brain disorders. Experimental evidences have shown that different regions of the brain have critical periods that occur at different times and are activated and regulated by distinct mechanisms (for a review, see Hensch, 2004). The primary visual cortex has been the main model used to study experience-dependent plasticity for 40 years as visual experience can be easily manipulated and the consequences of manipulations can be readily measured at the anatomical, physiological and molecular levels. Although the first, classic experiments have been performed in cats and primates, in the last decades rodents have been preferred for the simplicity of their visual system and the relative ease of genetic manipulation.

1.2 Disrupted visual experience during Critical Period.

The normal development of human vision depends on visual experience. It has been demonstrated that early visual impairments such as strabismus, uncorrected refractive errors and cataracts can impair visual acuity, global motion perception, contrast sensitivity and binocular vision if they remain beyond the end of the corresponding CP (Maurer et al., 1999; Lewis and Maurer, 2005). Uncorrected, these visual disability can lead to amblyopia, which is a frequent cause of vision loss in infants and young children, occurring naturally in about 2-4% of the population (Levi, 2006).

A widely used paradigm to study the role of experience on the development of the visual cortex is dark rearing (DR). In this protocol the animal is reared from birth in complete darkness, no visual information is available and only the spontaneous electrical activity is present along the visual pathways. Animals reared in DR show an abnormal functional and anatomical maturation of their visual cortex, that appears immature far beyond the end of the critical period. DR animals display several physiological deficits including: a rapid habituation of visual cortical neurons, i.e. the visual response tends to disappear with repetition of stimulus presentations (Sherman and Spear, 1982; Fagiolini et al., 1994); receptive fields of visual cortical cells are larger than normal, and neurons have a reduced orientation selectivity (Fagiolini et al., 1994); anatomically ocular dominance columns are immature (Sherman and Spear, 1982; Crair et al., 1998); the visual spatial resolution (visual acuity) of these animals is extremely low, as measured electrophysiologically and behaviourally ,(Fagiolini et al., 1994; Gianfranceschi et al., 2003). Other parameters of visual responses are changed: cell responsiveness is lower, latency of visual response is increased and spontaneous activity is altered (Pizzorusso et al., 1997). Indeed, the mean rate of spontaneous activity was increased in DR animals (Gianfranceschi et al., 2003). A total lack of visual experience also affects the fine

morphology of visual cortical neurons which exhibit alterations in dendritic arbors and in the size, morphology and density of dendritic spines (Valverde, 1971; Wallace and Bear, 2004). Light exposition, even if just for few hours, restored in the animals a regular development processes allowing the recovery of both neuronal response properties (Buisseret et al., 1978; 1982) and normal anatomical features (Wallace and Bear, 2004).

Another classic model used for understanding how experience-dependent activity can refine brain circuitry is monocular deprivation (MD), which consists in the closure of one eye by lid suture. Early life manipulation of afferent visual input by MD induced an ocular dominance (OD) shift leading to a loss of cortical physiological responses to the closed eye and an increase in the number of neurons responding preferentially to stimuli presented to the open eye: the binocularity properties of cortical neurons was dramatically disturbed. In parallel with this lack of ability to drive cortical neurons, the deprived eye shows a strongly reduced visual acuity and its contrast sensitivity is blunted; the deprived eye becomes amblyopic (Wiesel and Hubel, 1963a; Hubel and Wiesel, 1970; Olson and Freeman 1975; Movshon and Dürsteler, 1977; Olson and Freeman, 1980). Remarkably, spatial resolution in the deprived retina remained completely unaffected, suggesting that the modifications at the basis of the amblyopia occur at cortical level (Wiesel and Hubel, 1963b; Sherman and Stone, 1973; Kratz et al., 1979; Baro et al., 1990). Hubel and Wiesel observed that in kittens the susceptibility to MD changes with age, beginning abruptly near the start of the fourth week of life, being most robust during a specific time window (between sixth and eighth weeks) and then declining. It is possible to reverse the effects of MD by removing the eye occlusion but only during the critical period; with CP closure these effects become irreversible (Wiesel and Hubel, 1965a; Movshon, 1976; van Sluyters, 1978; Blakemore et al., 1981; Antonini et al., 1998; Berardi et al., 2000). There is little or no recovery from amblyopia in the adult. MD

starting in adulthood produces no detectable outcome (Hubel and Wiesel, 1970; Olson and Freeman, 1980).

At anatomical level, MD causes a reduction of territories innervated by lateral geniculate nucleus (LGN) afferents driven by the deprived eye in the cortical layer IV and a subsequent expansion of those driven by the open eye (Katz and Shatz, 1996). However, the reorganization of the geniculo-cortical projections may not be the first modification induced by MD as it was believed in the past years: anatomical and functional changes occur within one day from the start of MD in cortical layers II and III, but not in layer IV (Trachtenberg and Stryker, 2001; Oray et at., 2004). These findings suggest that MD elicits the first modifications at level of cortical neurons in the intracortical horizontal connections and that these changes are reflected in subsequent reorganization of the geniculo-cortical afferents. Several questions remain, however, with respect to the involvement of the connections between neurons of layer IV and II/III in OD plasticity and to the mechanism by which thalamo-cortical remodelling is guided by higher cortical stages (see Bence and Levelt, 2005).

MD effects on the OD of cortical neurons have been also demonstrated in rodents: the physiological responsiveness of neurons in the binocular zone of V1 shift towards the open eye, and this plasticity is confined to a well-defined critical period (Dräger, 1978; Fagiolini et al., 1994; Gordon and Stryker, 1996). As in other species, the OD shift in rodent is found in all layers, but it is more pronounced in extragranular layers than in layer IV (Gordon and Stryker, 1996), suggesting that intracortical as well as geniculo-cortical synapses undergo plasticity following MD. In the developing visual cortex of the mouse, this functional plasticity is accompanied by anatomical changes, as in higher mammals (Antonini et al., 1999). Moreover, it has been shown by in vivo two-photon microscopy that spine motility in the binocular region of V1 controlateral to the deprived eye, is 35% higher than motility in nondeprivated animals (Oray et al., 2004).

This increased spine motility may reflect structural destabilization which could precede a robust pruning of spine protrusions, probably correlated to the rapid reduction in the deprived-eye drive (Mataga et al., 2004).

1.3 Physiological mechanisms of visual cortex plasticity following MD.

Having defined the crucial role of experience during the development of visual system, the next step is to understand what changes in the nervous system following MD. Initially, Wiesel and Hubel proposed a mechanism in which OD plasticity results from competitive interactions between the two eyes for the control of cortical units: binocular lid suture was not effective to shift OD columns in mammals (Wiesel and Hubel, 1965; Sherman and Spear, 1982; Gordon and Stryker, 1996). An experiment performed by Stryker's laboratory supported this competitive view showing that an imbalance in the electrical activities of the two retinas is sufficient to shift OD also in visual deprivation conditions (Chapman et al., 1986). Besides, a reversible blockade of the activity of cortical neurons by intracortical infusion of sodium channel blocker tetrodotoxin (TTX) or muscimol completely prevents the OD shift that may normally occur after MD, or causes a paradoxical shift in favour of the deprived eye (Reiter et al., 1986; Reiter and Stryker, 1988; Hata and Stryker, 1994; Hata et al., 1999). The classic competition-based model is related to heterosynaptic mechanisms, involving interactions between two sets of inputs (i.e. from the two eyes) where open eye inputs drive down the synaptic efficacy of the deprived inputs (Miller et al., 1989; Harris et al., 1997). Active geniculate neuron corresponding to the open eye compete better than less active neurons, driven by the closed eye, so they become functionally and structurally strengthened.

Data published since 1990s proposed an alternative view of the processes underlying MD, suggesting the idea that OD plasticity is due to homosynaptic mechanisms, related to specific forms of synaptic plasticity. These mechanisms engage separately each eye's pathway (Blais et al., 1999; Heynen et al., 2003; Frenkel and Bear, 2004). Recently, chronic electrophysiological recordings in mice at the peak of the critical period have pointed out that binocular competition may actually be the consequence of separable processes with distinct time courses mediating depression of deprived-eye and potentiation of non deprived-eye response (Frenkel and Bear, 2004; Mrsic-Flogel et al., 2007; Kaneko et al., 2008a,b). There is, therefore, a depression of responsiveness of deprived eye, following by a potentiation of open eye responses. This model is known as Bienenstock-Cooper-Munro model (BCM theory) and postulates a bidirectional change of synapses: they can undergo homosynaptic long term potentiation (LTP) but also homosynaptic long term depression (LTD) (Kirkwood et al., 1996; Bear and Rittenhouse, 1999; Sengpiel and Kind, 2002; Heynen et al., 2003). Loss of responsiveness of deprived eye was proposed to be the result of homosynaptic depression, where spontaneous, residual activity coming from closed eye contribute to synaptic depression. To test this hypothesis, the effect of very brief MD by lid suture has been compared with that of monocular silencing by intra-ocular injections of TTX (Rittenhouse et al., 1999). Results showed that lid suture was more efficient in causing depression of deprived eye responses than blockade of all retinal activity by TTX. Other evidence indicates the involvement of a phenomenon of homosynaptic depression in the effects of MD; brief MD sets the same molecular and functional changes as the experimental model of homosynaptic LTD which accounts for the loss of responsiveness of deprived eye during MD (Heynen et al., 2003). On the other side, a delayed modification induced by MD is an experience-dependent potentiation of open eye responses. There is evidence supporting the idea that long-term potentiation of the

synapses driven by the open eye is important for ocular dominance plasticity. First, alphaCAMKII activity appears to be required for both LTP in vitro and MD plasticity in vivo (Kirkwood et al., 1997; Taha et al., 2002). Second, a form of in vitro LTP (white matter - layer II-III LTP) is developmentally regulated with a decline over time that mirrors that of the critical period for ocular dominance plasticity (Kirkwood et al., 1996). Monocular inactivation by TTX is also able to enhance potentiation of uninjected eye, as shown by Visual Evoked Potentials (VEPs) (Frenkel and Bear, 2004). However, other data are in disagreement with the view that MD effects during the critical period are entirely ascribable to LTP- and LTD-like mechanisms. Indeed, GAD65 knockout mice, which are not sensitive to brief MD, show no deficit in induction of LTP or LTD in layer II/III of mouse binocular visual cortex (Hensch et al., 1998). BDNF-overexpressing mice are sensitive to MD at least during an early phase of postnatal development, although BDNF prevents LTD in V1 (Huang et al., 1999; Jiang et al., 2003).

Thus, it is still unclear whether MD effects are completely modelled by homosynaptic mechanisms. Several alternative hypotheses have been also advanced to account for the phenomenology of OD plasticity; balanced levels of excitation and inhibition have shown to be critical for enabling plasticity (Hensch, 2005a; Hensch and Fagiolini, 2005). GABAergic circuits are ideally posed to lead the arrangement of activity-dependent synaptic modification. The mismatch in the maturation of excitation and inhibition may trigger phenomenon of activity-dependent plasticity; hyperexcitation as well as cortical silencing prevented the OD plasticity (Shaw and Cynader, 1984; Ramoa et al., 1988; Bear et al., 1990).

However, these manipulations do not provide mechanistic information and Hensch's group was the first to shed light on the role of local, inhibitory cortical circuits in OD plasticity (Hensch et al., 1998). Hensch and co-workers, using a knockout (KO) mouse, lacking the 65 kDa isoform of glutamic acid decarboxylase (GAD65), the GABA

biosynthetic enzyme, found no shift in their responsiveness in favour of the open. The enhancement of inhibition obtained by local delivering of diazepam produces a complete OD shift in the infused mutant visual cortex (Hensch et al., 1998). Remarkably, the rescue of plasticity is possible at any age, indicating that critical period onset is dependent on a certain level of inhibitory transmission (Fagiolini and Hensch, 2000). Indeed, the onset of the critical period can be accelerated by premature enhancement of GABA-mediated transmission (Fagiolini and Hensch, 2000; Fagiolini et al., 2004); in addition, in transgenic mice overexpressing BDNF the inhibitory circuits mature precociously and the critical period plasticity begins and ends earlier than normal (Huang et al., 1999). Importantly, GABA-releasing interneurons in the neocortex show precise connectivity (Somogyi et al. 1998, ; DeFelipe et al.,197) and one class of these that are particularly important for critical period plasticity are the parvalbumin positive cells. Specific blockade of a potassium channel (Kv3.1) that uniquely regulates the fidelity of fast-spiking behaviour (and thereby GABA release) from these cells decreases the OD shift after MD (Hensch, 2005). Hensch's group have also analyzed the role of GABAergic receptor in the OD plasticity, using a mouse "knockin" mutation to alpha subunits that renders individual GABAA receptors insensitive to diazepam: found that only GABAA receptors containing α1 subunit are responsible for critical period plasticity (Fagiolini et al., 2004). The key role of GABAergic transmission in the regulation of OD plasticity is not restricted to mouse, as it has been demonstrated that OD columns are disrupted in cat by manipulating inhibitory transmission (Hensch and Stryker, 2004). Thus, a certain threshold of inhibition is necessary to trigger plasticity, but a higher level of inhibition, reflecting maturation of circuits, is responsible for critical period closure (Huang et al., 1999). A recent study shows that pharmacological reduction of intracortical inhibition obtained through the infusion of either MPA (an inhibitor of GABA synthesis) or picrotoxin (a GABAA antagonist) directly into the visual cortex reactivates OD

plasticity in response to MD in adult rats (Harauzov et al., 2010). Moreover, also other manipulations resulting in reductions of cortical inhibition promote adult visual cortical plasticity (He et al., 2006; Maya-Vetencourt et al., 2008).

Among the physiological mechanisms proposed to explain visual cortex plasticity another form of synaptic plasticity has been proposed as a complement of Hebbian mechanisms. This view is based on the concept of homeostasis: neurons are able to maintain their responsiveness and synaptic strength within a certain range, despite perturbations of the levels of neuronal activity (Burrone and Murthy, 2003; Turrigiano and Nelson, 2004; Davis, 2006). The reduced visually-driven activity during MD could be counteracted by neurons in the visual cortex, using two strategies: deprived neurons could reduce the threshold for LTP induction (Kirkwood et al., 1996; Bear, 2003) and visual responsiveness could be enhanced directly by increasing synaptic strength or intrinsic excitability (Desai, 2003; Maffei et al., 2004; Turrigiano and Nelson, 2004). This mechanism, known as homeostatic response compensation, could include: the activity-dependent regulation on intrinsic neuronal firing properties (Desai, 2003; Marder and Prinz, 2003; Zhang and Linden, 2003); pre- and post-synaptic forms of excitatory synaptic plasticity, such as synaptic scaling, that adjust all of a neuron's excitatory synapses up or down in the right direction to stabilize firing (Turrigiano and Nelson, 2004; Davis, 2006); the balancing of excitation and inhibition within neuronal networks (Maffei et al., 2004); compensatory changes in synapse number (Kirov et al., 2004; Wierenga et al., 2006); and meta-plastic mechanisms that adjust the relative threshold of LTP and LTD induction (Bienenstock et al., 1982; Abraham and Bear, 1996). The studies on mechanism of homeostatic regulation have focused on the synaptic scaling of excitatory synapses which was first described in dissociated rat cortical cultures, where blockade of activity with TTX increases and blocking GABA-mediated inhibition decreases the amplitude of miniature excitatory postsynaptic currents (mEPSCs)

(Turrigiano et al., 1998). Homeostatic adjustments in synaptic strength may occur at both post-synaptic and pre-synaptic levels of synapse (Turrigiano et al., 1998; Murthy et al., 2001; Thiagarajan et al., 2005; Wierenga et al., 2005; 2006) and require that each neurons sense and translate changes in activity into compensatory changes in synaptic strength, but one point that remains to be clarified is if synaptic scaling is induced by postsynaptic changes in firing, presynaptic changes in neurotransmitter release or local dendritic changes in receptors activation or calcium influx.

Another crucial issue in homeostasis model concerns how changes in activity are signalled to synapses. The synaptic scaling could require widespread changes in network activity, perhaps through activity-dependent release of a soluble factor by many neurons or glia simultaneously, such as BDNF, cytokine tumor-necrosis factor α (TNFα) and the effector immediate-early gene product Arc (Rutherford et al., 1998; Stellwagen and Malenka, 2006; Shepherd et al., 2006; Turrigiano et al., 2007; Kaneko et al., 2008a). Recently, several molecules important for trans-synaptic signalling and cell adhesion have been implicated in synaptic scaling (Goddard et al., 2007; Cingolani et al., 2008). There is now increasing evidence that synaptic scaling in excitation and inhibition plays important roles during various critical periods of visual system development (Desai et al., 2002; Maffei et al., 2004; Maffei and Turrigiano, 2008a). Recently, it has been found, using two-photon calcium imaging in vivo, that a short MD led to a decrease of deprived-eye responses only in neurons with substantial open-eye input, while longer MD resulted not only in the strengthening of non-deprived eye activity, but surprisingly, also of deprived-eye responses in neurons largely lacking non-deprived eye inputs (Mrsic-Flogel et al., 2007). These findings demonstrate that the weak input of deprived eye is not able per se to induce response depression, which instead seems to be dependent on the input of the other eye. Interestingly, in monocular visual cortex, the population of neurons driven only by the deprived eye has homeostatic-mediated stronger responses after deprivation.

In support to the notion that synaptic scaling underlies the gain of the non-deprived eye, it has been found that the blockade of TNFα signalling in visual cortex has no effect on the loss of responsiveness to the deprived eye but prevented the gain of responsiveness to the non-deprived eye (Kaneko et al., 2008).

In conclusion, from this overview about the role of experience and the relative plastic modification in neuronal circuits of visual cortex emerged that there is not a single form of synaptic plasticity, but rather a complex interplay between multiple forms of change in synaptic strength, including modifications in inhibitory circuitry, homosynaptic depression and potentiation, and global changes in circuit gain.

1.4 Molecular substrate of visual cortex plasticity

A complete understanding of critical period plasticity requires to take into account the molecular mechanisms that make the changes described above possible. The molecular mechanisms that control the developmental plasticity of visual cortical connections are not yet fully understood.

The N-methyl-D-aspartic acid receptors (NMDARs), given their peculiar characteristics, might play a central role in visual cortex plasticity, acting as 'coincidence detectors' for Hebbian plasticity. Indeed, these receptors are not only transmitter and voltage-dependent, but they also allows influx of Ca2+ which acts as intracellular signal. The first experiments showed that the blockade of NMDARs in visual cortex prevented MD effects. However, these works were liable to criticisms, since their pharmacological manipulations had potent suppressive effects upon normal synaptic transmission (Bear et al., 1990; Roberts et al., 1998; Philpot et al., 2001). Subsequently, the use of different

NMDAR antagonists or antisense oligonucleotides to reduce expression of NR1 subunit of the NMDA receptor has overcome this problem, showing that it is possible to block the effects of MD without affecting visual responses (Roberts et al., 1998; Daw et al., 1999a). An interesting property of NMDARs is their subunit expression, which is developmentally and activity regulated. In particular their subunit composition varies in the visual cortex, from a dominant presence of the subunit NR2B to a high presence of the subunit NR2A, with a time course paralleling that of the critical period; the expression of subunit NR2A also correlates with the progressive shortening of the NMDA current (Carmignoto and Vicini, 1992; Roberts and Ramoa, 1999; Husi et al., 2000). It has been shown that the NR2A/NR2B ratio in DR animals, which show a delay in the critical period closure, is lower than in light-reared animals suggesting a delay in the development shortening of NMDARs current (Quinlan et al., 1999 a,b; Tongiorgi et al., 2003). However, mice with NR2B over-expression or with deletion of NR2A don't show an increased susceptibility to plasticity, respectively (Philpot et al., 2001; Fagiolini et al., 2003).

Thus, NR2A might be not necessary for critical period regulation, but might be involved in other properties of cortical maturation. Interestingly, a very recent study highlights a co-regulation of OD plasticity and NMDAR subunit expression in GAD65 knockout mice. In the visual cortex of these animals there are reduced NR2A levels and slower NMDA currents. In addition, application of benzodiazepines, which rescues OD plasticity in GAD65 knockout mice, also increases NR2A levels, suggesting that changes in inhibition would engage mechanisms that converge to regulate NMDA receptors, thereby enabling plasticity (Kanold et al., 2009).

There is a conspicuous number of observations suggesting that neurotrophins control visual cortical plasticity during the critical period. The pioneering experiments on neurotrophins have enabled to gain knowledge of the molecules mediating the action of

experience in plasticity. In 1990s, Maffei's group put forward the idea that competition for limited amounts of neurotrophins is the effector of activity-dependent plasticity in the cortex, and the conventional explanation for OD plasticity is that the deprived eye does not activate cortical cells as well as the open eye, thereby failing to stimulate them to release sufficient neurotrophins to sustain the deprived-eye pathway (Maffei et al., 1992; Domenici et al., 1997). Indeed, exogenous delivery of NT in the visual cortex during MD prevented competition, rescuing cortex from MD effects (McAllister et al., 1999; Berardi et al., 2000). With the exception of neurotrophin 3 (NT-3), all neurotrophins influence MD, but not all factors play the identical role on visual neuron properties: neurotrophin 4 (NT-4) and NGF prevent the shift induced by MD, and they have no effects on spontaneous or visually-driven activity (Gillespie et al., 2000; Lodovichi et al., 2000), while BDNF is less effective in preventing OD shift, and it changes both spontaneous and visually-evoked activity of cortical neurons (Lodovichi et al., 2000). A possible mechanism of action of neurotrophins on neural plasticity is the modulation of synaptic efficacy. In visual cortex synaptosomes, both NGF and BDNF potentiate glutamate and acetylcholine release, while only BDNF does so for GABA release. Like BDNF, NT4 potentiates GABA and glutamate release but is much less effective in enhancing acetylcholine release (Sala et al., 1998). Considering these information with data on the expression of trk receptors in the visual cortex and with data on retrograde transport of cortically injected NGF (Domenici et al., 1994b), it can be concluded that NGF is likely to act directly on cholinergic afferents from the basal forebrain and on a population of glutamatergic cortical neurons; BDNF targets are principally cortical glutamatergic pyramidal cells and inhibitory interneurons, whereas NT4 acts on glutamatergic thalamic afferents and probably pyramidal neurons and inhibitory interneurons.

Other key studies on the relationship between neurotrophins and the development of inhibitory processes were conducted in BDNF-overexpressing mice. These animals

have an accelerated postnatal rise of this neurotrophin which might be responsible of an acceleration of development of visual function, such as visual acuity, and of critical period time course. Notably, a strong link was established between BDNF and intracortical inhibition, because GABAergic circuit maturation is accelerated by overexpressing BDNF (Huang et al., 1999). Other studies have followed a complementary strategy antagonizing the action of endogenous neurotrophins showing that neurotrophins are important for normal visual cortical development and plasticity (Berardi et al., 1994; Domenici et al., 1994a; Cabelli et al., 1997).

In addition, neurotrophin production and release is developmentally regulated and depend on electrical activity representing a link between experience-dependent plasticity and their action (Castren et al., 1992; Bozzi et al., 1995; McAllister et al., 1999). Electrical activity can be modulate by NT at pre and post-synaptic level with fast actions by increasing transmitter release (Sala et al., 1998; Jovanovic et al., 2000) or by directly depolarizing neurons (Kafitz et al., 1999), and with slow actions by modulating gene expression (Poo, 2001). This reciprocal regulation between neurotrophins and neural activity might explain because active neuronal connections are selectively strengthened as observed during MD (Caleo et al., 1999; Kovalchuk et al., 2002). Indeed, neurotrophins seem to require the presence of electrical activity to exert their actions (Sala et al., 1998; Caleo et al., 1999; McAllister et al., 1999). From the literature it also emerged that in the classic view of the "neurotrophic hypothesis" the possibility of an anterograde action of neurotrophins must be included (Caleo et al., 2000; Kohara et al., 2001; von Bartheld, 2004).

The frame of thought significantly changes: not only cortex-derived factors guide stabilization of thalamic afferents on cortical neurons, but also thalamic fibers themselves release factors which promote and guide the formation and maintenance of their synapses

onto cortical neurons and that cortico-thalamic afferents may contribute to the development of the pattern of thalamo-cortical connectivity.

At this point, how do central neurons integrate electrical activity and neurotrophin signalling to control plasticity of cortical circuitry? Some studies have identified three important signalling kinases that can modulate synaptic strength and are critical for inducing OD plasticity: extracellular signal-regulated kinase 1,2 (ERK-1,2), cAMP-dependent protein kinase (PKA), and calcium/calmodulin-dependent protein kinase II alpha (CaMKIIα; Beaver et al., 2001; Di Cristo et al., 2001; Taha et al., 2002; Taha and Stryker, 2005). Each kinase is activated by specific pattern of extracellular signals however there is overlap and crosstalk among each pathway explaing also why the block of only one of these molecules affects OD plasticity. The possible targets of these kinases after visually driven activation are at two different levels: the cytoplasm and the nucleus. In the first case, their activation induce a direct phosphorylation of plasticity-regulating molecules at the synapse (such as glutamate or GABA receptors) or phosphorylation of substrates crucial for synaptic transmission, neuronal excitability and morphological stabilization (e.g. synapsin I, potassium channels, MAP2). In the second case, they may signal to the nucleus engaging gene transcription (Berardi et al., 2003). Indeed, rapid neuronal plasticity modifications are characterized by changes in synaptic efficacy but the long-lasting changes in neuronal circuitry need gene expression and a protein expression. The intracellular mechanisms mediated by kinase signalling can lead to the activation of cAMP-responsive element-binding protein (CREB), which in turn controls CRE-mediated gene expression of proteins essential for establishment and maintenance of plastic changes (Cancedda et al., 2003; Suzuki et al., 2004). It has been recently demonstrated that CRE-mediated transcription is upregulated by MD during the critical period in the visual cortex contralateral to the deprived eye and that CREB is necessary for OD plasticity (Pham et al., 1999; Liao et al., 2002; Mower et al., 2002). As with many

other molecules that mediate changes in plasticity, CREB levels also decrease with age (Pham et al., 1999).

In the last years, it became clear that neurons modify gene expression patterns in response to experience-dependent activity and that these mechanisms could explain how the brain produces long term changes in its circuits. Histone phosphoacetylation seems to be a good candidate to explain synaptic plasticity and activity-dependent gene transcription. Recently, the regulation of chromatin structure has emerged as one mechanisms regulating visual cortex plasticity, since it has been demonstrated the involvement of histone phosphoacetylation in OD plasticity (Putignano et al.,2007). The authors found that in juvenile mice, visual stimulation that activates CREB-mediated gene transcription also induces ERK-dependent MSK and histone H3 phosphorylation and H3-H4 acetylation, an epigenetic mechanism of gene transcription activation. Remarkably, this effect is developmentally regulated: in adult mice visual experience is able to activate ERK and other kinases, but it is unable to promote histone phosphoacetylation at a level comparable to that found in juvenile animals. Accordingly, stimulation of histone acetylation in adult animals by means of trichostatin is able to promote OD plasticity in adult mice (Putignano et al., 2007). The gene expression modifications deriving from the induction of histone acetylation could explain the way by which long-term changes of brain circuitry take place.

Downstream effectors that implement the program initiated by the signalling mechanisms described in the preceding section are largely unknown. However, it is becoming clear that the extracellular environment, and in particular the extracellular matrix (ECM), plays an important part in controlling spine dynamics and visual cortical plasticity. One of the factors involved in experience-dependent plasticity is represented by tissue plasminogen activator (tPA) which is induced by electrical activity as an immediate-early gene (Qian et al., 1993). Proteolysis by tPA increased in V1 after two

days of MD during the critical period, but not in adulthood or in GAD65 knock out mice (Mataga et al., 2002). Therefore, tPA inhibition impaired OD shift induced by MD (Mataga et al., 1996) and prevented recovery from MD following reverse occlusion (Muller and Griesinger, 1998). Targets of tPA include extracellular-matrix proteins, growth factors, membrane receptors, cell-adhesion molecules (Endo et al., 1999; Wu et al., 2000; Nicole et al., 2001), that could all be involved in cortical plasticity. Beside, it has been shown that tPA delivery in young animals trigger an increased spine motility suggesting a further evidence for a key role of tPA in plasticity (Oray et al., 2004).

Another component of ECM which has been investigated is represented by chondroitin-sulfate proteoglycans (CSPGs). These molecules are organized in typical structures, named perineuronal nets (PNNs), around soma and dendrites of parvalbumin-positive neurons. The time course of PNNs increase during development and their organization in the visual system is complete at the end of the critical period for the effects of MD (Hockfield et al., 1990; Koppe et al., 1997; Pizzorusso et al., 2002). In addition, the development of CSPGs is regulated by visual activity, since the process of PNN condensation is prolonged by dark rearing (Hockfield et al., 1990; Pizzorusso et al., 2002). The inhibitory action of CSPGs in cortical plasticity was remarked by Pizzorusso e coworkers which have shown that a degradation of CSPGs in adulthood by chondroitinase ABC is able to restore OD plasticity and to promote recovery from amblyopia (Pizzorusso et al., 2002; Pizzorusso et al., 2006). In addition, treatment with chondroitinase ABC coupled with reverse lid-suturing caused a complete recovery of spine density in the adult rats (Pizzorusso et al., 2006).

To date it has been accepted that the adult brain has a decreased ability to repair and that myelin exerts an active inhibitory role in these processes (Schwab, 2004). Few years ago it has been demonstrated that certain factors preventing brain repair, such as Nogo, a growth inhibitor associated to myelin, are involved in the closure of the critical

period. Mice with a mutation in Nogo receptors have an altered course of the critical period, showing a prolonged OD plasticity induced by MD (McGee et al., 2005). The Nogo pathway does not seem to affect GABAergic inhibition or tPA activity, that are indeed normal in mutant mice. Rather, its signalling involves the low-affinity neurotrophin receptor p75 and Rho pathway. Thus, myelinization is able to inhibit not only recovery from injury, but could also promote the decrease of plasticity observed at the end of the critical period (Sengpiel, 2005).

In the past few years, there was an intense research on molecular mechanisms underlying activity-dependent synapse remodelling focusing on possible candidate genes and gene expression programs. These approaches of genetic screens have opened the door for examination of new families of molecules in plasticity (e.g proteins related to IGF-I pathway or immune/inflammation system signals). Expression of most of these molecules is developmentally regulated and differentially altered by sensory experience (Ossipow et al., 2004; Majdan and Shatz, 2006; Tropea et al., 2006; Lyckman et al., 2008). From literature of OD plasticity emerges as a complex, interrelated set of mechanisms, involving a large number of molecules of different classes. For this reason, an important goal in the field of cortical plasticity is to understand how the many molecular mechanisms guiding plasticity are recruited, how they interact and converge to permit and instruct plasticity, and over which time scale they act.

1.5 Permissive or Instructive role of electrical activity.

The profound effect of experience-dependent electrical activity on cortical connections remodelling, as previously displayed in MD experiments, have led to

formulate the hypothesis that electrical activity was an essential requirement also for neuronal circuitry formation (Katz and Shatz, 1996). The issue of whether sensory-driven electrical activity has instructive or permissive roles for development during critical period is much debated.

A first series of experiments have suggested that visual activity might be instructive, namely it conveyed by temporal distribution of electric discharge, the information need to ocular dominance column construction (Weliky and Katz, 1997; Crair at al., 1999). However, recent evidences support that early emerging columns in the carnivore does not depend on visual experience (Crowley and Katz, 2000). Similarly, the normal layout of orientation and OD maps in the visual cortex initially proceeds in absence of visual inputs. However, long-term visual deprivation induces a progressive deterioration of orientation maps and OD columns (Crair et al.,1998). Besides, these functional maps are present at birth also in primate and their development, at least initially, proceed without visual-driven activity (Horton and Hocking, 1996). These observations suggest, therefore, that early establishment of the functional architecture of the visual cortex is based on intrinsic mechanisms independent from visual experience which is involved, later, in its maturation. As intrinsic mechanisms can be consider those factors that do not require for their activation experience-driven signals, such as spontaneous electrical activity, whose existence and temporal pattern depend, in turn, by genetic activation and occur in the developing visual neuronal circuitry well before birth. Indeed, in vivo recordings from animals in utero found action potentials in spontaneously active cells (Galli and Maffei, 1988; Weliky and Katz, 1997; Chiu and Weliky, 2001). Notably, recordings in anesthetized prenatal rats between embryonic days 18 and 21 demonstrated that the firings of neighbouring retinal ganglion cells are strongly correlated, resulting in highly correlated bursts of action potentials (Maffei and Galli-Resta, 1990). Correlation in the activities of neighbouring neurons in the retina could be

the basis of developmental processes such as refinement of retinotopic maps in the brain. To test the hypothesis that retinal waves drive segregation of axons in the dLGN, was blocked of spontaneous retinal waves were blocking in ferret pups by monocular intraocular injection of cholinergic agents. The projection from the active retina was greatly expanded into territory normally occupied by the other eye, and the projection from the inactive retina was substantially reduced altering the eye-specific lamination pattern of the lateral geniculate nucleus (Penn et al., 1998). In addition, increased retinal waves activity by elevated cyclic adenosine monophosphate (AMP) in one eye resulted in an expansion of the territories occupied by ipsilateral projection of that eye, suggesting an instructive role for retinal waves (Stellwagen and Shatz, 2002). However, every experiment wherein spontaneous retinal activity has been blocked or elevated, correlated ganglion cell activity was maintained.

Thus, although the relative level of activity in the two eyes is important for normal retinogeniculate development, it no permit to know whether normal spatiotemporal patterns of neural activity are necessary for eye-specific segregation: these experiments cannot conclude that spontaneous activity has instructive role. In a very difficult experiment, it has been demonstrated that disrupting the correlated activity of neighbouring ganglion cells in the developing ferret retina through immunotoxin depletion of starburst amacrine cells did not prevent normal eye segregation in the dLGN; if all spontaneous activity was blocked, segregation of projections from the two eyes failed to occur (Huberman et al., 2003). Therefore, some features of spontaneous neural activity are not required for the formation of eye-specific projections to the dLGN, but a threshold is necessary to reach a normal development. The debate is still open (Cook et al., 1999; Huberman et al., 2003). Experiments performed in ferrets indicated that retinal activity is not required for the initial formation of OD column, even if (Crowley and

Katz, 2000; Feller and Scanziani, 2005; Huberman, 2007) spontaneous activity plays a role in the refinement and maintenance of their initial structure.

Currently, it can be conclude that during development of visual system the neuronal circuitry, maps and networks were built under control of intrinsic factors such as spontaneous activity and that experience-driven activity is involved for maintaining and refining of final connectivity.

1.6 Disrupted early social experience and neural development.

As the development of the brain's sensory systems depends on sensory experience at certain critical stages also social behaviour depends on social experience at specific periods of neural development.

The first compelling evidence that early social interactions with other humans is essential for normal social development came in the 1940s from the work of the psychoanalyst Réne Spitz. Spitz compared the development of infants raised in a foundling home with the development of infants raised in a nursing home attached to a women's prison (Spitz, 1945). From this research emerged that two factors were crucially involved on the normal development of infants: amount of maternal care and surrounding social and sensory environment. The babies that received lesser amount of human contact (low maternal care) and lived under conditions of sensory and social deprivation, at the end of the first year showed a dramatically low motor and intellectual performance. Many of these children had developed a syndrome that Spitz called hospitalism, now often called analytic depression (Spitz RA., 1945; 1946).

The knowledge of the effects of an early social deprivation underwent one important step further in the 1960s when two psychologists, Harry and Margaret Harlow,

studied monkeys isolated for 6-12 months; these monkeys were physically healthy but behaviourally devastated (Harlow HF, 1958). These monkeys crouched in a corner of their cage and rocked back and forth like autistic children. They did not interact with other monkeys, nor did they fight, play or show any sexual interest. By comparison, isolation of an older animal for a comparable period didn't induce such drastic consequences. Harlow's research on affectional systems was also addressed to remedy the dramatic results of infant isolation. He thought a way to provide "mothering" to the isolated infants by developing surrogate mothers. It was found that the young monkeys clung to the terrycloth mother whether or not it provided them food and that the young monkey interacted with the wire surrogate only when it provided food (Harlow and Zimmermann, 1953). Harlow demonstrated that an infant's attachment to its surrogate mother was due much to "contact comfort" suggesting that for a mother-child bond to development it is necessary an intimate body contact.

Thus, the early experience of surrounding social environment and in particular the possibility to receive an "intimate body contact" by the mother or by a suitable surrogate mother can profoundly affect brain development. In the subsequent chapters, I will illustrate the effects of environmental enrichment and maternal care on brain development in rodent.

CHAPTER 2

THE INFLUENCE OF ENVIRONMENT ON BRAIN AND BEHAVIOUR: NEURAL CONSEQUECES OF ENVIRONMETAL ENRICHMENT

The old debate on the relative contribution of nature versus nurture to the construction and maintenance of brain architecture has led to the widely accepted consensus that genes and environment work in concert in shaping neural circuits and behaviour (Rutter et al., 2006). In the same years wherein Hubel and Wiesel began their studies on MD effects on the development of visual cortex, introducing the sensory deprivation as protocol to investigate the experience role during development, Rosenzweig and co-workers brought out environmental enrichment (EE) as an experimental protocol specifically devoted to investigate the influence of environment on brain and behaviour. This experimental protocol has permitted a relevant progress in understanding the influence of environmental experience on the development, refinement and maintenance of appropriate nervous system connections. A comprehensive definition of EE was provided for the first time by Rosenzweig et al. (1978) as 'a combination of complex inanimate and social stimulation'. Enriched animals are reared in large groups (6-10 individuals can be considered the most common used condition) and housed in wide stimulating environments where a variety of objects differently shaped (e.g. running wheels, platforms, boxes, toys, tunnels, shelters, stairs and nesting material) are present and changed frequently (specifically, the objects are completely replaced at least once a week; Fig.1). The purpose of EE is to improve the animals' quality of life by providing them with high levels of multi-sensory stimulation, increasing physical activity and social

interactions, stimulating natural behaviours and cognitive abilities (since the novelty due to frequent objects' replacement attracts the explorative curiosity of most laboratory animals). The richness of the environmental stimulation is viewed as a continuum ranging from the impoverished environment condition (IC; the animals is alone in a small cage with only available water and food), to standard environment condition (SC; the animals are housed with 2-5 individuals in laboratory standard cages where no particular objects are present except for food, water and litter) and to EE condition.

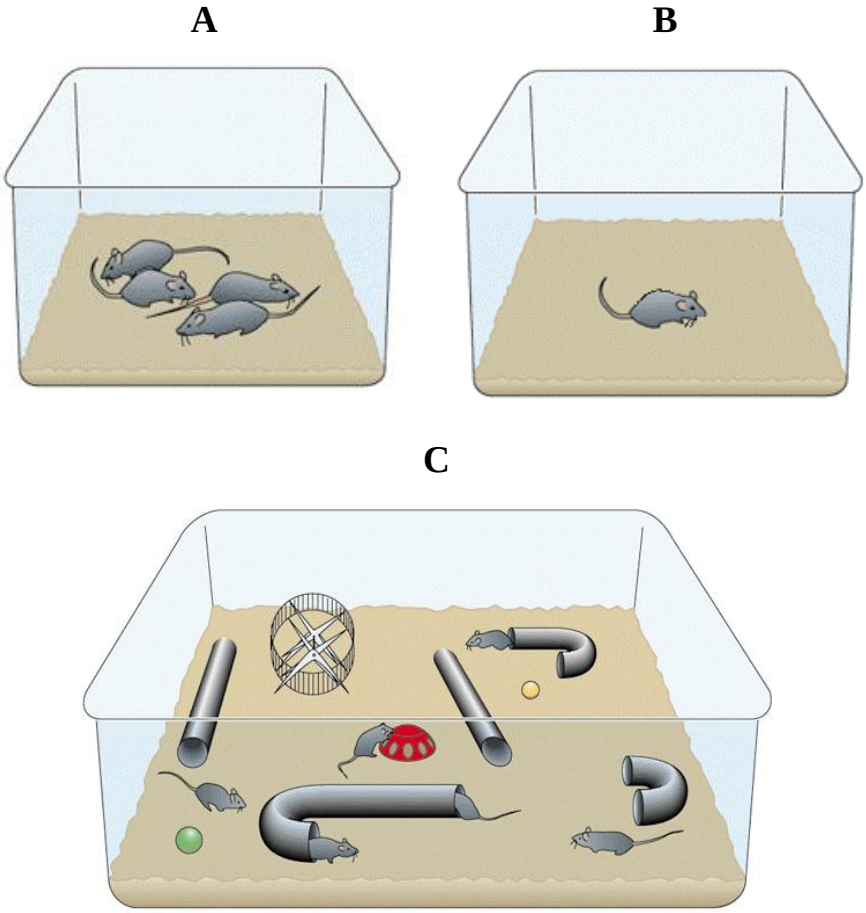


Figure 1

(A) Standard housing cage. The animals are housed as groups of 2-4 in regular size cages without any stimulus object. **(B)** Impoverished environmental condition. The animals are housed individually in regular size cages without any stimulus object. **(C)** Enriched environment. Enrichment consists of

social interactions (6-12 animals in big cages), stimulation of exploratory behaviour with different objects and a running wheel for exercise.

From theoretical point of view, it is usually assumed that EE condition is simply a way of rearing the animals in a setting more similar to the wildlife: a kind of semi naturalistic condition. However, the observation of rodents playing in an enriched environmental, choosing when and how much to run on the wheel and to explore the new objects indicated the different idea that EE is not just a way to reproduce more natural life conditions. Rather, EE implies a kind of challenge-free interaction with a stimulating surrounding. Although the activity of mice and rats in the wild is mostly driven by necessary, in an EE it is usually prompted by a combination of curiosity and play (Sale et al., 2009).

Many studies have tested the effects of the EE condition in several species of mammals (gerbils, ground squirrels, rabbits, cats and primates) and also in some avian species (Rosenzweig and Bennett, 1969; Cornwell and Overman, 1981; Hansen and Berthelsen, 2000; Kozorovitskiy et al., 2005; Lazic et al., 2007; Jansen et al., 2009), but the EE research has been mostly done upon rodents, showing that EE can elicit several plastic responses in the brain, ranging from molecular to anatomical and functional changes (for review, see Rosenzweig and Bennett, 1996; Diamond, 2001; van Praag et al., 2000).

Peculiarities of the environmental enrichment. The EE condition gives to animals the opportunity for enhanced social interactions, a continuous multi-sensorial stimulation provided by the novelty of new objects that encourage curiosity, exploration and visual-spatial memory exercise allowing to the organism to benefit from a balanced schedule of feeding and physical activity. Although the interest to separate different contributions exerted by these various components of EE on brain and behaviour is challenging, the

current opinion is that no single variable can completely account for the consequences of enrichment but rather these components might work in concert (see van Praag et al., 2000). For example, the component of increased sociality alone is not sufficient to account for all the effects of enrichment (Rosenzweig et al., 1978). Besides, morphological studies show that synaptic density, number of synapses per neuron and maximum length of synaptic contact zone are highest in enriched rats, intermediate in socially reared rats and lowest in isolates (Turner and Greenough, 1985; Sirevaag and Greenough, 1985). Thus, each single variable involved in enriched condition works in concert in the set up their effects on brain and behaviour.

However, there is a factor that has a major weight in the contribution to the EE effects: physical exercise. Indeed, animals reared in SC with the opportunity to increase their physical activity level thanks to presence of a running wheel or a treadmill, show results similar to those observed in the enriched animals (van Praag et al., 1999). Physical activity improves cognitive functions in rats and aging humans (Fordyce and Farrar, 1991; Kramer et al., 1999; van Praag et al., 1999 a, b; for review, see Churchill et al., 2002), attenuates motor deficits (Klintsova et al., 1998), increases neurogenesis and is neuroprotective, ameliorating neurological impairments in different neurodegenerative processes (Arkin, 1999; Petajan and White, 1999; Larsen et al., 2000; Mattson, 2000; Carro et al., 2000, 2001). Furthermore, physical exercise increases angiogenesis (Black et al., 1990; Isaacs et al., 1992) and enhances neurotrophin levels in the brain (Neeper et al., 1996; Oliff et al., 1998; Carro et al., 2000; Johnson et al., 2003; Farmer et al., 2004; Klintsova et al., 2004). Since these consequences are detected both in enriched environment and physical exercise alone, it has been suggested that they share common final pathways of cellular and molecular events. Another relevant element of EE is "learning"; enrichment including physical exercise seems to be more effective than exercise alone in enhancing memory functions (Bernstein, 1973). It is speculated that the

'learning' variable included in the enriched condition (due to increased exploration, exposition to novelty and to social interactions) is necessary to enhance subtle plasticity processes underlining learning and memory functions, while the increment of physical activity associated with EE can not always be sufficient to reproduce the effects elicited by running in an isolated cage.

The EE paradigm, therefore, can be consider as mild and natural treatment, which permits to perform studies of "gain of function" rather than "lost of function" as in MD protocol and the results obtained are of great interest and applicability also for humans in many different fields, from psychology to medicine. Human studies frequently demonstrate associations between environmental factors, particularly supportive social environments, and positive states of health (Winocur and Moscovitch, 1983; 1990; Winocour, 1998). The cognitive enrichment manifested in education, job complexity, and/or leisure activities, has beneficial effects which help to preserve several cognitive functions, and result in greater resilience to the effects of aging and dementia (for a review, see Kramer et al., 2004; Milgram et al., 2006).

2.1 Environmental enrichment effects on the adult brain

The idea of the 'enriched environment' arose in the 40s from studies performed by Donald Hebb. His research displayed that rats raised as pets in more complex environment than laboratory rodent's setting showed behavioural improvements in learning task (Rosenzweig et al., 1996). From these first observations began to flourish many other studies, mainly those of Rosenzweig and coworker, which stress how

different kinds of environments can produce changes in the brain throughout the life span at anatomical, physiological, molecular and behavioural level.

Anatomical changes. In the initial experiments were observed anatomical changes in animals exposed for 30 days at enriched environment protocol: different brain weights were found in littermates housed in EE and SC or IC conditions, not imputable to differences in body weight (Rosenzweig et al., 1962 a,b; Bennett et al., 1969). These changes occurred in the entire dorsal cortex, including frontal, parietal and occipital cortex. The conclusion that differential experience could produce measurable changes in the brain gained acceptance and further studies revealed increases in other measures, specially in occipital cortex making the visual cortex a preferred model. It has been widely reported that the cerebral cortex in EE animals is significantly thicker compared to littermates housed in impoverished and standard environments (Bennett et al., 1964a,b, 1970; Diamond et al., 1964, 1966, 1972; Walsh et al., 1971).

Subsequent studies have pointed out that exposure to EE leads an increment in size of cortical neurons' cell soma and nucleus (Altman and Das, 1964), dendritic branching and length (Holloway, 1966; Volkmar and Greenough, 1972; Globus et al., 1973; Greenough and Volkmar, 1973; Uylings et al., 1978; Green et al., 1983), number of dendritic spines (Globus et al., 1973), synaptic size and density (Mollgaard et al., 1971; West and Greenough, 1972; Diamond et al., 1975; Greenough et al., 1978; Bhide and Bedi, 1984; Turner and Greenough, 1985; Beaulieu and Colonnier, 1987), postsynaptic thickening (Diamond et al., 1964), gliogenesis (Diamond et al., 1966) and angiogenesis (Ekstrand et al., 2008). Further experiments revealed that significant cerebral effects of enriched versus impoverished (or standard) experience could be induced at any part of the life span and with relatively short periods of exposure (Bennett et al., 1964b; Riege, 1971; Ferchmin and Eterovic, 1986).

Another structure markedly sensitive to EE is the hippocampus, where changes similar to those reported for the cerebral cortex have been found for pyramidal cells in areas CA1 and CA3 and for dentate granule neurons (Walsh et al., 1969; Diamond et al., 1976; Fiala et al., 1978; Altschuler, 1979; Walsh and Cummins, 1979; Rosenzweig and Bennet 1996; Rampon et al., 2000a; Ekstrand et al., 2008). In particular, enriched living increases hippocampal neurogenesis and integration of the newly born cells into functional circuits: a short daily exposure to a complex environment for 14 days in adults is sufficient to induce a long-term increase in the rate of neurogenesis (Kempermann et al., 1997; Nillson et al., 1999; van Praag et al., 2000; Bruel-Jungerman et al., 2005). Indeed, using the proliferation marker bromodeoxyuridine (BrdU) it has been shown ≈70% increase in the number of newborn dentate gyrus cells in the enriched animals and most (80-85%) of these cells express a neuronal phenotype with a proportion similar to that observed in naïve rats. This suggests that EE effects are expressed independently of the cell lineage, resulting in a net increase in both neuronal and glial cells in the dentate gyrus (Nilsson et al., 1999). Moreover, EE appears to increase the number of surviving newly formed granule cells in the dentate gyrus rather than to act on proliferation of progenitor cells (Kempermann et al., 1997; Nilsson et al., 1999). These observations are explained by a result in differences apoptotic rates: apoptotic assessment using the TUNEL method revealed that EE reduced spontaneous apoptotic cell death in the rat hippocampus by 45% (Young et al., 1999). The functional significance and biological role of neurogenesis are not well understood. It is tempting to speculate that newborn granule cells in the dentate gyrus might be involve in memory formation supporting the improvement in the spatial learning tests observed of EE animals (Gould et al., 1999; Shors et al., 2001). No definitive conclusion on this issue has been reached ad yet.

Electrophysiological response. Few studies have addressed the possible relationship between enriched living and electrophysiological changes. The excitatory postsynaptic potential (EPSP) slopes and amplitudes in response to activation of medial perforant path (MPP) or cornu ammonis 1 (CA1) recorded in hippocampal slices, are greater in enriched housed rats with respect to age-matched control (Green and Greenough, 1986; Foster et al., 1996; Foster and Dumas, 2001; Irvine and Abraham, 2005). Similarly, a prolonged exposure to EE induced an increase of the EPSCs amplitude accompanied by a rise-time decrease and reduced pair pulse ratio in layer II/III of the auditory cortex (Nichols et al., 2007).

Other studies have found that a component of EE, exploration, could elicit patterns of electrical activity in hippocampal neurons of area CA1 that are similar to patterns of electrical stimulation used to induce LTP in hippocampal slices. In two recent studies, LTP and LTD, two different paradigmatic models of synaptic plasticity, are significantly enhanced in hippocampal area CA1 following 5-8 weeks of EE (van Praan et al., 1999; Duffy et al., 2001; Artola et al., 2006). Moreover, it is interesting to notice that enhancements in LTP and LTD, seen after a 5-weeks exposure to EE, are not reversed after 3-5 weeks exposure to standard housing (Artola et al., 2006). Two phenomena may contribute to this enhancement. First, paired-pulse facilitation is decreased in enriched rats compared with control animals, suggesting that exposure to enriched environment enhances transmitter release and thus, decreases the demand for presynaptic activation to reach the postsynaptic thresholds for inducing LTD and LTP. Accordingly, LTP induction requires a smaller number of high-frequency stimuli in enriched animals and it is very likely that enhanced LTD is also due, at least in part, to a facilitation of its induction. Second, environmental enrichment may also increase the dynamic range of synaptic modification. Indeed, repeated LTP and LTD induction produces larger synaptic changes in enriched than in control rats. These data reveal that

exposure to different environmental experiences can produce long-lasting effects on the susceptibility to synaptic plasticity, involving pre- and postsynaptic processes (Artola et al., 2006).

Although less is known about the changes induced by EE in other brain regions, in a recent work it has been demonstrated that in the anterior cingulate cortex (ACC), a cortical area that participates in cognitive functions including higher order emotional responses and several forms of memory, EE significantly increases LTP and largely diminishes LTD. Sensory experience changes synaptic plasticity in the ACC via postsynaptic mechanisms, by altering the dynamic regulation of NMDA receptor subunits: indeed, the component of NR2B-containing NMDA receptors is enhanced in EE-exposed animals (Shum et al., 2007).

Molecular changes. The initial experiments on the relation brain chemistry and EE condition in laboratory animals showed an increase in acetylcholinesterase activity (Rosenzweig and Bennett, 1962a, 1967) and subsequent studies confirmed this initial observation and extended it to other neurotransmitter systems which have diffuse projections to the entire brain. In particular, enriched living condition increased mRNA expression levels of serotonin 1A receptor, serotonin concentration in the cerebral cortex and hippocampus (Rasmuson et al., 1998; Galani et al., 2007; Brenes et al., 2008), noradrenaline concentration in the brain and potentiated β-adrenoceptor signalling pathway in the cerebral cortex, cerebellum and brainstem (Escorihuela et al., 1995; Naka et al., 2002).

Considering that neurotrophic factors (or neurotrophins, NTs) provide important extracellular signals regulating neural development, survival and plasticity and that these molecules respond to experience, their role in mediating EE effects was necessarily investigated. As expected, EE plays a key role in the modulation of synthesis and

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

New possibilities to further characterize brain molecular changes elicited by EE came from the recent development of gene chip analysis techniques and real-time PCR, allowing the simultaneous screening and comparison of differential gene activation in dependence on different environmental conditions. Although they produce only ‘snapshots’ of a highly dynamic process, such studies are instructive and suggest that a large number of genes change their expression levels in response to EE. The studies which have analyzed the effects of EE on gene expression in the mouse (Rampon et al., 2000a) and rat cerebral cortex and hippocampus (Keyvani et al., 2004) reported changes occurring after only 3 h of enriched environment exposure, but persisting until two weeks

from the start of the enriched housing procedure. A large number of genes was found to change expression levels in response to EE, with most of them being grouped in functional classes 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). Moreover, Pinaud et al. (2001) demonstrated that animals exposed daily, for 1 h, to EE exhibit a marked up-regulation in the cerebral cortex, hippocampus and striatum of the immediate early gene Arc mRNA, an activity-dependent neuronal marker involved in multiple forms of neuronal plasticity.

By comparing the gene expression profiles following a short experience in EE with those obtained after two weeks of EE, it was found that some group of genes are equally regulated (Keyvani et al., 2004): i) transcription factors, such as different zinc finger transcription factors (e.g. JunB, ElF-4E, Krox20, NGF1-B); ii) synapse-related molecules (e.g. synapsin, synaptogyrin, clathrin, Rho proteins); iii) proteolytic proteins and molecules mediating apoptosis (e.g. proteins belonging to Bcl family, ubiquitin-specific protease, ClpP protease, aspartyl aminopeptidase and prolidase). However, most of the genes regulated by a longer housing in EE are different from those involved in a brief experience of enriched condition. A number of genes associated with the regulation of neurotransmission and neuronal spiking activity are affected by EE. Indeed, EE dramatically modified the expression levels of different members of neurotransmitter (e.g. glutamate, GABA, dopamine and noradrenaline) receptors and of ion channels and transporters (e.g. Na,K-ATPase and Na-, K-channels; Keyvani et al., 2004). Rampon et al. (2000a) observed that the expression level of postsynaptic density 95 (PSD-95), important not only for anchoring the NMDA receptor at the postsynaptic membrane but also for coupling this receptor to pathways controlling synaptic plasticity, increases after 2 days and 14 days of enrichment. These authors have also investigated the influence of EE on knockout mice in which NMDA receptor was selectively deleted in the CA1

subregion of the hippocampus. These mice exhibited a learning deficit in hippocampus dependent behavioural tasks which could be rescue with two months of EE (Rampon et al., 2000a). Therefore, CA1 NMDA receptor activity seems not essential for experience-induced behavioural and synaptic plasticity and an explanation proposed by Tsien's group is that the compensation might be due to an enhancement in connectivity outside the functionally deleted hippocampus, for instance in the neocortex. Another study has supported this possibility showing that transgenic mice in which the NMDA receptor function is enhanced in the forebrain via overexpression of the NR2B subunit, had an overall improvement in their performances in learning and memory tasks. However, EE do not further increase their already augmented abilities and the occlusion of the effects induced by environmental stimulation suggests the existence of overlapping mechanisms between EE and genetic enhancement of the NMDA receptor functions (Tang et al., 2001).

A detailed dissection of the molecular mechanism underlying EE presented biochemical evidence that GluR1, NR2A and NR2B proteins in the forebrain begin to increase after 2 weeks of EE, indicating that NMDA and AMPA receptors functions might be directly modified by environmental experience. Besides, in the hippocampus was found an alteration in the expression of AMPA and NMDA subunit receptor following EE (Naka et al., 2005; Andin et al., 2007). EE triggers another neurochemical modification in the brain: indeed, very recent studies reported that differential rearing conditions affects also the opioid system, leading to sensitivity alterations in opioid receptor populations (Smith et al., 2003; 2005).

Other molecules might play an indirect role in the brain plasticity whose expression is regulated by enriched experience, i.e. metabolic enzymes (implicated in energy metabolism, oxidative stress and mithocondrial activity) and molecules involved in immune response (e.g. complement protein C1q, MHC class and T-receptor

molecules). Noticeably similar functional groups of genes were regulated in different brain areas, particularly in the hippocampus and striatum. However, different expression patterns were found in distinct brain areas at the individual gene level and there were only a few genes regulated in parallel in different brain regions: the higher responsiveness of the hippocampus to EE could be due to a more pronounced susceptibility of this structure for plasticity changes (Keyvani et al., 2004; McNair et al., 2007; Thiriet et al., 2008).

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

2.2 Environmental enrichment affects animals' behaviour

The changes previously observed at anatomical, electrophysiological and molecular level in animals housed in EE condition are accompanied by behavioural modifications. From the first descriptions performed by Hebb, subsequent findings have stressed how a more complex and stimulating rearing environment could enhance the performance on learning tasks in laboratory animals (Bingham and Griffiths, 1952; Forgays and Read, 1962).

After these pioneering observations, a large body of studies highlighted that environmental enrichment is able to modify animal's behaviour and in particular improves a complex cognitive functions as learning and memory (van Praag et al., 1999; for an exhaustive review, see Rampon and Tsien, 2000). Independently of the gender and age of tested animals, EE effects are especially evident in hippocampal-dependent tasks involving spatial memory, such as the Hebb-Williams maze (Kobayashi et al., 2002), the Lashley III maze (Greenough et al., 1972), the radial maze (Leggio et al., 2005) the Morris water maze (Tees et al., 1990; Falkenberg et al., 1992; Paylor et al., 1992; Moser et al., 1997; Kempermann et al., 1998a; Nilsson et a., 1999; Williams et al., 2001; Lee et al., 2003; Leggio et al., 2005; Fréchette et al., 2009) and the discrimination between different spatial contexts (Mitra and Sapolsky, 2008).

It has also found that enriched animals exhibited better performance in non-spatial learning and memory tasks as in the object recognition test, contextual and cued-fear conditioning (Rampon and Tsien, 2000; Duffy et al.,. 2001; Lee et al., 2003). However, in the olfactory discrimination memory test, that depend on the hippocampus, Rampon and Tsien noticed that enrichment has no effect on the social transmission of food preference.

The consequences of EE upon emotional reactivity are less documented and remained controversial for a long time: some authors reported no or inconsistent effects (Huck and Price, 1975; Fernandez-Teruel et al., 1997), others have observed modifications of emotional and stress reactions in EE animals (Chamove, 1989; Escorihuela et al., 1994). A more accurate analysis of the animal's behaviours in prenatally or postnatally stressed rats (Francis et al., 2002), aged mice (Thouvarecq et al., 2001) and mice considered as pathologically anxious (Chapillon et al., 1999; Roy et al., 2001; Iwata et al., 2007) led to assume that animals reared in enriched condition

exhibited a lower level of emotional reactivity than those reared in standard conditions (for a review, Chapillon et al., 2002).

The possibility to live in enriched environment can also modify the behaviour of strains of mice such as C57BL/6 and B6CBA, and of Wistar and Long-Evans rats. Indeed, it has been reported that environmental complexity decreases stress reactivity of these animals, as indexed by performance on an elevated plus maze (Fernandez-Teruel et al., 1997; Caston et al., 1999; Chapillon et al., 1999; Friske and Gammie, 2005; Zhu et al., 2006; Galani et al., 2007; Hoffman et al., 2009), defensive response to predators or intruders (Haemisch et al., 1994; Klein et al., 1994) and open field exploration (Widman and Rosellini, 1990; Chapillon et al., 1999; Nikolaev et al., 2002). Other works reported a significant differences in basal corticosterone levels between enriched and impoverished animals (Belz et al., 2003; Moncek at al., 2004; Welberg et al., 2006) and that BALB/c mice, a strain usually described as pathologically anxious (Trullas and Skolnick, 1993) displayed a decreased levels of anxiety after rearing in an enriched environment (Chapillon et al., 1999; Iwata et al., 2007), supporting the assumption of the EE anxiolitic action.

Furthermore, some recent experiments have shown that the increased level of corticosterone induced by a mild repeated immune challenge with lipopolysaccharides (LPS) injections can be abolished with enriched living condition (Mlynarik et al., 2004). Accordingly, animals reared in EE did not display typical signs of discomfort after LPS challenge, such as a transient decrease in body weight and suppression of grooming, suggesting that the failed physiological increased of corticosterone levels was advantageous and that EE animals could have a better ability to cope with stress. Besides, a mild electric shock significantly increases serum costicosterone levels only in mice housed in SC, but not in EE mice which display higher natural cells killer

cytotoxicity, an effect not abolished by stressing procedures (Benaroya-Milshtein et al., 2004).

These observation show that environment can affect the pathways of communication between the immune and the nervous systems suggesting important implications in the field of psychoneuroimmunology.

Other recent studies have demonstrated that EE is also protective for depressive disorders, leading to behavioural antidepressive-like effects in forced-swimming test both in normal rats and in animal models of depression (Brenes Sàenz et al., 2006; Llorens-Martin et al., 2007; Brenes et al., 2008). Finally, it has been shown that EE conditions can improve sensory information processing, leading to a difference in behavioural measures of visual acuity (Prusky et al., 2000a) and auditory spatial discrimination (Cai et al., 2009).

2.3 Environmental impact on developmental plasticity of the brain

Compared with the many paper on the neural and behavioural effects of environmental enrichment in adult animals, few works explore the influence of EE at early life stages and most of them apply post-weaning EE protocol. Nevertheless, many experimental evidences clearly suggest that the pre- and postnatal development of the nervous system are highly dependent on the interactions between the organism and its environment (reviewed in Chapillon et al., 2002).

Neonatal handling is a protocol used in many studies to investigate the effects of early postnatal manipulation at neurobiological and behavioural level. In this procedure, rat pups are removed from their mother and placed in a small container and then returned to their mother after a 10-15 minute separation period; this brief separation period occurs

daily over the first 2 or 3 weeks postpartum (Levine and Lewis, 1959). It has been reported that handled rats given have higher body weights (Denenberg & Karas, 1959) in adulthood as well as improved performance in cognitive tasks (Levine & Lewis, 1959; Lehmann et al., 2002; Kosten et al., 2007; Stamatakis et al., 2008) and a lack of cognitive decline in senescence (Meaney et al., 1988). Handling causes a variety of physiological changes, including alterations in plasma levels of norepinephrine and epinephrine (McCarty et al., 1981), increased glucocorticoid receptor (GR) expression in the hippocampus and frontal cortex (Meaney and Aitken, 1985; Meaney et al., 1989; Viau et al., 1993; Meaney et al., 1994), increased hippocampal NGF expression (Pham et al., 1997), reduced cell number and volume in the locus coeruleus (Lucion et al., 2003), and increased expression of immature GABA-A receptors in hippocampal neurons (Hsu et al., 2003). Although the handling procedure permit to have a lower output of corticosterone, prolactin and adrenaline in response to a mild stressor (Levine et al., 1967; Meaney et al., 1985; Meerlo et al., 1999; Nunez et al., 1996) and that it was described as therapeutic in reversing behavioural abnormalities induced by prenatal stress (Wakshlak & Weinstock, 1990) and prenatal exposure to alcohol (Lee & Rabe, 1999), no therapeutic advantage was observed following the preweaning handling of perinatal brain-injured rats (Gibb and Kolb, 2005). A comparison of neonatal stimulation with handling and adult EE shows that both treatments elicit some similar behavioural and neural consequences (reviewed in Fernandez-Teruel et al., 2002), but a direct comparison of early handling with early EE is still lacking.

The application of EE protocols during the preweaning period has attracted only a mild interest because it was believed that the sensory and motor systems immaturity prevented the possibility to engage sustained activities and in particular voluntary physical activity. Since increased levels of physical activity were thought to be an essential component of the enrichment protocol (van Praag et al., 2000), the possibility to

evoke neural and behavioural changes through preweaning enrichment were considered quite limited. For instance, we know that enriched living condition in adulthood stimulate hippocampal neurogenesis in mice and rats (Kempermann et al., 1997; Nilsson et al., 1999), but this effect was no found when pups from P7 to P21were put under enriched conditions (Kohl et al., 2002). However, in the literature some reports have shown how early enrichment can elicit experience-dependent plasticity processes. Indeed, more complex dendritic branching has been found in cortical pyramidal cells, in particular at the level of parieto-occipital cortex, following EE occurring either in the P10-24 period or starting postweaning (Venable et al., 1989; Kolb, 1995; Fernandez et al., 2003). In other studies, preweaning sensorimotor stimulation applied in rat pups from P5 to P21 increased neuronal cytodifferatiation in motor cortex and improved performance in behavioural adaptive responses, as measured in open field, narrow path crossing, hind limb support and ascending on a rope (Pascual and Figueroa, 1996).

A complete and systematic analysis of early enrichment effects as beneficial in pups born to stressed mothers, has been performed by Koo et al. (2003). They have shown that EE from birth can reverse the deleterious effects of prenatal stress on cognitive ability, cell proliferation and synaptic protein expression, and increased granular cell layer neurogenesis and hippocampal and cortical expression levels of NCAM, synaptophysin and BDNF compared with standard reared (not prenatally stressed) pups (Koo et al., 2003). These findings show that early EE can be therapeutic, attenuating adverse prenatal condition consequences, and to elicit a general improvement of brain functioning development under physiological conditions.

Recently, Simonetti et al. (2009) have found that enrichment limited to just the first three postnatal weeks (P0-P21) significantly increased not only exploratory behaviour in open field tests and improved motor coordination in swimming performance at P10, but influenced also adult acquisition of a spatial learning task; animals thus

enriched exhibited an acquisition rate similar to of mice enriched for their entire lives suggesting that preweaning enrichment may contribute to long-term measurable changes in complex behaviour. This observation is consistent with previous work in rats demonstrating that early experience has a life-long impact on performance of complex learning tasks (Hebb, 1947). As potential cellular basis of these behavioural changes, the authors have identified anatomical changes in a mammalian motor control pathway involving the striatum: the age-dependent increase in PNN formation within the matrix, as well as the decrease in striosomal CSPG cloud density, was accelerated in the striatum of enriched pups at P10 with respect to pups housed in standard condition (Simonetta et al., 2009).

With respect to the maturation of sensory systems, new evidence has been provided showing that EE has a remarkable impact on the developmental plasticity of the visual system. The most striking effect on visual system development elicited by an EE paradigm starting at birth is a marked acceleration in the maturation of visual acuity (VA), a very sensitive and predictive index of visual system maturation. This has been initially assessed in the mouse both electrophysiologically by visual-evoked potential (VEP) recordings and behaviourally by a discrimination task (visual water box task) (Cancedda et al., 2004), and then replicated in the rat (Landi et al., 2007a). The maturation of visual acuity in enriched animals is anticipated by 7 days with respect to control animals and considering that in the timescale of human visual development, it would be as a child reached his final visual acuity at around three years of age (i.e. approximately two years before the age at which children's acuity development normally ends), it is not difficult to understand the power of EE effects on brain development. This early maturation of visual acuity induced by EE is accompanied by a precocious developmental decline of the possibility to induce LTP of layer II-III field potentials after theta-burst stimulation of the white matter in the visual cortex, a well-established in vitro

model of developmental plasticity (Cancedda et al., 2004). At molecular level, the investigations on mediators underlying the effects of EE on visual system development have revealed one crucial factor: neurotrophin BDNF. Indeed, mice reared from birth in EE have increased levels of the BDNF protein in their visual cortex at P7 (Cancedda et al., 2004; Sale et al., 2004). The link between BDNF and accelerated maturation of visual acuity has been already suggested by Huang et al. 1999; the critical period plasticity begins and ends earlier than normal as consequence of an accelerated development of the inhibitory circuitry (Huang et al., 1999). Thus, precocious increase in BDNF levels would accelerate he development of the inhibitory GABAergic system, which, by affecting receptive field development and synaptic plasticity, could determine both the faster maturation of VA and the accelerated decline of synaptic plasticity. In line with this hypothesis, an increased expression of the GABA biosynthetic enzymes GAD65 and GAD67 has been found in EE pups at both P7 and P15 (Cancedda et al., 2004; Sale et al., 2004).

Another molecular factor crucially involved in EE effects on visual system development turned out to be IGF-I. IGF-I is increased postnatally in the visual cortex of enriched rats, and post-weaning administration of IGF-I in this structure mimics EE effects on VA acceleration. Furthermore, blocking endogenous IGF-I action in the visual cortex of developing EE subjects completely prevents EE effects on VA maturation (Ciucci et al., 2007). One of the targets of BDNF and IGF-I signalling is the activation of CREB. Cancedda et al. (2004) demonstrated that EE from birth accelerates the time course of CRE/CREB-induced gene expression and that treatment of non-EE mice with rolipram, a specific inhibitor of the high-affinity phosphodiesterase type IV that activate cAMP system, resulting in an increased phosphorylation of the transcription factor CREB, partially mimics EE effects on CREB pathway and on visual acuity development. Even if the work by Cancedda and colleagues focused on the visual system, it is very

likely that the EE effect is not specific to the visual cortex, as suggested by the influence on CRE-mediated gene expression observed also in the somatosensory cortex (Glazewski et al., 1998b, 1999; Barth et al., 2000

From these surprisingly findings, it emerges that EE affects BDNF and GABAergic inhibition before eye opening, indicating that some of the EE effects on visual system development could be totally independent of vision. This intriguing issue has been addressed by Bartoletti et al. (2004) in a study in which EE and dark rearing (DR) have been combined together. Studies in literature have demonstrated that lack of visual experience from birth in animals reared in standard condition prolonged the duration of the critical period and prevented the VA development (Blakemore and Price, 1987; Fagiolini et al., 1994).

These effects can be completely counteracted by providing DR animals with the opportunity to experience EE while in the dark: DR-EE rats show a normal closure of the critical period for OD plasticity and a normal VA development. Importantly, the action of EE on visual system development is very similar to that found in BDNF overexpressing mice (Gianfranceschi et al., 2003), and the influence of EE on GABAergic inhibitory circuits has been confirmed (Bartoletti et al., 2004). A more recent finding is the demonstration that also retina development, a structure traditionally consider less plastic than visual cortex, is affected by experience provided by EE both at electrophysiological and molecular level. Landi et al. (2007) monitored the development of retinal responses in enriched and non-enriched rats by pattern electroretinogram (PERG), a sensitive measure of retinal ganglion cells (RGCs) function. Retinal acuity development is sensitive to EE on the same time scale as cortical acuity (Landi et al., 2007a). Furthermore, enriched mice displayed a pronounced acceleration in the process of RGC dendrite segregation into ON and OFF sublaminae (Landi et al., 2007b). EE controls the development of retinal circuitry acting on BDNF and IGF-1. Higher BDNF levels were

found in the RGC layer of enriched animals and its retinal block by means of antisense oligonucleotides prevented the EE effects on retinal development (Landi et al., 2007a, b). A clear influence of EE on retinal development has also been reported during prenatal life. Recent data demonstrated that exposing pregnant females to EE (maternal enrichment) determines a marked acceleration of retinal anatomical development in the embryos, accelerating the migration of neural progenitors and anticipating the time-course of naturally occurring cell death (Sale et al., 2007). Interestingly, the documented anatomical modifications are accompanied by a marked increase in IGF-I expression in the retinas of enriched pups and in the milk of mothers. The neutralization of IGF-I in enriched mothers by means of administration of antiIGF-I antiserum prevents the action of maternal enrichment on retinal development, and chronic IGF-I injection to standard pregnant females mimics the effects of EE in the foetuses (Sale et al., 2007; Landi et al., 2009).

The paradigm of EE condition has been also applied in another sensory system; in a recent work the impact of an early auditory enrichment with music on auditory discrimination learning has been investigated of rats. This early enrichment protocol with music started at P14 and enhanced learning ability in auditory signal-detection task and in sound duration-discrimination task in adult rats. In parallel, a significant increase was noted in NMDA receptor subunit NR2B protein expression in the auditory cortex, suggesting that early auditory enrichment influences NMDA-mediated neural plasticity, which results in enhanced auditory discrimination learning. (Xu et al., 2009). In turn, it has been found that animals reared in a large cage of four levels with wheel and auditory enrichment have a global increase in the strength of auditory cortex responses revealed by recordings of tone-evoked potentials (Engineer et al., 2004).

In summary, the aforementioned studies demonstrate that CNS development is already responsive to the environment at very early stages. Indeed, early EE can elicit

experience-dependent plasticity processes, a general improvement in cognitive ability and it can also attenuate adverse prenatal condition consequences.

In addition, the classic concept by which "the development of visual functions is only visual experience dependent" has been challenged. Indeed, the increased levels of BDNF protein at P7 when the rat pups are completely dependent from their mother and the possibility to prevents the effects of dark rearing on the closure of the critical period and visual acuity maturation by EE suggest that the visual system development could be independent from vision experience. Therefore, it has been hypothesized that different levels of maternal care in the EE condition could act as an indirect mediator for the effects of EE on visual system development (Cancedda et al., 2004). A detailed analysis of maternal behaviour showed that enriched pups receive higher maternal care with respect to pups reared in standard condition, indeed enriched pups are never alone at the nest receiving a continuous physical contacts and high level of licking which means high level of tactile stimulation (Sale et al., 2004).

Considering these remarks, it would be interest to investigate whether an artificial maternal care protocol applied in the first weeks of life can affect the brain development and in particular the visual system development.

CHAPTER 3

EFFECT OF EARLY LIFE ENVIRONMENT ON BEHAVIOURAL AND NEURAL DEVELOPMENT: THE ROLE OF MATERNAL CARE

The first two weeks of life in rodents are characterized by a prevalent absence of interaction between the newborn and the external environment. Newborns spend their whole time in the nest, totally dependent on the mother, which is the most important source of sensory experience for the developing pup (Liu et al., 2000). It has been, therefore, suggested that during the first days of life enriched stimuli present in the environment affect pups development through the mediation of maternal behaviour (Cancedda et al., 2004).

The next step is to understand what is the role of maternal care on behavioural and neural development of pups considering their mother as a mediator between them and surroundings. This section addresses the main aspects of the phenomenology of maternal behaviour with particular reference to how variations in maternal care can induce immediate and long lasting effects on pup development and which are the mechanisms underlying these phenomena.

3.1 Survey of Maternal Behaviour.

Newborn mammals require nurturing care from the mother to survive and for most species the mother represents the prime source of food, warmth, protection and education essential for the development of normal social skills (Kuhn and Schanberg,

1998). The studies taken into consideration in this thesis are restricted to rats for two reason: they are a useful experimental model of early developmental events and their ontogeny is rapid and because reports on rats outnumber by far those any other species in this area of research. Thus, laboratory rats have proved to be a good model for the study of hormonal (Rosenblatt, 2002), sensory (Stern, 1996), neural (Numan and Sheehan, 1997), experimental (Li and Fleming, 2003), and developmental (Fleming et al., 2002) factors that control maternal behaviour, which is a highly organized behaviour that can be used as a model of social behaviour (for a review see Numan et al., 2006). Based on a long history of research in the area (Wiesner and Sheard, 1933[1]), there is a relatively complete picture of the phenomenology of maternal behaviour. In laboratories, lactating rats and mice display typical patterns of postpartum behaviour including nest building, retrieval, nursing, licking and grooming of pups and maternal defence of the nest (Rosenblatt, 1967[1], 1975[2]; Fleming and Rosenblatt, 1974a[1]; Gammie, 2005; Shoji and Kato, 2006). The retrieval and grouping of pups into the nest is necessary for thermoregulation, allowing the female to provide pups with ventral heat (Croskerry et al., 1978[2]; Leon et al., 1978[2]), and this positioning allows pups to gain access to the nipples for suckling (Stern and Johnson, 1990[1]).

The dam can assume two general types of nursing postures: hovering and crouching. Hovering is a posture in which a rat is positioned over some or all of the pups in the nest, but the female is not quiescent: she is actively licking pups, moving the nest material, self-grooming, or moving pups within the litter while hovering. Despite the mother rat being active, at least some pups have access to her teats. The second type of nursing, crouching, is considered to be a quiescent posture, and it usually occurs in response to sufficient stimulation by pups. Accordingly, the mother rat tends to stop other activities (although anogenital licking is sometimes observed) and develops a characteristic posture with her extremities spread out and back arched. This stance is

sometimes divided into low crouching and high crouching postures, depending on the degree of the arch of the spinal column. There is also another nursing posture that is rarely observed: a supine posture. The mother rat lies in her side, giving the pups access to her nipples.

Finally, fundamental components of maternal care are licking and grooming (LG) activities. These maternal stimulations, particularly of the anogenital region of pups without which they are not able to survive, are necessary for inducing urination and defecation (Rosenblatt and Lehrman, 1963[1]), increase the motor activity of the pups, which enhances their ability to attach to the nipples, and also serve to regulate brain and body temperature (Sullivan et al., 1988a,b[2]). For the mother, licking and grooming (LG) the pups provide a mechanism to reclaim salt and water that have been lost through lactation (Gubernick and Alberts, 1983, 1985[1]). Over the course of the preweaning period there are dramatic reductions in the frequency of time mothers spend in contact and LG pups corresponding to increased growth and maturation of pups (Gubernick and Alberts, 1983[1]; Champagne et al., 2003a). Overall, the behaviour of the mother provides care needed by the pups to survive and allows the mother to meet the physiological demands of prolonged care of young.

Maternal behaviour in mammalian species has often been characterized as a stereotyped, invariant or "innate" behavioural pattern that emerges from the combination of endocrine signals associated with the later stages of pregnancy and the stimulation arising from the young. An important question arises is maternal behaviour necessarily invariant? An early report of Myers et al. (1989[1])has described naturally occurring variations in maternal responsiveness in Long-Evans rats over the first week postpartum. This initial observation has been expanded showing that variations in maternal care exist and these individual differences are stable across litters and are reliably transmitted from mother to female offspring (Liu et al., 1997; Caldji et al., 1998, Francis et al., 1999;

Meaney et al., 2000; Champagne et al., 2003; Weaver et al., 2004; for a review see Champagne, 2008). Accordingly, a detailed analysis of naturally occurring variations in maternal care has been provided observing lactating female rats daily for the first 6-8 days postpartum. The focus was on LG and arched-back-nursing (ABN) which are two behaviour implicated in many aspects of development regulation in the rat (Myers et al., 1989[1]; Levine, 1994). The authors of this report have selected the females as High or Low LG-ABN mothers on basis of a cumulative frequency distribution in regard to the behavioural indexes analyzed (Champagne et al., 2003). It is important to note that the variations in maternal care should not be considered as a case of "good" and "bad" mothers, but rather these differences lie within the functional range of parental care. Moreover, these variations have been associated with effects on the pups (summarized in Table 1) and in particular on the development of the hypothalamic-pituitary-adrenal (HPA) axis and on behavioural responses to stress as well as certain forms of learning and memory (Caldji et al., 1998; Blaffer-Hrdy, 1999[2]; Liu et al., 1997, 2000; Meaney, 2001).

Finally, there are evidences that parental phenotype can be transmitted to females offspring. Cross-fostering studies show that the biological offspring of Low LG-ABN mothers reared by High LG-ABN dams resemble the normal offspring of High LG-ABN and viceversa, suggesting that variations in maternal behaviour serve as a mechanism for the nongenomic transmission of individual differences as it has been demonstrated in stress reactivity across generation (Flemming et al., 1999; Meaney, 2001; Francis et al., 1999). The issue of epigenetic mechanisms and the transgenerational effects of maternal care will be dealt with subsequently.

1. As quoted in: Ian Q. Whishow and Brian Kolb (Ed.), The behaviour of the laboratory rat. A handbook with tests (pp. 287-298). Oxford University Press, Inc. Neurobiology of the parental brain, Elsevier Inc.
2. As quoted in: Robert S. Bridges (Ed.), Neurobiology of the parental brain (pp. 20-59). Elsevier Inc.

Table 1

Summary of differences between offspring of High and Low LG dams (Champagne et al., 2003).

Measure	Maternal LG–ABN	Measure	Maternal LG–ABN
ACTH response to acute stress	High < Low	a2 adrenoreceptor	
CORT response to acute stress	High < Low	Locus ceruleus	High > Low
Hippocampal GC receptor		Nucleus tractus solitarius	High > Low
mRNA expression	High > Low	PVNh	High = Low
Protein expression	High > Low	Synaptophysin	
PVNh CRF mRNA expression	High < Low	Hippocampus	High > Low
GC negative-feedback sensitivity	High > Low	N-CAM	
Open-field exploration	High > Low	Hippocampus	High > Low
Morris water maze learning	High < Low	Acetylcholine Levels	
latency Novelty-suppression of feeding	High < Low	Dorsal hippocampus	High > Low
CBZ receptor		Vassopressin receptor (V1a)	
Central nucleus of the amygdala	High > Low	Central nucleus of the amygdala	High > Low
Lateral nucleus of the amygdala	High > Low	Oxytocin receptor binding (females)	
Locus ceruleus	High > Low	Central nucleus of the amygdala	High > Low
Nucleus tractus solitarius	High > Low	Bed nucleus of the stria terminalis	High > Low
Hippocampus	High = Low	Medial preoptic area	High > Low
Frontal cortex	High = Low	Lateral septum	High > Low
Medial prefrontal cortex	High = Low	V. nucleus of the hypothalamus	High = Low
CRF receptor		Estrogen receptor a (females)	
Locus ceruleus	High < Low	Medial preoptic area	High > Low
NMDA receptor (hippocampus)		V. nucleus of the hypothalamus	High < Low
NR2A subunit	High > Low		
NR2B subunit	High > Low		

3.2 Effects of early experience: the role of Maternal Care.

The previous remarks have underlined how during preweaning period environmental condition as EE or early postnatal manipulation as handling can actively contributes to the development of specific systems of rat pups. The effects of these early experiences were mediated by variations in parental behaviour, reinforcing the crucial role of maternal care (Liu et al., 1997; Sale et al., 2004). As reported at the beginnings of introduction, an intact and appropriate stimulating early social environment is essential for brain and behavioural development. In human, this is clearly shown by the consequences of its deterioration or impoverishment (Spitz, 1945; Harlow, 1958; Bowlby, 1968; Levine, 2005). Institutionally reared children, despite the marked improvements shown when removed from orphanage settings and placed in a family

environment, may present persistent behavioural problems and increased vulnerability to psychopathology (Hodges and Tizard, 1989; Wilson, 2003; Fries and Pollak, 2004; Gunnar et al., 2009). Other adverse early social experiences have been reported to have a negative impact on adult life as well. For instance, emotional abuse during childhood leads to higher risk of depressive disorders (Chapman et al., 2004) and suicide attempts in adulthood (Dube et al., 2003). Another example is low quality of parent–child interaction deriving from poverty, substance abuse by the mother or maternal depression that have been associated with a reduced ability to shape interpersonal relationships, limited emotional competencies and increased vulnerability to depression and anxiety disorders as well as with cognitive decline later in life (Korosi and Baram, 2009). Alternatively, an appropriate early social environment may lead to an increased resilience to stress and to stress induced illness (Smith and Prior, 1995; Wachs, 2006).

A deeper understanding of the consequences of maternal care variations on the development of brain and behaviour of infants has an important clinical relevance because it may potentially allow to design a feasible plan for babies which undergo a maternal care deprivation preventing pathological disorders.

Maternal Separation: short-term effects. In mammalian species, the importance of mother-infant attachment is commonly derived from deprivation studies. These reports have investigated whether the response of neonatal rats to separation from the dam is a unified process or whether each components of this response is mediated by independent pathways. A complex pattern of changes emerged: mother-infant interactions seem to regulate many behavioural and physiological parameters of pups (Kraemer, 1992[3]; Hofer, 1996b). The deprivation protocol triggers a pattern of reactions consisting of alterations in the sleep-wake states, activity level, sucking pattern, vocalization, heart rate, blood pressure, decrease in DNA synthesis and secretion of growth hormone and hormones

linked to stress response as adrenocorticotropic hormone (ACTH; Kuhn et al., 1978; Levine et al., 1991; Khun and Schanberg, 1998).

Physiological parameters are modulated through several different components of maternal care and each of these can selectively rescue the missing aspect if provided artificially. For example, if maternal warmth is provided to a deprived pup, the level of brain biogenic amine function underlying their general activity level is maintained but warmth has not effect on other systems such as the pup's cardiac rate (Hofer and Shair, 1980[3]). Indeed, heart rate falls 40% after 18 hours of separation and it has been found that this physiological index, normally maintained by sympathetic tone, is regulated by maternal provision of milk, which acts on chemical and neural receptors lining the pup's stomach (Hofer, 1970,1975[3]; Khun and Schanberg, 1998). In turn, a vigorous tactile stroking of maternally deprivated pups, miming maternal licking, prevents the marked fall of the enzyme ornithine decarboxylase (ODC), which is the rate-controlling enzyme in polyamine biosynthesis, and of growth hormone levels that occurs typically after 30 minutes from removal of the mother (Schanberg and Field, 1987; Schanberg and Kuhn, 1998).

Based on these findings, Hofer suggested a new way to understand the biology of maternal-infant separation. He proposed that the global, observable infant response to the absence of its mother actually results from loss of a group of "hidden regulators" provided by a collection of maternal stimuli (Hofer, 1994[3]). The author defines these maternal regulators as "hidden" because they were not evident in simply observing the mother-infant interaction but it is required a significant period of maternal deprivation for raising both short and long term responses of pups.

It has to be noted that maternal deprivation not only affects physiological parameters of pups but there is also evidence that this isolation produces an affective state in rat pups. Indeed, the first reply to isolation is expressed overtly in the rates of infant

calling by the ultrasonic vocalizations that cease within 10-15 minutes (USV, 40kHz; Kuhn and Schanberg, 1998). This reaction to detachment is attenuated or blocked in a dose-dependent manner by clinically effective anxiolytics that act at benzodiazepine and serotonin receptors; and conversely, USV rates are increased by compounds known to be anxiogenic in humans, such as benzodiazepine receptor inverse agonists and GABA-A receptors ligands (Hofer, 1996a). Neonatal rat USVs are powerful stimuli for the lactating rat, capable of causing her to interrupt an ongoing nursing bout, initiate searching outside the nest, and direct her search toward the source of the calls (Smotherman et al., 1978[3]).

Finally, several experiments have demonstrated that maternal factors are critical for the regulation of the HPA axis during development of pups (Levine, 2001). There is a period, in the developing rodent, from about day 4 to 14, when the adrenal response to stress is either minimal or non-existent and which has been designated as the stress hyporesponsive period (SHRP). During the SHRP, these is also a diminution of adrenal sensitivity, evidenced by the fact that the infant fails to show a significant elevation of CORT following administration of high doses of exogenous ACTH (Levine et al., 1967[3]). However, following 24hr of maternal deprivation at postnatal day 12, 16 and 20, the neonatal rat shows elevated basal levels of corticosterone (CORT) and exhibits a robust CORT and ACTH response to mild stress suggesting that for animals that are in the midst o the SHRP, prolonged MS resulted in a disinhibition of the distinctive pattern of HPA activity of the neonatal rat: the mother normally reduces infant HPA responses and separation eliminates this hyporesponsive state (Levine et al., 2001). Further, maternal deprivation (P8) determined higher stress-induced *c-fos* mRNA levels in the paraventricular nucleus (PVN) of deprived pups than non-deprived pups (Smith et al., 1997).

Noteworthy to mention, the effects of maternal deprivation are highly dependent on the time during development when the neonate experiences maternal deprivation:

there is an age-related progression of the effects of deprivation. Thus, at P4 there are few effects on the secretion of either ACTH or CORT and the pups remain relatively unresponsive to novelty or saline injection and the adrenal is refractory to ACTH beginning at about P4 (Levine et al., 2001). It is important to note that this hyporesponsive period is stimulus dependent, indeed it has reported a robust ACTH response to an injection of bacterial endotoxin as early as P3 (Witek-Janusek, 1988; Shanks and Meaney, 1994). Although maternal deprivation markedly alters the endocrine responses to a variety of stressful stimuli from P6 (Kent et al., 1996, 1997; Levine, 2001) a consistently response of HPA axis to mild stress provoking stimuli was seen at P8 (Smith et al., 1997).

Tracing these regulatory effects back to brain systems, Levine's group has found at least three aspects of maternal behaviour that play a role in the regulation of the HPA axis during development. Feeding is essential for reducing the sensitivity of the adrenal gland to ACTH in separated pups (Stanton et al., 1987[3]; Suchecki et al., 1993[3]). Passive contact with an anesthetized lactating dam eliminates the corticosteroid stress response to novelty in 12-, 16-, and 20-day-old rat pups. (Stanton et al., 1987[3]; Stanton and Levine, 1990[3]). Lastly, tactile stimulation appears capable of inhibiting most of the brain-related changes that occur following maternal deprivation. It has been found that an active contact as stroking procedure regulates the expression of the immediate-early gene *c-fos* mRNA in the PVN and the corticotrophin-releasing hormone (CRH) receptor mRNA expression as well in PVN, amygdale and other limbic system sites (van Oers et al., 1998b). Besides, repeated tactile stimulation of the deprived pups for as little as three 1minute periods prevented the increase in ACTH response (Levine et al., 1994).

Through this anatomical and molecular neuromodulator analysis, Levine and colleagues discovered that maternal licking and milk delivery during suckling,

independently exert a prolonged attenuating effect on the responsiveness of the HPA axis.

There are several biological similarities between maternal deprivation effects in rats and the growth retardation that occurs in some variants of human reactive attachment disorders of infancy. Starting from this new knowledge about the regulation of GH by stroking, it has been applied a combination of stroking and limb movement to prematurely born babies with low-birth-weight for three times a day for 15 minutes each time, and continued throughout 2 weeks hospitalization. Stimulated neonates averaged a 47% greater weight gain per day, were more active and alert during sleep/wake behaviour observations, and showed more mature habituation, orientation, motor, and range of state behaviour on the Brazelton scale, than control infants with beneficial effects discernible many months later (Field et al., 1986).

Thus, the interruption of mother-infant interaction activates powerful mechanisms at behavioural and emotional level, whose meaning is to promote the "nurturing touch" between pup and mother achieving re-establishment of their social bond.

Variation in Maternal Care: long-term effects. The pioneering work of Levine, Denenberg and their colleagues, and later Meaney, Plotsky and colleagues has shown that even quite subtle alterations in the experience of rats during the early postnatal period can have long lasting consequences for defensive behaviour, emotional and stress responsivity (Caldji et al., 2000a,b; Francis et al., 1999; Francis and Meaney, 1999; Liu et al., 2000a,b; Meaney and Szyf, 2005; for review see, Kaffman and Meaney, 2007).

3. As quoted in: J. B. Casey (Ed.), Developmental Psychobiology (Review of Psychiatry Series, Volume 23; Oldham JM and Riba MB, series editors). Washington, DC, American Psychiatric Publishing

Multiple variations of the maternal separation (MS) procedure have been employed with both the length and the number of the separation episodes varying across different laboratories. Across variations of the paradigm, a large number of studies has shown that separation of rat pups from their mothers during the early postnatal period produces permanent increases in anxiety-related behaviour such as in open-field test and elevated plus maze and in neuroendocrine stress response when offspring are tested as adults (Holmes et al., 2005; Aisa et al., 2007, 2009). Moreover, although less well-studied than emotional behaviour and stress reactivity, there is growing evidence that early life stress in rats also disrupts the development of neural systems mediating reward-related behaviour. Indeed, there is evidence that maternal deprivation increases voluntary ethanol consumption, exaggerates the behavioural and dopaminergic responses to psychostimulants, and alters dopamine and opiod signalling in the reward-related regions such as the frontal cortex and nucleus accumbens (for review see, Moffett et al., 2007).

In a series of interesting studies Meaney and his colleagues have used the maternal behaviour observational approach to directly test the maternal regulation of HPA axis function (Liu et al., 1997). They have characterized maternal behaviour in Long Evans hooded rats and reported large variability in levels of maternal care between individual dams and as mentioned in the paragraph of maternal behaviour a quantitative analysis has identified mothers with High LG-ABN and Low LG-ABN (Champagne et al., 2003). Subsequent investigations have stressed that offspring raised by High LG-ABN dams, in adulthood, showed decreased HPA activation in response to stress and were behaviourally less fearful in exploring a novel environment compared to those raised by Low LG-ABN. The latter have prolonged elevations in ACTH and CORT following restraint stress, reduced hippocampal glucocorticoid receptor (GR) mRNA and elevated hypothalamic CRH mRNA (Liu et al., 1997; Caldji et al., 1998).

These initial finding suggested that offspring of Low LG-ABN have elevated HPA activity as a consequence of decreased capacity to down-regulate the release of CRH and ACTH. A negative linear correlation was also demonstrated between the levels of maternal LG received and adult plasma levels of CORT coming after restraint stress. The release of CORT, which is subsequent to activation of the HPA axis, has negative-feedback effects in the stress response through interaction with hippocampal GR receptors (Sapolsky et al., 1984). Decreased levels of GR mRNA in the hippocampus as those shown by offspring raised by Low LG-ABN dams, result in a decreased capacity to return to baseline levels of CORT following the cessation of a stressor (Liu et al., 1997; Weaver et al., 2004, 2005).

Behaviourally, these neuroendocrine changes result in decreased exploratory behaviour and increased inhibition on tests such as the open-field and novelty-suppression of feeding, respectively (Caldji et al., 1998). Spatial learning and memory were also examined in the adult offspring of High and Low LG-ABN mothers using the Morris water maze test (Liu et al., 2000b; Bredy et al., 2003a, 2004). As adult, pups reared by High LG-ABN mothers showed significantly shorter latencies to locate the target platform and during probe trial with platform removed they increased searching in the target quadrant compared with the offspring of Low LG-ABN mothers (Liu et al., 2000b). These behavioural data are associated not only with increased hippocampal choline acetyltransferase (ChAT), synaptophysin and synaptic survival but also with increased expression of BDNF mRNA and levels of mRNA coding for the NR2A and NR2B subunits of the NMDA receptors in the hippocampus of the day 8 pups of High compared with Low LG-ABN mothers.

In a very recent work, the performance in contextual fear conditioning in adult High and Low LG-ABN offspring has been examined to assess the effects of maternal care on hippocampal-dependent learning during conditions of high stress: pups raised by

Low LG-ABN mothers displayed enhanced hippocampal-dependent learning under stressful conditions. Besides, variations in postpartum maternal care were associated with effects on morphology, synaptic functioning and CORT responsiveness of CA1 neurons: the adult offspring of High LG-ABN mothers exhibited longer dendritic branch length and increased spine density in CA1 neurons, alterations in electrophysiological properties at rest, enhanced LTP, and increased hippocampal expression of both mineralocorticoid receptor (MR) and GR, with no change in MR/GR ratio. Exposure to stress-like levels of CORT impaired LTP in the adult offspring of High LG-ABN mothers. In contrast, the same dose of CORT greatly enhanced LTP in Low LG-ABN offspring. A comparable effect was observed in vivo (Champagne et al., 2008). In addition to these effects, offspring of Low-LG dams have a decreased density of benzodiazepine receptors in the amygdala compared to the offspring of High-LG dams and GABA subunit expression is altered by maternal LG with implications for benzodiazepine binding (Liu et al., 1997, 2000a; Francis et al., 1999; Caldji et al., 2000, 2003). Neuronal survival in the hippocampus is decreased and apoptosis increased amongst the offspring of Low-LG dams associate with decreased levels of fibroblast growth factor (Bredy et at., 2003b).

Brain monoamine systems also appear to be altered by variations in maternal care. Rat pups exposed to High LG-ABN dams displayed increased α2-adrenoreceptor locus coeruleus-binding and attenuated noradrenergic responses to stress (Caldji et al., 1998; Liu et al., 2000a,b). Reduced noradrenergic activity to stress in offspring of High LG-ABN dams has, in turn, been linked to more effective GABAergic inhibition of these responses, as evidenced by increased binding to GABA-A and benzodiazepine receptors in the amygdala and locus coeruleus of these rats (Caldji et al., 1998, 2000a,b; 2003, 2004).

The quantity of maternal care can be experimentally manipulated not only to impair it but also to enhance it. Maternal care can be enhanced using “handling”

procedure. Studies in the 1950s showed that brief (3-15 min) daily removal of the pups from the dam during the first three weeks of life has profound and long-lasting effects on stress reactivity throughout life (Meaney et al., 2001). More precisely, adult animals handled during the postnatal period showed increased exploratory behaviour in novel environments and blunted HPA responses to stress as compared to non-handled animals (Koffmann and Meaney, 2007). Further work showed that handling during the first week of life, but not during postnatal days 14-21, is as effective as handling for the entire first three weeks of life suggesting that a brief separation during a specific period in development (i.e., the first week of life) is somehow necessary to alter the animal's response to stress in adulthood (Meaney and Aitken, 1985). Similarly to the adult rats reared by dams that naturally exhibit high levels of care, handling procedure suppress the stress response during adulthood, reduces CRH expression in the hypothalamus and increases GR in the hippocampus (Koffmann and Meaney, 2007). GABA(A) receptor was also increased in nucleus tractus solitarius, basolateral and central nucleus of the amygadala (Caldji et al., 2003). In addition, adult rats handled early in life were resilient to manipulations that lead to depressive-like behaviours and exhibited less anxiety-like behaviours in the elevated plus maze as well as improved performance in cognitive tasks (Meaney et al., 1988, 1991; McIntosh et al., 1999; Ploj et al.,1999; Liu et al., 2000; Tang, 2001; Fenoglio et al., 2005; Kosten et al., 2007; Stamatakis et al., 2008).

Interestingly, formal observations showed that when the dam is reunited with her pups, it immediately approaches the pups to restore maternal care and enacts a sustained increase in several forms of maternal behaviour and in particular of licking and grooming. A growing body of evidence indicates that some of the long-term consequences of handling are likely to be mediated by changes in tactile stimulation provided by the dam during the first week of life (for a review see, Kaffmann and Meaney, 2007). It has been demonstrated that stroking the pups with paintbrush affects

HPA reactivity, cognition and maternal care in a manner that resembles those of handling and exposure to High LG-ABN dams. Levine and his group investigated the role of maternal tactile stimulation stroking the separated pups during the separation period and showing that this manipulation was associated with blunted ACTH secretion in response to saline injection in these pups (Suchecki et al., 1993). It has been also reported that stroking pups with a brush over the first week of life acutely increased hippocampal GR expression in the neonatal rats (Jutapakdeegul et al., 2003). Similarly, as already previously mentioned, the physiology of normal growth and development as well as a blunted HPA responses to stress can be restored in the maternal deprived rat pups by tactile stimulation provided during separation period (Pauk et al., 1986; Schanberg and Field., 1987).

In addition, a recent study has been assessed whether the tactile stimulation protocol can prevent the behavioural effects of early adverse experiences as neonatal isolation. The authors have found that tactile stimulation can reverse the decreased locomotor activity in the open-field test, the enhanced anxiety-like behaviour in the elevated plus maze and the increased pain sensitivity in the hot-plate test of adult rats subjected to maternal deprivation as pups (Imanaka et al., 2008). It has also been investigated whether postnatal tactile stimulation could produce long-term beneficial effects on spatial working memory (SWM) in adulthood which is an important cognitive function depending on the integrity of the prefrontal cortex and hippocampus. In adulthood, it has been found that tactile stimulated pups showed better performance in SWM and exhibited enhanced in vivo LTP of the hippocampus-prefrontal cortical pathway, finding a more optimal activation of prefrontal D1 receptors when compared with controls that did not receive any manipulation (Zhang and Cai, 2008).

Finally, other researchers have developed another way to investigate the long lasting effects of tactile stimulation: an artificial rearing (AR) system. In this protocol 3

day old pups are removed from the dam, fed through a gastric canula and are reared at a control temperature in the complete absence of a dam (Lovic and Fleming, 2004). Using this procedure Fleming's group has shown that as adults, offspring raised by AR have significant deficits in a wide range of behavioural alterations including disrupted maternal and social behaviour, attention and emotionality (Lovic and Fleming, 2004; Gonzalez et al., 2001; Chatterjee et al., 2007). This group has also demonstrated that AR decreased the expression of various markers of plasticity such as synaptophysin and BDNF in several brain areas (Chatterjee et al., 2007). Importantly, most of the effects of artificial rearing can be reversed, in part or completely, by providing young pups with additional "licking-like" stimulation (tactile stimulation) during development (Burton et al., 2007; Chatterjee et al., 2007).

Together, these data provide direct evidence that tactile stimulation in rodents during the postnatal period (the first two weeks of life) can have long-lasting consequences in neurobiological and multiple behavioural domains such as cognition, affiliative behaviour and stress reactivity.

3.3 Epigenetic mechanisms and transgenerational effects of maternal care

The experience of maternal LG in infancy clearly has enduring effects on neurobiology and behaviour. One of the most intriguing questions to emerge from this research involves the mechanism mediating these effects: *how are early environment effects sustained into adulthood*?; *how does 'nurture' change the brain?* Recent works suggest that the answer to this questions involves understanding of epigenetic

modifications of gene expression in response to environmental cues (Kaffman and Meaney, 2007; Champagne and Curley, 2009).

Maternal influence on the epigenome. Converging evidence from rodent studies support the hypothesis that maternal environment has a profound influence on offspring phenotype and that this influence is mediated by changes in gene expression. Consequently, understanding the mechanisms governing these effects requires an investigation of the molecular mechanisms which regulate gene transcription and thus exploration of the epigenetics of gene expression.

DNA in cells is arranged in a complex three-dimensional structure composed of DNA, histone proteins, and RNA known as chromatin (for detailed reviews see Hsieh & Gage, 2004). Chromatin allows cells to condense the long linear DNA molecule into a more compact three-dimensional structure that fits into the cell nucleus, while at the same time maintaining specific regions of the DNA either accessible or 'closed off' (i.e., silenced) for transcriptional activation based on environmental and developmental needs. This condensed structure is maintained by complexes composed of four histone proteins: H1, H2, H3 and H4 (Champagne and Curley, 2009). In this state, DNA will not be transcribed and gene expression will be inhibited. To solve this problem, cells developed a complex machinery that is capable of opening and closing chromatin in a manner that provides access to some regions while maintaining others packed and inaccessible. Increased acetylation of histone promotes gene expression whereas inhibition of acetylation decreases gene expression: recruitment of histone acetyltransferases (HAT) or histone deacetylases (HDAC) to a specific location within the DNA plays an important role in DNA accessibility and gene (Champagne and Curley, 2009).

A second epigenetic process that has particular implications for long-term changes in phenotype is DNA methylation. DNA methylation involves a family of

enzymes known as DNA methyltransferases (DNMT). At a functional level, these proteins prevents access of transcription factors and RNA polymerase to DNA resulting in silencing of the gene. In addition, DNA methylation this process attracts other protein complexes which promote histone deacetylation (HDAC), further inhibiting the likelihood of gene expression (Champagne and Curley, 2009).

Though several examples of environmentally-induced changes in DNA methylation have been demonstrated, the question is whether the changes in gene expression that have been associated with postnatal mother-infant interactions are likewise associated with these epigenetic modifications (Champagne and Curley, 2009). Initially, the research has focused on the differences in hippocampal glucocorticoid receptor mRNA observed in the offspring of High and Low LG dams (Weaver et al., 2004). As mentioned above the hippocampus has a crucial role in the regulation of HPA response to stress being the cerebral region with the highest density of GR: higher levels of GR mRNA are associated with attenuated stress responsivity (Sapolsky et al., 1985; Jacobson and Sapolsky, 1991). Analysis of the level of DNA methylation within the GR 17 promoter region suggests that elevated levels of maternal LG are associated with decreased GR 17 methylation corresponding to the elevated levels of receptor expression observed in the hippocampus (Weaver et al., 2004). Site-specific analysis of the methylation pattern in this region indicates that the binding site for NGFI-A, a transcription factor induced by nerve growth factor, is differentially methylated in the offspring of High and Low LG dams and subsequent analysis indicated that the binding of NGFI-A to this region is reduced in hippocampal tissue taken from the offspring of Low LG dams (Weaver et al., 2004). A temporal analysis of the methylation of the GR 17 promoter indicates that differences between the offspring of High and Low LG dams emerge during the postpartum period and are sustained at weaning and into adulthood providing a possible molecular mechanism by which early maternal care could program

GR expression and vulnerability to stress throughout life (Weaver et al., 2004). The developmental time frame of the GR promoter remodeling is consistent with the ability of handling to alter stress reactivity in rodents when performed during the first five days of life (Champagne and Curley, 2009). This suggests that in rodents, the first week of life represents a critical period in which tactile stimulation provided by the dam is able to modify the development of circuits that control stress reactivity in a manner that persists into adulthood.

The role of epigenetic modifications in the mediating differences in gene expression and behaviour between of High and Low LG-ABN offspring is further supported by findings that these phenotype can be altered through pharmacological manipulation of DNA methylation. It has been hypothesized that if active demethylases are present in post-mitotic cells then HDAC inhibitors could trigger demethylation by making the chromatin structure more accessible. Intraventricular infusion of trichostatin-A (TSA) a histone deacetylase inhibitor that promotes demethylation, in adult animals has been demonstrated to reverse the effects of low levels of maternal LG (Weaver et al., 2004, 2006). Thus, the TSA-treated offspring of Low LG dams exhibit increased behavioural exploration, decreased levels of stress-induced corticosterone, increased NGFI-A binding and hippocampal GR mRNA expression compared to vehicle-treated offspring of Low LG dams and are indistinguishable from that of the offspring of High LG dams (Weaver et al., 2004, 2006).

Conversely, central administration of methionine, which give a methyl group inducing increased DNA methylation of the GR promoter, decreased expression of GR and increased HPA reactivity in animals raised by high LG-ABN (i.e., those with hypomethylated promoter) but had no effect on animals raised by low LG-ABN (Weaver et al., 2005). These data demonstrate that DNA methylation can be altered in postmitotic cells and are consistent with the notion that DNA methylation in post-mitotic cells exists

in a dynamic steady state that reflects a balance between rates of methylation and demethylation. Surprisingly, these broad pharmacological manipulations did not cause wide spread changes in gene expression in the hippocampus: microarray analysis of hippocampal samples revealed that infusion of TSA or methionine changed the expression of less than 3% of the hippocampal 'transcriptome' (i.e., genes normally expressed in the hippocampus; Weaver et al., 2006).

In addition to altering stress responsivity, maternal licking/grooming has consequences for the postpartum behaviour of female offspring associated with levels of estrogen receptor alpha (ERα) gene expression in the medial preoptic area of the hyppthalamus (MPOA; Francis et al., 1999; Champagne et al., 2003a,b). There is evidence that mothers and daughters are very similar in their styles of maternal care (Champagne et al., 2003b). Though this transmission of maternal care over generations, from mother to daughter, could be due to genetic variation, cross-fostering studies have shown that it is the quality of care received in infancy that predicts offspring maternal behaviour (Francis et al., 1999). Moreover, these effects can be passed on to subsequent generations of female offspring, such that mothers, daughters and granddaughters are similar in their patterns of maternal care. A wealth of neuroendocrine studies show that the expression of maternal behaviour in the rat is dependent upon the activation of ERα in the MPOA and downstream effects on oxytocin receptor binding (Kaffman and Meaney, 2007). Analysis of MPOA levels of DNA methylation within the ERα promoter indicate that low levels of maternal LG are associated with high levels of ERα methylation whereas high levels of LG are associated with low levels of ERα methylation amongst female offspring (Champagne et al., 2006). This differential methylation occurs at multiple regions within the ERα promoter and it is observed in infancy and maintained into adulthood, suggesting a long-term suppression of gene expression in response to low levels of LG (Champagne et al., 2006).

Moreover, MPOA levels of ERα appear to control expression of oxytocin receptor (OTR) in the MPOA, which in turn regulates several aspects of maternal behaviour such as frequency of LG-ABN; high levels of OTR are presented in offspring of High LG-ABN compared to Low LG-ABN. Direct administration of either estrogen or oxytocin into the MPOA increases maternal behaviour and central administration of OTR-antagonist on postnatal day 3 abolishes the differences in LG ABN between high and low dams (Kaffmann and Meaney, 2007). Thus the hypomethylated state of the ERα promoter of offspring exposed to high frequency of LG-ABN early in life provides a more efficient transcriptional mechanism to upregulate oxytocin receptor in response to increased estrogen levels associated with pregnancy (Fig.2).

In another words, maternal licking/grooming is associated with epigenetic effects in female offspring that mediate long-term changes in the expression of a gene involved in maternal behaviour and as such mediates the transmission of maternal care across generations (Champagne and Curley, 2009).

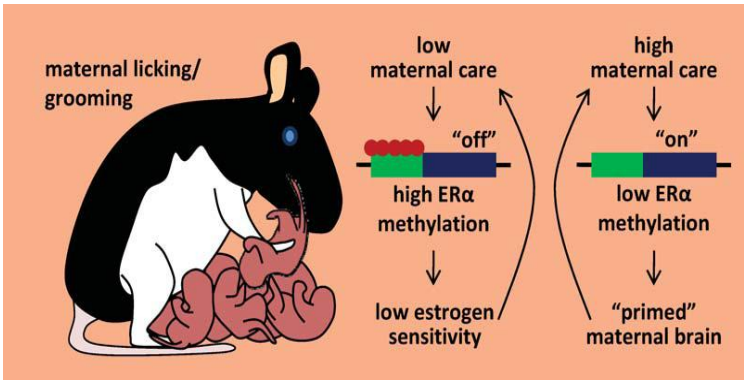


Figure. 2 Studies of maternal behavior in the rat suggest that mothering is transmitted "epigenetically" from mother to daughter through levels of methylation of the ERα gene promotor. The maternal care may shape the infant brain by turning genes 'on' or 'off' during development (Champagne, 2009).

Signalling pathways from maternal care to DNA methylation. In the rat, licking/grooming may serve as a critical source of tactile stimulation for the developing pup. The question is how this physical stimulation leads to the epigenetic changes in specific genes within hippocampal and medial preoptic area cells observed in offspring of High LG dams. Pups

provided with tactile stimulation in the form of stroking with a paintbrush or with maternal LG exhibit increases in hippocampal GR expression (Liu et al., 1997; Jutapakdeegul et al., 2003). These effects are mediated by increases in NGFI-A which is dependent on serotonergic activation of cAMP-coupled 5-HT_7 receptors (Mitchell et al., 1992; Meaney et al., 2000). Thus, the effects of tactile stimulation provided by mothers on GR expression can be mimicked by administration of a cAMP analogue and blocked by a 5-HT_7 receptor antagonist (Laplante et al., 2002). Recent evidences highlight the importance of NGFI-A as a downstream target of this pharmacological or behavioural treatment (Weaver et al., 2007). Hippocampal GR expression is not enhanced by 5-HT when an NGFI-A antisense is co-administered and both cAMP and 5-HT have been found to alter the methylation status of the NGFI-A binding site within the GR promoter region. Furthermore, increasing the levels of NGFI-A is associated with decreased methylation of the GR promoter and thus increased GR expression; however, if the NGFI-A binding site within the GR promoter is mutated, the effects of NGFI-A are blocked. Although the exact role played by NGFI-A in the demethylation of the GR promoter is not known, these findings suggest that through the stimulation of specific factors that bind to the steroid receptor promoter regions, maternal care can lead to a cascade of events that alter offspring development and result in stable patterns of adult gene expression and behaviour. This same principle of activation might be applicable also to the relationship between Stat-5 binding to the promoter of ERα estrogen receptor gene expression; up to now, however, this particular cascade has not yet been investigated.

Therefore, the level of maternal care and in particular of the tactile stimulation provided by the LG behaviour early in life might to play an important role in the development of several behavioural phenotypes that persist into adulthood. Recent data in rodents suggest that the stability of DNA methylation might provide a molecular

mechanism for explain these effects. These considerations open the door to a new way for understanding how genes and environment interact to modify neurodevelopment and provide a framework for studying the effects of childhood adversity in increasing the risk of psychopathology in adulthood (McGowan et al., 2009).

In summary, maternal behaviour in the first weeks of life leaves a profound trace in the developing CNS of rodent. An active physical contact provided by maternal licking and grooming affects brain structures, functions and molecules in rat pups creating a neuronal substrate that promote optimisation to environmental adaptation.

A protocol of tactile stimulation applied early in life on rat pups could be used as tool for investigating how a precious sensory experience affects the development of CNS and in particular of some crucial cognitive systems for adaptation as stress responsivity and recognition memory.

CHAPTER 4

AIM OF THE THESIS AND EXPERIMENTAL DESIGN

I have discussed how tactile stimulation provided by the mother can affect hippocampal structure and function, crucial molecules for plasticity such as BDNF and NMDA receptors, and can leave long-lasting traces in the offsprings' behaviour (Liu et al., 2000; Weaver et al., 2004, 2006, 2007; Meaney and Szyf, 2005; Champagne and Curley, 2009). The beneficial effects of tactile stimulation are also seen in preterm infants; very low birth infants exposed to massage therapy improved daily weight gain, had earlier discharge and presented less late onset sepsis compared to infants receiving standard care (Field et al., 2010; Procianoy et al., 2010). Infants receiving massage also showed less clinical signs of stress and lower plasma cortisol concentrations than controls (Acholet et al., 1993). An intriguing data comes from a 6month follow-up study which suggested improvements in weight gain and mental and motor development in preterm infants receiving massage intervention (Schanberg and Field, 1987). Recently, body massage and multisensory stimulation are increasingly included in neonatal care in human newborns, however, neither in animals nor in humans it is known whether massage may affect brain development. In the present work, I first explored the effects of massage protocol on brain development using the visual system as experience-dependent plasticity model.

I examined the effects of early tactile stimulation on the maturation of the visual cortex. I have assessed the developmental time course of the response to a flash by means visual evoked potential recorded from the primary visual cortex (fVEPs) between P14 and P18, when the maximum degree of latency shortening is normally observed. To

assess the effects of tactile stimulation on visual acuity, I also measured visual acuity at P25 by means of VEP recordings from the primary visual cortex and by means of the Prusky water box, a behavioural test, at P25 and P28. This experimental protocol allows to investigate the role of early sensory experience in the brain development and the results of these experiments demonstrate that the massage accelerates a maturation of visual cortex.

I then considered the molecular mechanisms underlying the acceleration of visual function development induced by massage procedure. I first focused on the IGF-1 molecule. Recently it has been found that IGF-1 is a key factor mediating EE effects on visual cortical development (Ciucci et al., 2007): EE increases the number of IGF-1-positive neurons in the visual cortex; increasing IGF-1 in the visual cortex of non-EE rats by means of osmotic minipumps mimics EE effects, accelerating visual acuity development while blocking IGF-1 action in the visual cortex of EE rats by means of the IGF-1 receptor antagonist JB1, blocks EE action on visual acuity development. Besides, growth hormone (GH) is affected by different levels of maternal care and regulates IGF-1 synthesis suggesting that IGF-1 could be a crucial molecule to mediate tactile stimulation effects on visual system development.

I investigated in massaged animals whether IGF-1 levels were increased in the brain and in particular in the primary visual cortex and in another sensory cortex, area Te1, the primary auditory cortex. In both areas, I quantified the presence of IGF-1 in terms of the number of IGF-1 positive cells normalized to the number of neurons and I found that massage led to increased number of IGF-1-positive neurons in the cortex of rat pups. In particular, antagonizing IGF-1 action blocked the effects of massage on the development of visual acuity in rat pups.

Afterwards, given the well-known role of cortical inhibitory circuits in the regulation of plasticity time (Hensch et al., 1998; Huang et al., 1999; Hensch, 2005), I

investigated whether the development of GABAergic intracortical inhibition was affected by massage assessing the presence of perisomatic inhibitory innervations. I quantified the expression of an isoform of glutamic acid decarboxylase (GAD65) in the presynaptic boutons of GABAergic interneurons around the soma of target neurons (puncta rings) at P25, the age of visual acuity assessment. I found that massage affects the density of inhibitory synapses in the visual cortex

In a second study, I investigated the role of massage and different protocols of environmental enrichment on stress response of the massaged and enriched pups once adult. I found that alterations in the experience of rats during the early postnatal period by massage or EE can have long lasting consequences for stress responsivity. A brief separation during a specific period in development (i.e., the first week of life) is somehow necessary to alter the animal's response to stress in adulthood. I also investigated the role of IGF-1 in the stress response; I performed subcutaneous injects of IGF-1 and its receptor antagonist JB1 in the first week of life in order to evaluate how early manipulation of IGF-1 pathway can affect a stress response in adulthood.

In a third study, I investigated the effects of massage and EE on the development of visual recognition memory in rat. I first obtained a detailed investigation on the development of recognition memory, and then showed that massage and enriched environment affect the maturational time course of this cognitive function with respect to untreated animals.

CHAPTER 5

MATERIALS AND METHODS

5.1 Animals 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 at 21 °C temperature, 12h/12h light/dark cycle, and food and water available *ad libitum*. Female rats were put with males (one male for every mating cage) in standard cages for reproduction (60X40X20 cm). Parturition was checked one time a day, and the day of birth was considered postnatal day 0 (P0). All behavioural assessments were conducted in a dedicated room different from where animals were housed.

5.2 Rearing environments

Pregnant female rats were assigned to either enriched environment (EE) or standard (SC) rearing conditions at least 7 days before delivery. With this procedure, both enriched and standard females received equivalent levels of stress due to cage transfer during pregnancy. After birth litters were reared in EE with their mother until the postnatal day required by the experimental protocol:

1) enriched condition until P12 (EE-P12): dams with their offspring live in large cages (100X50X82 cm) with two or more floors linked by stairs, containing several food

hoppers, two running wheels (one bigger for adults, the other smaller for post-weaning pups) to allow physical activity, and a range of differently shaped objects (tunnels, shelters, stairs) that were all substituted with others once a week (Fig.3). Every cage houses at least 4-5 female rats and their pups. When the offspring are P12, they are relocated in a standard laboratory cage (26X42X18 cm) with their mother until their weaning age (P22);

2) enriched condition until P45 (EE-P45): dams with their offspring live in large enriched cage (100X50X82 cm) with the same conditions describe above. At weaning age of pups, their mother and filler females are removed and are relocated in a standard laboratory cage while pups are left in the enriched cage. When enriched pups reach P45, they are relocated in a standard laboratory cage until the conclusion of experiments;

3) enriched condition until P60 (EE-P60): dams with their offspring live in large enriched cage (100X50X82 cm) with the same conditions describe above. At weaning age of pups, their mother and filler females are removed and are relocated in a standard laboratory cage while pups are left in the enriched cage. When enriched pups reach P60, they are relocated in a standard laboratory cage until the conclusion of experiments;

4) standard condition (non-EE); dams with their offspring live in standard laboratory cages (26X42X18 cm) until pups reach weaning age (P22). At P23, the animals are put in standard cage (26x42x18 cm), housing a maximum of three adult rats. Litter and food were the same in all environmental conditions; food and water were provided *ad libitum*.

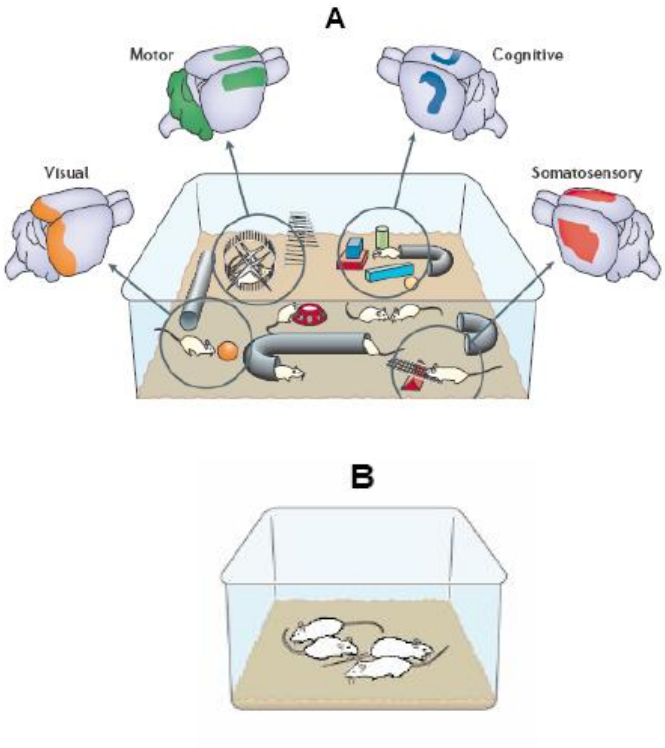


Figure 3

(A) *Enriched environmental condition*. Enriched environment provides social interaction (6-12 animals in big cages), stimulation of explorative behaviour and physical activity with different objects and running wheels. (**B)** *Standard environmental condition*. Animals are housed in small groups of 2-4 animals in regular size cage without any stimulus nor objects (modified from Nithianantharajah et al., 2006).

5.3 Maternal stimulation protocol

5.3.1 Experimental groups.

I used three groups of rat pups:

1) **massage group:** pups were separated from the mother three times per day (9:00 A.M., 2:00 P.M., and 7:00 P.M.) from P1 to P12 and received tactile stimulation;

2) **maternal separation group:** pups were separated from the mother for the same amount of time and at the same period of the day as the massage group but did not receive any stimulation;
3) **control group:** pups were left undisturbed with the mother.

For the massage and maternal separation groups the procedure was the following: the mother was temporarily put in another cage, and the entire litter of pups was removed from the home cage and put in a box over a warm plate (34°C) to recreate the temperature of the nest. Pups in the massage group were then individually subjected to the stimulation protocol, whereas pups in the maternal separation group were left in the box over the warm plate. At the end of each session, animals were replaced with their mother. The presence of the maternal separation group controls for the effects of removing pups from the cage and separating them from the mother.

5.3.2 The stimulation protocol.

To mimic the LG care of a mother rat, I used a modified protocol of tactile stimulation (Pauk *et al.*, 1986; Kuhn and Schanberg, 1998; Schanberg et al., 2003). It combined gently stroking and massaging to mimic maternal care. Each animal received 5 min of tactile stimulation: 2 min with a wet soft paintbrush on their back, on the head, on the limbs, and on the abdomen to mimic licking; 1.5 min massage with finger tips on both sides of their back combined with passive gentle movement of their limbs; 1.5 min with a soft toothbrush on the back and the abdomen to mimic grooming.

5.3.3 Body weight assessments

Both positive effects of handling and negative effects of separation from the mother on weight gain are reported in the literature (Hofer, 1970; Schanberg and Field, 1987); therefore I decided to evaluate whether our tactile stimulation protocol affected, positively or negatively, this physiological index.

At P12, the body weight was assessed in 108 animals, subsequently used, at later ages, for the electrophysiology, behaviour, or immunohistochemistry experiments described below. I found that both massage and maternal separation pups weighed more than control pups; the weight of separated pups was also higher than that of the massaged pups [Fig.3; one way ANOVA, p=0.001; F=15.218, multiple comparisons Holm–Sidak procedure, weight of control pups (n=35; mean± SEM, 23.8± 0.7 g) lower than massage and maternal separation pups (massage pups, n=30, mean± SEM, 26.1± 0.6 g; maternal separation pups, n=43, mean± SEM, 28.6± 0.6 g), and weight of massage pups lower than maternal separation pups (p=0.05)].

No difference in body weight between massage and maternal separation group was found at P25; rats in both groups weighed more than rats in control group [Fig.4; one-way ANOVA, F=7.39, p=0.004, multiple comparison procedure Holm–Sidak method, weight of control rats (n=7; three litters; mean± SEM, 53± 3 g) lower than massage and maternal separation rats (six litters, massage, n=11 pups, mean± SEM, 63.1± 1.7; maternal separation, n=11 pups, mean± SEM, 62± 1.5)].

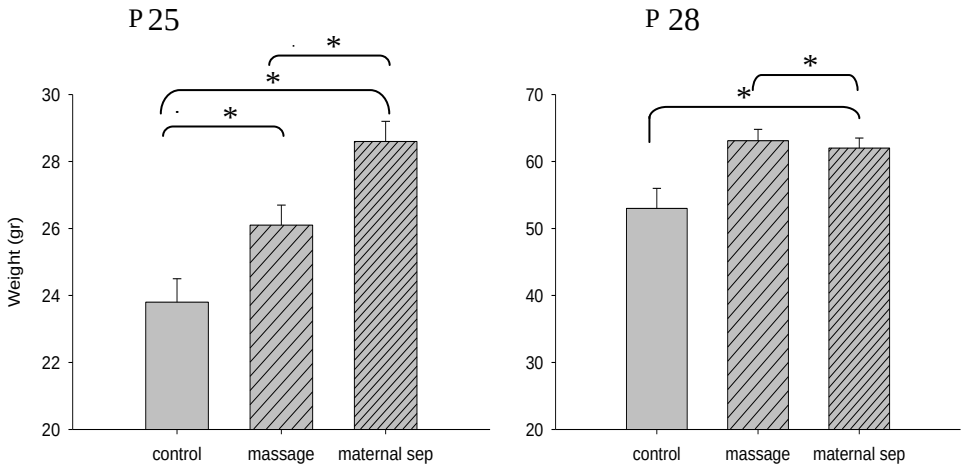


Figure 4

Left, mean body weight at P12 for massage, maternal separation and control groups. The body weigh in the massage and maternal separation groups is significantly higher than in the control group; weight of separated pups is also higher than that of the massaged pups (one way ANOVA, p=0.001, multiple comparisons Holm–Sidak procedure). Vertical bars denote SEM. Asterisk denote significant difference. **Right**, body weight at P25 for massage, maternal separation and control groups. There is no difference in body weight between massage and maternal separation group; rats in both groups weigh more than rats in control group (one way ANOVA, p=0.004, multiple comparisons Holm–Sidak procedure). Vertical bars represent SEM. Asterisk denote significant difference.

5.4 In vivo electrophysiology

5.4.1 Flash Visual Evoked Potentials (f-VEPs) recordings

Animals of massage and control group were anaesthetized by intraperitoneal injection of avertine (tribromoethanol, CBr3CH2OH; 1ml/hg) and body temperature was continuously monitored and maintained at 37°C by a thermostatic electric blanket.

Flash Visual evoked potentials (f-VEPs) were elicited using a full field flash generated on a monitor (20x22 cm, luminance 70 cd/m², flash rate 0,3 Hz, duration 0.66 sec) positioned 20cm from the rat eyes. Recording electrodes were positioned on the scalp overlying the primary visual cortex with the ground electrode on the front. Recorded activity was digitally band-pass filtered (DC-120 Hz) and 50 responses were averaged. The results were displayed scalp positive up (Fig.5). Flash VEPs were recorded at 14, 15, 16, 17 and 18 postnatal day. All recordings were carried out blind.

Transient f-VEPs in response to full field flash (0.3Hz) were evaluated in the time domain by measuring the peak latency of the major positive component: P1.(Pizzorusso et al., 2002).

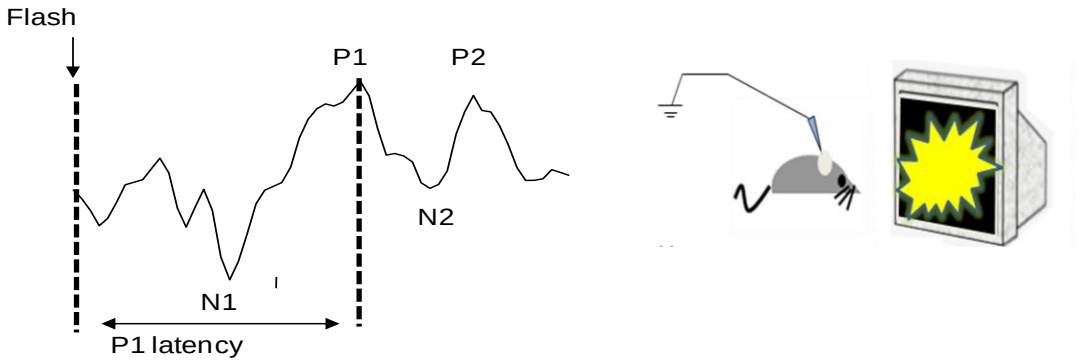


Figure 5

Schematic representation of the recorded signal for f-VEPs. The stimulus is elicited using a full-field flash on a monitor positioned 20 cm from rat's eyes. Recording electrode is positioned on the scalp overlying the primary visual cortex with the ground electrode on the front. The dotted line indicates the onset of visual stimulation and the continuous line is placed at the peak of positive wave P1.

5.4.2 Electrophysiological assessment of visual acuity

A total of 30 rats from 10 litters (Massage group: N= 11; Maternal separation group: N= 12; Control group: N= 7) was used. 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. For the entire duration of the recording session, the body temperature was monitored with a rectal probe and maintained at 37.0°C using a heating pad. Electrocardiogram was monitored. Visual stimuli were horizontal sinusoidal gratings of different spatial frequency and contrast generated by a VSG2/2 card (Cambridge Research System, Cheshire, UK) running a custom software (kindly provided by C. Orsini) and presented on a computer display (mean luminance=25 candles/m^2; area, 24X26 cm) placed 20 cm in front of the rat eyes. 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, band-pass filtered (0,1–120 Hz), and averaged (at least sixty events in blocks of ten event 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, visual

acuity was obtained by extrapolation to zero amplitude of the linear regression through the data points in a curve where VEP amplitude is plotted against log spatial frequency (Pizzorusso et al., 1996) (Fig.6).

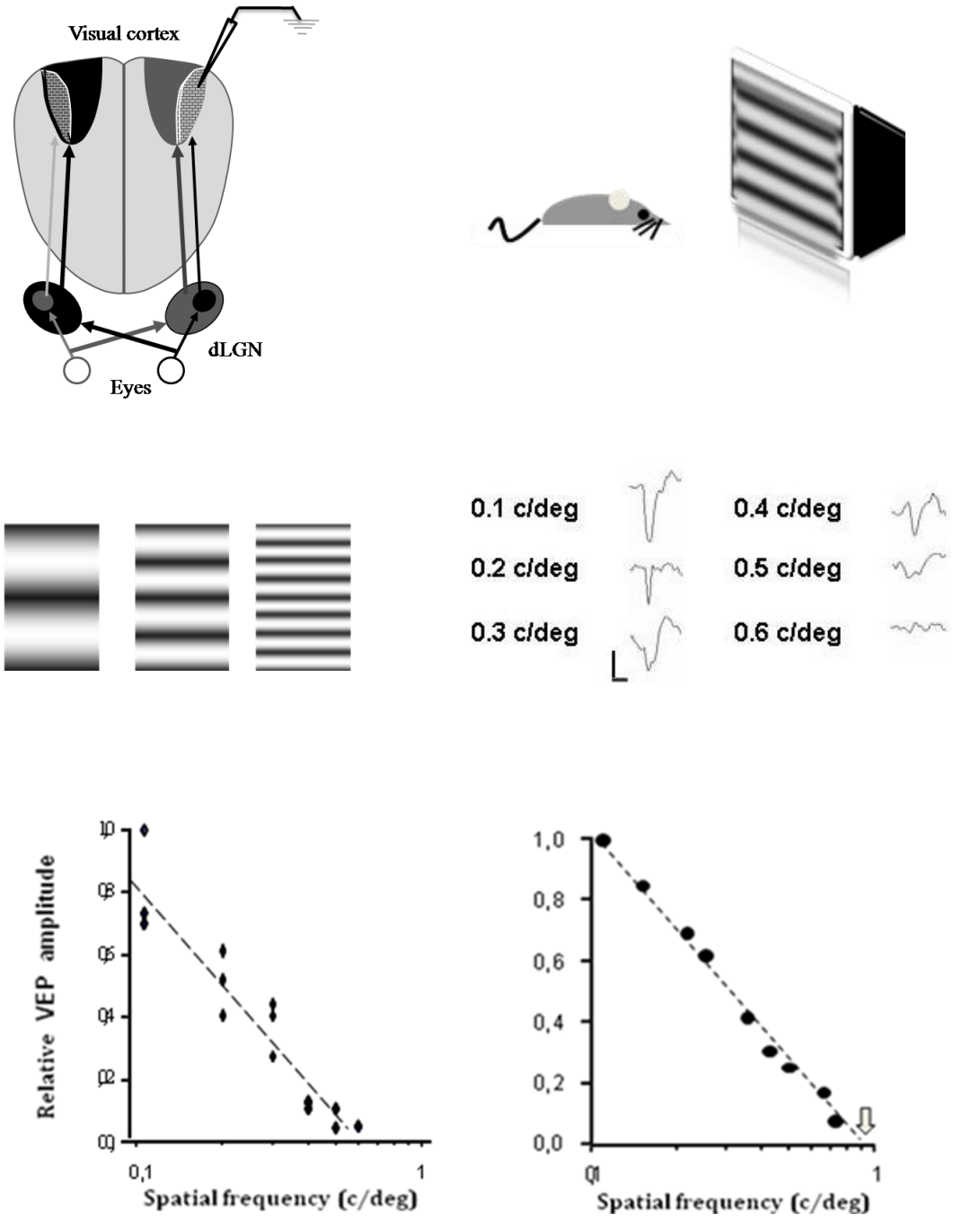


Figure 6

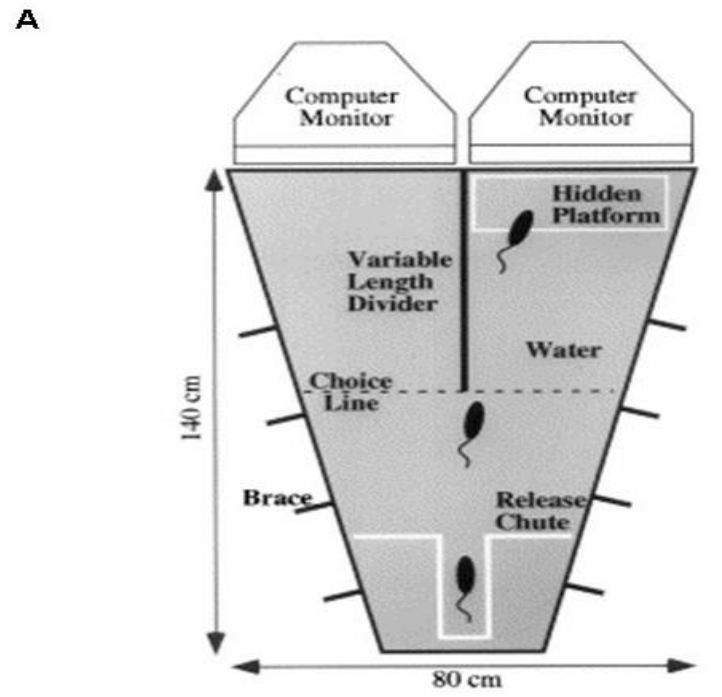
Top, schematic representation of the recording electrode position. VEPs activity was recorded from the binocular portion of the visual cortex. For VEP recordings typical visual stimuli were horizontal sinusoidal gratings of different spatial frequency. **Bottom**, visual acuity was obtained by extrapolation to zero amplitude of the linear regression through the data points in a curve where VEP amplitude is plotted against log spatial frequency. Note the different visual acuity value between P25 (c/d; on the left) and P44-45 (c/d; on the right) in normal rats. Calibration bars: 50 μV, 100 ms.

5.5 Behavioural assessment of visual acuity.

A total of 39 rats from 12 litters was used: massage group, n= 15 rats; maternal separation group, n= 11 rats; control group, n= 13 rats. Visual acuity was measured using a two alternative forced choice procedure (Prusky et al., 2000); behavioural assessment of visual acuity started at P19.

The basic apparatus consists of a trapezoidal-shaped pool with two computer-controlled monitors placed side-by-side at one end. The pool is made of 6 mm clear Plexiglas® and comprises a rectangular floor (140 cm long × 80 cm wide) and 55 cm (high) walls which are finished on the inside with flat black paint to reduce reflections; in the midline a short barrier (50 cm) was placed between the monitors into the pool (see Fig.7). The divider is painted black on both sides to make them opaque and reduce reflections. A portable escape platform (37 cm long × 13 cm wide × 14 cm high) is placed below one of the monitors. The pool is filled with tepid (22°C) water to a depth of 15 cm. White tempera dissolved in the water renders the platform invisible from water level. Visual stimuli are vertically-oriented square-wave gratings of different spatial frequency (90% contrast) displayed randomly on one of the two identical computer monitors, whereas the other displayed uniform gray of the same

mean luminance (gamma correction; mean luminance 25 cd/m2, area 24 x 26 cm) (Fig.7). The rationale of this task is to use the animal's ability to associate a grating with escape from water, as a strategy to measure its visual acuity. Animals must first be conditioned to distinguish a low spatial frequency from homogeneous grey of the same mean luminance with high reliability before the limit of this ability can be assessed at higher spatial frequencies.



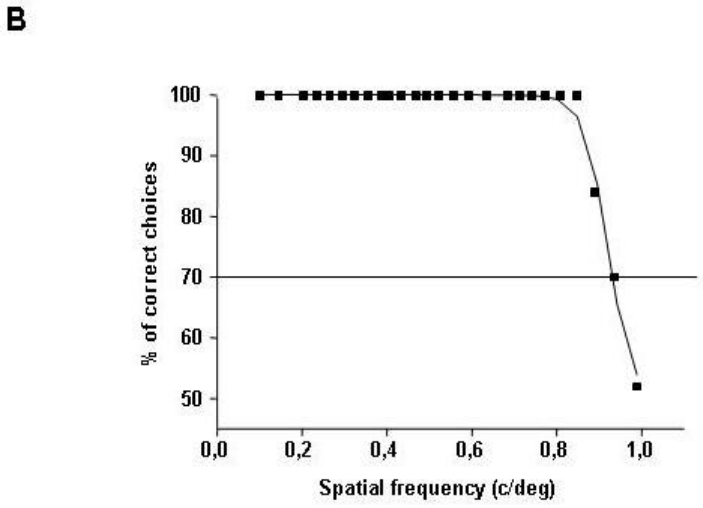


Figure 7

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

Rats were trained to swim from the release point, located at the end of the tank, to the grating side, in which they found the hidden platform. A short barrier was placed in the tank between the stimuli, creating a choice plane. The location of the grating on any trial was pseudorandomized. When performance with a low spatial frequency (0.117 c/°) reached at least 80% correct discrimination for two consecutive sessions, the training phase ended. To measure visual acuity, the limit of the discrimination was estimated by increasing the spatial frequency of the grating until performance fell below 70% accuracy. For each rat, several sessions of visual acuity estimate were run and a frequency of seeing curve was constructed from the total data (90–110 trials at least). The spatial frequency corresponding to 70% accuracy was taken as the acuity value. All measurements were performed blind.

5.6 Subcutaneous injection of JB1

A total of 14 rats from 4 litters was used. Injections of JB1 (catalog num.: H-1356, Bachem), an IGF-1 receptor antagonist (Fernandez et al., 1999; Ciucci et al.,

2007), were performed subcutaneously in massaged pups (n=8, massage plus JB1 group) from P1 to P9. For each pup, a single injection per day was performed, at 9:00 A.M. JB1 concentration was 50 ng/µl, and the volume of the injection was adjusted to the pup weight to reach a dose of 18 ng/g body weight. Six massaged pups were treated with subcutaneous injections of saline (massage plus saline group), as a control for the effects of subcutaneous injections in massaged rats. From P9 to P25 injected pups were allowed to wean according to the protocol already shown. At P25 the massage plus JB1 or saline groups were drawn from your cage and anesthetized as well from protocol of visual acuity recording.

5.7 Immunohistochemistry

A total of 48 rats from 10 litters was used (massage group, n=16 rats; maternal separation group, n=16 rats; control group, n=16 rats) Long–Evans hooded rats aged between P14 and P25 were used (P15, massage group, n=4; maternal separation group, n=4; control group, n=4; P18, massage group, n=4; maternal separation group, n=4; control group, n=4; P25, massage group, n=4; maternal separation group, n=4; control group, n=4). Protocol was as in the study of Ciucci et al. (2007).

Animals were deeply anesthetized with chloral hydrate and perfused transcardially with PBS 16followed by fixative (4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.4; PB). Brains were removed, post-fixed in the same fixative at 4uC, 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 I used polyclonal anti-IGF-1 antibody (1:500, IBT system) revealed with biotinylated secondary antibody goat anti-rabbit IgG (1:200 Vector Laboratories, Burlingame, CA), followed by fluorescein-conjugated extravidin (1:300, Sigma). For neuronal-specific nuclear protein (NeuN), I used a monoclonal antibody (1:500, Chemicon MAB377) revealed with Alexa 568 (1:400, Molecular Probes). For GAD65 I used monoclonal antibody anti GAD65 (1:500, Chemicon, MAB351) revealed with biotinylated secondary goat anti-mouse IgG (1:200, Vector Laboratories, Burlingame, CA) followed by incubation in fluorescein-conjugated extravidin (1:300, Sigma).

5.7.1 IGF-I immunoreactivity analysis

At all ages, images were acquired with a confocal microscope (Olympus) at 20X magnification (N.A. = 0,7, field 707x707 μm acquired at 1024x1024 pixels) to analyze the number of IGF-1 and NeuN positive cells. For each animal, at least three Oc1B sections and three Te1 sections were analyzed. Counts were done on the entire thickness of Oc1B and Te1. The number of IGF-1 positive cells was normalized to the number of NeuN positive cells. All analyses were performed blind.

5.7.2. GAD65 puncta rings quantification

A total of 12 rats from 4 litters were used at P25 (massage group, n=4 rats; maternal separation group, n=4 rats; control group, n=4 rats). 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 three sections 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., 2003). 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 was divided by the background intensity taken at the cell soma. A total sample of 70-120 neurons were analyzed for each cortex. All images acquisition and analysis were carried out in blind.

5.8 Plasma corticosterone assay

Corticosterone was measured by means of EIA kit from Chematil according to the provided protocol. Briefly, whole blood samples were collected from 18 rat pups from four litters at P14 (massage group, n=6 pups; maternal separation group, n=6 pups; control group, n=6 pups) by decapitation between 9:00 and 10:00 A.M. and allowed to settle on ice. Resulting samples were centrifuged (6000 rpm, 10 min, 4°C), and supernatants were stored at -80°C until use. Twenty five microliters of each sample were run in duplicate on polystyrene microtiters plate with goat anti-rabbit IgG immobilized to the inside wall of each well. One hundred microliter of rat corticosterone antiserum were added to each well, and the plate was shaken for 60 min at room temperature. After extensive washing, 100 µl of tetramethylbenzidine solution

were added and incubated for 15 min. The reaction was stopped with an additional 100 µl of stopping solution, and the plate was read with a microplate reader at 450 nm. Corticosterone amount was established by means of a calibration curve reporting absorbance at 450nm on abscissa VS log [standard concentration].

5.9 Anxiety response assessment: Elevated Plus Maze

A total of 94 rats from 15 litters (massage: n=16; massage plus JB1: n=16; massage plus IGF1: n=8; control: n=15; control plus vehicle: n=9; EE-P12: n=16; EE-P45: n=10; EE-P60: n=14) Long Evans hooded rats were employed in to study the anxiety responses at P60. The elevated plus maze is widely employed as a simple method for assessing anxiety responses of rodents (Walf and Frye, 2007). Unlike other behavioural assays used to assess anxiety responses that rely upon the presentation of noxious stimuli (i.e., electric shock, food/water deprivation, loud noises, exposure to predator odour, etc.) that typically produce a conditioned response, the elevated plus maze relies upon rodents' proclivity toward dark, enclosed spaces (approach) and an unconditioned fear of heights/open spaces (avoidance). Animals are placed in the intersection of the four arms of the elevated plus maze and their behaviour is video recorded for 5 min as it has been demonstrated that they show the most robust avoidance responses in the first 5 min after placement in the elevated open alleys (Walf and Frye, 2007).

The maze is made of black PVC and consists of four arms: two open without walls and two closed by 31 cm high walls; the open and closed arms were connected by a central square, 10x10 cm and each arms is 50 cm long and 10 cm wide (Fig.8). All arms of the maze are attached to sturdy plastic legs, such that it is elevated 55 cm above the floor level in a dimly lit room and a video camera was suspended above the

maze to record the movements for analysis. A video-tracking system (Ethovision System) is used to automatically collect behavioural data. The video camera is mounted overhead on the ceiling (Fig.8). The test is situated in separated part of the behavioural room that is illuminated with two halogen lamps in order to obtain similar levels of illumination on both open and closed arms. The legs of the maze are adjusted so that the maze is perpendicular to the ground and each arm is level.

The behaviours that are typically recorded when rodents are in the elevated plus maze are the time spent in and entries made into the open and closed arms. Behaviour in this task (i.e., activity in the open arms) reflects a conflict between the rodent's preference for protected areas (e.g., closed arms) and their innate motivation to explore novel environments, including the open arms.

The procedure consisted of six steps. First step, make sure maze is cleaned and dried before use and that video-tracking system is ready to be used. Fill out data sheets with subject number of animal, date, coded condition and initialed by the experimenter before testing. Second step, bring rodent, which is in its individual temporary transport cage, into the behavioral testing room. Third step, take rodent out of its cage and place at the junction of the open and closed arms, facing the open arm opposite to where the experimenter is. Make sure to handle the rodent in a consistent manner and place rodent in the elevated plus maze in the same position. Fourth step, start the video-tracking system and a timer set for 5 min when the rodent is placed in the maze. The video-tracking system is started after the animal is placed in the maze so that the behavior of each animal is consistently recorded 5 min. Fifth step, at the end of the 5-min test, remove the rat from the plus maze and place into a transport cage. Place back inside its home cage on the cart outside the room. Finally, clean the elevated plus maze with disinfectant and dry with paper towels before testing with another rodent.

Infrequently (in less than 1% of the rodents tested), rats run to the edge of the open arms and fall off. When this occurs, the animal is rapidly picked up and placed back onto the open arms of the maze. Behavioral data from an animal that does this are excluded from analyses. However, the experimenter continues to test the animal because it is important to make sure that exposure to the elevated plus maze is as consistent across animals as possible. All the experiments were carried out between 09:00 and 12:00 o'clock. All behavioural assessment was carried out in blind.

Figure 8

Left, top view of test during animal's performance. **Right**, front view shows opened and closed arms. Over the maze is placed a video camera in order to track the performance of animal.

5.10 Declarative memory assessment: Object Recognition Test

The object recognition test (ORT), introduced by Ennaceur and Delacour (1988), is a method to measure a specific form of recognition memory in rats and mice. It is based on the innate tendency of animals to spend more time exploring a

new, rather than a formerly encountered, object. The ORT is particularly attractive for several reasons: first, it requires no external motivation, reward or punishment; second, little training or habituation is required; and third, the task can be completed in a relatively short time. For these reasons, I have chosen it for investigating the effects of early different reared condition on development of rat recognition memory.

A total of 156 from 19 litters (EE-P45: n=32; massage: n=54; control: n=50) Long Evans hooded rats were employed in to study at different retention intervals. All experimental groups were tested at five age intervals: P18 (P17-P21); P25 (P23-P27); P30 (P29-P33); P40 (P39-P43); P60 (P59-P63). The apparatus consisted of clear Plexiglas® circular open-field (diameter, 1 m; height, 0.50 m) which is finished on the inside with flat white paint to obtain a homogeneous environment. it is used Plexiglas® cubes (15x15cm), as objects, on which different visual pattern (e.g., black and white shapes) were inserted. The cubes were placed at the same distance from the sidewalls along diameter of arena (Fig.9). The test is situated in separated part of the behavioural room that is illuminated with two halogen lamps in order to obtain similar levels of illumination on arena. A digital video camera is mounted overhead on the ceiling to record each trial of animals and an observer simultaneously quantifies the time spent exploring objects by means of timers. The protocol design consist of three phases: habituation, sample and choice sessions. Before to start these experimental procedures it is necessary to make sure that arena and cubes is cleaned and dried and that video-tracking system is ready to be used. After that, I fill out data sheets with subject number of animal, gender, birth date, experiment date, coded condition.

A day before to start the testing procedure, the animals were allowed to explore the apparatus without any objects for 5 minutes avoiding any interferences in the exploration activity in the following sessions. This phase is called **habituation phase (S0)**. Each animal is brought into the behavioural testing room with its

individual temporary transport cage. In the **sample phase (S1)**, the rodent is placed in the apparatus facing the wall opposite to where the experimenter is, with two identical objects (two identical visual pattern). It is important to handle the rodent in a consistent manner and place rodent in the arena in the same position. The video-tracking system is started after the animal is placed in the maze so that the behaviour of each animal is consistently recorded 5 min. At the end of the 5-min, I remove the rat from the arena and place into a transport cage. The animal come back inside its home cage on the cart outside the room. Before testing another rat, I clean the arena and cubes with disinfectant and dry with paper towels. In the **test phase (S2)**, the rat is reintroduced into the arena after a predetermined retention interval: 1, 24, 48 and 72 hours. The procedure is the same described above but in this session the visual pattern inserted in the cubes are different: one identical to the two pattern in the S1 (the old object) and one new (the new object). The criteria for considering a rat as exploring an object are strictly based on active exploration in which rats are reported to sniff the object at a distance of less than two cm and/or touching it with the nose and forepaws (Ennaceur et al, 1996). The location of the two choice objects is counterbalanced between rats and across phases and the visual patterns used had been previously validated to avoid any preferences' bias. The performance of the animals in exploring the old and new objects is quantified by following measurements: exploration time and discrimination index. The time spent exploring each object is assessed for the sample and the test phase at each retention interval. A discrimination index is computed for each retention interval as Tn-To/Tn+To, where Tn is the exploring time of new visual pattern and To is the old visual pattern. A value that is significantly above zero describes animals exploring more the novel than the familiar object. A value that is significantly below zero describes animals exploring more the familiar

than the novel object. A value of zero describes that animals explore equally both novel and old object (Ennaceur et al., 1996, 2005).

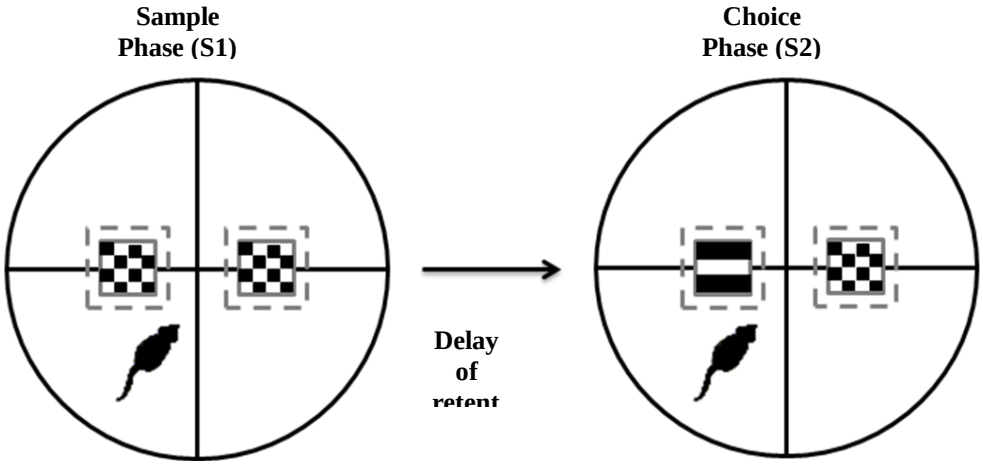


Figure 9

Top, object recognition task. A schematic representation of the sample (left) and choice (right) phase. The animal is placed in the arena with two identical objects (S1) and after a specific delay of interval it is reintroduced in the arena with two objects (S2): one familiar and one never seen before, a novel object. **Bottom,** a top view of test during animal's performance in the sample (left) and choice (right) phase.

5.12 Statistical analysis

Statistical analysis was performed with SigmaStat; parametric or nonparametric analysis was performed according to the results of the test for normality of the data. Significance level was 0.05. Effect size was calculated as Cohens's d and the difference between the two means divided by pooled SD for the data.

CHAPTER 6

RESULTS

6.1 Acceleration of visual system development by massage.

Recently, it has been shown that EE from birth leads to a conspicuous acceleration of visual system development in rodents, appreciable at the behavioural, electrophysiological and molecular level (Cancedda et al., 2004; Landi et al., 2007). It has been suggested that in the earliest phases of exposure to EE, the effects of EE do not stem from a direct action on the developing pups, but rather from a variation in maternal behaviour in enriched conditions (Cancedda et al., 2004; Sale et al., 2004), indeed these studies stressed that pups in EE received a higher level of stimulation through licking, grooming and physical contact. However, no data are available that tactile stimulation can influence visual system development.

6.1.1 Accelerated development of flash-evoked visual response in massaged rat pups.

As first index of early visual system maturation, I assessed the developmental time course of the response to a flash by means of Visual Evoked potentials recorded from the primary visual cortex (fVEP).

Normally in rats, the first light evoked responses in the visual cortex arise around P11, with a long latency negative wave; few days after the appearance of these first visual responses, the eyelids open around P14–P15 so that the animals begin to

experience pattern vision and approximately at P16 the negative-positive complex is similar to that obtainable in the adult rat (Rose, 1971) (Fig.10). During development the parameters of the negative-positive complex change and in particular it is observed a shorter latency components (Rose, 1968).

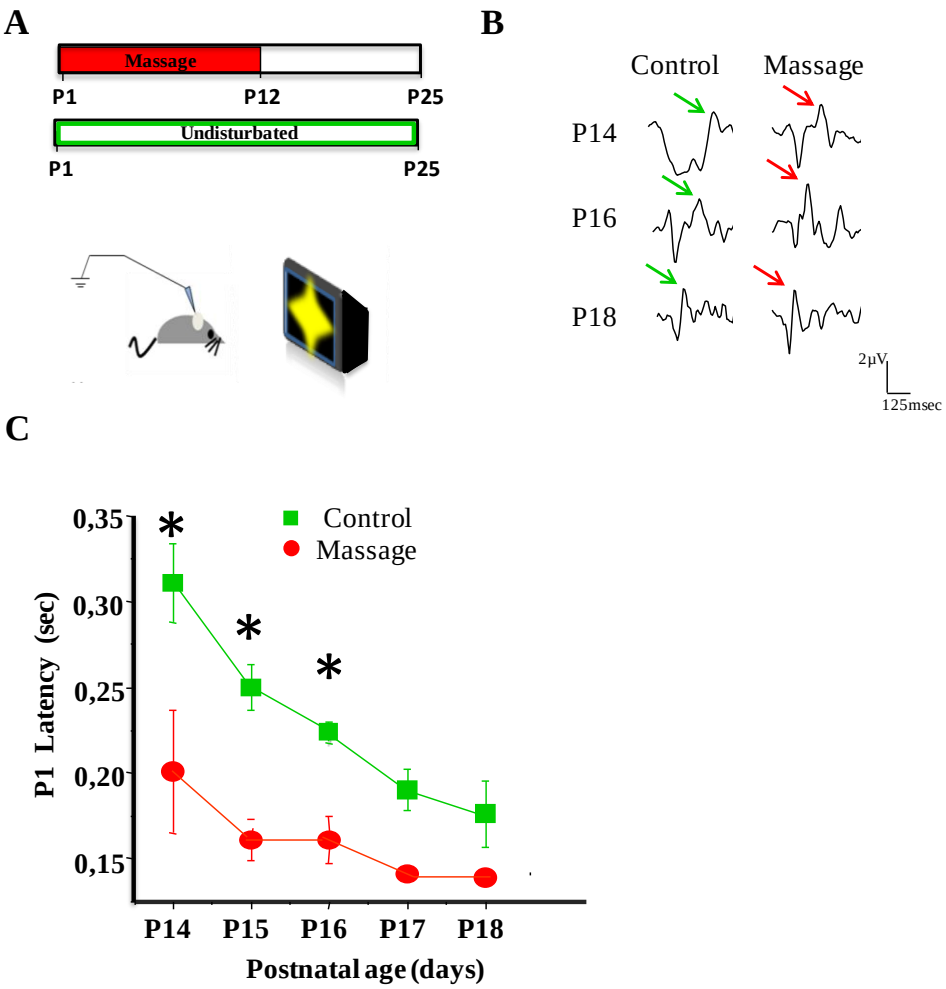


Figure 10.

(A) Experimental protocols. **(B)** Representative visually evoked potentials recorded from massaged and control rat pups at different ages ranging from P14 to P18. The narrows indicate the positive wave observed P1. **(C)** Massage in rat pups determines a significantly faster rate of reduction in the latencies of the flash VEP. Mean P1 latency plotted as a function of age for the groups of massaged and of control rats. The developmental shortening of P1 latency is significantly faster for massaged

rats than control rats (two-way ANOVA, treatment x age, factor age $p<0.001$, factor treatment $p<0.001$, n=4 for each age group both for massaged and controls, Holm–Sidak post hoc test). Asterisks denote significant difference between treatments at a given postnatal day. Vertical bars represent SEM.

I applied the massage protocol in newborn rats from P1 to P12 and recorded fVEP activity between P14 and P18, when the maximum degree of latency shortening is normally observed. I found a significantly faster rate of reduction of latencies in the massaged rats with respect to control rats. A significant difference in the two groups was found up to P16 (Fig.10; two-way ANOVA, age (df=4) x treatment (df=1), factor age significant, p=0.001 (F=8.269), factor treatment significant, p=0.001 (F=31.984), pairwise multiple comparison procedure Holm–Sidak, treatment within P14–P16, massage vs controls, p=0.05; treatment within P17 and P18, massage vs controls, p=0.05, n=4 for each experimental group at each age). This supports a role for massage therapy in the enhancement of fVEP maturation.

6.1.2 Accelerated development of visual acuity in massaged rat pups

A sensitive and predictive index of the visual system development is the maturation of visual acuity. I measured, therefore, the effects of tactile stimulation on VA development at P25 by means of VEP recordings from the primary visual cortex. Recordings were performed at P25 as it is the age at which visual acuity in EE animals start to differ from non-EE animals (Cancedda et al., 2004; Landi et al., 2007). I found that electrophysiological visual acuity was significantly higher in the massaged rats (n=11, mean visual acuity., 0.743 ± 0.02 c/deg) with respect to control rats (n=7; mean visual acuity, 0.63 ± 0.03 c/deg) (Fig.11). To rule out the possibility that these effects

were attributable to the separation from the mother, a third experimental group was introduced, consisting of rats that were separated from the mother for the same amount of time as the massaged rats but receiving no tactile stimulation (maternal separation group, n=12). No differences were found between this latter group (mean visual acuity, 0.66 ± 0.02 c/deg) and the controls, supporting the role of tactile stimulation per se in producing the observed effects on visual acuity development (one way ANOVA, factor treatment (df=2) significant, p=0.003 (F=7.38), multiple comparison procedure Holm–Sidak method, visual acuity of massage rats differs from that of control rats and of maternal separation rats, Cohen's d=1.827 and 1.64, respectively; the latter two do not differ, Cohen's d=0.64).

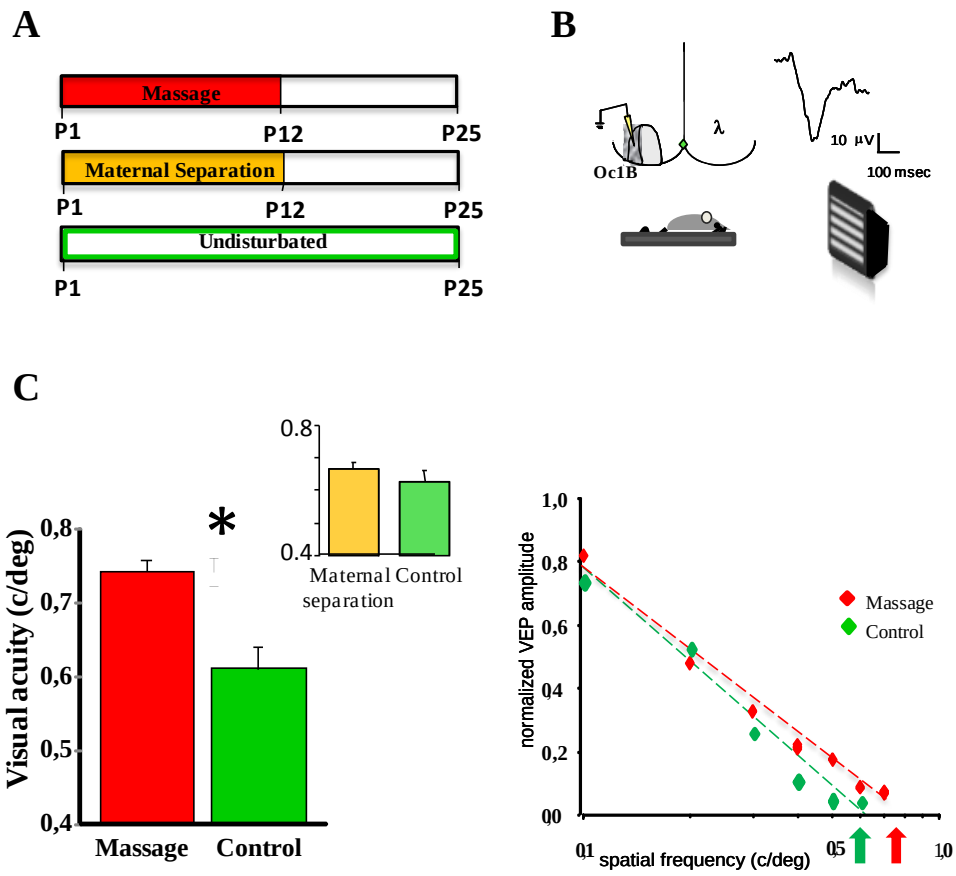


Figure 11.

(A) Experimental protocols. **(B)** Left, schematic representation of 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 contrast at 1 Hz. **(C)** Left, mean visual acuity determined at P25 by means of VEPs recorded from the primary visual cortex for massaged rats (n=11) and for control rats (n=7). Vertical bars are SEM. Massage group significantly differs from control group and from maternal separation group (n=12; inset) (one-way ANOVA, factor treatment significant, p=0.003, multiple comparison procedure Holm–Sidak method, visual acuity of massage rats differs from that of control rats and of maternal separation rats; the latter two do not differ). Right, example of visual acuity estimated in one massage (red) and control (green) animal. Experimental points are VEP amplitudes normalized to the mean amplitude of VEP at 0,1 c/deg; dashed lines are linear fits to the data. Estimated visual acuities (arrows) are taken as the extrapolation to 0 level of the fitting line.

To assess whether the effects of tactile stimulation on pup visual acuity were evident also at behavioural level, I measured visual acuity by means of the Prusky water box. All three experimental groups were trained from P19 to P22, and VA was measured from P23 until P25 and P28. No difficulty was encountered in training the animals of any group, indeed all rats reached 80% of correct choices at the third session. I observed that visual acuity in massaged rats (n=15) was significantly higher than in controls (n=13) and in maternal separation rats (n=11) at both P25 (Fig.12; mean±SEM visual acuity, 0.674 ± 0.009 c/deg in massage, 0.6 ± 0.01 c/deg in maternal separation, 0.59 ± 0.01 c/deg in controls) and at P28 (mean±SEM visual acuity, 0.76±0.009 c/deg in massage, 0.674±0.014 c/deg in maternal separation, 0.65±0.013 c/deg in controls); visual acuity in the latter groups did not differ [two-way repeated measures ANOVA, treatment (df=2) x age (df=1), factor age significant, $p<0.001$ ($F=48.86$), factor treatment significant, $p<0.001$ ($F=11.4$), visual acuity in massage group different from controls and maternal separation groups at both ages,

p=0.05; multiple comparison procedure Holm–Sidak method]. The fact that the effects of massage on visual acuity are apparent at both behavioural and electrophysiological level rules out the possibility that the effects on behaviour are simply attributable to aspecific effects of massage on pup attention or motivation and that the effects on visual cortex physiology are devoid of any behavioural relevance. A higher visual acuity in rats subjected to tactile stimulation was evident at an early stage of visual acuity development but was no longer found at older ages (mean±SEM visual acuity, 0.96±0.01 c/deg).

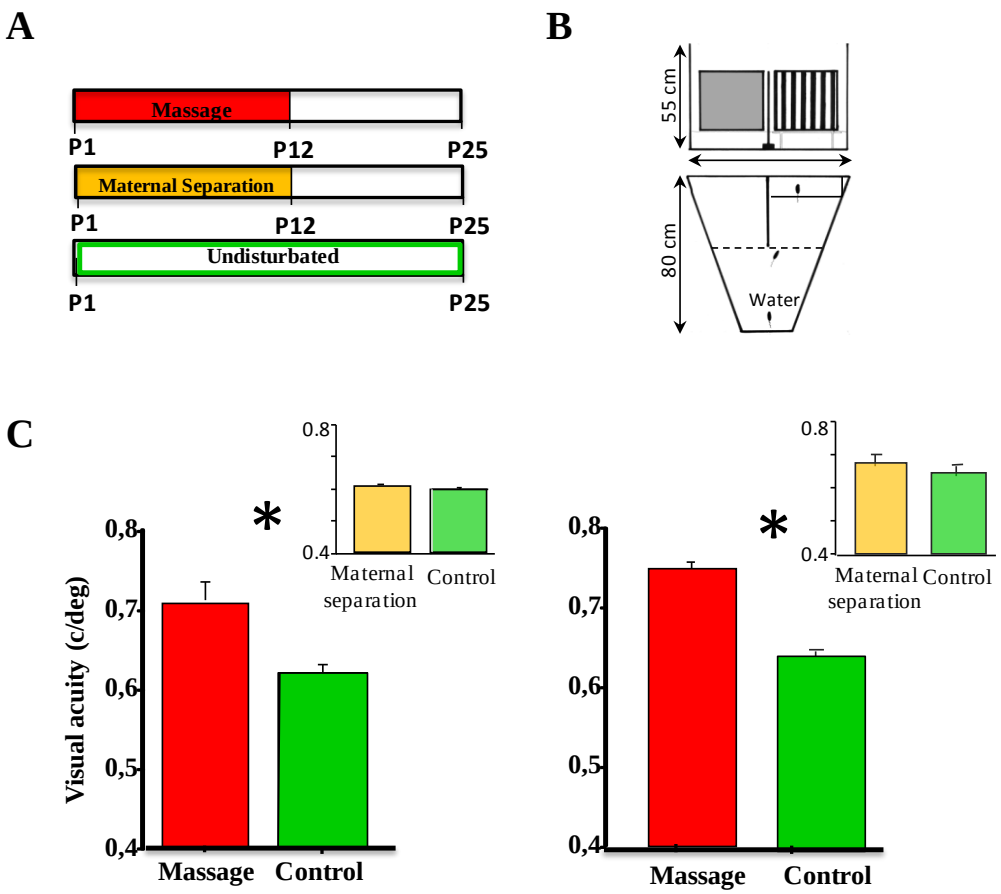


Figure 12.

(A) Experimental protocols. **(B)** Schematic diagram of the visual water box. **(C)** Mean behavioural visual acuity in P25 and P28 massage, controls, and maternal separation groups. Vertical bars

represent SEM. Visual acuity of massaged rats (n=15) is significantly higher than in control (n=13) or in maternal separation (n=11; inset) (two-way repeated measures ANOVA, treatment x age, factor age significant, $p<0.001$, factor treatment significant, $p<0.001$, visual acuity in massage group different from controls and maternal separation groups at both ages, $p<0.05$; visual acuity in controls and maternal separation group not significantly different, multiple comparison procedure Holm–Sidak method). Asterisks denote significant difference.

To compare the effects of massage on visual acuity development with those of exposure to environmental enrichment, it has been exposed another group of newborn rats to a time-restricted protocol of EE for a period of 12 days. The period during which massage protocol was applied (P0 –P12), it is also the time during which higher levels of maternal stimulation have been reported in EE pups compared with controls (Sale et al., 2004). It has been found that the effects of EE up to P12 on visual acuity development observed at P25 were not significantly different from those in massaged rats, both being less marked than those observed previously in P25 animals exposed to EE up to P25 (Cancedda et al., 2004; Sale et al., 2004; Landi et al., 2007) [Fig. 13; mean±SEM visual acuity for P25 rats EE up to P12, 0.74±0.01 c/deg, n= 5, difference with control P25 rats (n=7) significant, difference with massaged rats (n=11) non significant, difference with rats EE up to P25 (0.93±0.03 c/deg; n=5) significant, one-way ANOVA (df=3), $p<0.001$, F=33,558, multiple comparison procedure Holm–Sidak method)].

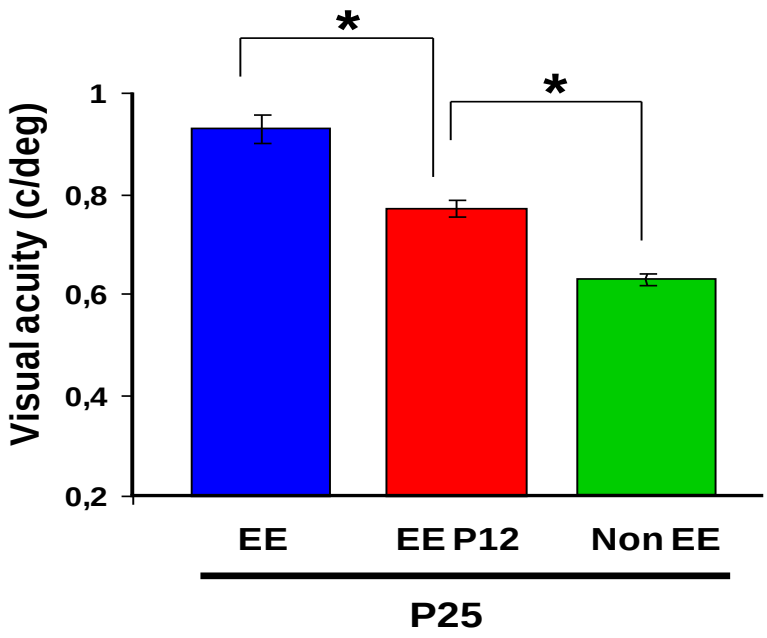


Figure 13

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 ($p<0,001$).

I thus conclude that tactile stimulation provided by maternal care is likely to be the main factor responsible for the effects on visual development of early exposure to EE, when pup interactions with the external environment are mainly represented by their interactions with the mother

6.1.3 The acceleration of visual system development in massaged rat pups is not due to a modulation of stress hormone levels.

Tactile stimulation in rodent pups reduces the increase in corticosterone caused by stressful events (Pauk et al., 1986). To control whether a modulation of stress hormone levels contributes to the documented effects on visual acuity development, I measured plasma corticosterone levels in P14 rats subjected to the protocol of stimulation, subjected to separation, or left undisturbed. I found (Fig.14) that corticosterone levels were significantly lower than in controls (n=6; mean±SEM, 306 ± 50 ng/ml) for both massaged (n=6; mean±SEM, 171 ± 30 ng/ml) and separated pups (mean±SEM, 171 ± 12 ng/ml), with no difference between themselves (one-way ANOVA (df =2), F =5.147, p±0.02, post hoc Tukey's test). Since only in massaged rats there was a significant effect on visual acuity development, I conclude that modulation of stress hormones does not contribute to tactile stimulation effects on visual development.

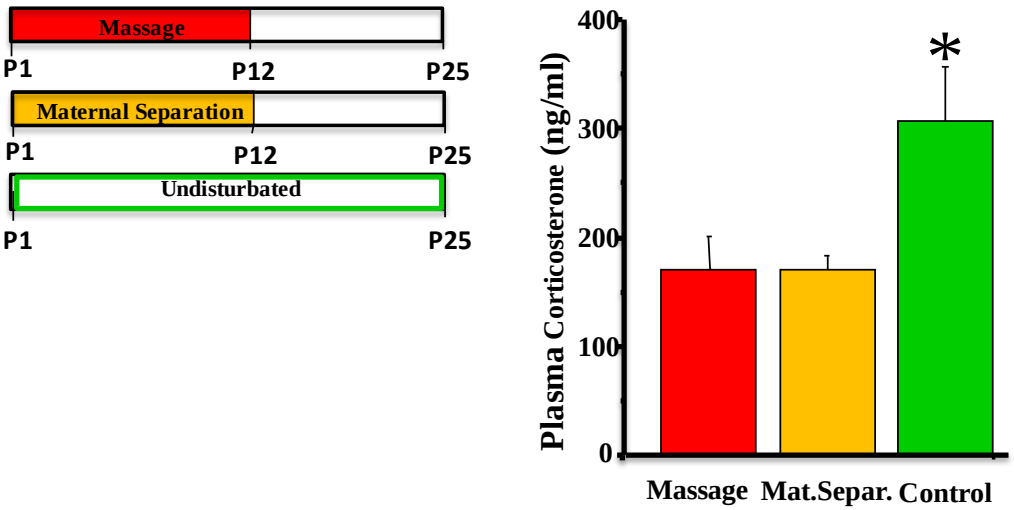


Figure 14

Left, experimental protocols. Right, mean plasma corticosterone levels at P14 in massage, maternal separation, and control groups. The level of corticosterone in the control groups is significantly

higher than in the maternal separation and massage groups (one-way ANOVA, p=0.02, n=6 for each group, post hoc Tukey's test). Vertical bars denote SEM. Asterisks denote significant difference.

6.1.4 Increased levels of IGF-1 in the cortex of massaged rat pups.

Increasing evidences strongly support the role of insulin-like growth factor 1 (IGF-1) as molecular mediator of central nervous system (CNS) development (for a review see D'Ercole at al., 1996; Folli et al., 1996; Anlar et al., 1999). It has been demonstrated that IGF-1 peak of expression is restricted to different time windows in different regions, according to the time course of their maturation which dovetail with periods of axon outgrowth, dendritic maturation and synaptogenesis (Bondy, 1991; Bondy et al., 1992; Barlett et al., 1991). Several works, both in human and in rodents, suggest that IGF-1 could be the key molecule modulating brain response to physical exercise (Schwarz et al., 1996; Wallace et al., 1999, Carro et al., 2000). In addition, IGF-1 crosses the blood-brain barrier and acting on neurons bearing its receptors present in the occipital cortex (Frolich et al., 1998) and in the retina (Rodrigues et al., 1988; Waldbilling et al., 1988) increases their electrical activity inducing the expression of molecules important for visual system plasticity (Carro et al., 2000).

Finally, it has been demonstrated that IGF-1 is increased postnatally in the visual cortex of enriched rats, post-weaning administration of IGF-1 in this structure mimics EE effects on visual acuity acceleration and blocking its action in the visual cortex of developing EE subjects completely prevents EE effects on visual acuity development (Ciucci et al., 2007). Interestingly, a role for IGF-1 in visual-cortical plasticity has independently emerged from a detailed genetic screening of factors controlled by visual experience during development (Tropea et al., 2006).

For these reasons, I considered IGF-1 as a good candidate to mediate massage effects on visual system development. To measure in the central nervous system IGF-1 protein I used a standard and reliable immunohistochemical protocol (Carro et al., 2000; Trejo et al., 2001; Carro et al., 2001; Ciucci et al., 2007).

I assessed in massaged animals whether IGF-1 levels were increased in the brain and in particular in the primary visual cortex. In both areas, I quantified the presence of IGF-1 in terms of the number of IGF-1-positive cells normalized to the number of neurons (NeuN-positive cells). In the primary visual cortex, IGF-1 presence was analyzed at P14, P18, and P25, as in the study by Ciucci et al. (2007). At P14 and P25, no difference was found between massaged, separated, and control rats (Fig.15). At P18, the presence of IGF-1 in the visual cortex of massaged rats was increased with respect to P18 separated or control rats; the latter two did not differ between themselves [Fig.15; two-way ANOVA, treatment (df=2) x age (df=2), factor treatment p=0.024 (F=4.2), factor age p=0.001 (F=9.27), post hoc Tukey's test]. Note that at no age the number of NeuN positive cells is increased by massage protocol with respect to maternal separation and control rats (two-way ANOVA, age x treatment, factor age not significant, p=0,105, factor treatment not significant, p=0,556); therefore, the increase in the density of IGF-1 positive neurons caused by massage protocol is due to an increased presence of IGF-1 labelled neuronal cells, not to an increase in the density of neurons.

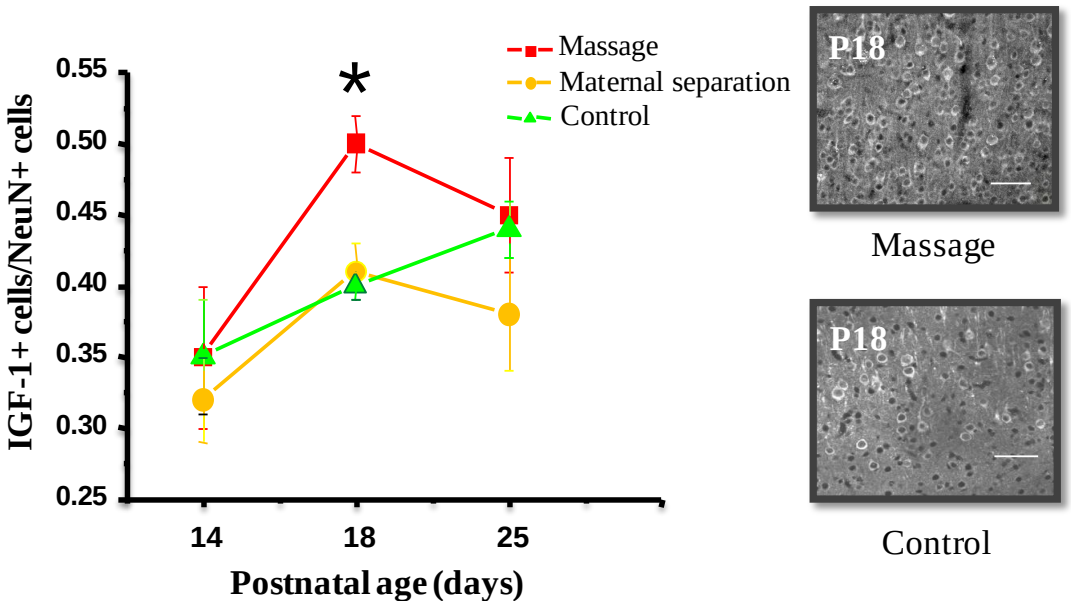


Figure 15

Left, mean number of IGF-1-positive cells in the visual cortex, normalized to the number of neurons (NeuN-positive cells) for each developmental age analyzed. n=4 for each age and each experimental group. Vertical bars represent SEM. The normalized number of IGF-1-positive cells increases significantly between P14 and P18; the normalized number at P18 in massaged rats is significantly increased with respect to maternal separation and control rats (two-way ANOVA, treatment x age, factor treatment $p=0.024$, factor age $p<0.01$, *post hoc* Tukey's test). Right, Examples of IGF-1 labeling at P18 for one massaged and one control rat. Scale bar, 50µm.

To control whether the effects of massage in IGF-1 brain levels were specific for visual areas, I assessed the presence of IGF-1 in the primary auditory cortex at P14. I found that IGF-1 content was significantly increased in massage pups (n=5) with respect to controls (n=3) also in the auditory cortex (Fig. 16; mean±SEM IGF-1-positive cells/NeuN-positive cells in massaged pups, 0.52 ± 0.022; in control pups, 0.43 ± 0.02; t test, $p=0.04$). Thus, the increase in IGF-1 in massage pups is not specific for the visual cortex. This is not surprising because it is known that early exposure to EE increases IGF-1 levels very precociously in the retina (up to P10) and in the

cerebellum (P1) of EE pups and higher levels of IGF-1 are found in the milk (Sale et al., 2007), supporting the idea that EE and massage act on brain development, not on development of specific brain regions.

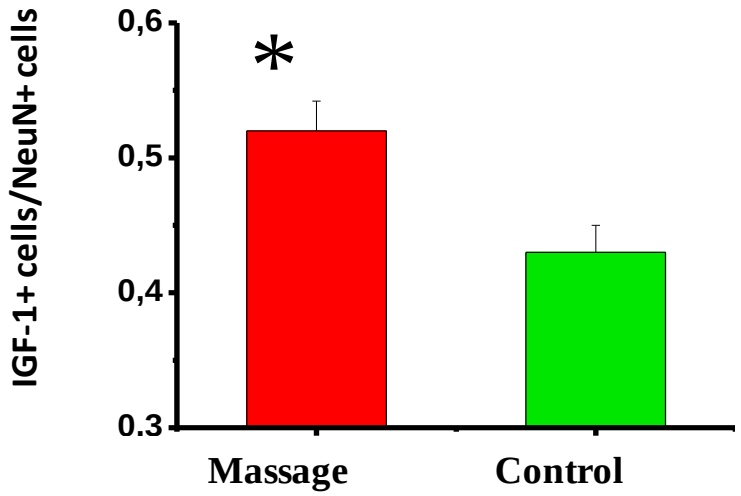


Figure 16

Mean number of IGF-1-positive cells in the auditory cortex, normalized to the number of neurons (NeuN-positive cells). The number of animals analyzed at P14 is: for massaged rats, n=5; for control rats, n=3. Vertical bars represent SEM. The normalized number at P14 in massaged rats is significantly increased with respect to control rats (t-test, p=0.04).

6.1.5 Block of IGF-1 action prevents the acceleration of visual acuity development in massaged rat pups.

To further reinforce these data it was necessary to prove that the regulation of IGF1 expression caused by massage was necessary for the effects of massage on visual development. It has been demonstrated recently that blocking IGF-1 action in the visual

cortex from P18 to P25 blocked EE effects on rat visual acuity development (Ciucci et al., 2007).

I assessed now whether blocking IGF-1 action more precociously and systemically blocks the effects of massage. I used the IGF-1 antagonist JB1, already used to antagonize circulating IGF-1 action (Carro et al., 2001) and, locally infused, to block IGF-1 action in the visual cortex (Ciucci et al., 2007).

I investigated whether blocking IGF-1 action by means of systemic JB1 injections from P1 to P10 could block the effect of massage on visual acuity development. JB1 injections were performed in massaged rats (massaged plus JB1 rats) subcutaneously every other day. Visual acuity was assessed electrophysiologically (VEPs) at P25. The results displayed that in massaged plus JB1 rats (n=8), visual acuity (0.61±0.03 c/deg) did not differ from that in control, non massaged rats and was significantly lower than in massaged rats (Fig. 17; one-way ANOVA (df=3), $p < 0.001$ ($F=7.907$) post hoc Holm–Sidak method, massaged vs massaged plus JB1, $p < 0.05$; control vs massaged, $p < 0.05$; control vs massaged plus JB1, $p > 0.05$). As control for aspecific effects of injections, another group of massaged rat pups was injected with saline with the same procedure used to inject JB1. As shown in Fig. 17 no difference between the visual acuity of these rats (n=4; massaged plus saline; visual acuity, 0.72 ± 0.02 c/deg), and the visual acuity of massaged rats was evident (one-way ANOVA (df=3) $p < 0.001$ ($F=7.907$) post hoc Holm– Sidak method, massaged plus saline vs massaged rats $p > 0.05$, massaged plus JB1 vs massaged plus saline $p < 0.05$).

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

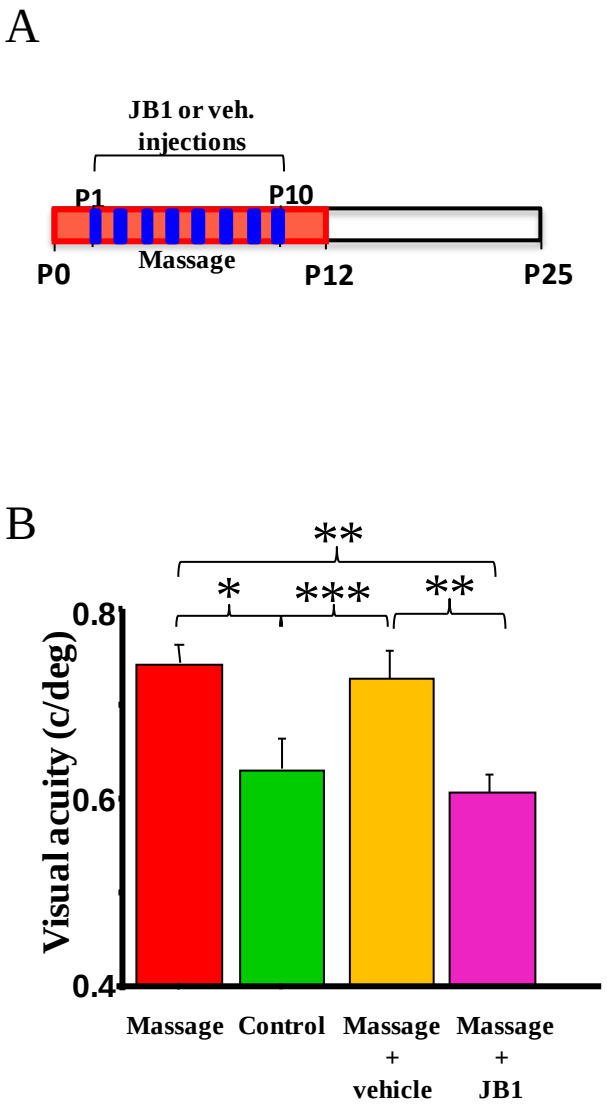


Figure 17

(A) Experimental protocol and schematic representation of recording site for VEPs. **(B)** Left, summary of visual acuity in all groups. Data are mean visual acuity and vertical bars represent SEM. Visual acuity of massaged plus JB1 animals (0.61±0.03 c/deg, n=8) is significantly lower than massaged animals (0.743 ± 0.02 c/deg n=11) or massaged plus vehicle animals (0.72 ± 0.02 c/deg, n=4); the latter two do not differ (One-way ANOVA , $p<0.001$; post hoc Holm–Sidak method, significance level 0,05). Asterisks denote significant difference (one asterisk, $p<0,001$, two asterisks, $p=0,003$, three asterisks $p=0,004$).

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

How could IGF-1 increase mediate massage 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). Recently, it has been also demonstrated that IGF-1 affects GAD-65 immunoreactivity in perisomatic innervations in the visual cortex suggesting that IGF-1 action in mediating EE effects could be exerted through the modulation of intracortical inhibitory circuitry (Ciucci et al., 2007).

I analyzed, therefore, the expression of GAD65, an isoform of glutamic acid decarboxylase that is concentrated in presynaptic terminals, in layers II-III of striate cortex by quantitative confocal microscopy (Huang et al., 1999). 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). The development of GABAergic perisomatic inhibition is not completed before the fifth postnatal week.

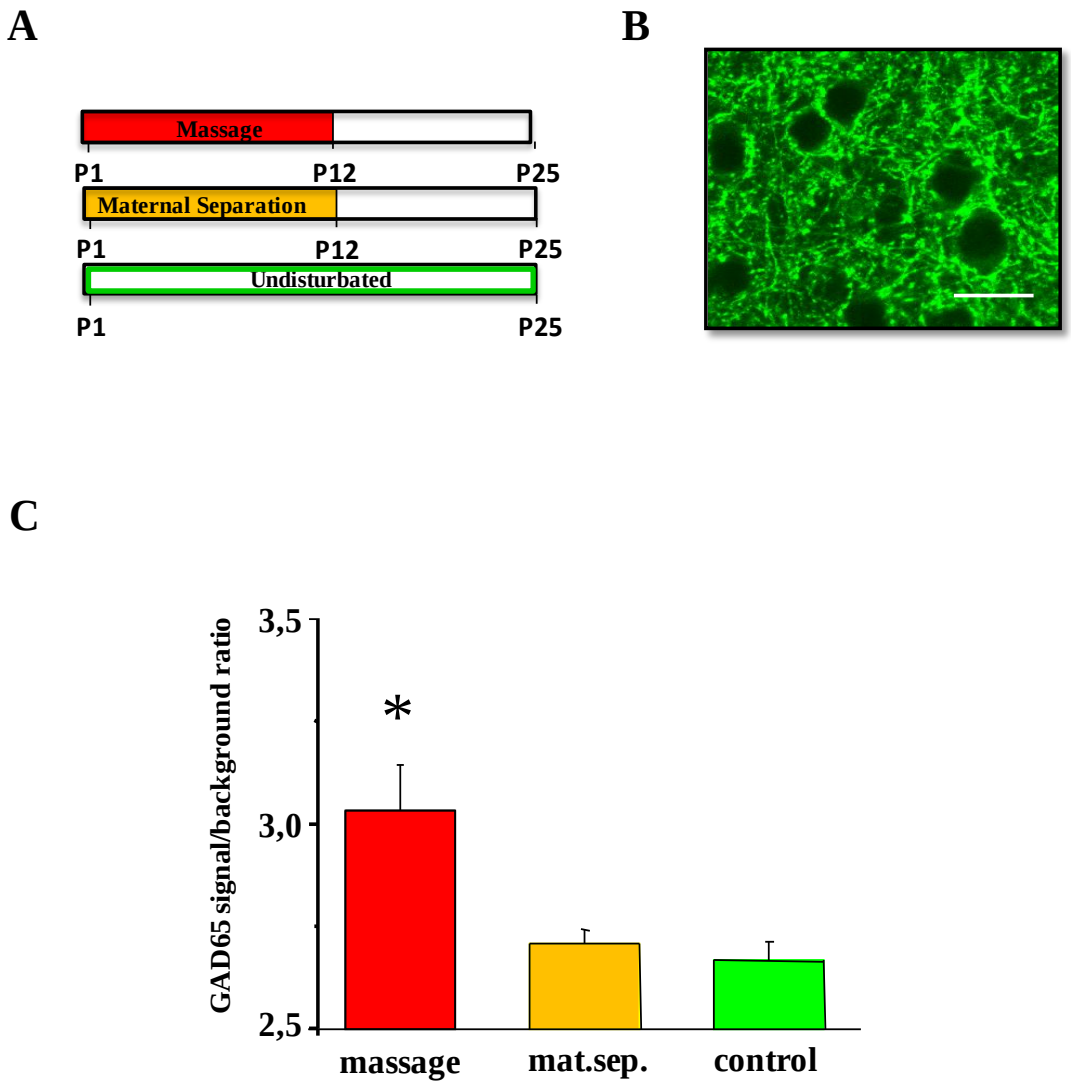


Figure 18

(A) Experimental protocols. **(B)** 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. **(C)** Summary of the GAD65 signal-to-background ratio (intensity of GAD65 label in puncta rings divided by the background staining in the soma) for the cells in each experimental group. GAD65 immunoreactivity is significantly higher in the massage group (n=5 mean± SEM signal/background ratio, 3,032± 0.11) than in the maternal separation (n=5, mean± SEM signal/background ratio, 2,668± 0.05) and control group (n=5; mean± SEM signal/background ratio, 2,71± 0.03) (one way ANOVA, p=0.008; F=7,428 multiple comparisons Holm–Sidak method, p<0,05). Vertical bars indicate SEM.

I 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 massaged (n=5), separated (n=5) and control (n=5) rats. Analysis of GAD65 fluorescence in "puncta-rings" surrounding the soma of cortical neurons indicated a higher GAD65 signal in visual cortex of the massage with respect to maternal separation and control rats; no difference was found in the GAD65 signal between maternal separation and control groups [Fig. 18; one way ANOVA, p=0.008; F=7,428 multiple comparisons Holm–Sidak method, GAD65 signal of massage animals (massage pups, n=5 mean± SEM signal/background ratio, 3,032± 0.11) higher than maternal separation and control animals (n=5; mean± SEM signal/background ratio, 2,71± 0.03; maternal separation pups, n=5, mean± SEM signal/background ratio, 2,668± 0.05), and no difference was found between GAD65 signal of maternal separation and control animals].

Thus, IGF-1 increase could be a mediator of massage effects on visual acuity development via an effect on inhibitory system development.

6.2 Massage and EE affect anxiety-like behaviour in adult rat: a role for IGF-1.

As outlined in the Introduction, the pioneering works of Levine, Denenberg and their colleagues, and later Meaney, Plotsky and colleagues have shown in rats that experience during the early postnatal period can have long lasting consequences for defensive behaviour, emotional and stress responsivity (for review see, Kaffman and Meaney, 2007); adult animals handled during the postnatal period showed increased exploratory behaviour in novel environments and blunted HPA responses to stress as compared to non-handled animals (Levine et al., 1957, 1967; Meaney et al., 1989, 1992; Meaney and Aitken, 1985). It has been proposed that the effects of postnatal handling are mediated by changes in mother-pup interactions, with increased levels of maternal care received by handled pups in respect to undisturbed pups (Levine, 1975, for review see Denenberg, 1999). The question about how this maternal mediation might occur and whether such factors might contribute to naturally occurring individual differences in HPA responses to stress was answered by Meaney and collaborators (Liu et al., 1997). It has been shown that mothers of handled pups display enhanced levels of licking/grooming (LG) and of arched back nursing (ABN), which are some of the most critical behavioural modules in the repertoire of maternal behaviour toward the offspring. Accordingly, adult rats reared by dams that naturally exhibit high levels of care, showed decreased HPA activation in response to stress compared to those raised by dams that naturally exhibit low levels of care (Liu et al., 1997; Caldji et al., 1998).

By converse, a large number of studies has shown that separation of rat pups from their mothers during the early postnatal period produces permanent increases in anxiety-related behaviour such as in open-field test and elevated plus maze and in

neuroendocrine stress response when offspring are tested as adults (Holmes et al., 2005; Aisa et al., 2007, 2009).

A large body of studies highlighted that environmental enrichment is able to modify animal's behaviour and in particular to improve a complex cognitive function as learning and memory (Falkenberg et al., 1992; Kempermann et al., 1997; Nilsson et al., 1999; Rampon and Tsien, 2000; Rampon et al., 2000b). The consequences of EE upon emotional reactivity are less documented and remained controversial for a long time (Huck and Price, 1975; Chamove, 1989; Escorihuela et al., 1994; Fernandez-Teruel et al., 1997). A more accurate analysis of the animal's behaviours in prenatally or postnatally stressed rats (Francis et al., 2002), aged mice (Thouvarecq et al., 2001) and mice considered as pathologically anxious (Chapillon et al., 1999; Roy et al., 2001; Iwata et al., 2007) led to assume that animals reared in enriched condition would exhibit a lower level of emotional reactivity than those reared in standard conditions (for a review, Chapillon et al., 2002).

This suggestion is also consistent with the evidence that pups born in EE receive higher levels of maternal care (Sale et al., 2004). A direct evidence that animals born in EE show a reduced anxiety-like behaviour as adult is however lacking.

Maternal care in rodents comprise behavioural modules with a high levels of tactile stimulation, such as licking and grooming (LG). Recently, it has been shown that enriching environment in terms of massage affects brain development, and in particular visual development, both in human infants and in rat pups (Guzzetta et al., 2009). The results in rat pups are superimposable to those obtained with an EE protocol restricted to the preweaning period (up to P12). This would suggest that tactile stimulation could produce effects similar to those produced by high level of

maternal care, including lower anxiety-like behaviour in adults. Also in this case, a direct experimental evidence is lacking.

The molecular mediators of these long-lasting effects of the levels of maternal care are under investigation. It has been shown that different levels of maternal LG during the postnatal period is associated with changes in forebrain GR levels that persist into adult. The offspring raised by high LG dams, like the handled animals, show increased levels of glucocoticoids receptor (GR) in the hippocampus and an increased sensitivity to the inhibitory effects of glucocorticoids on stress-induced HPA activity as compares to offspring raised by low LG dams (Liu et al., 1997; Weaver et al., 2004, 2005).

I therefore, explored the effects of massage and different protocols of enriched environment on anxiety-like behaviour in adult rat by means of elevated plus maze (EPM) test.

6.2.1 Massage and EE decrease anxiety-like behaviour in adult rat.

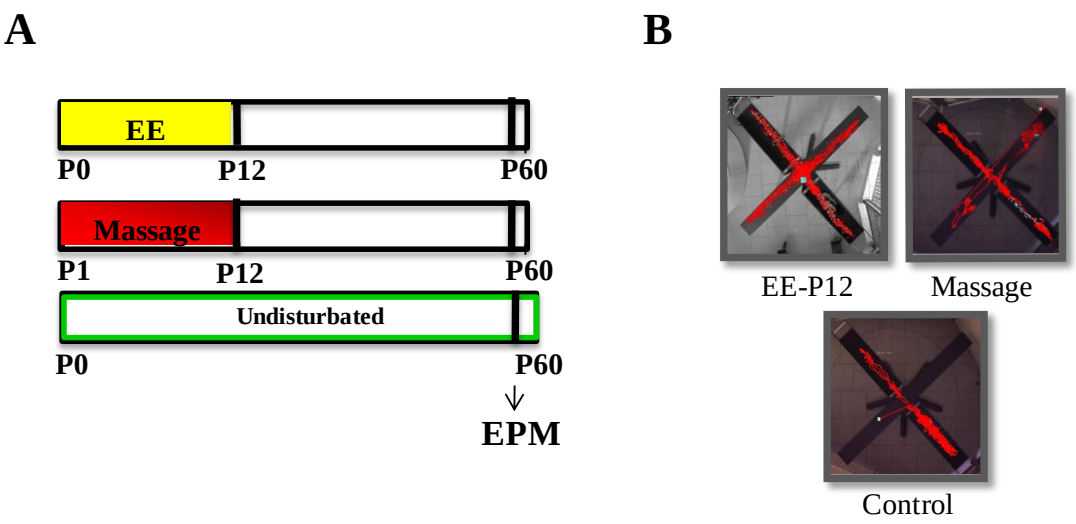
Comparison between massage and EE protocol up to P12.

I assessed the impact of an early environment manipulation on anxiety like behaviour by comparing performance of massage and EE rats, once adult, in the EPM with respect to control animals. Rat pups reared in standard condition received a body massaged three times a day from P1 to P12; EE was applied up to P12, to test the effects of early exposure to EE, the most likely to be mediating by the levels of maternal care.

The behaviour of each experimental group was tested at P60 and anxiety-like behaviour was measured by percentage of time spent on the open arms and percentage of open arm entries. As observed in figure 19, massaged and EE rats show decreased

anxiety levels, spending significantly longer time in the open arms [one-way ANOVA (d=2), factor treatment significant p=0.005 (F=5.825), post hoc Holm–Sidak method, massaged rats vs control rats, p<0.05; EE-P12 rats vs control rats, p<0.05; massaged rats vs EE-P12 rats, p>0.05] and increasing significantly the frequency of entries in open arms than control rats [one-way ANOVA (d=2), factor treatment significant p<0.001 (F=12.455), post hoc Holm–Sidak method, massaged rats vs control rats, p<0.05; EE-P12 rats vs control rats p<0.05; massaged rats vs EE-P12 rats, p>0.05].

Thus, the performance of massaged and enriched up to P12 rats were comparable suggesting that high levels of maternal care in environmental enrichment are crucial component of the effects of early EE protocol itself in programming the adult anxiety response.



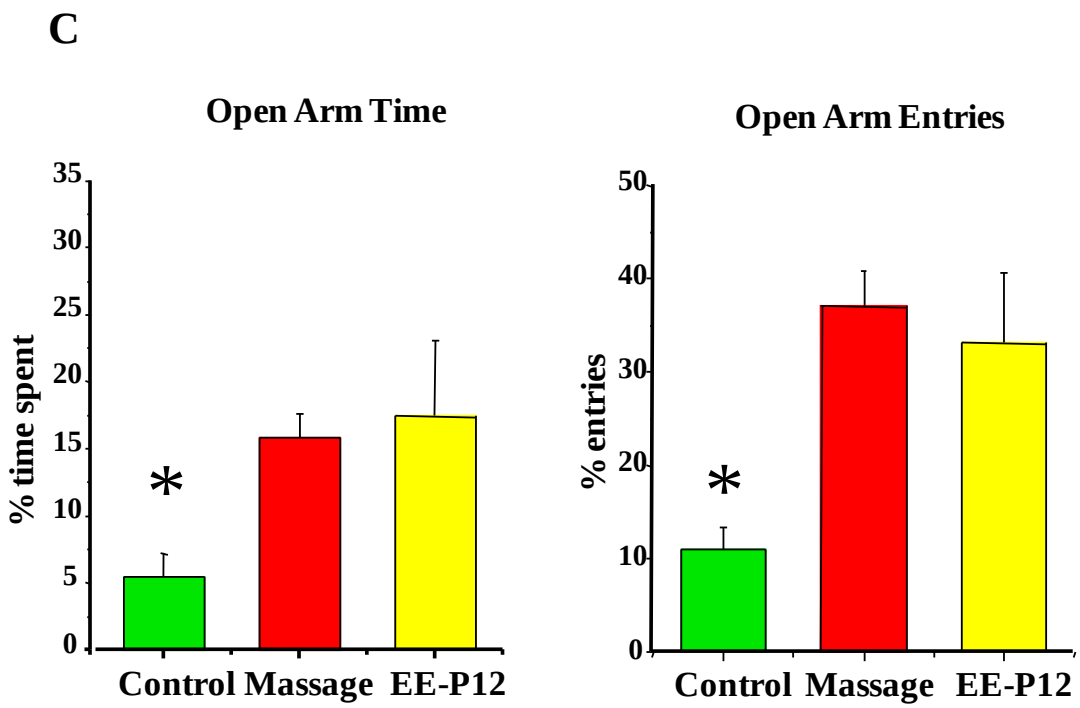


Figure 19.

(A) Experimental protocols. **(B)** Computer generated tracks of movement during the test trial. It was quantified the time spent and the entry frequency into open and closed arms. In these representative video images, the tracings (red lines) show a different amount of tracking in the open arms; at P60 massaged rats and EE-P12 rats were tracked more frequently in the open arms than control rats indicating a lower level of anxiety. **(C)** Left, mean percentage open arms time at P60 of massaged rats (n=11), enriched up to P12 rats (n=) and control rats (n=7). Massage and EE-P12 groups significantly differs from control group (one-way ANOVA, factor treatment significant, p=0.005, multiple comparison procedure Holm–Sidak method, percentage open arms massaged and enriched rats differs from that of control rats) Asterisks denote significant difference between. Vertical bars are SEM. Right, mean percentage open arms entries at P60 of massaged, enriched up to P12 and control rats. Massage and EE-P12 groups significantly differs from control group (one-way ANOVA, factor treatment significant, $p<0.001$, multiple comparison procedure Holm–Sidak method, percentage open entries of massaged and enriched rats differs from that of control rats). Asterisks denote significant difference between treatments. Vertical bars are SEM.

Comparison between massage and EE protocol until P45 or P60.

As second step, I compared the anxiety like behaviour of massaged rats with that of rats reared in EE until P45 or P60.

Massage, EE-P45 and EE-P60 groups spent longer time exploring open arms than control group [Fig. 20; one-way ANOVA (d=3), factor treatment significant $p=0.005$ ($F=4.772$), post hoc Holm–Sidak method, massaged rats vs control rats, $p<0.05$; EEp45 rats vs control rats $p<0.05$; EEp60 rats vs control rats $p<0.05$; massaged rats vs EEp45 rats, $p>0.05$; massaged rats vs EEp60 rats, $p>0.05$]. At the same time, the statistical analysis of open arm entries confirmed that massaged and enriched rats have lower anxiety level than control animals [Fig. 20; one-way ANOVA (d=3), factor treatment significant, $p<0.001$ ($F=9.083$), post hoc Holm–Sidak method, massaged rats vs control rats, $p<0.05$; EEp45 rats vs control rats $p<0.05$; EEp60 rats vs control rats $p<0.05$; massaged rats vs EEp45 rats, $p>0.05$; massaged rats vs EEp60 rats, $p>0.05$].

From these findings emerged that performance of animals exposed to EE until P45 and P60 were comparable with those obtained from EE-P12 and massaged rats strengthening the observation that the first weeks of postnatal life are critical for the stress response in adulthood.

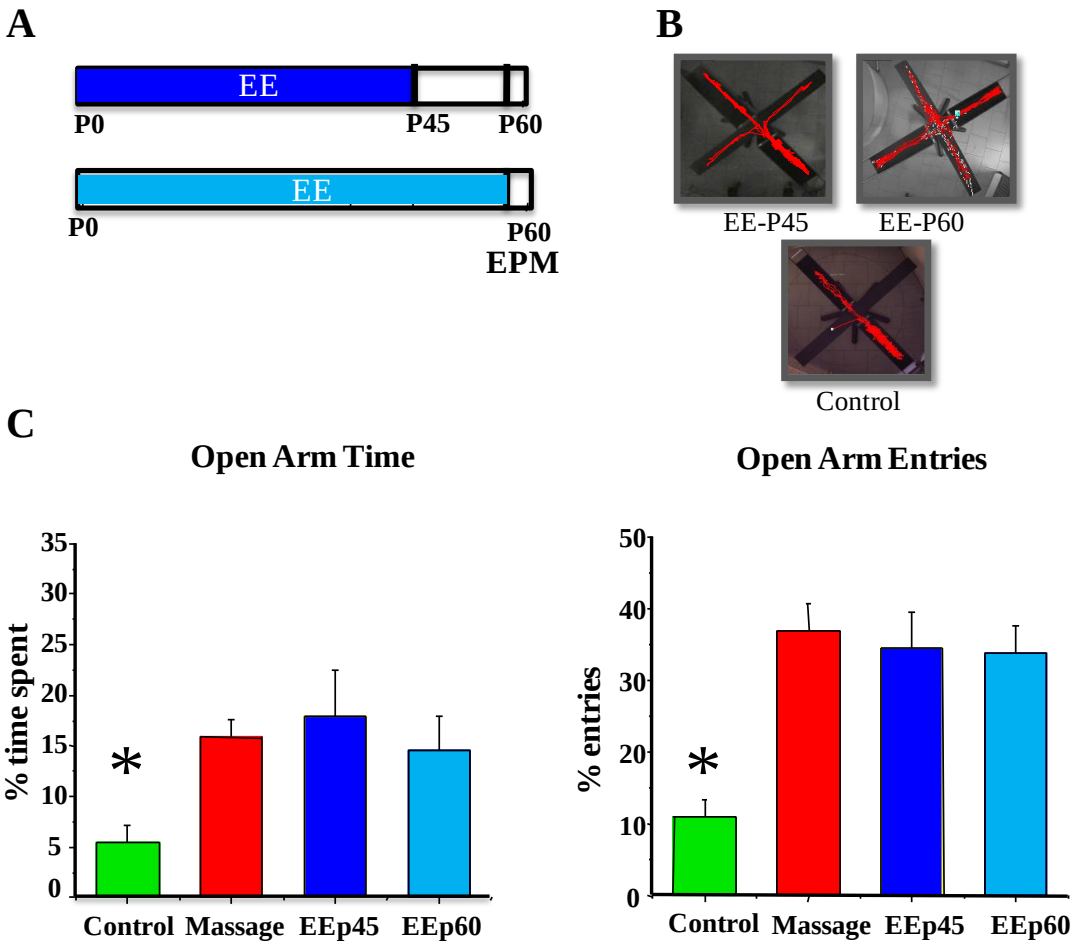


Figure 20.

(A) Experimental protocol. **(B)** Computer generated tracks of movement during the test trial. These representative tracings (red lines) show that enriched up to P45 rats were tracked more frequently in the open arms than control rats indicating a lower level of anxiety. **(C)** Mean percentage open arms time (left) and mean percentage of open arm entries (right) at P60 were significantly higher in EE-P45 (n=11) and EE-P60 (n=10) animals than control rats (n=7); no differences were found between massaged and enriched rats (left, one-way ANOVA, factor treatment significant, p=0.005, multiple comparison procedure Holm–Sidak method, percentage open arms enriched rats differs from that of control rats; massage and enriched rats do not differ) (right, one-way ANOVA, factor treatment significant, $p<0.001$, multiple comparison procedure Holm–Sidak method, percentage open entries of enriched rats differs from that of control rats; massage and enriched rats do not differ). Asterisks denote significant difference between treatments. Vertical bars are SEM.

Besides, the performance of three EE groups (EEp12, EEp45 and EEp60) are comparable [one-way ANOVA (d=2), factor treatment no significant p=0.859 (F=0.152), post hoc Holm–Sidak method, EEp12 vs EEp45; EEp12 vs EEp60; EEp45 vs EEp60, p>0.05].

Together these results suggest that the experience in the first two weeks of life may effect the stress response in adult rats supporting a central role of maternal care in the development of this function.

It was also measured the locomotor activity of each experimental groups assessing the total arm entries and distance moved during the performance in EPM at P60. In this way, the effects observed, without alterations in locomotor activity, could supposedly be strictly attributed to treatments or protocols applied in aforementioned experiments. As shown in the figure 21, the total arm entries [one-way ANOVA (d=4), factor treatment no significant p=0,276 (F=1.296) post hoc Holm–Sidak method, p>0.05] and the distance moved [one-way ANOVA (d=4), factor treatment no significant p=0.990 (F=0.0727) post hoc Holm–Sidak method, p>0.05] of each experimental group do not differ.

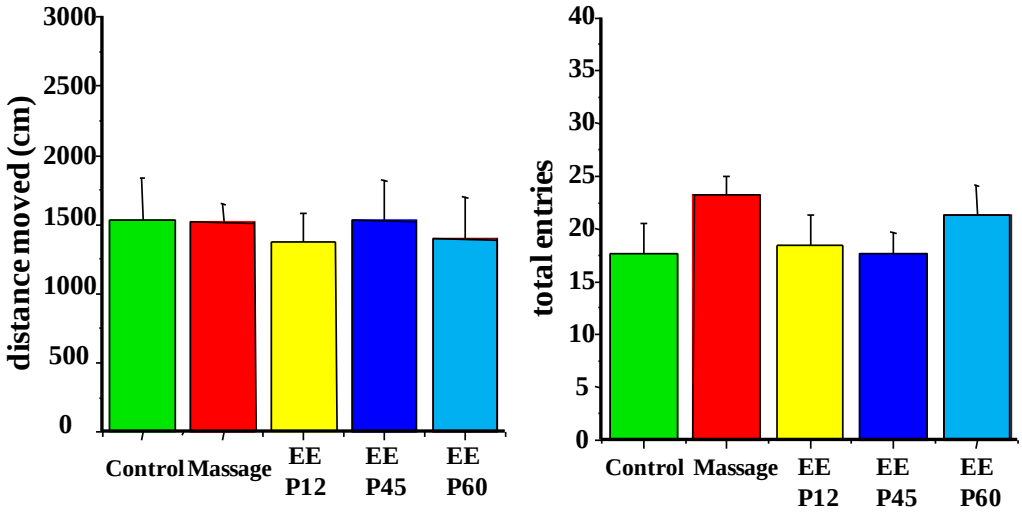


Figure 21.

Left, mean distance moved (cm) of all experimental groups during test session in elevated plus maze. No difference was found between groups (one-way ANOVA, factor treatment no significant, $p=0.276$, multiple comparison procedure Holm–Sidak method, $p<0.05$). Vertical bars are SEM. Right, mean total arm entries of all experimental groups during test session in elevated plus maze. No difference was found between groups (one-way ANOVA, factor treatment no significant, $p=0.990$, multiple comparison procedure Holm–Sidak method, $p<0.05$). Vertical bars are SEM.

6.2.2 IGF-1 subcutaneous administration decreased anxiety-like behaviour in adult rat.

Which molecular mechanisms could mediate long-lasting effects of early postnatal stimulation on emotional reactivity? IGF-1 could be a good candidate for mediating the EE and massage effects as showed in the first part of the results. I have described how rat pups subjected to massage show a higher IGF-1 immureactivity in the visual cortex, comparable to that found in EE rat pups, and in the auditory cortex. Moreover, there are evidences that early exposure of rat pups to EE increases IGF-1 protein levels not only in the retina (Landi et al., 2007a) but also in the cerebellum and maternal milk (Sale et al., 2007). This correlated IGF-1 increase in different brain regions and in the gastric content of EE rat pups would support the idea that enrichment of environment (massage and EE) result in a higher level of circulating IGF-1. Indeed, higher blood IGF-1 levels are found in the massaged preterm infants (Field al., 2008) and to antagonize the circulating IGF-1 blocks the massage effects on visual acuity development in rats (Guzzetta et a., 2009).

From these remarks, I assessed whether systemic subcutaneous injections of IGF-1 in rat pups, reared in a standard condition, could mimic the effects induced by massage and EE on stress response at adulthood. The treatment consisted in a daily

subcutaneous injection of IGF-1 or vehicle in rat pups from P1 to P10 as previously described for massaged rat pups injected with JB1; rats are then tested at P60.

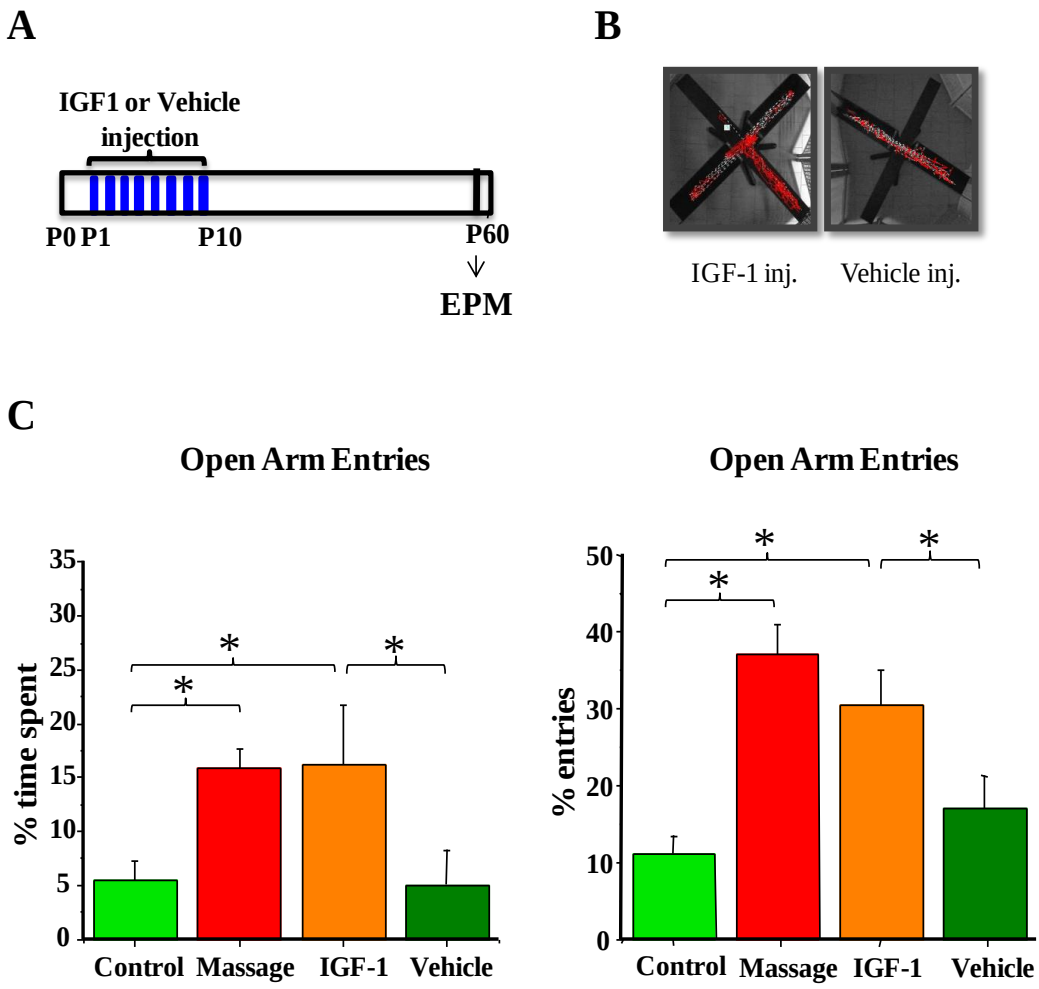


Figure 22.

(A) Experimental protocol. **(B)** Computer generated tracks of movement during the test trial. The tracings (red lines) show that IGF-1 injected rats were tracked more frequently in the open arms than control and vehicle injected rats. **(C)** Early injections of IGF-1in rat pups mimic massage and EE effects on stress response in adult rats. Left, mean percentage open arms time at P60 of IGF-1 groups significantly differs from control and vehicle groups; massaged and IGF-1 injected animals do not differ (one-way ANOVA, factor treatment significant, $p<0.001$, multiple comparison procedure Holm–Sidak method, $p<0.05$). Vertical bars are SEM. Right, mean percentage open arms entries at P60 of IGF-1 treated animals significantly differs from those of control and vehicle

treated groups; no difference was found between massage and IGF-1 injected rats (one-way ANOVA, factor treatment significant, $p<0.001$, multiple comparison procedure Holm–Sidak method, $p<0.05$) Vertical bars are SEM. Asterisks denote significant difference between treatments.

I found that IGF-1 injected rat pups decreased their anxiety-like behaviour, once adult (Fig. 22); indeed percentage of time spent in open arms and percentage of open arm entries in P60 IGF-1-treated animals are significantly higher than those in vehicle treated or in untreated animals; the latter two do not differ [one-way ANOVA ($d=3$), factor treatment significant $p<0.001$ ($F=7.545$), post hoc Holm–Sidak method, IGF-1 treated rats vs vehicle treated rats and IGF-1 treated rats vs control rats, $p<0.05$; vehicle treated rats vs control rats $p>0.05$]. The effects of IGF-1 treatment are comparable with those produced by massage, indeed the performance of IGF-1 treated rats does not differ from that of massaged rats at P60 [one-way ANOVA ($d=3$), factor treatment significant $p<0.001$ ($F=7.545$), post hoc Holm–Sidak method, IGF-1 treated rats vs massaged rats $p>0.05$].

6.2.3 Block of IGF-1 prevent decreased anxiety-like behaviour in massaged and enriched rat once adult

To assess whether IGF-1 is a crucial factor mediating massage and EE effects on stress response development, I also performed the experiment of antagonizing IGF-1 action in massaged and EE rats.

I blocked IGF-1 action by means of systemic JB1 injections from P1 to P10; JB1 injections were performed in massaged (massage plus JB1 rats) and enriched until P45 rats (EE-P45 plus JB1rats) subcutaneously every other day and their anxiety behaviour assessed by EPM at P60. I found that JB1 treatment blocks massage and EE effects on stress response in adult rats.

As shown in figure 23, percentage of time spent in open arms of JB1 treated massaged animals and JB1 treated EE animals is comparable with percentage of control and it is significantly lower than massaged [one-way ANOVA (d=3), factor treatment significant $p<0.001$ ($F=6.191$), post hoc Holm–Sidak method, massaged rats vs massage plus JB1 rats; massaged rats vs control rats, $p<0.05$] and enriched animals [one-way ANOVA (d=3), factor treatment significant $p=0.002$ ($F=5.924$), post hoc Holm–Sidak method, EEp45 rats vs EEp45 plus JB1 rats; EEp45 rats vs control rats, $p<0.05$].

The percentage of open arm entries also show that the performance of JB1 treated massaged animals and JB1 treated EE animals is significantly lower than massaged [Fig. 23; one-way ANOVA (d=3), factor treatment significant $p<0.001$ ($F=10.689$), post hoc Holm–Sidak method, massaged rats vs massage plus JB1 rats; massaged rats vs control rats, $p<0.05$] and enriched rats [Fig. 24; one-way ANOVA (d=3), factor treatment significant $p<0.001$ ($F=7.549$), post hoc Holm–Sidak method, EEp45 rats vs EEp45 plus JB1 rats; EEp45 rats vs control rat, $p<0.05$]; no difference are found between JB1 treated groups and control groups.

Thus, antagonizing IGF-1 action in the first week of life completely prevents massage and EE effects on stress response in the adult animals supporting the idea that early interactions with the environment might programme the stress response in the adult age.

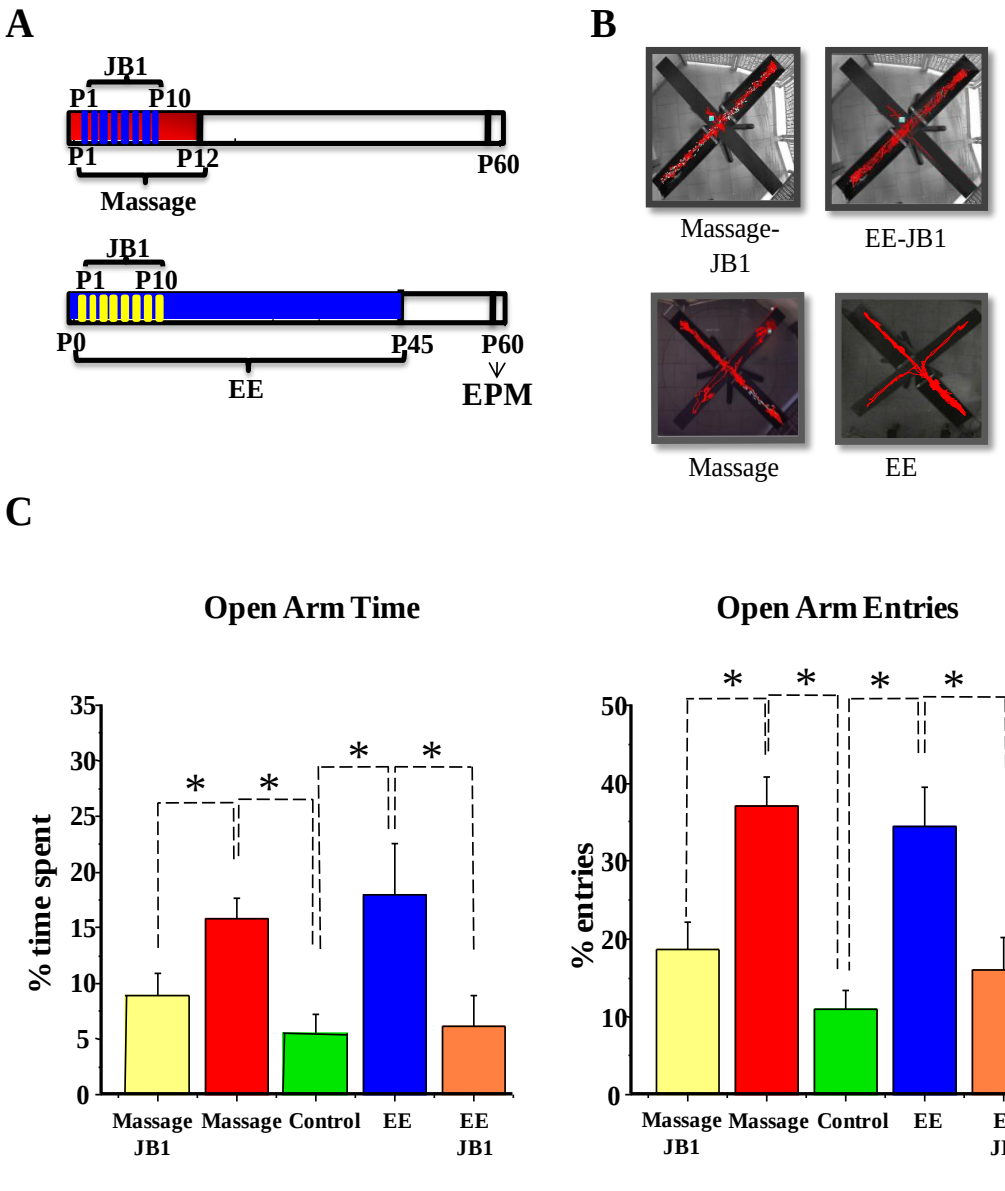


Figure 23.

(A) Experimental protocols. **(B)** Computer generated tracks of movement during the test trial. The tracings (red lines) show that enriched JB1 injected rats and massaged JB1 injected rats were tracked less frequently in the open arms than massaged and enriched rats. **(C)** Blocking IGF-1 action early in life of rat pups prevents the effects of massage and EE on stress response once adult. Top, mean percentage open arms time at P60 of massage JB1 treated and enriched JB1 treated groups was significantly lower than mean percentage of massage (one-way ANOVA, factor treatment significant, $p<0.001$, multiple comparison procedure Holm–Sidak method, $p<0.05$) and EE groups (one-way ANOVA, factor treatment significant, $p=0.002$, multiple comparison

procedure Holm–Sidak method, p<0.05); massaged plus JB1, enriched plus JB1 and control animals do not differ Vertical bars are SEM. Bottom, mean percentage open arms entries at P60 of massage JB1 treated and enriched JB1 treated groups was significantly lower than mean percentage of massage (one-way ANOVA, factor treatment significant, p<0.001, multiple comparison procedure Holm–Sidak method, p<0.05) and EE groups (one-way ANOVA, factor treatment significant, p<0.001, multiple comparison procedure Holm–Sidak method, p<0.05); massaged plus JB1, enriched plus JB1 and control animals do not differ Vertical bars are SEM. Asterisks denote significant difference (one asterisk, p<0,001, two asterisks, p=0,002).

To control that the changes in open arm exploration observed in IGF-1 and JB1 treated animals were not due to general changes in exploration activity, I measured total arm entries and distance moved during the performance in EPM at P60. Total arm entries was comparable between each group; indeed no difference were found in the statistical analysis [Fig. 24; one-way ANOVA (d=3), factor treatment significant p=0.533 (F=0.743), post hoc Holm–Sidak method, p>0.05].

Another index that I considered is the distance moved. As shown in the figure 24 all experimental groups do no differ in this variable [one-way ANOVA (d=3), factor treatment significant p=0.999 (F=00103), post hoc Holm–Sidak method, p>0.05].

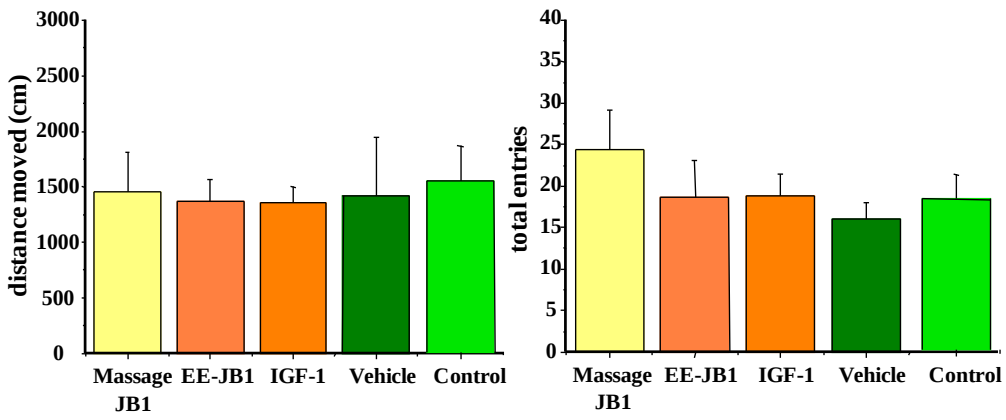


Figure 24.

Left, mean distance moved (cm) of all experimental groups during test session in elevated plus maze. No difference was found between groups (one-way ANOVA, factor treatment no significant, $p=0.533$, multiple comparison procedure Holm–Sidak method, $p<0.05$). Vertical bars are SEM. Right, mean total arm entries of all experimental groups during test session in elevated plus maze. No difference was found between groups (one-way ANOVA, factor treatment no significant, $p=0.999$, multiple comparison procedure Holm–Sidak method, $p<0.05$). Vertical bars are SEM.

These findings permit, therefore, to rule out that effects of treatments or protocols applied were dependent on low level of locomotor activity or just because the animals moved less in the maze.

6.3 Massage and EE affect developmental time-course of Visual Recognition Memory

As outlined in the Introduction, studies on rodent have shown that early life experiences based on mother–infant interactions, in particular licking/grooming (LG), with its tactile stimulation components (Jutapakdeegul et al., 2003), have a profound impact on neuronal development that subsequently regulates behavioural, cognitive and neuroendocrinological function in adulthood (Lehmann et al., 1999; Lovic et al., 2004; Kalinichev et al., 2002; Champagne et al., 2003; Menard et al., 2004; Levine at al., 2005; Zhang et al., 2005; Kaffmann and Meaney, 2007).

Despite this bulk of information, behavioural longitudinal studies about influence of standard environment and particular environmental inputs such as massage or multisensory stimulation on development of recognition memory are not available.

I investigated this issue by studying the longitudinal development of visual recognition memory (RM) using a behavioural tests: the object recognition task (ORT). Firstly, I assessed the performance of naïve rats at five age intervals: P18 (P17-P21), P25 (P23-P27), P30 (P29-P33), P40 (P39-P43) and P60 (P59-P63) with different retention intervals; their performance allowed to trace a developmental baseline of visual recognition memory in rat. Following, I evaluated the effects of tactile stimulation and EE on the maturation of this cognitive function.

6.3.1 Developmental time-course of recognition memory in naive rats

Recognition memory is comprised of both familiarity detection and recollection (Aggleton and Brown, 2006; Fortin et al., 2004). These functions are primarily localized within the medial temporal lobe (MTL) (Bachevalier and Vargha-Khadem, 2005; Brown and Aggleton, 2001), structures that undergo substantial postnatal development and reorganization in rats, monkeys and humans. Currently there is controversy over the role of the hippocampal complex versus the perirhinal cortex in object recognition abilities (Reger et al., 2009). Better understanding of the ontogeny of recognition memory in conjunction with studies of neurodevelopment would shed light on the debated neurophysiology.

Given the extensive developmental work in primates, the relative dearth of maturational literature on the ORT in rats was surprising. Although, several studies have used the ORT as part of a panel of behavioural tests in adolescent/juvenile aged (postnatal day >25–42) rats to demonstrate neurocognitive deficits due to perinatal iron deficiency (Wu et al., 2008) or early disruptions of circadian light cycles (Toki et al., 2007), there are only two papers that directly applying the ORT to the immature rat. In the first study the authors reported a decreased preference for novel object in

preweanlings (18 days old) at retention intervals of 1 min and 2hr compared to adults which showed stable performance across these intervals (Anderson et al., 2004). In the second study, Reger et al. (2009) have investigated the ontogeny of rat recognition memory by ORT: weanling (P20–23), juvenile (P29–40), and adult (P50+) rats were tested after 15 minutes, 1, 24, and 48 hr delays. The findings showed that adult and juvenile rats exhibited comparable performance. While weanling rats were able to exhibit robust object recognition across shorter delays; they showed inferior long-term memory retention (Reger et al., 2009).

I decided, therefore, to explore more in details the ontogeny of rat RM increasing the range of retention intervals and manipulating the early rearing conditions in order to investigate how this early postnatal manipulations may affect the development of that cognitive ability of rat.

I measured two dependent variables to assess the maturation of RM: exploration time and discrimination ratio (d.r.). These indexes permit to evaluate if the animal recognize, or not, the old object. Briefly, each testing session included an habituation session (S0), a sample session (S1) and a choice session (S2) which is performed at 1, 24, 48, 72 and 96hr after the respective sample sessions. The animals were tested at five different ages in order to analyze pre-weaning (P18), post-weaning (P25 and P30) and young-adulthood phases (P40 and P60).

At P18, the analysis of exploration time indicated that only at 1h retention interval naive animals spent significantly longer time exploring the novel rather than the familiar object [Fig. 25A; pared t-test, 1h p=0.016 (n=10), 24hr p=0.327 (n=10), 48hr p=0.828 (n=8); 72hr p=0.754 (n=9)].

At P25, the exploration time for the new and the old objects were significantly different only at 1h; no difference were detected for the remaining intervals [Fig. 25B; pared t-test, 1h p=0,02 (n=11), 24hr p=0,297 (n=11), 48hr p=0.235 (n=10), 72hr

p=0.196 (n=10)]. Thus, at P18 and P25 rats can recognize familiar object only 1h after having seen it.

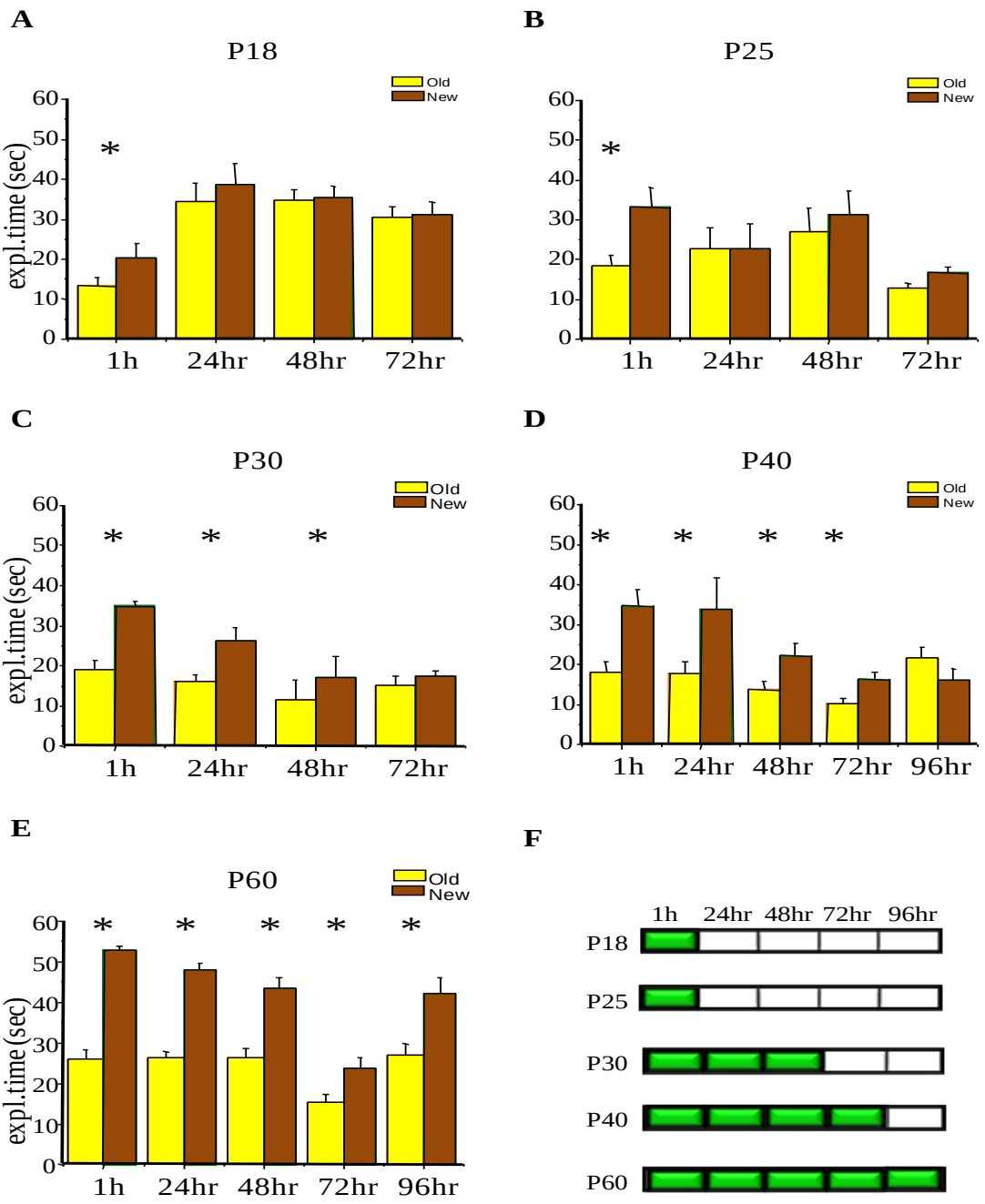


Figure 25.

(A–E) exploration time (sec) towards old object (yellow) and new object (brown) as function of retention intervals (1h, 24/48/72/96 hr). **(A-B)** control rats at P18 and P25 recognize familiar object only at 1h (paired t-test, at P18 p=0,016 and at P25 p=0,02). **(C)** at P30 the animals spent

significantly longer time to explore new object at 1h (p<0,001), 24hr (p=0,003) and 48hr (p=0,0119 but not at 72hr (p=0,196). **(D)** at P40 control rats are able to recognize familiar object also at 72hr retention intervals (p<0,001); however the performance of control rats fall down at 96hr(p=0,068). **(E)** at P60 the development of cortical areas delegated to RM function permits to recognize the familiar object at all retention intervals observed (paired t-test, 1h p<0.001, 24hr p<0.001, 48hr p<0.001, 72hr p<0.001, 96hr p=0,013). Vertical bars are SEM. Asterisks denote significant difference. **(F)** summary of the development of visual recognition memory observed in naive rats.

At P30, the animals spent longer time exploring novel objects not only at 1h but also at 24hr and 48hr retention intervals [Fig. 25C; paired t-test, 1h p=<0.001(n=10); 24hr p=0.003 (n=9); 48hr p=0.011 (n=9), 72hr p=0.196 (n=10)] showing that memory traces were now present for longer retention time.

At P40, the exploration times of novel object were significantly higher than those of familiar object at 1h, 24hr, 48hr and 72hr retention intervals, while the explorative behaviour at 96hr did not show any preference [Fig. 25E; paired t-test, 1h p=0.004 (n=11), 24hr p=0.023 (n=10), 48hr p<0.001 (n=12), 72hr p<0.001 (n=10), 96hr p=0.068 (n=6)).]

At P60, the performance of animals showed a significantly preference for new object than familiar object at all retention intervals [Fig. 25F; paired t-test, 1h p<0.001 (n=6), 24hr p<0.001 (n=8), 48hr p<0.001 (n=8), 72hr p<0.001 (n=6), 96hr p=0,013 (n=6)]. The rats reared in standard condition, therefore, are able at P60 to recognize the familiar object at all retention intervals examined suggesting a marked maturation in cortical areas delegated to RM function.

These findings are consistent with the visual recognition memory literature across the species; it corresponds to the general observation of ‘‘infantile amnesia’’ on learning and memory tasks in which young rats forget more quickly than older rats reflecting a difference in short-term and long-term memory abilities (Rudy &

Morledge, 1994). Furthermore, recognition memory ability is the earliest primate learning and memory function to appear and yet primate infants are not proficient on the visual paired comparison at longer delays until they are several months old (Alvarado et al., 2000).

6.3.2 Developmental time-course of recognition memory in EE rat.

I repeated the same previous procedure to study the development of RM on rat pups reared in EE until P45.

At P18, the enriched animals spent significantly longer time to explore a new object at 1h and 24hr; no difference was found at 48hr and 72hr intervals [Fig. 26A; pared t-test, 1h $p<0.001$ (n=9), 24hr $p=0.004$ (n=10), 48hr $p=0.148$ (n=8), 72hr $p=0.832$ (n=6)].

At P25, I found the same trend, indeed the examination of exploration time confirmed a significantly higher preference for new object than familiar object at 1h and 24hr [Fig. 26B; pared t-test, 1h $p<0.001$ (n=9), 24hr $p=0.002$ (n=5), 48hr $p=0.091$ (n=6), 72hr $p=0.469$ (n=7)]. Thus, at P18 and P25 enriched rats can recognize familiar object at 1h and 24hr intervals showing an acceleration in the ability to detect it than control rats.

The following age interval, P30, is a crucial for the development of RM in enriched rats. Although there are only 5 days respect of the previous age interval, it possible to find substantial changes in the rat performance. The statistical analysis of exploration time indicates a preference versus novel object at short retention intervals (1h and 24hr) and at long retention intervals (48hr, 72hr and 96hr) [Fig. 26C; pared t-test, 1h $p<0.001$ (n=6), 24hr $p<0.001$ (n=8), 48hr $p=0.003$ (n=5), 72hr $p<0.001$ (n=6),

96hr p<0.001 (n=11)]. Enriched rats, therefore, at P30 recognized the familiar object also after longer delay until a 96hr growing away from control rats.

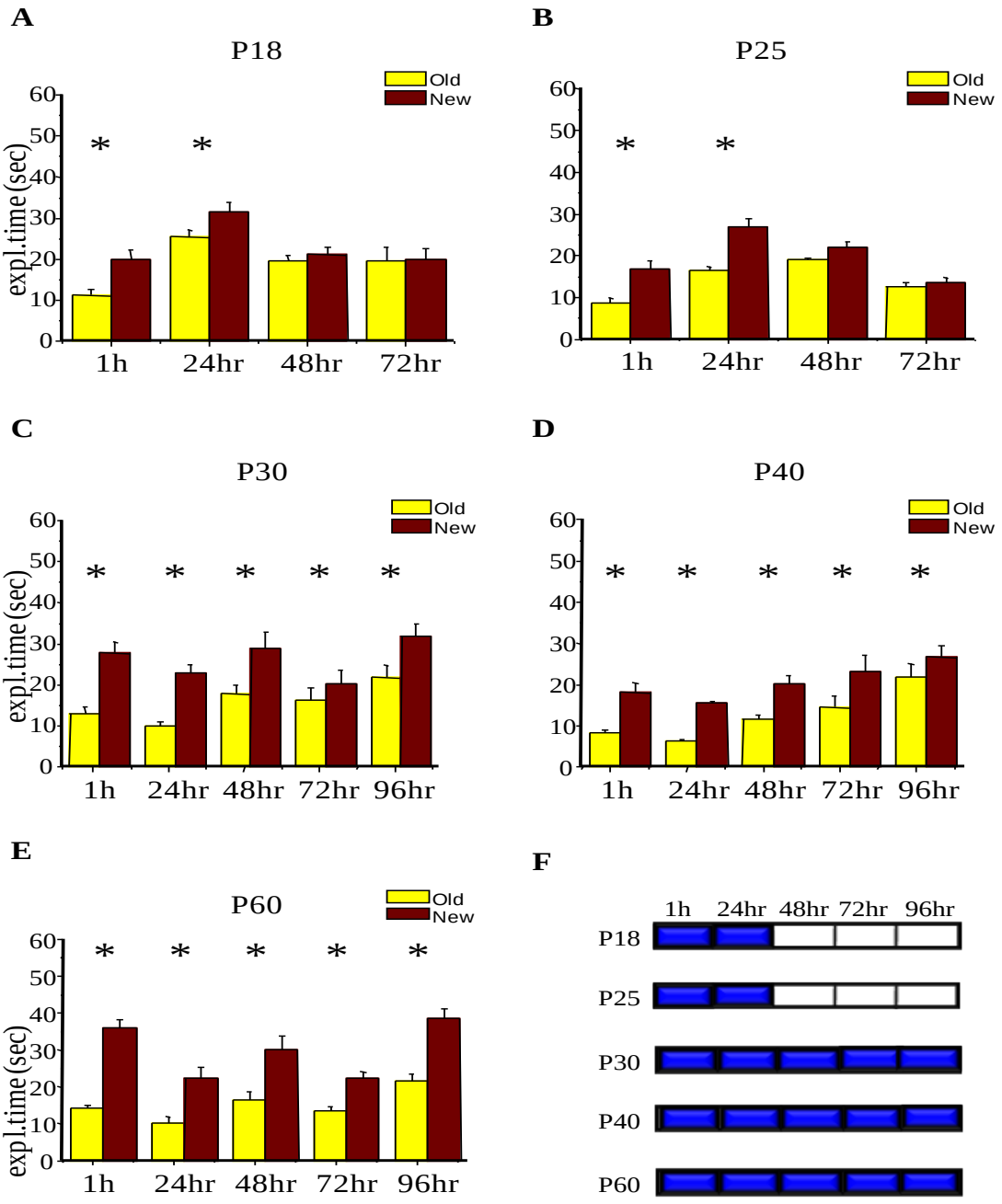


Figure 26.

(A–E) exploration time (sec) towards old object (yellow) and new object (brown) as function of retention intervals (1h, 24/48/72/96 hr). **(A-B)** enriched rats at P18 and P25 recognize familiar object at 1h (paired t-test, at P18 p<0,001 and at P25 p=0,004) and 24hr (paired t-test, at P18

p<0,001 and at P25 p=0,002). **(C)** at P30 the performance of enriched animals show a marked improvement exploring significantly longer time new object at all retention intervals (pared t-test, 1h p<0.001, 24hr p<0.001, 48hr p=0.003, 72hr p<0.001, 96hr p<0.001). **(D-E)** enriched rats at P40 and at P60 confirmed the results showed at previous age interval (pared t-test, P40: 1h p<0.001, 24hr p<0.001, 48hr p<0.001, 72hr p=0.002, 96hr p=0.007; P60: 1h p=0.004, 24hr p<0.001, 48hr p=0.001, 72hr p=0.008 , 96hr p<0.001). Vertical bars are SEM. Asterisks denote significant difference. **(F)** summary of the development of visual recognition memory observed in naive rats.

At P40 and at P60, the exploration time analysis confirmed the previous results; the animals showed a higher significantly preference for new object than one familiar [Fig. 26D-E; **P40:** pared t-test, 1h p<0.001 (n=8), 24hr p<0.001 (n=6), 48hr p<0.001 (n=6), 72hr p=0.002 (n=7), 96hr p=0.007 (n=10); **P60:** pared t-test, 1h p=0.004 (n=10), 24hr p<0.001 (n=10), 48hr p=0.001 (n=9), 72hr p=0.008 (n=9); 96hr p<0.001 (n=5)].

6.3.3 Developmental time-course of recognition memory in massaged rat.

I decided to introduce a third experimental group for studying the effects of a shorter environmental manipulation than EE up to P45 on the recognition memory ontogeny. I applied the same procedure on rats reared in standard condition which received body massage three times per day from P1 to P12.

As observed in the figure 27A, the preweaning animals spent longer time to explore novel object than familiar object at 1h and 24hr; no difference was found at 48hr and 72hr [pared t-test, 1h p=0.002 (n=10), 24hr p=0.031 (n=8), 48hr p=0.075 (n=9); 72hr p=0.982 (n=9)].

At P25, the ability of massaged rats to recognize familiar object is shown again at 1h and 24hr retention intervals, while after longer delay the explorative behaviour is

random [Fig. 27B; pared t-test, 1h p=0.002 (n=8), 24hr p<0.001 (n=9), 48hr p=0.650 (n=8); 72hr p=0.366 (n=8)].

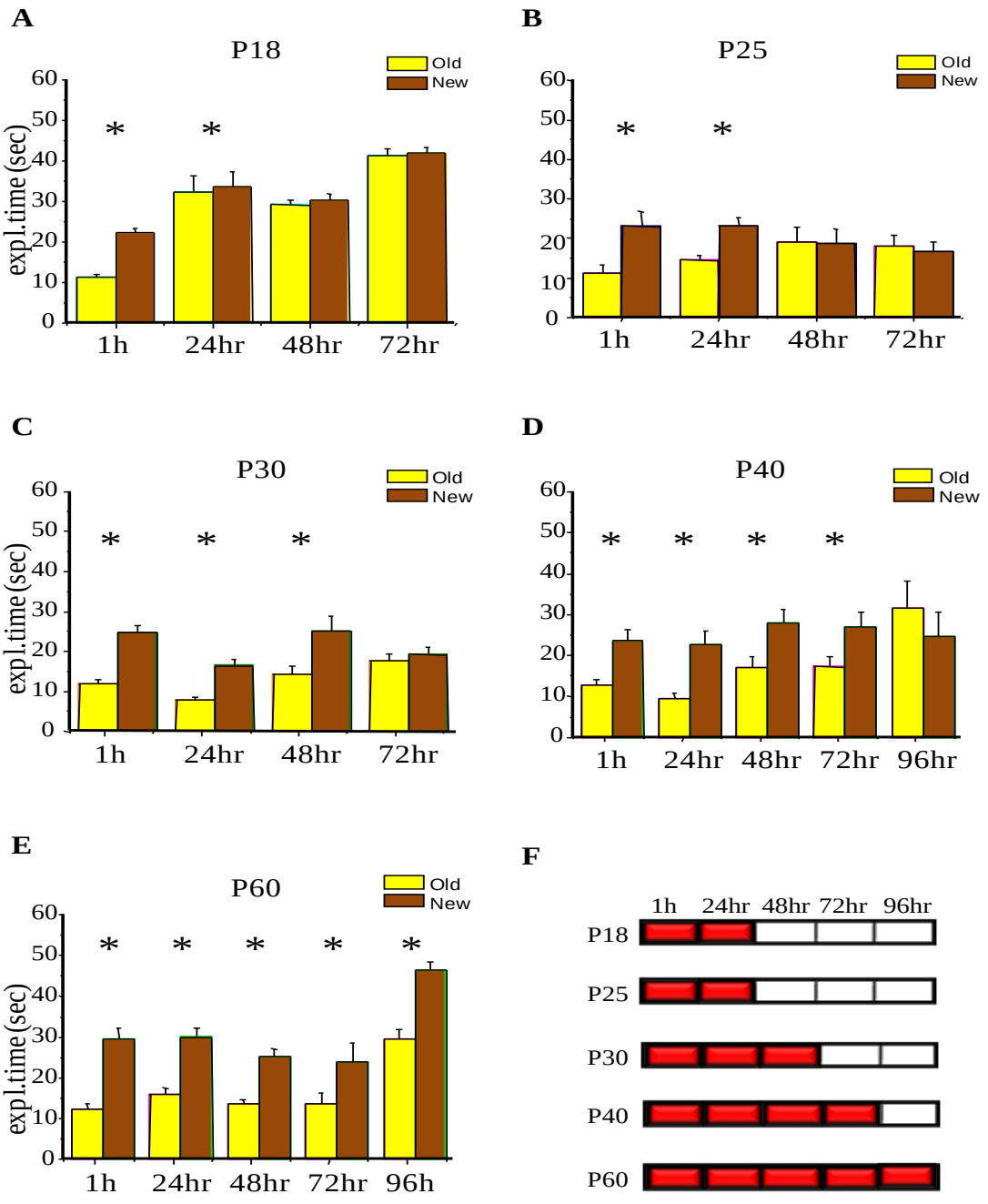


Figure 27.

(A–E) exploration time (sec) towards old object (yellow) and new object (brown) as function of retention intervals (1h, 24/48/72/96 hr). **(A-B)** massaged rats at P18 and P25 recognize familiar object at 1h (paired t-test, at P18 p=0,002 and at P25 p=0,031) and 24hr (paired t-test, at P18

p=0,002 and at P25 p<0,001). **(C)** at P30 the animals spent significantly longer time to explore new object at 1h (p<0,001), 24hr (p=0,003) and 48hr (p=0,0119) but not at 72hr (p=0,196). **(D)** at P40 control rats are able to recognize familiar object also at 72hr retention intervals (p<0,001); however the performance of control rats fall down at 96hr (p=0,068). **(E)** at P60 the development of cortical areas delegated to RM function permits to recognize the familiar object at all retention intervals observed (paired t-test, 1h p<0.001, 24hr p<0.001, 48hr p<0.001, 72hr p<0.001, 96hr p=0,013). Vertical bars are SEM. Asterisks denote significant difference. **(F)** summary of the development of visual recognition memory observed in naive rats.

Thus, massaged rats at P18 and P25 recognized the old object after a delay of 1h and 24hr as observed in enriched animals suggesting an acceleration in the development of RM function compared to animals reared in standard condition.

At P30, the examination of exploration behaviour display that the animals are able to recognize the old object at 1h, 24hr and 48hr but not at 72hr [Fig. 27C; pared t-test, 1h p=0.001 (n=9), 24hr p=0.002 (n=9), 48hr p=0.003 (n=8); 72hr p=0.061 (n=7)]. Thus, the developmental time-course of RM in massaged rats is in line with that in control rats; the developmental trend of RM in enriched rats is markedly separated from that showed in control and massaged rats.

At P40, it is possible to see an improvement in the performance of massaged rats; indeed they spent significantly longer time exploring novel object also at 72hr [Fig. 27D; pared t-test, 1h p=0.001 (n=9), 24hr p=0.001 (n=9), 48hr p<0.001 (n=8), 72hr p<0.001 (n=9)]. However, they were not able yet to recollect the information previously storage at 96hr interval [p=0.138 (n=7)]. The findings indicate a further maturation of the cerebral structures delegated to recognition memory; however it is not complete since the performance fell down at 96hr retention interval.

Finally, when the animals became a young adult (P60) the analysis of exploration time illustrated that adult rats recognized the familiar object after all delay

investigated [Fig. 27E; pared t-test, 1h $p<0.001$ ($n=10$), 24hr $p<0.001$ ($n=12$), 48hr $p<0.001$ ($n=12$), 72hr $p=0.001$ ($n=11$), 96hr $p<0.138$ ($n=7$)] suggesting a complete maturation of RM function for the retention intervals observed.

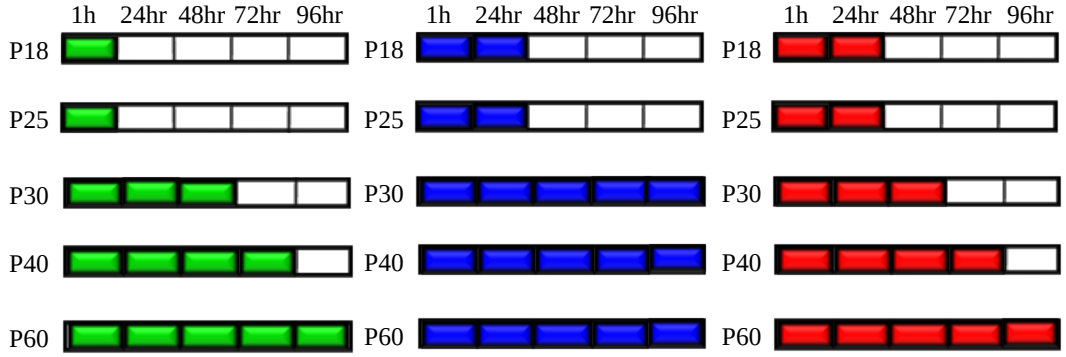


Figure 28.

Summary of the development of recognition memory in the control, EE and massage groups. The green, blue and red box represents the ability of animals to recognize the familiar object exploring preferentially the new object. The analysis of exploration time shows an accelerated maturation of the RM in enriched and massaged rats for 24hr at P18 and P25. Starting from P30, the developmental time course of RM in massaged and control animals is the same while in enriched rats continues to have a marked acceleration with respect to other two groups.

In these paragraphs I have clarified separately the ontogeny of the RM in the control, enriched and massaged rats (Fig. 28). In other words, I investigated how different quality of environmental experience could effect the maturation of rat's ability to discriminate which stimulus was familiar or novel. In the next section, I directly compare the discrimination ratio values obtained in these experimental groups at each age as function of retention intervals.

6.3.4 Acceleration of recognition memory development by EE and massage.

By summarizing each animal performance with the recognition index, I have analyzed the performance of animals of all age, all experimental groups for all retention intervals (Table 2).

Table 2
Performance at different ages using different delay intervals (1, 24, 48, 72, 96hr)

		P18	P25	P30	P40	P60
Mean values (S.E.M.) of discrimination ratio (d.r.) during choice session (s2)						
control	1h	0,21 ±0,01	0,29 ±0,06	0,30 ±0,06	0,31 ±0,07	0,34 ±0,04
control	24h	0,06 ±0,05	0,14 ±0,03	0,22 ±0,05	0,27 ±0,07	0,29 ±0,02
control	48h	-0,04 ±0,04	0,04 ±0,05	0,23 ±0,04	0,22 ±0,03	0,25 ±0,02
control	72h	-0,02 ±0,03	0,05 ±0,04	0,09 ±0,04	0,19 ±0,04	0,25 ±0,05
control	96h				-0,06 ±0,06	0,17 ±0,04
EE	1h	0,33 ±0,03	0,32 ±0,02	0,37 ±0,02	0,36 ±0,02	0,42 ±0,01
EE	24h	0,10 ±0,02	0,24 ±0,02	0,39 ±0,01	0,43 ±0,02	0,38 ±0,02
EE	48h	0,04 ±0,05	0,07 ±0,03	0,24 ±0,01	0,27 ±0,01	0,29 ±0,01
EE	72h	0,03 ±0,03	0,05 ±0,01	0,12 ±0,02	0,23 ±0,01	0,25 ±0,01
EE	96h			0,15 ±0,03	0,13 ±0,04	0,29 ±0,02
massage	1h	0,33 ±0,01	0,34 ±0,01	0,35 ±0,02	0,30 ±0,02	0,41 ±0,02
massage	24h	0,03 ±0,01	0,23 ±0,01	0,36 ±0,02	0,32 ±0,05	0,31 ±0,02
massage	48h	0,02 ±0,01	-0,02 ±0,02	0,27 ±0,02	0,25 ±0,02	0,30 ±0,02
massage	72h	-0,002 ±0,01	-0,01 ±0,04	0,04 ±0,02	0,22 ±0,003	0,27 ±0,01
massage	96h				-0,11 ±0,07	0,23 ±0,04

Analysis of discrimination ratio in control group.

As showed in the Fig. 29, the performance were comparable at 1h interval: the memory trace can be retrieved at 1h at all ages including P18 [two-way ANOVA, age (d=4) x interval (d=3), factor age significant, $p<0,001$ ($F=16.404$), factor interval significant, $p<0.001$ ($F=14,055$), interaction age x interval no significant $p=0.774$ ($F=0,676$); multiple comparison procedure Holm–Sidak, age within 1h, $p>0.05$].

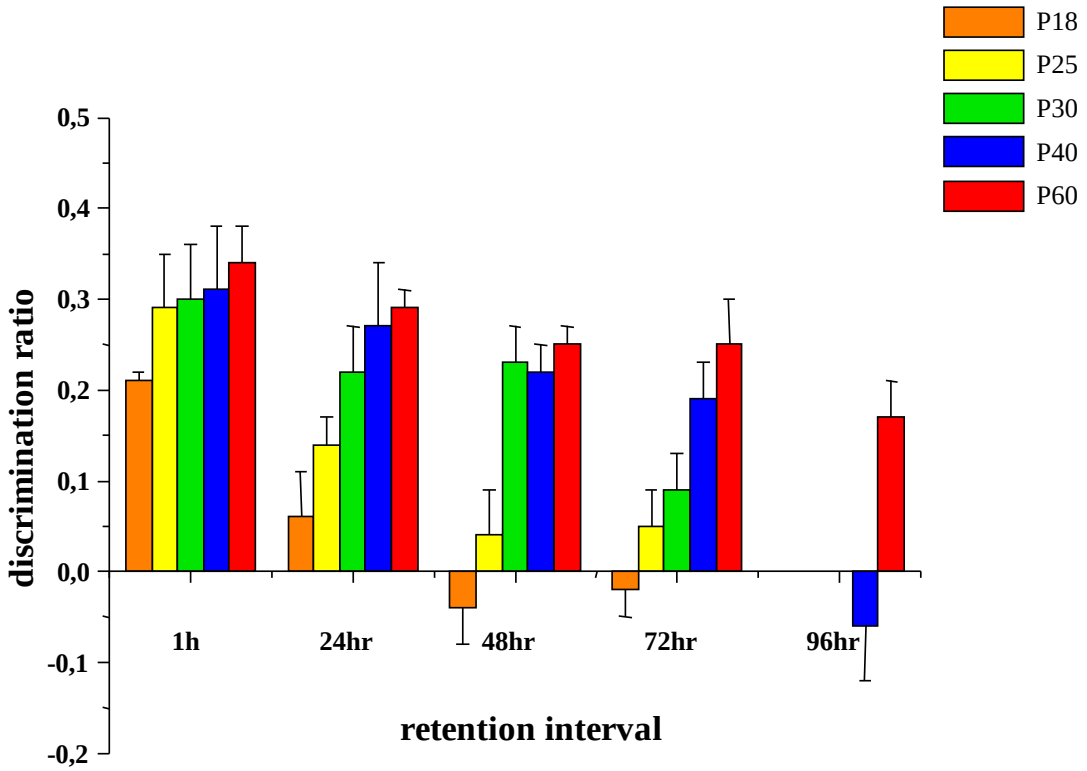


Figure 29.

Summary of the development of recognition memory in the control animals. Mean discrimination ratio of each age is plotted as function of retention intervals 1h, 24hr, 48hr, 72hr and 96hr. Vertical bars are SEM. At 1h the performance are comparable; at 24hr there is a gradual improvement and the performance at P40 and P60 are significantly different than that at P18. At 48hr a clear break point in the performance emerged; the performance begin to be significant difference starting from P30. At 72hr the performance progressively increases getting different starting from P40. Finally, at 96hr the control rats recognize familiar object only at P60 [two-way ANOVA, age (d=4) x interval (d=3), factor age significant, p<0,001 (F=16.404), factor interval significant, p<0.001 (F=14,055), interaction age x interval no significant p=0.774 (F=0,676); multiple comparison procedure Holm–Sidak, p<0,05]. Asterisks denote significant difference.

For the retention interval of 24hr, the age became a crucial factor. Indeed, performance was significantly higher starting from P40 (Fig.29); the discrimination ratio, at P18, was significantly lower with respect to that at P40 and P60 [two-way ANOVA, age (d=4) x interval (d=3), factor age significant, p<0,001 (F=16.404), factor interval significant, p<0.001 (F=14,055), interaction age x interval no

significant p=0.774 (F=0,676); multiple comparison procedure Holm–Sidak, age within 24hr, P40 and P60 vs P18 p<0.05].

The time-course of the RM development for a retention interval of 48hr was not gradual as seen above for 24hr interval: there was a point-break at P30 [Fig.29; two-way ANOVA, age (d=4) x interval (d=3), factor age significant, p<0,001 (F=16.404), factor interval significant, p<0.001 (F=14,055), interaction age x interval no significant p=0.774 (F=0,676); multiple comparison procedure Holm–Sidak, age within 48h, P30, P40 and P60 vs P18; P40 and P60 vs P25 p<0.05]. Accordingly, the study of exploration time confirmed that at P18 (p=0,828) and P25 (p=0,235) the information couldn't to be recover after 48hr while the performance improved at P30 (p=0,011) when the animal began to recognize the familiar object (Fig.25).

As expected, at 72hr interval the values of d.r. progressively increased as a function of age: the performance at P40 and P60 were significantly different with respect to that at P18 [Fig.29; two-way ANOVA, age (d=4) x interval (d=3), factor age significant, p<0,001 (F=16.404), factor interval significant, p<0.001 (F=14,055), interaction age x interval no significant p=0.774 (F=0,676); multiple comparison procedure Holm–Sidak, age within 72h, P40 and P60 vs P18; P60 vs P25 p<0.05]. Note that the performance at P40 and P60 were comparable suggesting that in these 20 days no further maturational changes occurred.

Finally, at 96hr retention interval only the animals at P60 were able to recognize familiar object spending significantly longer time exploring familiar object [Fig.29; two-way ANOVA, age (d=4) x interval (d=3), factor age significant, p=0,025 (F=5.268), factor interval significant, p<0.001 (F=5.825), interaction age x interval no significant p=0.099 (F=2,041); multiple comparison procedure Holm–Sidak, age within 72h, P60 vs P40 p<0.05].

Analysis of discrimination ratio in the experimental groups.

To summarize the results on RM development, I have plotted for each intervals the discrimination ratio as a function of age for the experimental groups observed.

Comparison of experimental groups at 1h

All experimental groups discriminate familiar object from one new at all age intervals after delay of 1h. However, at P18, the d.r. values of the treated groups (enriched and massaged rats) was higher with respect to recognition index of the control groups (naïve rats); no difference was found in the other ages [Fig. 30; two-way ANOVA, treatment (d=2) x age (d=4), factor treatment significant, p=0,007 (F=5,302), factor age significant, p=0,010 (F=3,537), interaction age x interval p=0.808 (F=0,559); multiple comparison procedure Holm–Sidak, interval within P18, EE vs control; massage vs control, $p<0.05$]. Thus, although each experimental group was able to retrieve the information previously stored after 1h at all ages, an acceleration of maturation of the RM in enriched and massaged rats occurred early at P18.

Comparison of experimental groups at 24hr

The manipulation of multisensory experience by environmental enrichment or by tactile stimulation protocol induced a marked change in the performance of rats at 24hr. In the previous paragraph, it has been demonstrated that enriched and massaged rats were able to recognize familiar object at 24hr starting from preweaning age P18 (Fig.28). The control rats, instead, arrived to spent significantly longer time exploring new object only at P30. Statistical analysis shows that d.r. values in EE and massage groups are significantly higher than d.r. value in control group at P30 and P40; at P60

there is no difference [Fig. 30; two-way ANOVA, treatment (d=2) x age (d=4), factor treatment significant, p<0,001 (F=13,629), factor age significant, p<0,001 (F=36,793), interaction age x interval p=0.279 (F=1,250); multiple comparison procedure Holm–Sidak, interval within P30 and P40, EE vs control; massage vs control, p<0.05].

These findings, therefore, support an acceleration in the development of cortical structures delegate to RM in treated groups in regard to the recognition ability for 24hr interval.

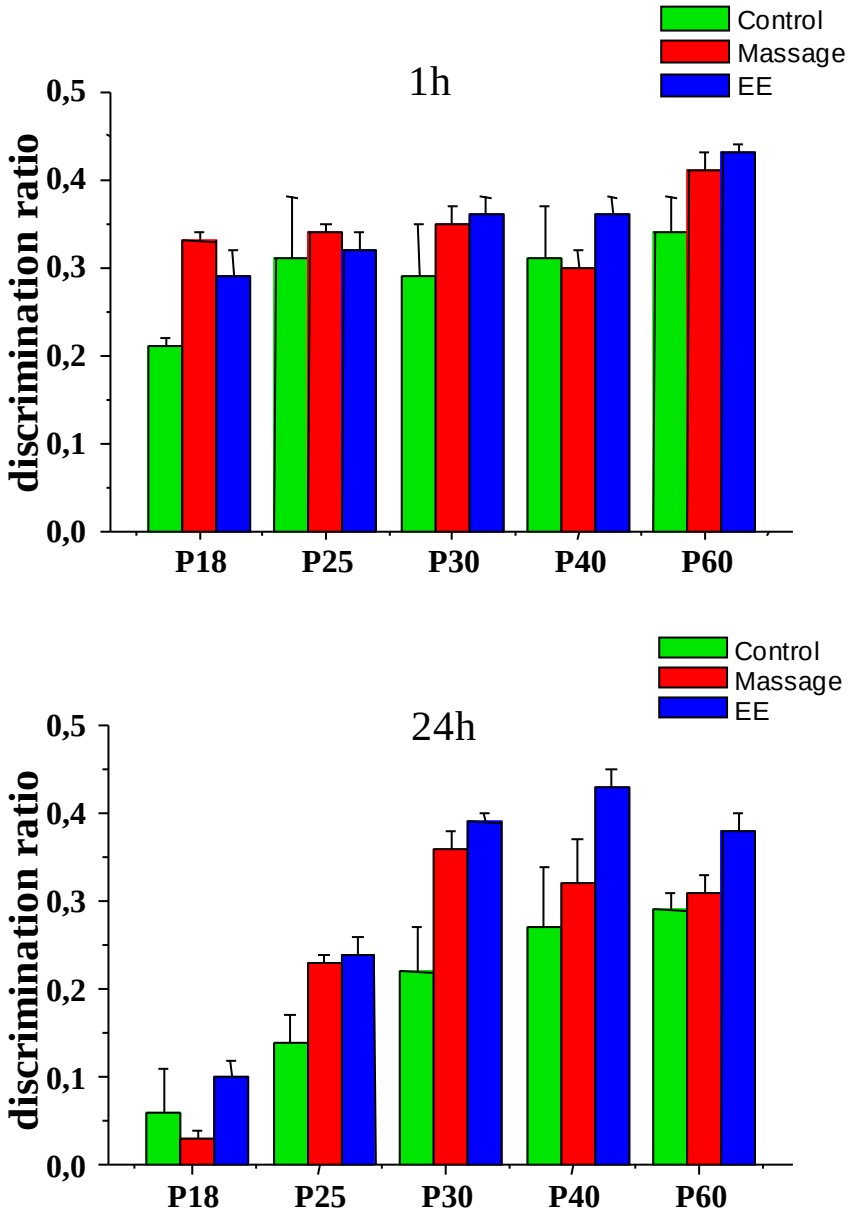


Figure 30.

Summary of the recognition memory maturation in the control, enriched and massaged rats. Mean discrimination ratio of 1h and 24hr retention intervals is plotted as function of age P18, P25, P30, P40 and P60. Vertical bars are SEM. **Top,** mean value of d.r. at 1h in enriched and massaged rats is significantly higher than in control rats at P18; the performance are comparable in the other age intervals [two-way ANOVA, treatment (d=2) x age (d=4), factor treatment significant, p=0,007 (F=5,302), factor age significant, p=0,010 (F=3,537), interaction age x interval p=0.808 (F=0,559); multiple comparison procedure Holm–Sidak, p<0,05]. **Bottom,** the performance of EE and massage groups are higher from P25; mean value of d.r. at 24hr in a treated groups is significantly higher than in control group at P30 and P40 [two-way ANOVA, treatment (d=2) x age (d=4), factor treatment significant, p<0,001 (F=13,629), factor age significant, p<0,001 (F=36,793), interaction age x interval p=0.279 (F=1,250); multiple comparison procedure Holm–Sidak, p<0,05].

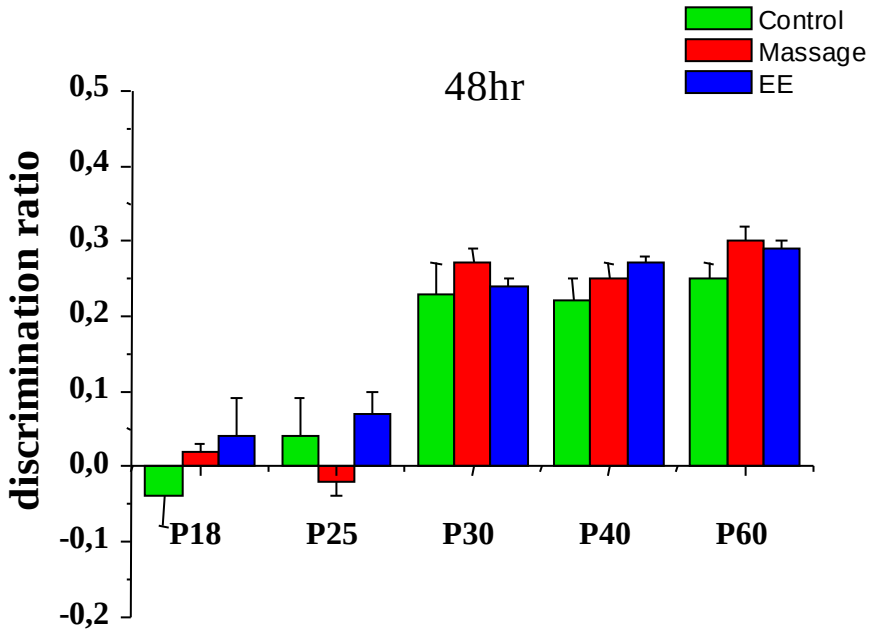
Comparison of experimental groups at 48hr

As showed in the figure 31, at 48hr no difference was found between groups at all age intervals [two-way ANOVA, treatment (d=2) x age (d=4), factor treatment no significant, p=0,145 (F=1,974), factor age significant, p<0,001 (F=55,305), interaction age x interval p=0.880 (F=5,360); multiple comparison procedure Holm–Sidak, p<0.05]. This result is congruent with analysis of exploration time that underlines how all experimental groups become able to recognize familiar object at 48hr only from P30 (Fig. 28). This latter is a crucial age for each experimental groups, treated and untreated, and the performance in the follow ages are comparable.

Comparison of experimental groups at 72hr

The analysis of discrimination ratio at 72hr confirmed the difference emerged in the exploration time examination; the d.r. value of enriched animals is significantly higher than those of massaged and control animals at P30; the other ages do no differ [Fig.31; two-way ANOVA, treatment (d=2) x age (d=4), factor treatment significant,

p<0,001 (F=42,191), factor age significant, p=0,026 (F=3,795), interaction age x interval p=0.717 (F=0,669); multiple comparison procedure Holm–Sidak, within P30, EE vs control; EE vs massage, p<0.05]. These data are in agreement with those of exploration time where only EE group at P30 is able to recognize familiar object while massage and control groups acquire this competence only at P40 (Fig. 28). Interestingly, at this retention interval emerges a significant difference also between the performance of enriched and that of massaged rats suggesting that it is necessary a longer multisensory stimulation protocol to induce a more marked acceleration in the development of RM.



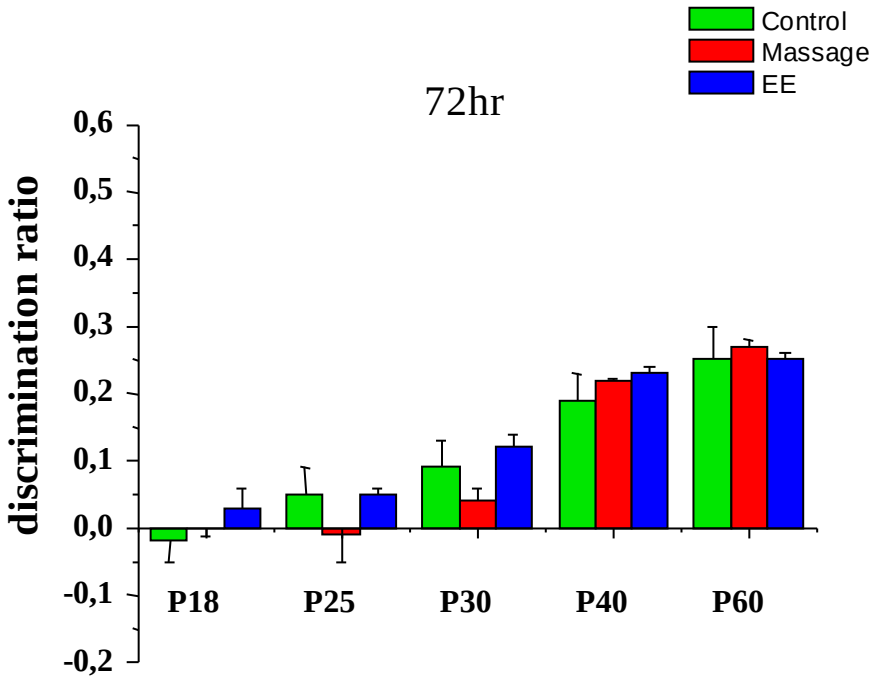


Figure 31.

Summary of the recognition memory maturation in the control, enriched and massaged rats. Mean discrimination ratio of 48hr and 72hr retention intervals is plotted as function of age P18, P25, P30, P40 and P60. Vertical bars are SEM. **Top,** mean value of d.r. at 48hr is comparable in all experimental groups at all ages observed [two-way ANOVA, treatment (d=2) x age (d=4), factor treatment no significant, p=0,145 (F=1,974), factor age significant, p<0,001 (F=55,305), interaction age x interval p=0.880 (F=5,360); multiple comparison procedure Holm–Sidak, p<0.05]. **Bottom,** mean value of d.r. in enriched animals is significantly higher than those of massaged and control animals at P30; the other ages do no differ [two-way ANOVA, treatment (d=2) x age (d=4), factor treatment significant, p<0,001 (F=42,191), factor age significant, p=0,026 (F=3,795), interaction age x interval p=0.717 (F=0,669); multiple comparison procedure Holm–Sidak, p<0.05].

Comparison of experimental groups at 96hr

Finally, the performance of experimental groups are compared after delay of 96hr at P40 and P60. As expected, the d.r. value of EE group was significantly higher at P40 than those of massage and control groups [Fig.32; two-way ANOVA, treatment

(d=2) x age (d=4), factor treatment significant, p=0,006 (F=6,085), factor age significant, p<0,001 (F=31,889), interaction age x interval p=0.188 (F=1,762); multiple comparison procedure Holm–Sidak, within P40, EE vs control; EE vs massage, p<0.05]. Accordingly, the analysis of exploration time shows that massaged and control rats are able to recognize familiar object only at P60 while the enriched rats spent longer time exploring new object since P30.

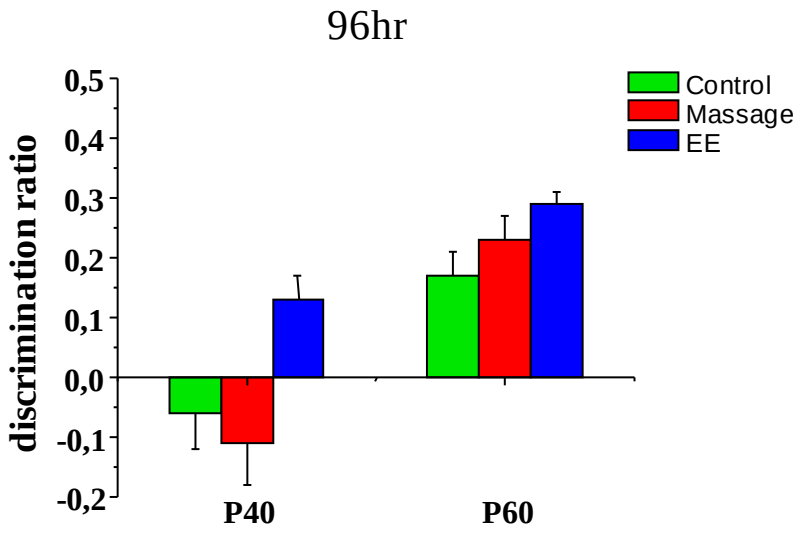


Figure 32.

Mean discrimination ratio of 96hr in control, EE and massage groups at P40 andP60. Vertical bars are SEM. Animals reared in EE condition show a significant higher d.r. than those of massaged and control rats at P40; at P60 all experimental groups are comparable [two-way ANOVA, treatment (d=2) x age (d=4), factor treatment significant, p=0,006 (F=6,085), factor age significant, p<0,001 (F=31,889), interaction age x interval p=0.188 (F=1,762); multiple comparison procedure Holm–Sidak, p<0.05].

In summary, environmental enrichment at short or long term induces an acceleration in the development of recognition memory. The analysis of exploration time have highlighted that enriched and massaged rats raised the ability to recognize

familiar object at 24hr before (P18) than rats reared in a standard condition (P30). This result suggests that EE and massage permit an acceleration in the development of RM for a short retention interval as 24hr. For longer retention intervals the landscape changes. Indeed, at P30 enriched rats explore preferentially new object also at 48hr, 72hr and 96hr retention intervals, while the performance of massaged rats are in line with those of rats reared in standard condition (Fig. 28). At P60 all experimental groups show a complete development of their recognition memory.

The discrimination ratio is an index that permits to analyse the level of consolidation of the memory traces; in another words d.r. measures the robustness of the remembrance. In agreement with exploration time analysis, enriched and massaged rats show a memory traces significantly more robust for 24hr interval at P30 and P40. No difference emerges for 48hr interval and this result is plausible since the ability to recognize familiar object after a delay of 48hr appears at the same time for all experimental groups (P30). At longer retention intervals such as 72hr and 96hr, enriched animals present a more robust memory than massaged and control animals, respectively at P30 and P40. At P60 the level of consolidation of memory for all experimental groups at each retention intervals are comparable.

DISCUSSION

CHAPTER 7

Enriched environment is a powerful experimental paradigm which deeply affects brain development. Important results about the consequences of exposing animals to enriched living conditions during development came 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., 2007a,b) 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). Recently, it has been also demonstrated that 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 (Ciucci et al., 2007).

From the aforementioned studies an intriguing evidence emerges: the environment acts on visual system development not necessarily by increasing levels of visual stimulation (Cancedda et al., 2004; Bartoletti et al., 2004; Landi et al., 2007a). The first two weeks of life in rodents are characterized by a prevalent absence of interaction between the newborn and the external environment and pups spend their whole time in the nest: the mother can be considered the most important source of

sensory experience for the developing pups (Hofer, 1984; Liu et al., 2000). A quantitative analysis of maternal behaviour has shown that enriched pups receive a continuous physical contact due to the presence of adult females in the nest and higher levels of licking, provided from both the dam and the filler females, than pups reared in standard condition (Sale et al., 2004). In the first days of life EE may affect visual system maturation indirectly through maternal care and in particular through the tactile stimulation provided with licking and grooming (Sale et al., 2004).

The first aim of this thesis was to gain insight into the early experience triggering the faster maturation of visual cortex and to investigate the molecular factors involved in the changes detected in the visual system in response to a tactile stimulation protocol. First, I have examined the effects of massage on development of visual function at electrophysiological and behavioural levels. Second, I have investigated the involvement of the IGF-1 and intracortical inhibitory circuitry in mediating the acceleration of visual acuity development by massage.

7.1 Massage accelerates visual system development in rat pups

In the present work, I addressed a new issue: enriching the environment in terms of body massage and multisensory stimulation ("massage therapy") affects brain development and in particular visual system maturation in rat pups. This not only underlines the importance of the environment as a driving force in early postnatal development but suggests that the environment acts by modulating the level of endogenous factors such as IGF-1 and the intracortical inhibitory circuitry.

7.1.1 Massage accelerates the development of visual function in rat pups

To investigate the hypothesis that early enriched experience in terms of body massage may affect visual system development in rat pups, I have applied an artificial tactile stimulation protocol which is widely used in literature as surrogate of maternal licking and grooming to prevent deleterious effects of maternal deprivation on developing rat pups (Pauk et al., 1986; Schanberg and Field, 1987; Kuhn and Schanberg, 1998; van Oers et al., 1998b; Schanberg et al., 2003; Burton et al., 2007; Chatterjee et al., 2007).

As early index of the maturation of physiological visual functions, I recorded the fVEP response between P14 and P18. Normally in rats, the first light evoked responses arise around P11-P12 in the visual cortex with a long latency negative wave and approximately 2-4 days after eyes opening (P16-P18) emerges a negative-positive complex similar to that obtainable in the adult rat (Rose, 1968a, 1971). I found an accelerated development of flash-evoked visual response in massaged rat pups; a faster rate of reduction of latencies up to P16 was shown supporting a role for massage therapy in the enhancement of fVEP maturation.

Subsequently, I assessed the effects of massage on visual acuity development which is a very sensitive and predictive index of visual system maturation (Huang et al., 1999; Porciatti et al., 1999). I found that tactile stimulation protocol applied in the first two weeks of life induced an acceleration of the visual acuity development in P25 rat pups at electrophysiological and behavioural level. Moreover, the effects of massage in accelerating visual acuity development in rat pups are not attributable to the pup manipulation or to the simple removal from the nest or to modulation of stress hormone levels, because they are absent in pups separated from the mother for the same amount of time as massaged pups but not subjected to massage. This supports the importance of massage *per se* in promoting brain development. These results also

support the hypothesis that the level of tactile stimulation provided by licking/grooming is an important regulator of brain development (Liu et al., 2000; Weaver et al., 2004, 2006, 2007; Meaney and Szyf, 2005). This is the first time that body massage is shown to influence visual development. The studies previously conducted in rat pups using tactile stimulation assessed enzyme ornithine decarboxylase, growth hormone (Evoniuk et al., 1979; Schanberg and Field, 1987; Wang et al., 1996; Kuhn and Schanberg, 1998) and levels of stress hormones such as CORT, ACTH and CRH (van Oers et al., 1998b; Kuhn and Schanberg, 1998), but did not investigate massage effects on brain development.

7.1.2 Massage affects the IGF-1 expression levels in the developing brain

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. Interestingly, 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).

Running in the wheel produces an increment of circulating IGF-I and its brain uptake affecting different cerebral areas (Carro et al., 2000). It has been demonstrated that also environmental enrichment affects IGF-1 pathway: EE has been shown to up-regulate IGF-I receptor gene in the adult rat hippocampus and sensorimotor cortex (Keyvani et al., 2004). IGF-I is considered the mediator of the effects of exercise (Carro et al., 2000) and of EE on functional recovery from spinal cord injury (Koopmans et al., 2006) and a key molecule related to functional and anatomical

plasticity in the brain (for review see Torres-Alemann, 1999, 2000 and 2005; Aberg et al., 2006). It has been also shown that EE accelerates the development of the retina anda crucial factor involved in this process, with BDNF, is IGF-I (Landi et al., 2007, 2009). Besides, exposure of rat pups and their mother to EE increases IGF-I protein levels in the cerebellum and in maternal milk respectively (Sale et al., 2007). Finally, in a recent work Ciucci et al. (2007) have demonstrated that IGF-I mediates the EE effects on the visual system development and its expression in the visual cortex is affected by EE.

I decided, therefore, to investigate whether IGF-1 expression could be affected by tactile stimulation at cortical level mainly in the visual cortex and in another cortical area as auditory cortex. I found that IGF-I protein expression in the visual cortex increases between P14 and P25 in 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-I in experience-dependent visual cortical plasticity suggested by Tropea et al. (2006) whom show an up-regulation of insulin-like growth factor 1 binding protein 5 (IGFBP-5) and a down-regulation of IGF-1R after monocular deprivation. I provided evidence that the expression of IGF-I protein in the visual cortex is affected by massage; IGF-I immunoreactivity at P18 is higher in massaged rat pups than in separated and control rats.

To assess whether the effects of massage in IGF-I brain levels were specific for visual areas, I quantified the presence of IGF-I positive cells in the primary auditory cortex at P14. I found that IGF-1 content was significantly increased in massaged pups with respect to controls also in the auditory cortex.

These findings showed that the increase of IGF-1 in massaged pups is not specific for the visual cortex. This is in accordance with aforementioned data: in rats precociously

exposed to EE, which receive a higher level of tactile stimulation through licking and grooming during the early phases of their postnatal development (Sale et al., 2004), were found an increase in IGF-1 levels not only in the visual cortex but also in the retina and in the cerebellum (Ciucci et al., 2007; Sale et al., 2007). All these results support the idea that both EE and massage act on brain development, not on development of specific brain regions. The hypothesis is that both EE and massage induce an increase of IGF-1 circulating affecting the whole organism.

7.1.3 IGF-1 mediates the effects of massage on visual cortical development

In the previous section, it has been shown that massage is effective in increasing IGF-1 levels in the cortex and in particular in the visual cortex at P18. Such an increase could promote the changes necessary to accelerate visual acuity development. Indeed, in a recent study exogenous administration of IGF-1 from P18 to P25 in the rat visual cortex is sufficient to accelerate visual acuity development, mimicking EE effects, and that blocking IGF-1 action in the visual cortex of rats exposed to EE prevents the acceleration of visual acuity development (Ciucci et al., 2007).

To assess if the enhanced IGF-1 levels observed in the visual cortex of massaged rats are responsible for the acceleration of visual acuity development, I performed a block of IGF-1 action subcutaneously injecting the IGF-1 receptor antagonist JB1 (Pietrzkwoski et al., 1992), during the application of tactile stimulation protocol from P1 to P10.

In this study, I found that blocking IGF-1 action prevents the effects of massage, showing that IGF-1 is also crucial for the effects of massage on rat visual acuity development. This strongly suggests that IGF-1 is one of the mediators of massage therapy effects on visual development.

7.1.4 Massage affects the density of inhibitory synapses in the visual cortex

The action of an increase in IGF-1 on visual acuity development is likely to be mediated by the accelerated maturation of intracortical inhibitory circuitry (Ciucci et al., 2007); the development of visual acuity is correlated with a decrease in cortical receptive field size (Fagiolini et al., 1994) and with postnatal development of intracortical inhibitory circuits that shape visual cortical receptive fields (Sillito, 1975; Hensch et al., 1998). Indeed, also the accelerated development of visual acuity in EE mice is accompanied by a precocious increase in the expression of GABA biosynthetic enzymes, GAD65/67, in the visual cortex. In line, visual acuity development is faster in BDNF-overexpessing mice that show an accelerated development of inhibition in the visual cortex (Huang et al., 1999).

I provided evidence that massaged rat pups at P25 show in visual cortex an increase in GAD65 immunoreactivity than quantification observed in separated and control animals; a raised inhibitory tone may explain the acceleration in visual acuity maturation. I have hypothesized that IGF-1 affects GAD65 immunoreactivity in puncta-rings as it has been demonstrated in a recent study where inhibitory interneurons respond to IGF-1 with a GAD65 increase in their synaptic terminals (Ciucci et al., 2007), 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).

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).

In sum, these findings demonstrate that massage has an influence on brain development and in particular on visual development and suggest that its effects are

mediated by specific endogenous factors such as IGF-1 and its action could be exerted through the modulation of intracortical inhibitory circuitry.

Interestingly, a research group that have collaborated with us have explored the effects of body massage in preterm infant. It has been found that massage affects the brain development accelerating the maturation of electroencephalographic activity and of visual function, in particular visual acuity in preterm infants (Guzzetta et al., 2009). The most conspicuous change in the EEG is the transition from discontinuous to continuous activity, with a progressive reduction of the duration of the intervals between bursts of activity (Stockard-Pope et al., 1992; Scher et al., 1994). There is a much larger degree of shortening of the interburst intervals between the two assessments in massaged infants with respect to controls. Moreover, the effects of massage on visual acuity development were evident at 3 months of age in infants, that is more than 2 months after the end of the massage protocol, but were no longer present at 7 months of age.

This is the first time that body massage is shown to affect brain development in human infants. The studies previously conducted in infants using massage therapy assessed body weight, levels of stress hormones such as cortisol, growth hormone (Schanberg and Field, 1987; Vickers et al., 2004), and IGF-1 (Field et al., 2008), but did not investigate massage effects on brain development. These results on infants are in agreement with those previously exposed on rat pups suggesting that massage effects are not specific for the visual system. Indeed, they find changes in the maturation of cerebral electrical activity in infants that are evident at all EEG electrodes, not only at the occipital ones.

Moreover, the massaged preterm infants show higher levels of circulating IGF-1 and IGFBP3 and higher reduction of cortisol levels than control subjects as reported in literature (Schamberg and Field, 1987; Field et al., 2008).

Taken together, these results on infants and on rats demonstrate for the first time that massage therapy may affect brain development and suggest that its effect are mediated by IGF-1 (Guzzetta et al., 2009).

7.2 Massage and EE protocols decreased anxiety-like behaviour in adult rat.

The development of the nervous system is highly dependent on the interactions between the organism and its environment. Human experiences and animals experiments suggest that social conditions in early childhood can influence adult emotional and cognitive behaviour.

The pioneering studies of Spitz (1945), Harlow (1958) and Bowlby (1968) have clearly shown how an adequate maternal care was a crucial early environmental experience not only for the growing but mainly for the surviving of the newborns. Low quality of parent–child interaction has been associated with a reduced ability to shape interpersonal relationships, limited emotional competencies and increased vulnerability to depression and anxiety disorders (Korosi and Baram, 2009), as well as with cognitive decline later in life (Kaplan et al., 2001; Wilson et al., 2007). However, an appropriate early social environment may lead to an increased resilience to stress and to stress induced illness (Smith and Prior, 1995; Wachs, 2006). In other words, positive affective states as those observed during mother-infant interaction induce resilience to depression and anxiety and lead to increase in overall health (Lyubomirsky et al., 2005). Moreover, individuals who have high levels of positive emotion are at less risk to developing anxiety disorders, depression and global health problems (Fredrickson et al., 2003; Lyubomirsky et al., 2005).

However, the molecular mechanisms underlying these long-lasting effects on resilience to depression and anxiety at adulthood are not completely understood.

7.2.1 Enriched early-life experiences and reduced anxiety-like behaviour: a role for IGF-1.

The employment of rodent models have allowed comprehensive investigations of the effects of early environmental manipulations on neural development.

As widely reported in the Introduction, some early experiences of "enrichment" such as handling and high levels of LG provided by dam can affect emotional reactivity making rodents more reliance to stress at adulthood (Levine et al., 1957, 1967; Meaney and Aitken, 1985; Meaney et al., 1989; Escorihuela et al., 1994; Liu et al., 1997; Caldji et al., 1998; Pena et al., 2009).

A growing body of evidence indicates that a better coping with stressful situations later in life of rat are likely to be mediated by changes in the amount of tactile stimulation provided by dam during postnatal period In particular, offspring raised with high levels of tactile stimulation showed increased exploratory behaviour in novel environments, decreased HPA activation in response to stress and were behaviourally less fearful compared to those raised with low levels of tactile stimulation, at adulthood (for a review see, Kaffaman and Meaney, 2007).

In addition, some works have also demonstrated that stroking the pups with a fine paintbrush (miming maternal tactile stimulation) affects HPA reactivity in a manner that resembles those of handling and exposure to High LG dams (Pauk et al., 1986; Suchecki et al., 1993; Kuhn and Schanberg, 1998; Jutapakdeegul et al., 2003). Finally, it has been demonstrated that living in EE can modify the fearful behaviour of strains of mice such as C57BL/6 and B&CBA and Wistar and Long-Evans rats

supporting the assumption of the EE anxiolitic action **(**Widman and Rosellini, 1990; Haemisch et al., 1994; Klein et al., 1994; Fernandez-Teruel et al., 1997; Caston et al., 1999; Chapillon et al., 1999; Nikolaev et al., 2002; Belz et al., 2003; Moncek at al., 2004; Friske and Gammie, 2005; Zhu et al., 2006; Welberg et al., 2006; Galani et al., 2007; Iwata et al., 2007; Hoffman et al., 2009).

However, in literature there is a relative dearth of studies on the positive effects of a tactile stimulation protocol applied in the first two weeks of animal's life. Indeed, the most part of studies utilizes this protocol for reverting the short and long lasting negative consequences triggered by maternal deprivation in rat pups. At the same time, EE protocol is more frequently employed at beginning of postweaning age and scarcely restricted only at preweaning age.

Therefore, although the effect of neonatal handling before and maternal High/Low LG levels later on the adult animal's physiology and behaviour has been extensively studied, a direct comparison about the effects of tactile stimulation protocol miming maternal licking and grooming with restricted EE condition at preweaning age and at longer periods is still lacking.

Thus, several studies have elucidated how increased tactile stimulation levels provide by dam, or triggered by handling procedure, can influence behaviour and stress reactivity in a manner that persist into adulthood. It has been revealed that handling and High maternal LG levels are associated with robust upregulation of GR expression in the hippocampus and prefrontal cortex. Levels of hippocampal GR regulate the HPA response to stress though a negative feedback relationship with higher levels of GR mRNA associated with attenuated stress responsivity (Meaney and Aitken, 1985; Meaney et al., 1989; Liu et al., 1997; Weaver et al., 2004, 2005). Pups treated with a tactile stimulation protocol in term of stroking with paintbrush also exhibit an acute increases in hippocampal GR expression (Jutapakdeegul et al., 2003).

These effects are mediated by increase in NGFI-A which is dependent on serotonergic activation of cAMP-coupled 5-HT_7 receptors (Meaney et al., 2000; Mitchell et al., 1992; Laplante et al., 2002; Weaver et al., 2007).

The molecular mechanisms by which such diverse changes can be induced by maternal care are poorly known.

A molecular factor that could be involved in mediating tactile stimulation effects on stress response in adult rats is IGF-1. In rats, IGF-1 has been shown to be elevated by hedonic exercise and induce resilience to depression and anxiety. The antidepressant effect of voluntary exercise in rats appears in part to be mediated by exercise-induced increased in brain IGF-1 levels (Trejo et al., 2005). These effects have been found to be IGF-1R-specific given that the administration of an anti-IGF-1 antibody blocks the antidepressant effects (Duman et al., 2009). IGF-1 administration has also antidepressant effects in the forced swim test and tail suspension test, and anxiolytic effects in the elevated plus maze test in both rats and mice (Hoshaw et al., 2005; Malberg et al., 2007).

The aim of this study is to uncover the molecular basis of positive effects of early massage and different enriched environmental protocols on anxiety-like behaviour in rats by means of elevated plus maze (EPM), and to evaluate the possible crucial role of IGF-1.

My results show that massage in rat pups may lead to an increased resilience to stress in the adulthood. An increased tactile stimulation experience limited to the first twelve postnatal days (P12) significantly decreases anxiety-like behaviour in adult rats. To extend the EE protocol to the end of adolescence (P45) or to the beginning of adult age (P60) did not improve their stress response in adulthood supporting the idea that only the first postnatal weeks, before weaning, are crucial for programming the future behaviour in stress conditions of adult animal. These data are in line with

aforementioned studies; maternal tactile stimulation (liking and grooming) affects stress response system of the offspring making them more resilient to stressful events.

At molecular level, I have provided evidence that IGF-1 is crucially involved in the tactile stimulation effects on stress response in the adult rat. Indeed, subcutaneous injects of IGF-1 from P1 to P10 in the rat pups mimed the effects of massage and EE protocols: adult rats spent more time exploring open arm showing lesser anxiety-like behaviour than control rats. Accordingly, subcutaneous injections from P1 to P10 of IGF-1 receptor antagonist JB1 blocked the massage and EE effects on behavioural response to stress. These results, therefore, demonstrate for the first time that tactile stimulation (massage therapy) early in life may affect stress response in the adult rat through IGF-1 pathway suggesting an its programming action on adult coping with stressful situations.

A role for IGF-1 in shaping adult response to stress is supported to its antidepressant and anxiolytic effects. A current hypothesis is that neurotrophins or drugs that modulate plasticity-related proteins or growth factors may provide the next generation of antidepressant or anxiolytic drugs (Malberg and Schechter, 2005): neurotrophic factors as IGF-1 and BDNF play a significant role in mood disorders such as depression and anxiety (Hoshaw et al., 2005). Indeed, it has been demonstrated that increasing level of IGF-1 in the CNS via an IGFBP inhibitor produces both anxiolytic and antidepressant-like effects in the adult mouse, similar to those observed after IGF-1 administration. Further, these effects are blocked by administration of an IGF-1R antagonist (JB1) suggesting that these effects may be mediated by activation of the IGF-1R (Malberg et al., 2007).

Interestingly, a recent work of Panksepp's group (2010) have shown that IGF-1 plays a functional role in the generation of positive affective states as indexed by hedonic 50-kHz ultrasonic vocalizations (USVs) produced by adult rats during rough-

and-play activity. Hedonic rough-and-play increased cortical mRNA and protein levels of IGF-1, IGF-1R and IGFPB2. Positive affect was shown to specifically elevate cortical IGF-1 protein levels controlling for social interaction, somatosensory stimulation, age and arousal. Cortical IGF-1 protein levels were also shown to be elevated in rats bred for high rates of hedonic USVs in a locomotor activity independent manner. Conversely, negative affective stimuli reduced cortical IGF-1 protein levels. IGF-1 was shown to promote the generation of hedonic USVs via its action on the IGF-1R using direct lateral ventricle administration of IGF-1 alone and in concert with a silent dose of the IGF-1R antagonist JB1 (Burgdorf at al., 2010).

IGF-1 signals through the IGF-1R via a multiprotein signalling complex (Ye and D'Ercole, 2006). This receptor signalling pathway activates both the Ras/mitogen-activated protein (MAP) kinase pathway and the PI3 kinase-AKT pathways (Brunet et al., 2001; Ye and D'Ercole, 2006). These pathways share a high degree of overlap with the signalling pathways of 5-HT and BDNF which are also implicated in antidepressant action (Mattson et al., 2004; Hoshaw et al., 2005). It has been also shown that chronic icv infusion of IGF-1 increases hippocampal 5-HT levels (Malberg et al., 2005). IGF-1R activation may involve other growth factors such as FGF, GH and VGF which have been also shown to be elevated by positive affective states and/or induce resilience to depression and anxiety (Gordon et al., 2003; Stouthart et al., 2003; Hunsberger et al., 2007; Turner et al., 2008). Another neurotrophin involved in the vulnerability to depression and anxiety is NGF. Indeed, early life events, such psychophysical stress, affect NGF levels and induce dysregulation of the HPA axis (for review see Alleva and Francia, 2009). Additionally, both the mesolimbic dopamine system and opioids play a functional role in the generation of positive affective states (Burgdorf and Panksepp, 2006; Le Merrer et al., 2009).

Clinically, reduced serum IGF-1 levels are seen in patients with growth hormone (GH) deficiency and depressive symptoms have been reported in this population (Wallymahmed et al., 1996). In these patients, GH therapy is the standard treatment, which normalizes IGF-1 levels. Interestingly, Pavel et al. (2003) have demonstrated that adult patients who received GH treatment had an improvement in mood. In contrast, cessation of GH treatment, which produced a decrease in IGF-1 levels, also produced an increase in depression and other negative symptoms and complaints (McMillan et al., 2003; Stouthart et al., 2003; Lasaite et al., 2004). This effect can be counteracted in young adults by restarting GH treatment and once again normalizing IGF-1 levels (Sthouthart et al., 2003). In addition, children with GH deficiency (compared to children with normal GH but short stature) exhibit both depression and anxiety, and these conditions are reduced by GH treatment (Stabler et al., 2001). Besides, i.v. IGF-1 injections reduce levels of depression and anxiety in humans (Thompson et al., 1998; Graham et al., 2007).

Taken together, these studies have shown that IGF-1can induce resilience to depression and anxiety in humans and in rodents. The answer to the question related to the molecular mechanism by which postnatal maternal care might be able to affect a behavioural phenotype as stress response, I suggest that this might be due to an increased tactile stimulation levels that affect expression of neurotrophic factors, such as IGF-1, that in turn not only have a role in the neurobehavioural responses to stress but also in vulnerability and resilience to stress-related neuropsychiatric disorders. To further elucidate the molecular mechanisms that could underlie the effects of massage on stress response at adulthood, it could be interested use focused microarray platform that, when coupled with appropriate bioinformatics tools, provide a systematic approach for identifying significant gene families associated with massage. These investigations could be corroborated by quantitative mRNA and protein assays.

Finally, it would be interest to asses whether subcutaneous injections of IGF-1 are able to revert the negative long lasting consequences in rat pups subject to maternal deprivation protocol.

Another hypothesis related to positive effects of massage on anxiety-like behaviour in adult rats could implicate the vagal activity. Several studies on human infants suggest that vagal activity is associated with both infant growth and socioemotional development (Field and Diego, 2008; Field et al., 2010). Vagal activity has been noted to increase following the stimulation of pressure receptors as in massage therapy and that, in turn, stimulates gastric motility which mediates weight gain in infants (Diego et al., 2005). Besides, vagal activity has been also notably elevated during synchronous mother-infant interactions and positive affect, providing confirmatory data for the Porges "Social Engagement System" model (Porges, 2001). In contrast, low vagal activity has been noted in prenatally depressed mothers and prenatally angry and anxious mothers and their infants, as well as in children with autism (Field and Diego, 2008). The massage therapy model proposed by Field's group indicate that baroreceptors and mechanoreceptor within the dermis are innervated by vagal afferent fibers that permit, in the last analysis, a parasympathetic control of the gastrointestinal system (Chang et al., 2003). Direct stimulation of the vagus nerve can regulate gastric motility, can enhance food digestion and can increase the availability of nutrients (Chang et al., 2003). Vagal stimulation also promotes the release of insulin and insulin has been shown to stimulate the synthesis and release of IGF-1 (Field and Diego, 2008). The stimulation of pressure receptors can also promote the release of insulin (Marchini et al., 1987). These results, therefore, suggest that stimulation of pressure receptors (as in massage) increases vagal activity and vagal stimulation facilitates the release of insulin and can significantly predict growth velocity in preterm infants (Field et al., 2010). Recently, it has been proposed a

potential gastrointestinal link between enhanced postnatal maternal care and reduced anxiety-like behaviour in adolescence rats (Weber et al., 2009).

A deeper understanding of the consequences of early experience variations and in particular of maternal care levels on the development of brain and behaviour of infants has an important clinical relevance because it may potentially allow to design a feasible plan for babies which undergo a maternal care deprivation preventing pathological disorders.

7.3 Developmental time-course of Rat Recognition Memory: environmental and tactile stimulation influence.

Recognition, a judgment of the prior experience with objects is thought to be a critical component of human declarative memory. People with normal memory may engage this ability hundreds of times each day, but impaired recognition occurs in many memory disorders, in particular in patients affected by neurodegenerative diseases (as AD) or who have suffered brain injury (Winters et al., 2008). The ability to distinguish between an object one has encountered previously and one that is new is so fundamental to normal memory function that understanding its neural base seems necessary to develop a comprehensive picture of how the brain remembers things. Such knowledge may also contribute to better methods of diagnosing and treating certain memory disorders.

Rats readily distinguish between objects they have previously seen and ones they have not. The extent to which object recognition memory involves similar processes in rats and humans is not entirely clear. Standardized tasks for assessing

object recognition in rats, the effects of various brain lesions, and effects of drugs on this ability all suggest similar processes**.** However, there is a relative dearth of maturational literature on the object recognition memory in rats with respect to developmental works in primates. Besides, a direct comparison of developmental time-course of recognition memory in rats as function of different rearing conditions is still lacking.

A large body of studies highlighted that environmental enrichment is able to modify animals' behaviour and in particular improves a complex cognitive functions as learning and memory (van Praag et al., 1999; for an exhaustive review, see Rampon and Tsien, 2000). EE effects are especially evident in hippocampal-dependent tasks involving spatial memory, such as the Morris water maze (Falkenberg et al., 1992; Paylor et al., 1992; Moser et al., 1997; Kempermann et al., 1998a; Nilsson et a., 1999; Williams et al., 2001; Lee et al., 2003; Leggio et al., 2005; Fréchette et al., 2009), and enriched animals also exhibited better performance in non-spatial learning and memory tasks as in the object recognition test (Rampon and Tsien, 2000; Duffy et al.,. 2001; Lee et al., 2003).

The purpose of this study was to investigate a rat's ability to recognize a previously explored object across development at different retention intervals by means of the object recognition task (ORT). Moreover, once elucidated the maturational steps in the rat reared in a standard condition, I have assessed how different rearing conditions, in particular EE from birth up to P45 and tactile stimulation protocol from P1 to P12, could affect the normal developmental time-course of recognition memory in rats.

7.3.1 Ontogeny of recognition memory measured by ORT in naive rat.

I have chosen the object recognition task for investigating the recognition memory because the ORT is particularly amenable to developmental work. Indeed, it is free from response contingencies and require no pretraining and relies upon a rat's intrinsic exploratory drive to investigate novel stimuli. The ORT also lacks overt stress components, such as forced swimming or food deprivation. A second strength of the ORT is that simple nonrule-based tests of object recognition ability are well understood in humans (including preverbal infants) and monkeys (Alvaro and Bachevalier, 2000; Bachevalier and Vargha-Khadem, 2005).

In literature, prior to the present study, only two papers had been published directly applying the ORT to the immature rat. In the first study the authors reported a decrement in preweanlings (P18) on object recognition with retention intervals between 1 min and 2hr, while adults showed stable performance across these intervals (Anderson et al., 2004). However, a substantial number of the preweanlings were dropped from analyses because they did not perform the task. In the second study, Reger et al. (2009) have resolved this question using an age-appropriated arenas. Consequently, the authors were able to investigate the performance in the ORT at younger ages over a wider range of retention intervals. They found that adult (>P50) and juvenile (P29-P40) rats exhibited comparable ORT performances; while weanling (P20-23) rats were able to exhibit robust object recognition across shorter delays (0.25 and 1h), and showed inferior long-term memory retention (24hr). All groups failed to show a novel object preference after a 48hr retention interval (Reger et al., 2009).

My results elucidate more in details those of Reger et al. (2009). I modified the size of the arena and visual pattern cube to be age-appropriate. I decided to investigate the development of recognition's ability in rat at five different age intervals: preweaning age (P17-P21), postweaning age (P23-P27), early adolescence(P29-P33), late adolescence

(P39-P43; the adolescence phase in rat is conservatively defined as P28 to P42; Spear, 2000) and young adult (P59-P63). Finally, I extended the range of retention intervals assessed enhancement the long-term intervals at 72hr and 96hr.

I found that preweaning and postweaning rats were able to recognize a novel object only at 1h, while at longer retention intervals their performance fall down. They began to exhibit robust object recognition at 24hr and 48hr between P29 and P33, while at longer delay as 72hr and 96hr this ability appeared in the late adolescence and adult age, respectively. Thus, an age-dependent change in long-term memory ability was detected. These results are in agreement with the work performed by Reger et al. (2009). These authors found that juvenile (P29-P40) and adult (+P50) rats showed comparable, significant object recognition at 0,25, 1h and 24hr while the weanlings were able to recognize the novel object only at shorter intervals but couldn't at longer delay as 24hr.

However, their findings are not completely in agreement with my results. In fact, it has been found that all experimental groups didn't explore preferentially the novel object after a delay of 48hr (Reger et al., 2009). Instead, my analysis shows that in the early adolescence (P29-P33) the rat is able to explore preferentially the new object also at 48hr; this ability is maintained in the following age intervals. A possible explanation could be found in the different objects used; while Reger et al. utilized common life objects, I employed standardized visual pattern inserted in the Plexiglas's cube. Moreover, I provided evidences, for the first time, that the ability to recognize novel object at 72hr and 96hr is acquired, respectively, in the late adolescence (P39-P43) and in the early adulthood (P59-P63). Accordingly, the analysis of discrimination ratio confirmed the previous results. At all age intervals, the performance were comparable at 1h, while the performance at longer retention intervals were markedly affected from the age of animals: age became a crucial factor starting from 24hr interval suggesting that the long-term memory begins to emerge between P29-P33.

My findings are consistent with the visual recognition memory literature across species, and more in general with the principle of ‘‘infantile amnesia’’ (Bachevalier and Beauregard, 1993). Recognition memory ability is the earliest primate and human learning and memory function to appear and yet primate and human infants are not proficient on the visual paired comparison task (VPC) at longer retention intervals (Alvarado et al., 2000). The age-dependent discrepancy may be rooted within the medial-temporal lobe (MTL), the area of the brain responsible for recognition memory. The MTL (characterized by the interconnected structures of the hippocampus/dentate complex, peri- and entorhinal cortices and the parahippocampal gyrus) undergoes a protracted period of postnatal development in humans, monkeys, and rodents, with its different structures maturing along different time continuums and the learning and memory functions subserved emerging differently in time as well (Alvarado et al., 2000). The hippocampus was originally thought to be the seat of recognition memory and some authors have reported hippocampal related long-term ORT deficits in the face of intact short-term performance (Clark et al., 2000; Brown and Agglenton, 2001; Hammond et al., 2004; Bruel-Jungerman et al., 2005). However more recent publications have argued against this point (Mumby, 2001; Mumby et al., 2002; Murray et al., 2007; Winters et al., 2004) and have instead placed importance primarily on the perirhinal cortex (for review see, Winters et al., 2008). Neurodevelopmentally, the emergence in rats of hippocampus-dependent behaviours coincides with the end of neurogenesis and synaptogenesis in the dentate of the hippocampal complex at around the time of weaning. Proficient ORT performance emerged sometime between weaning and postnatal day 29 (beginning of the operationally defined juvenile period). Little is known about the development of the perirhinal cortex, although in primates its maturation has been shown to lag behind the entorhinal cortex (Alvarado et al., 2000). Nevertheless, MTL structures are highly

integrated and perhaps the perirhinal cortex is sufficient for the processing and recognition of object attributes after short retention intervals but the hippocampus is involved in long-term object recognition.

It has been suggested that MTL damage is similar to MTL immaturity and therefore uncovering the neurophysiological underpinnings of recognition memory across rat development will be important to related models of pediatric disease and dysfunction. Traumatic brain injury (TBI) is the number one cause of death and disability in children and adolescents (Weiner and Weinberg, 2000), many of whom exhibit recognition memory deficits (Levin et al., 1988). A lack of decrement in procedural or implicit memory (a known non-MTL mediated memory functions) after pediatric TBI has also been observed, lending support to the idea that MTL learning and memory are especially vulnerable to insult in children (Ward et al., 2002). Future ORT characterization studies should incorporate preweanlings with an age-appropriate design as well as investigate how the development of the hippocampal complex and perirhinal cortex are correlated with performance.

7.3.2 Environmental enrichment and neonatal massage modulate the developmental time-course of the rat recognition memory.

I found an acceleration in the development of the long-term recognition memory ability in enriched and massaged rat even if their developmental time-course presents marked differences. In particular, these experimental groups show a competence to recognize novel object after a delay of 24hr already at preweaning age while in rat reared in a standard condition this proficiency emerges only between P29-P33. Further, surprisingly, at age interval P29-P33 enriched rats show not only to recognize a new object at 48hr as massage and control rats, but also at 72hr and 96hr. Instead, massaged

rats follow the same maturational trend previously showed by control rat; in the early adolescence are able to recognize novel object only until a retention interval 48hr and then adult proficiency of recognition memory is achieved gradually. The analysis of discrimination indexes, however, underlines that robustness of memory trace at 72hr and 96hr in enriched rats is age-dependent. Indeed, d.r. observed at 72hr and 96hr improved across development reaching an adult-like value in the follow age intervals (late adolescence and adulthood respectively), just when in massaged and control rats emerged these specific competences.

Environmental manipulations, therefore, can accelerate the ontogeny of recognition memory and improve its performance in a "dose-dependent" manner. A protocol of EE until the end of adolescence accelerate the maturation of recognition memory reaching adult-like proficiency more early. Instead, an early enrichment protocol as massage effects partially the developmental time-course of recognition memory. These findings are in agreement with effects observed in sensory system development and in hippocampal ontogeny: their maturation is influenced by experience as neonatal handling/exposure to novelty and peripubertal EE (Meaney et al., 1985, 1988; Fernandez-Teruel et al., 1997; Pham et al., 1997; Tang, 2001; Tang and Zou, 2002; Bredy et al., 2003b).

7.3.3 Which mechanism could underlie the effects of EE and massage on recognition memory development?

As describe before, many studies stress a contributing role for MTL cortical regions outside the hippocampus in the performance of object recognition tasks (for a review see, Winters et al., 2008). Indeed, serious object recognition memory deficits can result from damage to perirhinal (PRh) and related cortex even when the hippocampus is

fully intact (Murray and Mishkin, 1986; Zola-Morgan et al., 1989c). There are evidences from neuronal recordings, immediate early-gene imaging and animal lesions studies that the hippocampal and perirhinal cortex contributions are different and can be dissociated; a system centring on the PRh cortex is concerned with discriminating the familiarity and recency of occurrence of individual stimulus items, whereas a system centring in the hippocampus is concerned with spatial arrangements of stimuli and paired associate learning (for review see, Brown and Aggleton, 2001). Besides, a recent study have shown a role of the PRh neuronal activity in encoding, retrieval, and consolidation of the object memory trace suggesting an involvement in object recognition memory measured by ORT (Winters and Bussey, 2005c).

The main parahippocampal region crucially involved in the recognition of object information *per se* appears to be the PRh, representing the anatomical structure where to investigate possible factors regulating PRh-mediated object recognition memory.

The maturation of specific anatomical areas is associated with the emergence of particular functions. It has been demonstrated that PRh develops in a manner similar to neocortical regions but its morphology and laminar distribution of cell type is unique (Furtak et al., 2007b). In particular, the majority of cells first observed in PRh are of the two largest types-pyramidal and multipolar neurons which are excitatory and increase their number until P10-P12 with an apparent net decrease after P17. Moreover, pyramidal cells continue to grow: dendritic length continue to increase between P28-P32 and P35-P40.

Smaller, nonpyramidal cell types become more numerous after P6; small round neurons and aspiny stellate cells, which are presumed to be inhibitory based on their morphology and firing pattern, develop in different temporal pattern: stellate neurons are the very last cell class to appear. Their late development suggests that this presumed source of inhibitory control of neuronal activity has a delayed onset relative to the

formation of much of the rest of the PRh circuitry. Note that eye-opening coincides with the increase in the number of stellate neurons; the first large increase in the number of stellate neurons occurred at P14-P17 and the peak is not reached until P28-P32 (Furtak et al., 2007).

Interestingly, at this age interval I found that enriched rats showed a better long-term recognition memory than that observed in naive and massaged rats. It could be hypothesized an acceleration of the PRh morphological development and their neural circuits that control the processing, storage and retrieval of information within the medial temporal lobe.

At physiological level, it is widely believed that changes in synaptic strength support long-term memory storage in the brain (Martin et al., 2000). Electrophysiological studies of PRh slices *in vitro* have indicated that both incremental (LTP) and decremental (LTD) forms of long-term synaptic plasticity can be observed within PRh under appropriate stimulation conditions (Cho et al., 2000; Brown and Bashir, 2002). In these forms of synaptic plasticity are most commonly implicated the glutamate receptors: NMDA receptors for LTP (Cho et al., 2000; Massey et al., 2004) and a conjoint activation of NMDA and merabotropic glutamate receptors (mGluRs) group I and II for LTD (Cho et al., 2000; Brown and Bashir, 2002). Besides, it has been found by electrophysiological recordings that PRH neurons in rats responded less vigorously to familiar visual stimuli than to novel visual stimuli (Brown and Aggleton, 2001; Brown and Bashir, 2002). These results induce to speculate that enriched rats could present this decremental responses of PRh neurons at long-term retention interval more precociously than naive and massaged rats. Moreover, it would be interest to analyse the expression of gene encoding for glutamate receptors implicated in the mechanism of synaptic plasticity in PRh as LTP and LTD and synaptophysin expression as a measure of synaptic density since it has been

found that EE protocol and high level of LG increase expression of these receptors in the hippocampus (Rampon et al., 2000a,b).

Although glutamate receptors are most commonly implicates in synaptic plasticity mechanisms, other neurotransmitters are known to affect synaptic efficacy under certain conditions. One such neruotrasmitter is the neuromodulator acetylcholine (ACh). ACh has long been implicated in learning and memory and electrophysiological studies have indicated that it may play important roles in cortical and hippocampal synaptic plasticity (Segal and Auerbach, 1997; Rasmusson, 2000); besides, early studies report that EE increases the neurotransmitter Ach (Rosenzweig et al., 1962; 1967). Some authors have suggested that a processes as LTD could provide the mechanism underlying the decremental neuronal responses towards familiar object (Cho et al., 2000; Brown and Bashir, 2002). Massey et al. (2001) showed that activation of muscarinic cholinergic receptors in rat PRh slices induced a form of protein synthesis-dependent LTD, which did not require activation of NMDA receptors; application of the NMDA receptor antagonist AP5 did not block the cholinergic-induced LTD. Moreover, systematic injections or intra-PRh infusions of scopolamine, an antagonist of muscarinic cholinergic receptors, impaired object recognition memory and disrupted the normal decremental responses of PRh neurons to familiar versus novel visual pattern (Warburton et al., 2003).

Thus, a cholinergic mechanisms of synaptic plasticity within PRh may play a role in the induction or expression of activity-dependent LTD and EE protocol could effect the expression of muscarinic cholinergic receptors in PRh supporting an acceleration in the emergence of long-term recognition memory ability. Moreover, recent research with cholinergic immunotoxins has implicated the cholinergic basal forebrain input to PRh in object recognition memory in rats and monkeys (Winters and Bussey, 2005b).

Finally, there is good evidence that *zif268* may be part of a signalling cascade involved in the regulation of synaptic plasticity processes required for aspects of object

recognition memory. A research has indicated that manipulations that disrupt the functions of the mitogen-activated protein kinase/extracellular signal-related kinase (MAPK/ERK) and the cAMP response element-binding protein (CREB) can prevent the expression of late phase, protein synthesis-dependent LTP in the hippocampus and produce deficits in long-term object recognition memory tasks (Bozon et al., 2003). In addition, a recent study has demonstrate the importance of CREB protein phosphorylation in PRh LTP and long-term object recognition memory: CREB inhibition within rat PRh impaired spontaneous object recognition with a long (24hr) but not a short (15min) retention delay (Warburton et al., 2005). Moreover, PRh slices taken from rats treated with the CREB inhibitor had impaired LTP (Warburton et al., 2005). It would be interesting to see expression of *zif268* mRNA in PRh in rats following sample phase as function of age and retention intervals in each experimental group as predictor of a long term consolidation of the memory trace.

However, I cannot exclude a hippocampal component in the accelerated development of the long-term recognition memory observed particularly in enriched rats. It has been demonstrated that hippocampal neurogenesis in enriched rats is crucially involved in the enhanced long-term recognition memory. The reduction of neurogenesis during EE protocol completely prevented the memory-enhancing effect at longer retention intervals (24hr and 48hr) in the preweaning enriched rats (Bruel-Jungerman et al., 2005). It would be interest to investigate the role of hippocampal neurogenesis and its functional activity in mediating an accelerated maturation of recognition memory following EE protocol.

7.4 Conclusion

A fine interplay exists between sensory experience and innate genetic programs leading to the sculpting of neuronal circuits during brain development (Fagiolini et al., 2009). Importantly, CNS development is already responsive to the environment at very early stages.

EE from birth leads to a conspicuous acceleration of visual system development in rodents, detectable at the behavioural, electrophysiological, and molecular level (Cancedda et al., 2004; Landi et al., 2007). It has been suggested that, in the earliest phases of exposure to EE, the effects of EE do not stem from a direct action on the developing pups but rather from a variation in maternal behaviour during enriched conditions (Cancedda et al., 2004; Sale et al., 2004): i.e. pups in EE receive a higher level of tactile stimulation through licking, grooming, and physical contact (Sale et al., 2004).

Our results show for the first time that enriching the environment in terms of body massage accelerates brain development and in particular the maturation of visual function in rats. Indeed, we show that early body massage affects visual acuity development and we suggest that IGF-1 is one of the mediators of those effects and that its action could be exerted through the modulation of intracortical inhibitory circuitry. These findings are replicated in preterm infants underling the role of early environmental stimulation as a crucial factor for early postnatal development also in humans. Massage therapy could be a good implementation of normal intensive treatment reserved for preterm babies aimed at more efficaciously counteracting the onset of neurological pathologies associated with a precocious delivery.

Moreover, pre-weaning massage and EE decrease anxiety-like behaviour in adult rats and IGF-1 is a key factor in mediating these long-lasting effects supporting the hypothesis that IGFI-dependent signalling could be a potential therapeutic target for the treatment of anxiety.

Finally, I demonstrated that environmental manipulations can accelerate the ontogeny of recognition memory and improve its performance in a "dose-dependent" manner. Indeed, rat pups reared in EE from birth to P45 show an acceleration in the development of recognition memory. Instead, an early enrichment protocol as that of massage only partially affects the developmental time-course of recognition memory.

Taken together, these results underline how early experience can induce lifelong consequences including electrophysiological, molecular and behavioural features. But, "early experience is not necessarily destiny" (R.M. Sapolsky, 2004). An intriguing open issue is to investigate what molecular mechanism(s) mediates the long-lasting effects of early experience to find, then, possible ways for changing the "destiny".

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ACKNOWLEDGEMENTS