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ENRICHED EXPERIENCE, VISUAL CORTEX PLASTICITY AND RECOVERY FROM AMBLYOPIA IN ADULT RATS: EFFECTS OF VISUAL PERCEPTUAL LEARNING

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INDEX

CHAPTER 1: PERCEPTUAL LEARNING **4**

1.1 VISUAL PERCEPTUAL LEARNING **5**

1.1.1 NEURAL CHANGES UNDERLYING VISUAL PERCEPTUAL LEARNING 10

1.1.2 PERCEPTUAL LEARNING AND TOP-DOWN INFLUENCE 15

1.1.3 POSSIBLE CELLULAR MECHANISMS UNDERLYING PERCEPTUAL LEARNING 25

CHAPTER 2: AMBLYOPIA **28**

2.1 NEURAL MECHANISM UNDERLYING AMBLYOPIA **32**

2.2 TREATMENT FOR AMBLYOPIA IN CHILDHOOD **46**

2.3 TREATMENT FOR AMBLYOPIA IN ADULTHOOD **48**

2.3.1 PERCEPTUAL LEARNING AS TREATMENT FOR AMBLYOPIA 52

CHAPTER 3: AIM OF THE THESIS AND EXPERIMENTAL DESIGN **55**

CHAPTER 4: MATERIALS AND METHODS **59**

4.1 ANIMAL TREATMENT AND SURGICAL PROCEDURES **59**

4.2 BEHAVIORAL TASKS **60**

4.2.1 PERCEPTUAL LEARNING TASK 60

4.2.2 BEHAVIORAL ASSESSMENT OF VISUAL ACUITY 64

4.3 IN VITRO ELECTROPHYSIOLOGY: LTP **65**

4.4. IN VIVO ELECTROPHYSIOLOGY **66**

4.5. ANALYSIS OF NEUROTRANSMITTER RELEASE IN V1 SYNAPTOSOMES **68**

CHAPTER 5: RESULTS **70**

5.1 VISUAL PERCEPTUAL LEARNING IMPROVES VISUAL DISCRIMINATION ABILITIES IN ADULT RATS 70
5.2 VISUAL PERCEPTUAL LEARNING TASK IS SELECTIVE FOR STIMULI ORIENTATION 71
5.3 VISUAL PERCEPTUAL LEARNING CAUSES LTP-LIKE CHANGES IN PRIMARY VISUAL CORTEX 73
5.4 VISUAL PERCEPTUAL LEARNING OCCLUDES LTP IN PRIMARY VISUAL CORTEX 75
5.5 VISUAL PERCEPTUAL LEARNING DOES NOT INVOLVE CHANGES IN PRIMARY SOMATOSENSORY CORTEX 77
5.6 VISUAL PERCEPTUAL LEARNING TASK RESTORES VISUAL FUNCTIONS IN AMBLYOPIC ADULT RATS 79
5.6.1 RECOVERY OF VISUAL FUNCTION MEASURED WITH ELECTROPHYSIOLOGICAL TECHNIQUE 80
5.6.2 RECOVERY OF VISUAL FUNCTION MEASURED WITH BEHAVIOURAL TECHNIQUE 81
5.7 RECOVERY FROM AMBLYOPIA ELICITED BY VISUAL PERCEPTUAL LEARNING IS ASSOCIATED WITH REDUCED INHIBITION/EXCITATION BALANCE IN THE PRIMARY VISUAL CORTEX 84

CHAPTER 6: DISCUSSION 87

6.1 VISUAL PERCEPTUAL LEARNING INDUCES LTP IN THE VISUAL CORTEX 87
6.2 VISUAL PERCEPTUAL LEARNING PROMOTES RECOVERY FROM AMBLYOPIA IN ADULT RATS 90

REFERENCES 95

APPENDIX: SYSTEM CONSOLIDATION OF SPATIAL MEMORIES IN MICE: EFFECTS OF ENRICHED ENVIRONMENT 134

ACKNOWLEDGEMENTS 158

Chapter 1

Perceptual learning

The brain strategies we use to perceive the world are constantly modified by experience. With practice, we become better at identifying familiar objects or distinguishing fine details in our environment. Everyone can enjoy the taste of a glass of wine, but only wine tasters can detect its distinctive features; everyone can appreciate a figure skating performance, but only an Olympic judge can evaluate the quality of a double toe loop jump. The development of this expertise falls into perceptual learning (PL). PL can be defined as "an increase in the ability to extract information from the environment, as a result of experience and practice with stimulation coming from it" (Gibson, 1969).

Experts in many domains, including radiologists, wine tasters, and Olympic judges, develop specialized perceptual tools for analyzing objects within their domains of expertise. Much of the training and expertise involves not only developing a database of cases or explicit strategies for dealing with the world, but also tailoring perceptual processes to represent the world more efficiently. The improvement of perceptual processes allows an organism to respond quickly, efficiently, and effectively to stimuli without dedicating on-line attentional resources. PL is a form of implicit memory, involving improvement in sensory discrimination or detection by repeated exposure to sensory stimuli. Contrary to the declarative forms of learning, and similar to motion learning, PL seems to directly modify the neuronal pathways active during the task, and not to require an intermediate consolidation storage, such as the hippocampus for declarative memory. Moreover it does not consist of consciously memorized facts or events and it does not lead to conscious insights that can be easily communicated (Leritz et al., 2006; Speekenbrink et al., 2008). In addition, the fact that PL involves long lasting changes in perceptual abilities distinguishes it from other implicit forms of learning (Schacter, 1987 and 1992). Contrary to associative learning (Pavlov, 1927) PL does not bind together two separated processes but improves discrimination between stimuli that could not be discriminated before the learning. PL differs also from adaptation which usually implies an adjustment within a predefined working range, with no long-term

neural changes. Adaptation seems to be a more basic mechanism and differs from PL in being induced by exposure to stimuli rather than by task-specific practice (Bedford, 1993; Dodwell and Humphrey, 1990). The type of implicit learning most closely related to PL is priming. Priming describes the effects of a stimulus on subsequent perception of stimuli and/or behavioral response. Perceptual priming is based on the form of the stimulus and is enhanced by the match between the early and later stimuli, and it is also sensitive to the modality and exact format of the stimulus (Wiggs and Martin, 1998; Squire, 1992). However, in perceptual priming, the presentation of prime stimulus alters the detection or identification of a subsequent probe stimulus and the effect is not a result of practice to discriminate features of the stimulus.

PL is the improvement in performance on a variety of simple sensory tasks, following practice. Such perceptual tasks range from simple discriminations along a single dimension to complex categorizations, which typically involve the integration of several dimensions. At one end of the spectrum, it can involve discrimination of visual orientation (e.g., Vogels and Orban, 1985; Shiu and Pashler, 1992; Schoups et al., 1995), auditory pitch (e.g., Fitzgerald and Wright, 2011; Recanzone et al., 1993), and tactile frequency (e.g., Jenkins et al., 1990; Recanzone et al., 1992). At the other end, perception of more complex forms, objects, and faces improves through practice (e.g., McLaren, 1997; Hussain et al., 2009; Espinet et al., 1999; Fine and Jacobs, 2000; Furmanski and Engel, 2000). Visual classification, such as sexing of young chickens (Biederman and Shiffrar, 1987), also improves as a result of training (Hock et al., 1987; Wills and McLaren, 1998). The improvement in the performance tends to persist over months, even years, and the changes are due to environmental inputs. Moreover, PL can be quite specific for the exact task trained.

Considering the specificity of learning among sensory modalities, henceforth only the visual PL will be discussed.

1.1 Visual perceptual learning

PL is the improvement in performance on a variety of simple sensory tasks, following practice. In visual perception, such tasks, often called discrimination tasks, involve identifying small differences in simple visual attributes, such as position, orientation, texture or shape.

PL has been documented in a wide range visual perceptual task: stimulus orientation discrimination (Vogels and Orban, 1985; Shiu and Pashler, 1992; Schoups et al., 1995; Matthews and Welch, 1997; Matthews et al., 1999), motion direction discrimination (Ball and Sekuler, 1982, 1987; Ball et al., 1983; Matthews and Welch, 1997), differences in the waveforms of two sinusoidal stimuli discrimination (Fiorentini and Berardi, 1980, 1981; Berardi and Fiorentini, 1987), detection of visual gratings (DeValois, 1977; Mayer, 1983) texture discrimination (Ahissar and Hochstein, 1996; Karni and Sagi, 1991, 1993), discriminate changes in spatial frequency within simple or complex plaid pattern (Fine and Jacobs, 2000), ability to detect small differences in the depth of two targets (Fendick and Westheimer, 1983; Westheimer and Truong, 1988), ability to perceive depth in random-dot stereograms (Ramachandran and Braddick, 1973), ability to discriminate between 10 band-pass Gaussian filtered noise texture (Gold et al., 1999a), ability to discriminate object (Furmanski and Engel, 2000) and face recognition (Gold, et al., 1999b). Training can improve the discrimination of small differences in the offset of two lines (Vernier acuity) even though initial thresholds are already in the hyperacuity range (McKee and Westheimer, 1978). In addition, a number of studies indicates that the spatial resolution of visual acuity can improve with practice up to the domain of hyperacuity (Poggio et al., 1992; Fahle and Edelman, 1993; Beard et al., 1995; Saarinen and Levi, 1995; Fahle and Morgan, 1996; Bennett and Westheimer, 1991).

Time course and longevity of visual perceptual learning

An important component of PL is the rate at which learning occurs. PL follows a time course, which can be specific for each task. For some visual tasks, the learning effects have been found to take place within an hour or two of training (Fiorentini and Berardi, 1980 and 1981; Shiu and Pashler, 1992; Fahle, Edelman and Poggio, 1995; Liu and Vaina, 1998). In some of these studies, learning is practically complete after a few hundreds of trials (Fiorentini and Berardi 1980, 1981), showing fast saturation. For other tasks, there is also an initial fast saturating phase of learning, followed by a slow phase where the performance continues to improve from one daily session to the next one until a stable optimal level is reached (Karni and Sagi 1991). Karni and Sagi (1993) also found that improvement between sessions occurs only if the two sessions are separated by at least six-eight hours, suggesting a consolidation period. Otherwise, some visual tasks showed a relatively long-term learning processes by requiring that training was carried out for at least four sessions or thousand of trials (Ball and

Sekuler, 1987; Fahle and Edelman, 1993; McKee and Westheimer, 1978; Mayer, 1983; Fendick and Westheimer, 1983; Matthews and Welch, 1997).

PL certainly gives rise to long-term memory, since improvement obtained through PL lasts for months (e.g, Ball and Sekuler, 1987; Fiorentini and Berardi, 1981) and, in certain tasks, the improvement in performance lasts for years. For example, in Karni and Sagi's experiments on texture discrimination (Karni and Sagi, 1993) subjects achieved a significant improvement in performance over four to five days. However, once subjects learned the task, they maintained their improved level of performance for at least three years without needing further practice.

Specificity and generalization of visual perceptual learning

Visual PL shows a high specificity for the features of the stimuli used in the task. Many studies reported that visual performances are typically improved on test trials using the same stimuli as those used during training, but these performances often return to baseline levels when test trials use stimuli mildly different from training stimuli. Specificity of learning was found for the orientation of lines or gratings (Fahle and Edelman, 1993; Fiorentini and Berardi, 1980; Fiorentini and Berardi, 1981; Karni and Sagi, 1991, Poggio et al., 1992, Ramachandran and Braddick, 1973; Schoups et al., 1995; McKee and Westheimer, 1978) or the direction of motion (Ball and Sekuler, 1987 and 1982) and for the retinal location of the stimuli used in the learning procedure (Ball and Sekuler, 1987; Fiorentini and Berardi, 1981; Karni and Sagi, 1991, Schoups et al., 1995; Shiu and Pashler, 1992).

Fiorentini and Berardi (1980) found that practice improved discrimination between complex gratings, but that improvement did not transfer to stimuli rotated by 90°. Poggio, Edelman and Fahle (1992) described a similar orientation specificity for a vernier discrimination task where observers had to indicate whether the right segment of a horizontal vernier was above or below the left segment, or whether the lower segment of a vertically oriented vernier stimulus was offset to the right or to the left relative to the upper segment. Both groups of observers improved performance significantly during training but their performance returned to base level when the stimulus was rotated by 90°. Similarly, the ability to discriminate similar directions of motion gradually improves with training, and is restricted to the trained direction and other similar directions. The gain in performance with learning at one location is lost when the task is moved more than a couple of degrees from the

trained location (Ball and Sekuler, 1987). Eventually, Ramachandran and Braddick (1973) reported that learning to perceive depth in stereograms consisting of obliquely oriented line elements does not transfer to stereograms whose elements are oriented at the orthogonal oblique direction. PL of complex grating discrimination is also selective for the chromatic attributes of the stimuli. Learning to discriminate gratings having a pure luminance contrast does not transfer to isoluminant gratings defined by a chromatic contrast, and moreover, learning does not transfer from gratings with a certain chromatic contrast to gratings with a different chromatic contrast (Fiorentini and Berardi, 1997; Morrone et al., 2004). In most cases PL is not restricted to the eye employed for training, if the training is monocular: learning transfers completely or partially to the untrained eye (Ball and Sekuler, 1982, Fiorentini and Berardi, 1981; Schoups et al., 1995; Beard et al., 1995) indicating that the learning process occurs centrally to the site where the inputs from the two eyes converge. Texture discrimination is exceptional, showing little interocular transfer of learning (Karni and Sagi, 1991, Schoups and Orban, 1996).

Retinotopy of PL is very precise in some cases. For instance, discrimination of complex gratings with different luminance profiles, is restricted to the trained area of 1° width, at a retinal eccentricity of 1°, and no transfer of learning effects is found for gratings located at 2° eccentricity flanking the trained area with no overlap (Berardi and Fiorentini, 1987). For discrimination of motion direction in random dot patterns there is some degree of transfer if the test stimuli overlap at least partially the trained area (Ball and Sekuler, 1987). In a texture discrimination task (TDT), where the location of the training stimuli varies within a retinal quadrant from one trial to the next, the learning has been proved to be restricted to the trained quadrant (Karni and Sagi, 1991). Shiu and Pashler (1992) found the same no interhemispheric transfer of PL for an orientation discrimination task. Lastly, improvement with a stereoacuity discrimination task presented in the periphery fails to transfer when the task is presented 3° away (Westheimer and Truong, 1988). Even if the visuotopic specificity of PL suggests involvement of early cortical stages, it is not so strict for all PL tasks. Learning in a three-line bisection task, for example, transfers to positions up to about 8° away, a distance larger than the size of receptive fields (RFs) in primary visual cortex (V1), opening the possibility of the involvement of longer range spatial interactions across V1 (Crist et al., 1997). Recently, Xiao et al. (2008) used a novel double-training paradigm that employed conventional feature training (e.g., contrast) at one location, and additional training with an irrelevant feature/task (e.g., orientation) at a second location, either simultaneously or at a

different time. They showed that this additional location training enabled a complete transfer of feature learning (e.g., contrast) to the second location.

Improvement of performance on visual discrimination tasks depends on the specific configuration of the elements composing the stimulus used for training and, therefore, it is specific not just for simple attributes but for complex shapes. For example, improvement on a specificity vernier acuity task fails to transfer between a stimulus composed of lines and one composed of dots (Poggio of et al., 1992). There is no transfer of learning between a bisection task, with the test stimulus composed of three dots configuration or composed of two similar lines placed in a side by-side configuration, and a vernier task, with stimuli composed of a configuration of dots or line in the same visual field location or with the same orientation. In the same manner, learning did not transfer to a number of discrimination tasks that were composed of stimuli with similar orientation and spatial position, though presumably both stimuli would have activated overlapping population of neuron in V1 area (Fahle and Morgan, 1996; Crist et al., 1997; Westheimer et al., 2001). Polat and Sagi (1994) found that contrast threshold for an oriented Gabor signal can be enhanced by positioning two high contrast flanking signals (masks) at a distance of about three times the target wavelength. These interactions were found only when the masks were placed in the direction defined by the target orientation or orthogonal to it. Then they showed that practice can increase the range of these interactions by a factor of six, but only along the cardinal directions. In this case, there is a facilitatory interaction between the Gabor elements and the mask element. Similarity, transfer of improvement in detection of difference in depth of stimulus element depends on the spacing of the element and upon their surrounding context (Fendick and Westheimer, 1983, Fahle and Westheimer, 1988, Westheimer and Truong, 1988).

Contrary to the dependence of PL on the orientation or the location of stimulus elements, the specificity of learning to more complex features requires a mechanism that is context dependent. Furthermore, the specificity or generalization obtained in a task depend on task difficulty (Ahissar and Hochstein, 1997; Liu and Weinshall, 2000; Liu and Vaina, 1998). Ahissar and Hochstein (1997) showed that the degree of specificity depends on the difficulty of the training conditions. In easy conditions, learning generalizes across orientation and retinal position, matching the spatial generalization of higher visual areas. As task difficulty increases, learning becomes more specific with respect to both orientation and position, matching the fine spatial retinotopy exhibited by lower areas.

Finally, visual training through reading text seems to modify the way people perceive printed words: reading-related training improves perception of words but not of non-words (Nazir et al., 2004). Moreover, Chung et al. (2004) found that PL can expand visual spans, and that this expansion is accompanied by an increase in maximum reading speed. Improvement generalizes to untrained retinal locations and is retained for at least three months following training.

1.1.1 Neural changes underlying visual perceptual learning

The specificity for orientation, position, and especially for the eye trained points to an early location in the visual system of the neuronal changes underlying PL. For some time, adult V1 was considered to be a hard-wired first stage of analysis (Marr, 1982), however more recent evidence points to plasticity even in the adult V1 (Gilbert et al., 2001; Gilbert and Wiesel, 1992; Chino et al., 1992; Eysel et al., 1998; Godde et al., 2002), supporting the hypothesis that PL may involve plasticity even in V1. However, it has been argued that perceptual improvement determined by PL could not be based exclusively on bottom-up processes permanently changing signal processing in V1 and that PL might take place at levels beyond V1, in spite of the high specificity for stimulus parameters (Mollon and Danilova, 1996; Morgan, 1992). The effects of PL would in this case be attributed to changes at higher levels of visual cortical information processing than V1.

Changes in the primary visual cortex

The selectivity of visual PL for basic attributes of the stimuli, such as orientation (Fahle and Edelman, 1993; Fiorentini and Berardi, 1980, 1981; Karni and Sagi, 1991; Poggio and Edelman, 1992; Ramachandran and Braddick, 1973; Schoups et al., 1995; McKee and Westheimer, 1978), motion direction (Ball and Sekuler, 1987, 1982) and even retinal location (Ball e Sekuler, 1987; Fiorentini and Berardi, 1981; Karni and Sagi, 1991; Schoups et al., 1995; Shiu and Pashler, 1992), suggests the involvement of early stages in cortical visual processing, where neurons have relatively small RFs and are selective for stimulus features such as orientation, size, chromatic properties, direction of motion, etc, and the visual topography is most precisely mapped. Cells in the early part of the visual pathway, particularly in V1, are highly selective for the orientation of visual contours (Hubel and Wiesel, 1959), and orientation is an important parameter in the columnar architecture of V1

(Hubel and Wiesel, 1977). At later stages of the visual pathway, cells have receptive fields with broader orientation tuning and visual areas are characterized by a functional architecture organized around more complex stimulus attributes (Tanaka, 2003). Therefore, forms of learning that show specificity for the orientation of the trained stimulus are likely to be mediated by mechanisms present at early stages of the visual pathways where a more accurate representation of the orientation of stimulus elements is present. After the convergence of inputs from the two eyes, which creates the binocular cells present in V1 and in subsequent areas, information about the eye of origin is most likely lost. Therefore, PL restricted to the eye used during monocular training would also suggest that the learning effects are mediated by mechanisms located at or before the level of input to V1. Indeed, a lack of interocular transfer has been reported in a few studies of PL (Karni and Sagi, 1991; Poggio et al., 1992). However, these reports are difficult to reconcile with the orientation specificity of the learning process, because orientation tuning first appears in cortical cells that are also binocular. However, it has been seen that, in other studies, PL did show interocular transfer (Fiorentini and Berardi, 1980, 1981; Ball and Sekuler, 1987; Schoups et al., 1995; Beard et al., 1995).

With regard to early visual representations, it has been suggested the involvement of V1 in humans. Using a neurophysiological method, Fahle and Skrandies (1994) found that the improvement of performance in a motion-detection task results in multichannel evoked-potential recordings that changed significantly in component latency. Significant differences between the potential distributions occur for potentials at latencies of less than 100 ms over the occipital pole, suggesting an involvement of plasticity in V1 of human adults. Pourtois et al. (2008) recorded the C1 component of visual evoked potentials (VEP), an evoked response in V1 implicated in visual early processing (Clark et al., 1995; Foxe and Simpson, 2002), while humans participants performed a Texture Discrimination Task (TDT) on targets presented at either the trained location or an untrained location. They showed that TDT learning can modify the early (<85 ms) sensory response evoked within visual cortex for stimuli presented at the trained location. Functional imaging studies have revealed increases in neuronal activity at the representation of the location in the visual field that is trained (Kourtzi et al., 2005; Yotsumoto et al., 2009; Yotsumoto et al., 2008). Furmanski et al. (2004) found that after subjects are trained to detect low-contrast oriented stimuli, a specific improvement in detection at the trained orientation and location is accompanied by an increase in the functional magnetic resonance imaging (fMRI) signals from V1. Changes in signals localized to V1 were also found after training on the more complex task of illusory

contour detection (Maertens and Pollmann, 2005). Using fMRI, Schwartz et al. (2002) measured neural activity 24 h after a single session of intensive monocular training on TDT, performed in one visual quadrant. They reported an increased response in calcarine cortex to target-texture stimuli presented to the trained eye, relative to stimuli presented at the same retinal regions in the untrained eye, demonstrating learning-dependent reorganization at early processing stages in the visual cortex of adult humans. They also reported that learning-dependent changes were not associated with an increased engagement of other brain areas remote from early visual cortex. In addition to measuring brain activity with fMRI, transcranial magnetic stimulation (TMS) has been used to disrupt activity either broadly in all visual areas or specifically in the V1. Neary et al. (2005) delivered a single pulse TMS to human occipital cortex at time delays of 70-154 ms after the onset of an odd-element, line orientation discrimination task. They found that, when TMS was delivered at a delay of 84 ms or later, the accuracy of visual discrimination was transiently degraded in subjects. Moreover, if visual PL improves the efficacy or strength of trained visual channels in occipital cortex, then it may concomitantly decrease the amount of transient TMS suppression of early visual processing; in fact they also demonstrated that while TMS impaired initial performance in line orientation discrimination, training on this task significantly reduced disruptive effects of TMS.

In non-human primates, electrophysiological studies of visual learning found an increase in neuronal sensitivity in V1 that is specific for relevant stimulus attributes (e.g., location, orientation) (Schoups et al., 2001), as well as a modulation of neuronal responses by contextual influences via intrinsic long-range horizontal connections (Crist et al., 2001). Schoups et al. (2001) linked the behavioural improvement in orientation-discrimination task to an improved neuronal performance of trained, compared to naive neurons. This improvement results in an enhancement of the tuning specificity of cells in V1 that are involved in discrimination of orientation. Another study (Crist et al., 2001) indicated that PL did not cause modification of the basic RF properties of V1, such as RF size or orientation tuning, but affected the contextual interactions, through long-range horizontal connections. Li et al. (2004) trained two monkeys to perform two visual discrimination tasks (bisection task or vernier task) based on different attributes of the stimuli. They found that neurons in the striate cortex changed their responses in a task-dependent manner, suggesting that RF properties in V1 are adaptive, and can change according to task demands. Li et al. (2008) reported that training monkeys in a contour detection task induces strong contour-related

responses specific to the trained retinotopic region. These responses were most robust when animals performed the task but disappeared under anesthesia, suggesting that even the process of contour integration in V1, which is generally believed to be a hard-wired process, can strongly depend on PL and top-down influences.

Few studies involving PL have been performed in rodents. Frenkel et al. (2006) described a form of experience-dependent response enhancement (stimulus-selective response potentiation SRP) in the visual cortex of awake mice. They found that repeated presentations of grating stimuli of a single orientation result in a persistent enhancement of responses evoked by the test stimulus. Response potentiation was specific to the orientation of the test stimulus, developed gradually over the course of several training sessions, and occurred in both juvenile and adult mice. The observation that SRP induced through the ipsilateral eye did not transfer to the contralateral eye, suggests a modification of synaptic transmission early in the visual pathway, before inputs from the two eyes are mixed. In addition, the cortical locus for SRP is also suggested by the fact that SRP is orientation selective, since orientation selectivity first emerges in V1. This is confirmed by experiments in which the administration of an NMDA receptor antagonist and the block of new insertion of AMPA receptors in V1 was shown to prevent SRP. SRP lacks however the active component of PL, being a passive viewing of stimulus presentation.

Changes beyond the primary visual cortex

The specificity of learning for basic visual features does not implicate that the representations of learning occur only in the early stage of the visual system. In fact, empirical evidence for V1 plasticity has sometimes been difficult to obtain and, when obtained, the extent of plasticity was often surprisingly small (Ghose et al., 2002). The cortical changes associated with PL can also occur in middle visual stages. For example, changes in the tuning properties of cells in V4 in monkeys were found after training on an orientation discrimination task, whereas no such tuning changes were observed in V1 (Yang and Maunsell, 2004, Ghose et al., 2002). Yang and Maunsell (2004) first demonstrated that PL modifies basic neuronal response properties at an intermediate level of visual cortex (V4). They found that orientation discrimination task changes the response properties of V4 neurons: after training, neurons in V4 with RFs overlapping the trained location had stronger responses and narrower orientation tuning curves than neurons with RFs in the opposite, untrained hemifield. Moreover, the changes were most prominent for neurons that preferred orientations close to the trained range

of orientations. Furthermore, some types of PL depend on perceptual constancy, i.e. the stable representation of certain properties of an object despite variable visual input (Garrigan and Kellman, 2008). In this case, PL may involve changes in early sensory analyzers, but such changes may, in general, be constrained by categorical distinctions among the high-level perceptual representations to which they contribute. Such representation is thought to occur in the middle visual stage.

The idea that changes associated with PL occur exclusively in early or middle visual areas has recently been challenged by the results of some neurophysiological studies in monkeys (Law and Gold, 2008; Chowdhury and DeAngelis, 2008). In one of these studies (Law and Gold, 2008), learning to evaluate the direction of visual motion did not change the responses of cells in the middle temporal area (MT), a region highly responsive to motion, but did change the responses of cells in the lateral intraparietal area (LIP), a region that is known to represent the transformation of visual motion signals into responses by saccadic eye movements. Despite that, changes in MT subsequent to PL have been reported. Zohary et al. (1994) studied the simultaneous activity of pairs of neurons recorded with a single electrode in MT while monkeys performed a direction discrimination task, exploring the relationship between inter-neuronal correlation and behavioral and stimulus parameters. They reported that spike counts from adjacent neurons were noisy and only weakly correlated but that even this small amount of correlated noise can affect signal pooling, suggesting a relationship between neuronal responses and psychophysical decisions.

Even in the inferotemporal (IT) cortex, known to be involved in object recognition, training can give cells a specificity for very simple features. Discrimination training leads to changes in response of properties of cells in IT (Sakai and Miyashita, 1994; Vogels and Orban, 1994). Sakai and Miyashita (1994) examined the hypothesis that form representation in IT is acquired through learning, using transformed computer-generated Fourier descriptors as visual stimuli in a discrimination task. They found that response selectivity of IT neurons can be influenced by visual experience: they proposed that the neuronal tuning mechanism in this area subserves category-related recognition that requires fine discrimination among similar items in each category. Vogels and Orban (1994) found that animals trained to discriminate the orientation of single lines will have cells in IT cortex that are selective for such stimuli.

1.1.2 Perceptual learning and top-down influence

As reported above, PL's improvement is often very specific for the task trained, precise stimulus orientation, stimulus position in the visual field, and the eye used during training (Fiorentini and Berardi, 1997). This specificity indicates location of the underlying changes in the nervous system at least partly at the level of V1 (Gilbert et al., 2001). Despite its specificity, PL is unlikely to rely only on permanent modifications of RF properties of 'early' cortical neurons (e.g., by sharpening their orientation tuning). Some studies find that PL can lead to involvement of higher visual areas (Sasaki et al., 2010). Thus, PL seems to rely on changes at a relatively early level of cortical information processing, such as V1, but under the influence of top-down selection and shaping influences.

The role of attention

Attention exerts a significant influence on many types of PL. An important role of conscious effort was demonstrated by research examining the effects of giving subjects feedback on the correctness of their responses on PL. The nature of effective feedback is interesting, because block feedback (that is when the percentage of correct response is given at the end of a block of *n* trials) is as effective as trial-by-trial feedback. Shiu and Pashler (1992), using a discrimination task with a pair of straight lines differing by 3°, found that subjects learned at the same pace with trail-by-trial feedback and with block feedback. Instead, in the condition with no feedback provided, observers did not show much improvement within each session but from one session to the next. On the other hand, fake or random feedback signals, without any correlation to the correctness of the response, effectively prevented improvement through training if observers assumed that they received correct error feedback (Herzog and Fahle, 1997).

Although feedback can facilitate learning, learning can occur in the absence of feedback. Fahle and Edelman (1993), and Herzog and Fahle (1997), using a vernier discrimination, found that the lack of feedback did not prevent learning but only slowed down the process. McKee and Westheimer (1978) obtained a practice effect in vernier acuity whether or not they gave their subject feedback. Ball and Sekuler (1987) also found that for cardinal directions of movement subjects' accuracy in direction discrimination improved at the same rate whether they were provided of trial-by-trial feedback or no feedback. A further indication that feedback is not necessary for learning comes from the results of Karni and Sagi (1991). In their experiments, subjects performed letter discrimination followed by texture

discrimination in the same complex stimulus. Their results show significant learning in the texture task, even though feedback was given only for the letter discrimination. These results strongly suggest that PL can proceed in an unsupervised manner, although at a slower pace.

Some studies of PL found that a conscious effort to direct focused attention plays a fundamental part in gating visual plasticity, suggesting that focused attention must be directed to a feature in order to be learned (Shoups et al., 2001; Shiu and Pashler, 1992; Herzog and Fahle, 1998; Gilbert et al., 2001; Ahissar and Hochstein, 1993). For instance, little or no transfer of learning effects was found between two tasks that involved judgments on different stimulus attributes (either orientation of local elements or global shape) of the same stimuli (Ahissar and Hochstein, 1993). It was also reported that the ability of subjects to discriminate the orientation of a line did not improve when brightness rather than orientation of the line was attended (Shiu and Pashler, 1992). Additionally, a single-unit recording study in monkeys found neuronal plasticity manifested as a change in the orientation tuning curves of V1 cells, with RFs overlapping the spatial location of the training task. No plasticity was found for cells with RFs overlapping the location of task-irrelevant stimuli presented at a different location from those relevant to the task (Shoups et al., 2001). Furthermore, some studies indicated that PL is task-dependent; learning of a particular feature did not transfer from the task on which the subject was originally trained to another task involving the same or similar stimuli but using a different procedure (Huang et al., 2007; Li et al., 2004). These findings can lead to the conclusion that conscious effort, such as focused attention to the task relevant feature during training (or to the task procedure), is necessary for the feature (or the task) in order to be learned.

However, recent evidence from studies of "task-irrelevant" learning (Seitz and Watanabe, 2003; Watanabe et al., 2001; Nishina et al., 2007) show that PL can occur in the absence of focused attention to the learned feature. In the Watanabe's study (2001), subjects were asked to identify a letter in the centre of a display, while the background motion, consisting of moving dots, was presented in a peripheral field. The weak background motion signal was below their perceptual thresholds (only 5% of the dots moved coherently and the remaining dots moved randomly). The subthreshold background motion was also irrelevant to a central task that engaged the subject's attention. The percentage of dots moving coherently was so small that subjects were able to discriminate or detect the coherent-motion direction with only chance-level performance before (pre-test) and after (post-test) the exposure period. Nevertheless, when subjects were subsequently tested with a supra-threshold (10%) coherent

motion display, their ability to discriminate or detect coherent motion was enhanced for motion in the direction to which they were previously exposed. So, despite being below the threshold of visibility and being irrelevant to the central task, the repetitive exposure improved performance specifically for the direction of the exposed motion when that direction was subsequently tested. A follow-up study demonstrated that this task-irrelevant learning was highly specific to local motion of the exposed stimuli, as opposed to the global motion, and that learning was retained for months after training (Watanabe et al., 2002). These findings indicate that focused-attention is not necessary for PL, but task-irrelevant learning might not occur simply as a result of exposure to a stimulus.

Recently, Seitz and Watanabe (2003) found that a sensitivity enhancement occurred as the result of temporal pairing between the presentation of a subliminal, task-irrelevant, motion stimulus and a task-target. The four different directions of motion were presented an equal number of times during the exposure stage of the experiment, with a 'designated direction' always being 'paired' with the targets of the letter-identification task and the other directions being randomly associated with the letter-task distractors. If PL were due only to passive exposure, motion direction thresholds should improve equally for all of the presented directions; but if learning occurs only for features to which attention is directed, no improvement should be found for any presented direction. In fact, learning was found only for the motion direction that was temporally-paired with the task targets, not for the other motion directions. These results have to be reconciled with the studies that found no learning outside the focus of attention. The solution could be that the studies failing to find task irrelevant learning lacked a consistent relationship between the task-irrelevant features and the target presentation. For example, in Ahissar and Hochstein (1993), two different orientations (vertical/horizontal) of the global texture array (the task-irrelevant features) were paired with the target display and the blank display with equal probability. In Shiu and Pashler (1992), each of two line orientations (the task-irrelevant feature) could be paired with each of two degrees of line brightness (the task-relevant features). Task-irrelevant learning was not observed in these studies. By contrast, in Watanabe et al. (2001 and 2002), when a target was presented, the same directional coherent motion was present. In Seitz and Watanabe (2003), a unique motion direction was consistently paired with a target whereas three other directions were paired with distractors. Task-irrelevant learning was observed in these studies. These results suggest that a necessary condition of task-irrelevant learning is for the task-irrelevant feature to uniquely coincide with the target presentation.

Seitz and Watanabe (2005) proposed a model for task-irrelevant learning that can also explain task-relevant learning. PL occurs through the coincidence of diffusive signals driven by a task activity (reinforcement signals) and signals induced by the presentation of a stimulus (stimulus-driven signals). On the one hand, if a task target and a task-irrelevant feature are presented with an appropriate temporal relationship, such as temporal coincidence, then task irrelevant learning occurs. On the other hand, because the task target itself causes both reinforcement signals and stimulus-driven signals, these two signals always temporally coincide and result in task-relevant learning. These two different signals could fit with the attentional framework proposed by Posner (Posner and Petersen, 1990). This framework is composed of different attentional subsystems and each one has distinct effects on stimulus processing. The alerting, orienting and executive function are dissociable subsystems and can replace the vague concept of attention. The alerting system controls a non-specific arousal state; the orienting system directs resources to a specific spatial cue or feature; the executive control system is involved in solving a task involving conflict. The orienting and executive control systems might be more selective to regions of space (spatial attention), individual features (feature-based attention) or objects (object-based attention) regarded to be task-relevant items. During alerting, a phasic but non-specific signal increases general processing based on the time during which important stimuli are thought to be present. Thus, when a subject is performing a task, the orienting attention subsystem directs attentional resources to the location of the task target. This orienting signal aids in target detection. The alerting attention subsystem is activated by a temporal cue, such as the occurrence of the task target, and serves as a temporal signal that enhances processing of a large extent of the scene, including the task-irrelevant features.

Each of these attention subsystems has been linked with different neuromodulatory signals (Fan et al., 2002): orienting with the acetylcholine system (Davidson and Marrocco, 2000), alerting with the norepinephrine system (Coull et al., 1996; Marrocco et al., 1994; Witte and Marrocco, 1997) and executive with dopamine (Fossella et al., 2002). A considerable amount of behavioral and neurophysiological data show that learning is formed as a result of diffusely released modulatory neurotransmitters, which have been shown to result in sensory plasticity (Schultz, 2000; Dalley et al., 2001). For instance, pairing a tone with stimulation of the ventral tegmental area, which releases dopamine (Schultz, 2000), results in increased representations of the paired tone in the primary auditory cortex (A1) (Bao et al., 2001). Similarly, pairing a tone with stimulation of the nucleus basalis of the basal

forebrain, which releases acetylcholine, results in an increased representation of the paired tone in A1 (Kilgard and Merzenich, 1998). Likewise, norepinephrine, which is released from the locus coeruleus, has been shown to be involved in learning both at the behavioral (Dalley et al., 2001; Usher et al., 1999) and neuronal level (Gordon et al., 1988).These findings suggest that PL might be regulated through the release of neuromodulators, such as acetylcholine, norepinephrine, and dopamine, which gate learning and thus restrict sensory plasticity and protect sensory systems from undesirable plasticity.

For a better understanding of the role of neuromodulatory signals in PL, Seitz et al. (2009) developed a reward-learning technique for studying human visual learning, in which human subjects, deprived of food and water, passively viewed visual stimuli while receiving occasional drops of water (reward) paired with the "trained orientation" in a effective temporal relationship. Using this procedure, Seitz and coworkers could examine the hypothesis that reward-related learning signals are sufficient to cause improvements in visual sensitivity for visual stimuli paired with rewards. Thus they reported that stimulus-reward pairing is sufficient to cause learning even in the absence of awareness of the learned stimuli or stimulus-reward contingencies, demonstrating that visual learning in humans can be driven by reward signals and the dopamine might be the candidate mechanism that may underlie the learning.

The idea of a diffusive reinforcement signal has been used also in another model developed by Roelfsema and van Ooyen (2005). The Attentional-gated reinforcement learning (AGREL) is a reinforcement learning model that learns by trial and error. This model is based on two factors that determine synaptic plasticity: (i) a reinforcement signal that is homogeneous across the network and depends on the amount of reward obtained after a trial, and (ii) an attentional feedback signal from the output layer that limits plasticity to those units at earlier processing levels that are crucial for the stimulus-response mapping. Therefore, AGREL uses, to guide the learning, a global neuromodulatory signal that informs all synapses whether the outcome of a trial is better or worse than expected, and a complementary feedback signal from the response selection stage that restricts plasticity to those synapses that were responsible for the behavioral choice. The network receives a reward for a correct choice, whereas it receives nothing if it makes an error. After the action, neuromodulators are released into the network to indicate whether the rewarded outcome is better or worse than expected. If the network receives more reward than expected, the neuromodulators cause an increase in strength of the connections between active cells, so that this action becomes more

probable in the future; the opposite happens for actions with a disappointing outcome. The second signal is the attentional feedback during action selection that ensures the specificity of synaptic changes. Although the neuromodulators are released globally, the synaptic changes occur only for units that received the attentional feedback signal from the response selection stage during action selection. AGREL causes feedforward and feedback connections to become reciprocal, in accordance with the anatomy of the cortico-cortical connections. Consequently, the neurons that give most inputs to the winning action also receive most feedback. As a result, only sensory neurons involved in the perceptual decision change their tuning. In this case, attention to a feature determines which representations undergo plasticity and which do not. The most likely route is through feedback connections that run from the higher areas back to lower areas of the visual cortex.

Theories about the mechanisms for learning can appear to be contradictory in explaining why the task irrelevant PL was found in some studies but not in others. It is evident that the models agree about the role of neuromodulatory signals but also that they appear to contradict each other regarding the role of selective attention. Some studies demonstrated an important role for attention in learning whereas others demonstrated learning for unattended, irrelevant and even imperceptible stimuli. However the neuromodulatory reward signals and selective attention can act in concert to implement learning rules in the cortex: the neuromodulatory signals reveal whether the outcome of a trial is better or worse than expected, while the attentional feedback signal highlights the chain of neurons between sensory and motor cortex responsible for the selected action. While AGREL stresses the importance of attention, the model by Seitz and Watanabe indicates that the coincidence of a visual feature and an internal or external reward is sufficient for learning. Roelfsema et al. (2011) proposed that the attentional feedback signal that enhances plasticity of task-relevant features in the visual cortex also causes inhibition of task-irrelevant features so that their plasticity is switched off; in this way, they have combined attentional learning theories, like AGREL (Roelfsema et al., 2005), and theories that emphasized the importance of neuromodulatory signals, like the model of Seitz and Watanabe (Seitz et al., 2005), into a single framework. It has been further proposed that stimuli that are too weak to be perceived escape from the inhibitory feedback signal so that they are learned if consistently paired with the neuromodulatory signal. This proposal can explain why studies using stimuli close to or below the threshold for perception observed task-irrelevant PL whereas studies using suprathreshold stimuli invariably implicate selective attention in learning. A recent study

(Tsushima et al., 2008) directly compared task irrelevant learning for a range of stimulus strengths and indeed observed that learning only occurred for motion strengths at or just below the threshold for perception but not for very weak or strong stimuli. The very weak motion signals are not learned because they hardly activate the sensory neurons; the strong motion signals could interfere with the primary letter detection task and are therefore suppressed by the attentional feedback that also blocks plasticity. Threshold stimuli, however, might stay "under the radar" of this attentional inhibition mechanism so that they are not suppressed and can be learned if consistently paired with the neuromodulatory signal.

Recent results of Tsushima, Sasaki and Watanabe (2006) provide further support for this view. They measured the interference caused by irrelevant motion stimuli in a letter detection task and found an unexpected dependence on signal strength. Weak motion stimuli interfered more than suprathreshold motion stimuli, and an fMRI experiment revealed that they caused stronger activation of motion sensitive area MT+. The enhanced activation of MT+ by threshold stimuli can be explained by the involvement of dorsolateral prefrontal cortex (DLPFC), a region that generates attentional inhibition signals. The suprathreshold stimulus activated DLPFC, which then suppressed MT+, whereas the threshold stimulus did not. These psychophysical and fMRI results, taken together, indicate that weak motion signals can indeed escape from the attentional control system so that they can be learned.

Further evidence for how attention can suppress task irrelevant PL is shown by Choi et al. (2009). Subjects performed a task reporting the direction of arrows, which were presented above the fixation point. These arrows served as exogenous attentional cues directing subjects' attention towards the location to which the arrows pointed. Task irrelevant dynamic random dot motion stimuli where displayed at the left and right of fixation at subthreshold levels of motion coherence. One direction of motion was always presented at the location to which the arrows pointed (attended motion display) and another direction of motion was presented at the location away from which the arrows pointed (unattended motion display). Learning was found only for the unattended motion display, not for the attended one.

The Reverse Hierarchy Theory

The top down-driven learning component is a crucial factor in the Reverse Hierarchy Theory (RHT) proposed by Ahissar and Hochstein (2002 and 2004). The RHT of PL states that perceptual improvement largely originates from a gradual top-down-guided process which begins at high-level areas of the visual system, and progresses backwards to the input

levels; this process is subserved by a cascade of top-to-bottom level modifications that enhance task-relevant and prune irrelevant information through a better signal-to-noise ratio.

As repeatedly stated, the visual system is organized in a serial, hierarchical pathway: the cortical areas processes basic visual features information and progressively generalize over these parameters and become responsive to more and more complex stimuli such as objects, categories and concepts. If PL affects performance, it must modify representations at one or more of these levels. If modification occurred at low-levels, improvement of learning would not transfer to new stimulus conditions and the subject performance would be degraded towards initial levels, requiring a process of re-learning. On the other hand, if learning resulted from high-level modifications, it would largely transfer to novel positions and orientations. Ahissar and Hochstien (1993) concluded that easy task conditions (with large signal-to-noise ratios) are learned at higher cortical levels along the visual pathways, where RFs generalize across position and orientation. Difficult conditions, on the other hand, are learned at lower-levels where RFs are more specific to both retinal position and orientation. In the model, initial vision at a glance depends on high-level object and category representations. In this way, initial high-level learning transfers over basic stimulus parameters. Later vision with scrutiny is a return to simple feature details available at low levels, with the low-level modification guided by feedback connection. An important RHT prediction is that PL will depend on task-specific attention, and many studies, using 'pop-out' detection (Ahissar and Hochstein, 1993), orientation and texture discrimination (Karni and Sagi, 1993; Shiu and Pashler, 1992), and vernier acuity (Herzog and Fahle, 1997) are consistent with this prediction. However, recent studies (Watanabe et al., 2001; Seitz and Watanabe, 2003) pointed out the possibility of a PL without attention to the task. In order to respond to this possibility, Ahissar and Hochstein (2004) observed that bottom-up induced modifications are possible, but they also claimed that it is the practice in a perceptual task that leads to a top-down cascade of weight retuning.

The RHT proposes a gradual transition from the naïve (untrained) performers, governed by representations at the top of the visual hierarchy without changes on low-level representations, to the expert performers, in which the low-level changes were chosen by top-down guidance. The idea that naïve perception reflects high levels, whereas trained performance with respect to local attributes is more closely related to lower level activity has received strong support from recent fMRI findings in humans (Schwartz et al., 2002; Furmanski et al., 2004; Sigman et al., 2005; Mukai et al., 2007). For example, Sigman et al.

(2005) found that when observers were trained to search for a local T-shaped target, initial performance level was correlated with activity in a higher order area, whereas subsequent performance was correlated with activity at earlier, retinotopically organized, areas. Similarly, Furmanski et al. (2004) showed that after practicing for a month on the detection of low-contrast oriented patterns, V1 response for the practiced orientations significantly increased.

Interaction between feedback connections and local cortical circuits: possible role of long-range horizontal connections

Gilbert and Li (2009) proposed that functional changes, associated with PL, can involve both long term modification of cortical circuits during the course of learning, and short term dynamics in the functional properties of cortical neurons. In this view, top-down influences of attention, expectation and perceptual task interact with the experience-dependent modification at the early level of the visual system. A key to understanding the nature of experience dependent changes in the visual cortex is the higher order, context-dependent properties of visual cortical neurons. The basic property of orientation selectivity discovered by Hubel and Wiesel (1959 and 1962) is a framework upon which one can understand their selectivity for more complex features. If one uses a simple stimulus such as an oriented line as a visual stimulus to map a neuron's RF, the measured extent of the RF is quite small. However, both anatomical and physiological evidence shows that even in V1, neurons integrate information over relatively large parts of the visual field, much larger than what one would expect by RF maps obtained with single short line stimuli. Cortical pyramidal cells have axonal arbors that extend for distances up to 8mm parallel to the cortical surface (Gilbert and Wiesel, 1979; Rockland and Lund, 1982; Gilbert and Wiesel, 1983; Stettler et al., 2002). This means that their targets are capable of integrating information over an area of visual space much larger than their RFs as measured by a single line stimulus. The horizontal connections also show a specific relationship with the cortical orientation columns. Moreover, lateral connections account for the majority of the inputs that neurons receive, with over 76% of excitatory inputs arising from outside their resident hypercolumn (Stepanyants et al., 2009). These long range connections endow neurons with selectivity for features more complex than the ones predicted from their response to a single line stimulus placed in and around their RFs. Their responses are context dependent, and the contextual influences play a role in contour integration as well as in sensitivity to the configuration of complex stimuli. These horizontal connections features fit with the perceptual characteristics of contour integration, as seen in

the phenomenon of contour saliency. If a contour made of a series of collinear line segments is embedded in a complex background of randomly oriented and positioned line segments, it will easily be distinguished from the background, and will 'pop out'. Contour saliency is subject to PL: practice on this detection task can lead to an improvement in the ability to detect contours, so that observers can detect contours composed of fewer line segments, or contours with greater separation between the constituent line segments (Li and Gilbert, 2002; Li et al., 2008). The neuronal properties related to contour detection are subject to task-dependent top-down influences: Li et al. (2008) observed that monkeys showed no detection contour facilitation when they attended to the contour location, but were not performing the contour detection task. Thus, the top-down influence required for facilitation is not spatial attention but the task of contour detection itself. Moreover, as previously described, learning effects depend on the precise configuration of the stimulus used in training (Fahle and Morgan, 1996; Crist et al., 1997; Westheimer et al., 2001). This leads to the hypothesis that the response characteristics of neurons, even in V1, are as dependent on the behavioural context as they are on the visual stimuli themselves. Furthermore, information conveyed by top-down influences is not simply attention to the location of the stimulus, but also information about the task that the animal is performing. Thus functional significance of neuronal responses change on the moment, according to the perceptual tasking being executed. However learning affects only the subset of neuronal inputs that are active under a specific stimulus context, and neuronal responses are unaffected when a different context is presented. Therefore, training on one stimulus configuration does not influence neuronal responses to other configurations. Gilbert and Li (2009) proposed that many contextual influences can be mediated by long range horizontal connections within each cortical area, and that the top-down effects select the lateral inputs, according to context. In fact the functional selectivity of the horizontal connections is not fixed, but can be selectively expressed on the moment, according to task demands. This allows the same population of neurons to mediate multiple tasks. In this view, PL involves establishing an interaction between cortical feedback and intrinsic connections, in order to address the appropriate subset of horizontal inputs by the feedback connections that are active during the execution of a given task (Gilbert and Sigman, 2007).

1.1.3 Possible cellular mechanisms underlying perceptual learning

Despite recent progress in localizing the visual areas involved in PL, elucidation of the mechanisms at the cellular level remains a challenge. Learning is supposed to rely on changes in neuronal circuits in brain areas specific for the practiced task, leading to long-lasting modifications in synaptic efficacy (synaptic plasticity). While the notion that synaptic plasticity underlies learning is widely accepted for declarative memory processes mediated by temporal lobe areas or for implicit forms of memory such as classical conditioning (Kandel, 2009), the specific role of synaptic plasticity in PL, a form of implicit memory, remains unclear. It has been shown that skill motor learning leads to long-lasting synaptic plasticity changes in the primary motor cortex (M1) (Rioult-Pedotti et al., 2000) and, in the visual system, changes in V1 activity have been documented following visual PL both in monkeys and humans (e.g., Schoups et al., 2001; Li et al., 2008; Yotsumoto et al., 2008). At present, however, there is no conclusive evidence for the presence of synaptic plasticity phenomena in V1 in correlation with visual PL.

Several possible cellular mechanisms have been proposed to account for the effects of PL. One possibility is that the number of neurons representing the learned stimulus increases after training; this mechanism has been found mainly in the auditory (Recanzone et al., 1993) and somatosensory (Recanzone et al., 1992) cortex. In the visual system, PL appears to be mediated primarily by changes in the response strength or tuning of individual neurons, rather than large-scale spatial reorganization of the cortical network, as found in the auditory and somatosensory systems.

When Schoups et al. (2001) examined changes in V1 orientation tuning accompanying improved performance in orientation discrimination in adult monkeys, they found no increase in the proportion of neurons tuned to the trained orientation. Instead, there was an increase in the slope of the tuning curve at the trained orientation for neurons whose preferred orientations were 10°–20° from the trained one. The authors suggested that since the firing rates of these neurons are most sensitive to small changes near the trained orientation, they may be the most relevant for the learned discrimination task. Thus, tuning sharpening can lead to improved discrimination of trained attributes, particularly for those cells for which the steepest parts of tuning curves coincide with the value of trained attribute. By sharpening tuning, neurons decrease the overlap in their responses to a range of different stimuli. In contrast, a similar study by Ghose et al. (2002) found that PL caused little change in the response properties of V1 and V2 neurons, aside from a small reduction in the response

amplitude of the cells tuned to the trained orientation. They suggest that the psychophysical change is mediated by top-down influence for the trained task, and not by an improved neural representation of orientation in early visual areas. The differences between these two studies at the physiological level could be related to their differences at the psychophysical level. For example, it has been shown that the difficulty of the visual task can affect the extent of V1 involvement in learning (Ahissar and Hochstein, 1997). Thus, differences in the exact task design could lead to differences in the underlying visual areas. Notably, the learning process observed by Schoups et al. (2001) was eye and location specific, which is consistent with a neural change in early visual areas. In contrast, Ghose et al. (2002) found transfer of the perceptual improvement between eyes and across retinotopic locations. Thus, their results do not necessarily argue against V1 as the locus for spatially specific PL.

Another form of PL was found to be associated with changes in the contextual modulation of V1 responses (Crist et al., 2001; Li et al., 2004). After training in a three-line bisection or vernier task, monkeys showed significant improvement in determining the location of the middle test line relative to the reference lines. Similar to the case of orientation discrimination (Schoups et al., 2001; Ghose et al., 2002), the perceptual improvement was not accompanied by any obvious change in basic V1 RF properties such as location, size, or preferred orientation. Instead, there was a significant change in contextual modulation. The responses of the neurons near the trained retinal location showed higher sensitivity to the positions of the line stimuli outside of the classical RF, and this effect existed only when the monkey was performing the relevant task. The mechanism of this specific, context-dependent learning may involve a modulation of subsets of horizontal inputs to a cell, at a more refined level than that observed following retinal lesions. In this view, modulation of inputs from cells with RFs arrayed along an axis perpendicular to the target line could account for specificity in the localization to references placed along that axis as opposed to those placed along the colinear axis. This modulation of subsets of inputs would permit both specificity in learning to a particular stimulus configuration and storage of information about a large number of visual discrimination tasks, without having the multiple learned tasks interfering with one another and allowing for retention in the learning for an extended period of time.

While in primates the neural substrate involved in PL may have a deep dependence on training specificity, in rodents the relationship between learning and neural changes may be more simple. As previously described, Frenkel et al. (2006) found that repeated exposure of awake mice to stimuli of a certain orientation induced a specific potentiation of the V1

response to the trained orientation. This is consistent with the cortical change expected for PL, although no performance of any task was required for the effect. Interestingly, such cortical change observed in the mouse is more similar to the training-induced increase in fMRI response in the human visual cortex (i.e., Furmanski et al., 2004) than to the effects measured with single-unit recordings in monkey V1. In addition to changes in their visual responses, cortical neurons may also develop sensitivity to nonvisual inputs that are paired with visual stimuli in a learning task. After training freely moving rats in a task that associates different reward times with visual stimuli to the two eyes, a significant proportion of visual cortical neurons developed firing patterns that are correlated with the expected reward time (Shuler and Bear, 2006).

If PL were to promote neural plasticity in early visual areas, possibly determining the potentiation of the visual connections active during learning, it could be exploited to facilitate recovery from conditions in which deficits in a set of visual neural connections leads to visual impairments. In the last two decades, there has been a progressive increase in studies that have tested and developed visual rehabilitation programs based on PL. I shall now discuss the possible application of PL in treating amblyopia.

Chapter 2

Amblyopia

The development of brain circuitry in the visual system depends on the interaction between genetic programs and experience-driven plasticity processes (Goodman and Shatz, 1993; Katz and Shatz, 1996). Before the time of eye opening, targeting of visual connections is controlled by genetic programs and spontaneous activity (Galli et al., 1988; Crowley and Katz, 1999; Sur and Leamey, 2001; Crowley and Katz, 2002; Sur and Rubenstein, 2005). Sensory experience is required for a proper development of the visual system: the environment drives the refinement of neural circuitries, selecting the correct inputs from a wider array of possibilities (Weliky, 2000; Lewis and Maurer, 2009). There are time windows in early postnatal life, named critical periods (CPs), during which plasticity is enhanced and neural circuits display a heightened sensitivity to acquire instructive and adaptive signals from the external environment. In these periods, proper experience is required at fixed developmental times and results in irreversible changes in brain function (Knudsen, 2004). Sensitive periods for experience-dependent plasticity occur in virtually every species, from Drosophila to humans (Berardi et al., 2000). The existence of CPs for experience-dependent plasticity has been demonstrated for the visual, auditory and somatosensory systems, but also for many other functions, including song in birds and language in humans (Berardi et al., 2000; Hensch, 2004; Doupe and Kuhl, 1999; Doherty, 1997). Interestingly, CP duration is tightly correlated with average life expectancy (Berardi et al., 2000).

It is now clear that there are different sensitive periods for different functions (even within the same sensory system; e.g. Harwerth et al., 1987, 1990), and for different parts of the brain (even within different layers of the primary visual cortex, V1; Levay et al., 1980), and distinct sensitive periods for recovery from and for induction of sensory deprivation effects (Berardi et al., 2000). However, the CP is not a simple, age-dependent maturational process but is rather a series of events itself controlled in a use-dependent manner. There is evidence that a total absence of sensory input leads to a delay in the functional and anatomical

maturation of the visual cortex, that appears still immature far beyond the end of the CP. For example, the visual cortex of adult animals reared in darkness from birth (dark rearing, DR) displays serious physiological deficits including reduced orientation and direction tuning, lower cell responsiveness and increased latency, larger RF sizes, altered spontaneous activity, rapid habituation to repeated stimulus presentation, immature ocular dominance (OD) distribution and lower visual acuity (Fregnac and Imbert, 1978; Timney et al., 1978; Benevento et al., 1992; Fagiolini et al., 1994; Pizzorusso et al., 1997). Moreover, animals reared from birth in complete darkness express a delayed critical period time course with plasticity persisting into adulthood (Fagiolini et al., 1994; Iwai et al., 2003; Mower, 1991).

It has been known for a long time that a modification in visual experience, during the CP, caused by unbalanced inputs from the two eyes leads to the development of a pathology known as *amblyopia.*

Amblyopia (from the Greek, *amblyos*-blunt; *ops*-vision), also called "lazy eye", is one developmental abnormality usually associated with physiological alterations in the visual cortex occurring early in life (Ciuffreda et al., 1991; Holmes and Clarke, 2006). In humans, this pathology occurs in approximately 1-5% of the population and the presence of amblyopia is generally associated with an early history of abnormal visual experience: binocular misregistration (strabismus), image degradation (high refractive error and astigmatism and anisometropia) or form deprivation (congenital cataract and ptosis). Strabismus is by far the most common contributing factor. Refractive errors, the second most common contributing factor, can be difficult to detect. Amblyopia resulting from refractive error, is divided into two types: anisometropic and isometropic. Anisometropic amblyopia develops when the refractive errors in the two eyes are unequal. Severe refractive errors in both eyes, even if symmetric, may cause bilateral isometropic amblyopia, especially if optical correction is delayed. Finally, the rare amblyogenic condition called congenital or early-acquired media opacity, causes a form of amblyopia called deprivation amblyopia, the most severe and damaging type of amblyopia. In this case cataracts, corneal lesions, or ptosis block or distort retinal image formation.

Regardless of the etiology, amblyopia is usually unilateral: the visual acuity of one eye is reduced with respect to the other eye. Associated symptoms include poor stereoscopic depth perception, low contrast sensitivity, and reduced motion sensitivity. The damage produced by amblyopia is generally expressed in the clinical setting as a loss of visual acuity in an apparently healthy eye, despite appropriate optical corrections; however, there is a great

deal of evidence showing that amblyopia results in a broad range of neural, perceptual and clinical abnormalities (Barrett et al., 2004; Kiorpes, 2006; Levi, 2006). Despite some early indications that the retina may be the primary site of amblyopia (Hess, 2001), the preponderance of evidence from animal studies and human electrophysiological investigations has firmly established that the retina and the LGN demonstrate essentially normal physiological function in the presence of amblyopia. The current consensus is that the primary site of neural loss in amblyopia is found at the level of the striate or V1.

In primates, the first sign of impaired cortical function resulting from prior visual deprivation has been seen in the input layers (IVc) of the striate visual cortex (Blakemore and Vital-Durand, 1986). Following these initial studies, there has been an increasing evidence that the striate cortex is neurologically anomalous in amblyopia (Hess, 2001). Early visual evoked potential (VEP) studies routinely observed reduced and distorted VEPs above the occipital lobe (Levi and Harwerth, 1978), indicating that human amblyopia affected striate or extrastriate cortex. More recent quantitative evaluations using fMRI have shown that areas of the striate cortex responding to input from the amblyopic eye display reduced neural activity (Barnes et al., 2001).

Clinicians are aware that amblyopia does not develop after 6-8 years of age (Worth, 1903; von Noorden, 1981) suggesting that there is a "sensitive period" for the development of the disease. The notion that there is a sensitive period (or periods) for the development of amblyopia has often been taken to indicate that there is also a CP for the treatment of this pathology. Lewis and Maurer (2009) suggest the existence of three sensitive periods. "The period of visually driven normal development" is the classic definition of the sensitive period, during which there are developmental changes in an organism raised with visual input that do not occur if the visual input is missing. However, for some aspects of vision, abnormal visual input can have a permanent deleterious effect even when the abnormal input starts after the system acquired an adult like functionality. Thus, a second sensitive period, called "sensitive period for damage", indicates the time of vulnerability and it could also occur after completion of normal development. A third sensitive period is the time during which the visual system displays the potential to recover from the deleterious effect of deprivation; this period is also known as "the sensitive period for recovery". To examine these sensitive periods in humans, the visual development of normal children has been compared to that of children who were deprived of visual experience at some point during development because they were born with, or developed, cataracts in one or both eyes.

The specific timing of the sensitive period of visual development varies depending on the specific aspect of vision being considered. Visual capabilities continue to improve after early infancy, but the age at which children's vision is as good as adults' varies widely with the aspect of vision under study. For example, by 6 to 7 years of age, children are as accurate as adults on measures of acuity, contrast sensitivity, holistic and featural face processing, and sensitivity to global motion (Ellemberg et al., 1999a; Ellemberg et al., 2002; Mondloch et al., 2002; Mondloch et al., 2007; Parrish et al., 2005). In contrast, at age 6, children are not as accurate as adults on sensitivity to biological motion (Freire et al., 2006) and sensitivity to global form (Lewis et al., 2004). On some measures such as distinguishing faces based on the spacing of internal features and integrating elements with a similar alignment when presented in a background of noise, even 14-year old children are not as accurate as adults (Kovacs et al., 1999; Mondloch et al., 2003).

Early visual input is necessary to preserve the neural infrastructure for later visual learning, even for visual capabilities that will not appear until later in development. Visually normal young infants see only low spatial frequencies (Atkinson et al., 1977; Banks and Salapatek, 1978) and they do not begin to see high-spatial frequencies of 20 cpd, even at maximum contrast, until at least 2 years of age (Mayer et al., 1995). Yet, most children born with cataracts in one or both eyes whose deprivation ended within the first 6 months of life later fail to develop normal sensitivity to those high-spatial frequencies in the aphakic eyes (Ellemberg et al., 1999b; Ellemberg et al., 2000). Critical flicker fusion frequency -the fastest rate of high-contrast flicker that can be perceived- is adult-like at 2 months of age, (Regal, 1981) whereas grating acuity, the narrowest high contrast stripes that can be differentiated from gray, is not adult-like until 4 to 6 years of age (Mayer and Dobson, 1982; Ellemberg et al., 1999a). Visually normal infants appear to process faces in a piecemeal fashion with the first evidence of a type of holistic processing at 4 months of age (Cashon and Cohen, 2003; Cashon and Cohen, 2004) and of sensitivity to large differences in the spacing of features at 5 months of age (Bhatt et al., 2005). Yet, children born with cataracts in both eyes whose deprivation ended by 2 to 3 months of age fail to later develop normal holistic face processing or sensitivity to spacing of features (Le Grand et al., 2001; Le Grand et al., 2004).

Despite its symptoms, amblyopia is often diagnosed in routine ocular examination and eye tests, and children (and parents and teachers, etc.) may not be aware of the condition because of compensation by the other eye. Currently, there is no positive diagnostic test for amblyopia. Instead, amblyopia is diagnosed by exclusion: the diagnosis of unilateral

amblyopia is made when reduced visual acuity is recorded in the presence of an amblyogenic factor, despite optimum refractive correction (i.e., best-corrected visual acuity) and not explained by another ocular abnormality. The evaluation can begin with an ocular history based on the patient's age and parental observations, and a vision assessment should be performed as part of the physical examination. The eyes and eyelids should be inspected for ptosis, corneal lesions, and cataracts. The ocular alignment should be examined carefully. A critical component of amblyopia diagnosis is the measurement of visual acuity. In newborn to two years children, the visual acuity assessment relies on preferential looking techniques (Teller acuity cards) (Getz et al., 1996), Kay pictures (Kay, 1983), and Cardiff cards (Hazell, 1995). Children older than three years can complete optotype visual acuity testing (identifying symbols or letters), allowing quantification of visual acuity on a Snellen scale: in general, the tumbling E, HOTV, Lea and Allen charts should be used for children three to five years of age, and Snellen letters or numbers should be used for children six years and older (Committee on Practice and Ambulatory Medicine, Section on Ophthalmology, 2003). The severity of amblyopia appears to be associated with the degree of imbalance between the two eyes (e.g. dense unilateral cataract results in severe loss of visual acuity) and to the age at which the amblyogenic factor occurred. How these factors precisely interact with each other is still unknown, but it is evident that different early visual experiences result in different functional losses in amblyopia (Mckee et al., 2003) along with the presence or absence of binocular function.

2.1 Neural mechanism underlying amblyopia

Much of our current understanding of the neural mechanisms underlying this disorder derives from studies on animal models, revealing that the major pathological changes in amblyopia occur at cortical level.

A reduction of the inputs from one eye by lid suture (MD), during development, dramatically decreases binocularity and shifts the physiological responsiveness of cortical neurons towards the open eye. As a direct consequence, the deprived eye becomes amblyopic: its visual acuity is strongly reduced and its contrast sensitivity blunted (Wiesel and Hubel, 1963a; Hubel and Wiesel, 1970; Olson and Freeman 1975; Movshon and Dürsteler, 1977; Olson and Freeman, 1980). As previously reported, physiological responses in the deprived retina and thalamus remain unaffected (Wiesel and Hubel, 1963; Sherman and Sgtone, 1973;

Kratz et al., 1979; Baro et al., 1990). Hubel and Wiesel observed that in kittens the susceptibility to the effects of MD starts suddenly near the beginning of the fourth week of life, remains robust between the sixth and eighth weeks, and then declines completely after the third month, thus defining a CP for MD effectiveness. MD starting in adulthood produces no detectable outcome (Hubel and Wiesel, 1970; Olson and Freeman, 1980). The effects of MD and the existence of a CP for ocular dominance plasticity have been subsequently described also in several species of mammals (Hubel et al., 1977; Blakemore et al., 1978; LeVay et al., 1980; Horton and Hocking, 1997; Van Sluyters and Stewart, 1974; Emerson et al., 1982; Fagiolini et al., 1994; Issa et al., 1999). The effects of deprivation can be reversed to a limited extent during the CP by reversing the visual deprivation, but they later become irreversible (Wiesel and Hubel, 1965a; Movshon, 1976; van Sluyters, 1978; Blakemore et al., 1981; Antonini and Stryker, 1998).

Similar to higher mammals, MD in rodents shifts the physiological responsiveness of neurons in the binocular zone of V1 towards the open eye, and this plasticity is confined to a well-defined CP (Dräger, 1978; Fagiolini et al., 1994; Gordon and Stryker, 1996). At least in the mouse, this is at first due to a rapid weakening of deprived-eye response, and later to strengthening of open-eye response (Frenkel and Bear, 2004). Interestingly, the OD shift is found in all layers, but it is more pronounced in extragranular layers than in layer IV, with the greatest shift in infragranular cells (Gordon and Stryker, 1996), suggesting that in rodents, as in other species, intracortical as well as geniculo-cortical synapses undergo plasticity with MD. Anatomical changes accompany functional plasticity in the developing visual cortex of the mouse, as they do in higher mammals (Antonini et al., 1999). Moreover, the advent of two-photon microscopy allowed the in vivo study of visual cortex spine dynamics during development and after visual deprivation: Oray et al. (2004) showed that spine motility in the binocular region of V1, contralateral to the deprived eye, is 35% higher than motility in control, nondeprived animals, indicating that sensory deprivation in a plastic cortex is able to initiate a rapid sequence of events that leads to increased structural dynamics at the level of individual spines. Such an increase in spine dynamics may reflect structural destabilization of a population of spines whose function is affected by visual deprivation. This, in turn, could precede a robust pruning of spine protrusions, probably correlated to the rapid reduction in the deprived-eye drive (Mataga et al., 2004).

Physiological mechanisms

Wiesel and Hubel proposed a mechanism in which OD plasticity operates through a competitive interaction between inputs from the two eyes for the control of cortical neurons, depending on the activity state of the postsynaptic neurons. This hypothesis was supported by the fact that binocular lid suture is not effective to shift OD columns in mammals (Wiesel and Hubel, 1965b; Gordon and Stryker, 1996; Antonini and Stryker, 1998). In addition, a reversible blockade of the discharge activities of cortical neurons by intracortical infusion of tetrodotoxin (TTX) or muscimol completely prevents the OD shift that would normally be seen after MD, or causes a paradoxical shift in favour of the deprived eye (Reiter et al., 1986; Reiter and Stryker, 1988; Hata and Stryker, 1994; Hata et al., 1999). However, the mechanism underlying binocular competition has remained elusive. The classic competition-based model is related to heterosynaptic mechanisms, where open eye inputs drive down the synaptic efficacy of the deprived inputs (Miller et al., 1989; Harris et al., 1997). Previous studies have implicated activity-dependent uptake of neurotrophins, as mediators of binocular competition (Maffei et al., 1992; Cabelli et al., 1995); subsequent experiments have shown that neurotrophins actually have cell specific effects such as the regulation of the inhibitory circuitry development, which may provide an alternative explanation for their importance for OD plasticity (Berardi and Maffei, 1999; Huang et al., 1999).

The homosynaptic view

There are two categories of changes, not mutually exclusive, that could account for the reduction in the deprived-eye responses. First, excitatory drive into the cortex from the deprived eye could be weakened, thereby decreasing the responses driven by the deprived eye. Second, intracortical inhibition of deprived-eye inputs could increase following MD, thereby suppressing or “masking” visual responses evoked by the deprived eye. A large body of classic work in kittens has supported the former idea, demonstrating that MD leads to a reduction in cortical innervations by excitatory thalamocortical axons subserving the deprived eye (Shatz and Stryker, 1978), which is accompanied, physiologically, by considerable weakening of deprived-eye inputs (Singer, 1977; Tsumoto and Suda, 1978; Mitzdorf and Singer, 1980). It has been proved that MD induces two mechanistically distinct modifications: long-term potentiation (LTP) of non-deprived eye synapses and long-term depression (LTD) of deprived eye synapses (Frenkel and Bear, 2004; Kirkwood et al., 1996; Rittenhouse et al., 1999; Heynen et al., 2003). The induction of LTP has been extensively demonstrated at

multiple synapses of the visual cortex ex vivo, although the mechanism appears to vary across layers (Wang and Daw, 2003). Homosynaptic LTP has been described in visual cortex (Heynen and Bear, 2001; Kirkwood and Bear, 1994), suggesting that, possibly at thalamortical synapses, it can mimic the effects of open-eye potentiation after MD. Moreover it has been found that many manipulations, known to disrupt homosynaptic LTP, cause the disruption of OD plasticity (Gordon et al., 1996; Taha et al., 2002; Sawtell et al., 2003; Frenkel et al., 2006).

Stronger evidence exists that LTD-like mechanisms influence depression of deprived-eye responses. The biochemical signature of LTD (in terms of AMPA receptor phopshorylation and cell-surface expression) has been used as a 'molecular fingerprint' to ask whether similar changes occur in visual cortex, following a period of MD. This has been examined in the rat visual cortex and the results support the hypothesis that MD induces this type of LTD in visual cortex (Heynen et al., 2003; Yoon et al., 2009). A second approach to address whether LTD is induced by MD is to ask whether naturally occurring synaptic depression in vivo occludes LTD ex vivo. This issue has been examined in rodents: LTD measured in slices is reduced (occluded) by three days of MD in vivo in both layer IV and II/III (Heynen et al., 2003; Crozier et al., 2007). Furthermore, the reduction in deprived-eye responses after lid suture is likely due to hebbian processes, as monocular inactivation with TTX (which prevents decorrelated inputs) blocks this depression (Frenkel and Bear, 2004). However, the question of the relative contribution of this synaptic modification to the functional consequences of MD is still controversial. Several genetic mutations in mice have been shown to independently disrupt OD without altering LTD. It has been found that GAD65 knockout mice, which lack normal OD plasticity, show no deficit in induction of LTD in layer II/III of mouse binocular visual cortex (Hensch et al., 1998a), while similar studies at younger ages show absence of LTD (Choi et al., 2002). In addition, a mutant that disrupts metabotropic glutamate receptor (mGluR)-dependent LTD does not alter the normal OD shifts in response to MD (Renger et al., 2002), though mGluR LTD is not the only form of synaptic depression in visual cortex. Moreover, loss of one PKA regulatory subunit (RIβ knockout mouse) disrupts LTD, but not OD (Hensch et al., 1998b), while loss of a different subunit (RIIβ knockout mouse) leaves LTD intact but disrupts OD plasticity (Rao et al., 2004). By contrast, RIIβ knockout mice exhibit normal LTP at the same synapse, but lack both LTD and OD plasticity (Fischer et al., 2004). Finally, LTD is intact whereas OD

plasticity is lost in mice that conditionally overexpress the calcineurin, the only known Ca^{2+}/calmodulin-activated protein phosphatase (Yang et al., 2005)

LTD/LTP mechanisms alone are unlikely to account for the OD plasticity. It has been shown that endogenous brain-derived neurotrophic factor (BDNF) prevents LTD in the visual cortex (Jiang et al., 2003) but does not block loss of deprived-eye input in transgenic mice overexpressing it (Huang et al., 1999). Conversely, early LTP and LTD that remain in the presence of protein synthesis inhibitors are inadequate to sustain shifts of OD in vivo (Frey et al., 1993; Taha and Stryker, 2002). Indeed, several alternative hypotheses have also been advanced to account for the phenomenology of OD plasticity. Balanced levels of excitation and inhibition have shown to be critical for enabling plasticity (Hensch, 2005; Hensch and Fagiolini, 2005).

Excitatory-inhibitory balance

In recent years, the question of the relative contribution of excitation and inhibition in MD-induced changes has been readdressed with new approaches. In all species tested so far anatomical and physiological evidence indicates that synaptic inhibition matures later than excitatory transmission in the neocortex (Blue and Parnavelas, 1983; Luhmann and Prince, 1991; Benevento et al., 1992; Guo et al., 1997; Micheva and Beaulieu, 1997; Gao et al., 2000; Mower and Guo, 2001; Murphy et al., 2005). By controlling excitation, GABAergic circuits are ideally posed to control the engagement of activity-dependent synaptic modification. Thus, the mismatch in the maturation of excitation and inhibition may define a window of opportunity for activity-dependent plasticity to occur. Taking advantage of gene-targeting technology, it has been shown that reduction in GABAergic transmission (GAD65 knockout mice) in juvenile mice prevents induction of OD plasticity, but normal OD plasticity can be rescued by infusion of diazepam to potentiate inhibitory transmission (Hensch et al., 1998a). The deficit in plasticity can be rescued by diazepam infusion in young mice, which results in OD plasticity before the traditionally recognized CP; however, similar plasticity cannot be induced by diazepam in adult animals once the CP has passed (Fagiolini and Hensch, 2000). On the other hand, diazepam infusion can trigger OD plasticity at any time in life for GAD65 mice, where the inhibitory threshold is not normally reached. An earlier triggering of the CP by diazepam in these mice, however, precludes later plasticity (Fagiolini and Hensch, 2000). Furthermore, accentuated excitation, by preventing the natural developmental switch of NMDA receptor subunit composition, also weakens the response to MD (Fagiolini et al.

2003). In NR2A knockout mice, synaptic NMDA responses remain prolonged in the absence of NR2A, yielding increased charge transfer (Fagiolini et al. 2003). An acute infusion of benzodiazepine agonists concomitant with MD restores full plasticity to NR2A knockout mice, proving a decisive role for excitatory-inhibitory balance (Fagiolini et al. 2003).

Interestingly, not all GABA circuits are involved in CP regulation. Maturation of parvalbumin-positive interneurons parallels CP onset (Del Rio et al. 1994; Gao et al. 2000). Large basket cells, in particular, extend a wide, horizontal axonal arbor that can span OD columns in cat visual cortex (Buzas et al., 2001). Moreover, these electrically coupled networks of fast-spiking cells offer a system sensitive to timing (Connors, 2004; Galaretta and Hestrin 2001). Systematic use of the mouse "knock-in" mutation has further shown that only the interneurons containing the $GABA_A$ receptors α1-subunit (i.e., basket cells) drives cortical plasticity and are preferentially enriched at somatic synapses opposite to parvalbumin-positive large basket cell terminals (Fagiolini et al., 2004; Klausberger et al., 2002).

Spike timing-dependent plasticity has recently emerged as an attractive alternative based on natural, realistic, millisecond-scale sequences in the temporal order of pre- and postsynaptic action potentials (Bi and Poo, 2001; Froemke and Dan, 2002). Inhibitory regulation of spike-timing could instruct the direction of plasticity (Song et al., 2000). Among the vast diversity of GABAergic interneurons in neocortex, parvalbumin-containing cells target the axon initial segment and soma (DeFelipe, 1997; Somogyi et al., 1998), where they can control back-propagation (basket cells), required for synaptic plasticity in the dendritic arbor. Furthermore, prolonged discharge in both NR2A and GAD65 knockout mice would impair plasticity by altering the pattern of neural activity encoding visual input. The normal development of inhibitory circuitry, as well as diazepam infusion in transgenic mice, improve temporal processing of sensory input, allowing OD shift in response to MD to take place (Hensch and Fagiolini, 2005).

Thus, development of inhibition seems to be a determinant of the time-course of plasticity decline during development and of the CP closure. The available findings suggest that inhibition levels cross two thresholds during development: the first setting the point after which inhibition levels are enough to allow OD plasticity to be expressed (beginning of CP); as development proceeds further, the inhibitory tone further increases and crosses a second threshold, after which inhibition drastically reduces the potential for plasticity (closure of CP) (Feldman, 2000). Accordingly, Harauzov et al. (2010) reported that pharmacological reduction of GABAergic transmission in the adult rat visual cortex reactivates OD plasticity

in response to MD, suggesting that the adult level of inhibition actively restricts cortical plasticity.

Homeostatic synaptic plasticity

Homeostatic synaptic plasticity mechanisms are emerging as important complements to Hebbian forms of plasticity in the activity-dependent refinement of synaptic connectivity (Turrigiano and Nelson, 2004; Davis, 2006; Turrigiano, 2008). Homeostatic plasticity acts to stabilize the activity of a neuron or neuronal circuit against perturbations that alter excitability, providing a robust mechanism for generating stability in network function in the face of experience-related changes in synaptic input. The best studied mechanism of homeostatic regulation is synaptic scaling of excitatory synapses, which was first described in dissociated rat cortical cultures, where blockade of activity with TTX increases and blocking GABA-mediated inhibition decreases the amplitude of miniature excitatory postsynaptic currents (mEPSCs) (Turrigiano et al., 1998). Interestingly, the rules for synaptic scaling depend on the synapse type: inhibitory synapses onto pyramidal neurons are scaled in the opposite direction from excitatory synapses, suggesting that the firing rate is regulated through reciprocal changes in excitation and inhibition (Kilman et al., 2002; Swanwick et al., 2006). Finally, synaptic scaling could require widespread changes in network activity, perhaps through activity-dependent release of a soluble factor by many neurons or, simultaneously, by glia, such as BDNF and TNFα (Rutherford et al., 1998; Stellwagen and Malenka, 2006; Kaneko et al., 2008a).

Synaptic scaling has been most thoroughly studied in vivo in the visual system, using standard visual deprivation paradigms to mimic in vivo the activity blockade in culture. There is now increasing evidence that synaptic scaling in excitation and inhibition plays important roles during various CPs of visual system development (Desai et al., 2002; Maffei et al., 2004; Maffei and Turrigiano, 2008a). In particular, it has been suggested that the potentiation of non deprived-eye responses following MD might arise through homeostatic mechanisms that boost the excitability of cortical neurons in response to a drop of sensory input. A recent study using in vivo calcium imaging to monitor eye-specific activation of individual neurons within binocular layer II/III of visual cortex reported that binocularly driven neurons maintain their overall level of responsiveness to the two eyes, so that the decrease in the responsiveness to the deprived-eye stimulation is compensated by an increase in responsiveness to non deprived-eye stimulation. Interestingly, in monocular visual cortex, the population of neurons

driven only by the deprived eye has homeostatic-mediated stronger responses after deprivation, as do all neurons after binocular deprivation (Mrsic-Flogel et al., 2007). In support of the notion that synaptic scaling underlies gain of responsiveness to the non-deprived eye, blocking TNFα signalling in visual cortex either pharmacologically or genetically has no effect on the loss of responsiveness to the deprived eye but prevented the gain of responsiveness to the non-deprived eye (Kaneko et al., 2008a). Complicating the interpretation of these studies is the recent report that the mode of homeostatic plasticity within layer II/III of the visual cortex during the CP depends strongly on the method of visual deprivation: lowering visual drive through TTX or DR induces synaptic scaling, whereas eyelid suture causes an increase in the intrinsic excitability of monocular cortex pyramidal neurons (Desai et al., 2002; Maffei and Turrigiano, 2008b).

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

Molecular substrate

The development of treatments promoting visual function recovery after the end of the CP requires a deep understanding of the molecular mechanisms involved in amblyopia onset. Several mechanisms are currently thought to underlie the impact of visual deprivation, leading to amblyopia development.

Glutamatergic receptors

The properties of NMDA receptors (NMDARs) suggest that these molecules might play a central role in visual cortex plasticity, acting as ‘coincident detectors’ for Hebbian plasticity. The involvement of NMDARs in OD plasticity has been repeatedly proposed by pharmacological experiments (Kleinschmidt et al., 1987; Gu et al., 1989; Bear et al., 1990), but such manipulations have potent suppressive effects upon normal synaptic transmission. Successively, use of different NMDAR antagonists or antisense oligonucleotides to reduce expression of NR1 subunit of the NMDA receptor has overcome this problem, showing that it is possible to block the effects of MD without affecting visual responses (Roberts et al., 1998;

Daw et al., 1999a). The direct dependence of OD plasticity on NR1 subunits has been further demonstrated using conditional NR1-knockout mice (Sawtell et al., 2003). An interesting property of NMDARs is that their subunit expression, determining the calcium influx, is developmentally and activity regulated. In particular, subunit composition varies in the visual cortex, from low to high NR2A/NR2B ratio, with a time course paralleling that of functional visual cortical development and the CP (Roberts and Ramoa, 1999). It has been shown that in dark-reared animals the NR2A/NR2B ratio is lower than in light-reared animals (Quinlan et al., 1999 a,b; Tongiorgi et al., 2003). However, it has been demonstrated that NR2B over-expressing animals do not show an increased susceptibility to plasticity (Philpot et al., 2001) and in mice with the deletion of NR2A subunit the sensitivity to MD is weakened, even if restricted to the normal CP (Fagiolini et al., 2003). Interestingly, a recent study highlights a co-regulation of OD plasticity and NMDAR subunit expression in GAD65 knockout mice. In the visual cortex of these animals there are reduced NR2A levels and slower NMDA currents. In addition, application of benzodiazepines, which rescues OD plasticity, also increases NR2A levels, suggesting that changes in inhibition would engage mechanisms that converge to regulate NMDA receptors, thereby enabling plasticity (Kanold et al., 2009).

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

Neurotrophins

Several observations have suggested that neurotrophins control visual cortical plasticity during the CP (Domenici et al., 1991, 1992; Lodovichi et al., 2000; Pizzorusso et al., 1999; Berardi et al., 1994; Domenici et al., 1994; Cabelli et al., 1997). In addition, neurotrophin production and release are developmentally regulated and depend on electrical activity, in particular to visual activity (Castren et al., 1992; Bozzi et al., 1995; McAllister et al., 1999). Moreover, neurotrophic release plays a key role in the modulation of synaptic transmission

and electrical activity at both presynaptic and postsynaptic levels (Carmignoto et al., 1997; Berardi and Maffei, 1999; Poo, 2001) and it can also play a fast action, by increasing transmitter release (Sala et al., 1998; Jovanovic et al., 2000) or by directly depolarizing neurons (Kafitz et al., 1999), or a slow action, by modulating gene expression (Poo, 2001). The "neurotrophic hypothesis" can explain the possible mechanism of neurotrophins' action given the fact that 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). This hypothesis states that competition for limited amounts of neutrotrophins is the effector of activity-dependent plasticity in the cortex, and the conventional explanation for OD plasticity is that the deprived eye does not activate cortical cells as well as the open eye, thereby failing to stimulate them to release sufficient neurotrophins to sustain the deprived-eye pathway (Thoenen, 1995; Bonhoeffer, 1996; McAllister et al., 1999). The possibility of an anterograde action of neurotrophins as opposed to target-derived action has also emerged from literature (Caleo et al., 2000; Kohara et al., 2001; von Bartheld, 2004). The available experimental data, however, underline another possible mechanism of action of neurotrophins on OD plasticity, as an orchestrated modulation of synaptic efficacy, rather than a direct effect on thalamocortical afferents alone. It has been shown that NGF is likely to act directly on cholinergic afferents from the basal forebrain and on a population of glutamatergic cortical neurons; BDNF targets are principally cortical glutamatergic pyramidal cells and inhibitory interneurons, whereas NT4 acts on glutamatergic thalamic afferents and probably pyramidal neurons and inhibitory interneurons (Berardi and Maffei, 1999).

BDNF infused in the cat visual cortex paradoxically results in the expansion of connections subserving the deprived eye, as previously observed with the intracortical infusion of the GABA receptor agonist muscimol (Reiter et al., 1986; Carmignoto et al., 1993; Fiorentini et al., 1995; Galuske et al., 2000; Gillespie et al., 2000; Silver et al., 2001). The relationship between neurotrophins and the development of inhibitory processes has been investigated in BDNF overexpression transgenic mouse: BDNF overexpression causes an acceleration of the maturation of intracortical GABA-mediated inhibition, paralleled by a precocious development of visual acuity with respect to wild type, and an accelerated time course of the CP, resulting in an early shift of the CP of about one week (Huang et al., 1999). It should be noted that recent studies of a mutant mouse heterozygous for the null allele of BNDF demonstrate that a 50% reduction in the BDNF levels has no effect upon OD plasticity (Bartoletti et al., 2002). Similarly, Stryker and colleagues, using a conditional transgenic

mouse, show that TrkB inactivation does not affect the induction of OD plasticity following MD (Kaneko et al., 2008b). However, since the redundancy of neurotrophin action on the modulation of synaptic transmission, these data do not exclude that neurotrophic factors play a fundamental part in the plasticity of visual cortex: the compensating action of other neurotrophins could account for the absence of alterations in visual cortex plasticity in these mutant mice.

GABAergic inhibition and BDNF signaling

GABA-mediated inhibition regulates cortical plasticity on multiple fronts. Maturation of cortical inhibition is strongly involved in the timing of CP for OD plasticity (Hensch, 2005) as well as OD column development in the cat (Hensch and Stryker, 2004). There is now considerable evidence that a minimal level of cortical inhibition is necessary for the initiation of OD plasticity and that factors that influence the development and the extent of GABA transmission (such as BDNF, benzodiazepines, PSA-NCAM and fluoxetine) can control the plastic properties of visual cortical circuitry (Hensch and Fagiolini, 2005; Jiang et al., 2005; Di Cristo et al., 2007; Maya Vetencourt et al., 2008). These recent pharmacological studies have focused much attention toward a specific subset of GABAergic neurons, the parvalbumin-positive cells (which include fast-spiking basket cells), for their role in visual plasticity (Fagiolini et al., 2004; Maffei et al., 2006; Tropea et al., 2006). Maturation of these cells is regulated by BDNF (Huang et al., 1999) and the benzodiazepine-sensitive $GABA_A$-α1 subunits are localized on receptors that specifically receive parvalbumin-positive afferents (Klausberger et al., 2002). Mice lacking these receptors have more sustained GABA currents, an effect similar to the administration of benzodiazepines (Ponomarev et al., 2006). In addition, fast-spiking basket cells have been shown to mediate potentiation of inhibition in visual cortex *in vitro*, suggesting an important feed-forward mechanism that contributes to the rapid deprived-eye depression following MD (Maffei et al., 2006). There is also structural evidence for a role of GABAergic transmission in synaptic development and plasticity (Mataga et al., 2004; Heynen et al., 2003).

Neuromodulatory systems

It has been known for over 30 years that agonists of adrenergic and cholinergic systems facilitate the onset of OD plasticity (Kasamatsu and Pettigrew, 1976; Bear and Singer, 1986; Gu, 2003). Successively an analogous function was also described for the serotoninergic

system (Gu and Singer, 1995). As with many other molecules involved in cortical plasticity, the distribution of different receptors and fibres is developmentally regulated (Foote and Morrison, 1984: noradrenergic fibres) and is dependent on cortical input (Prusky et al., 1988: cholinergic receptors).

The role of serotonin in OD plasticity has been chronically investigated by infusing a specific neurotoxin into the visual cortex of kittens undergoing MD: the results showed that serotonin depletion prevents the susceptibility to experience-dependent modifications. Equally, the combined infusion of two broad serotonergic receptor antagonists reduces OD plasticity (Gu and Singer, 1995). In addition, it has been demonstrated that the serotonin 5-HT2c receptor subtype plays a key role in activity-dependent synaptic modifications in the visual cortex (Wang et al., 1997). To explain the facilitatory action of serotonin in OD plasticity, it has been proposed also in this case a mechanism associated with NMDA receptors (Nedergaard et al., 1987; Reynolds et al., 1988; Panicker et al., 1991; Hoyer and Martin, 1997). Moreover, it has been demonstrated that different muscarinic receptors activate distinct intracellular pathways (Origlia et al., 2006); thus, the same stimulus may alter plasticity in unique ways depending on the relative contribution of neuromodulatory systems. Finally it has been shown that administration of the selective serotonin reuptake inhibitor fluoxetine restore OD plasticity in adult animals. The effects induced by fluoxetine are associated with a marked reduction of GABAergic inhibition, thus suggesting that also serotonin might affect visual cortical plasticity modulating intracortical inhibition (Maya Vetencourt et al., 2008).

Intracellular signalling of cortical plasticity

Experiments using transgenic mice and/or pharmacological manipulation have identified three signalling kinases that can modulate synaptic strength and are critical for inducing OD shifts: extracellular signal-regulated kinase 1,2 (ERK, also called p42/44 mitogen-activated protein kinase), protein kinase A (PKA), and calcium/calmodulin-dependent protein kinase II alpha (CaMKIIa) (DiCristo et al., 2001; Taha et al., 2002; Berardi et al., 2003; Taha and Stryker, 2005). These kinases may rapidly promote OD plasticity by directly phosphorylating plasticity-regulating molecules at the synapse (such as glutamate or GABA receptors), thereby modulating synaptic strength, or they may signal to the nucleus to mediate changes in gene transcription. The intracellular mechanisms mediated by kinase signaling can lead to the activation of cAMP-responsive element binding protein (CREB) which in turn controls CRE-

mediated gene expression of a host of synaptic signaling molecules (Cancedda et al., 2003; Suzuki et al. 2004). Visual manipulation, like MD, induces activation of CREB (Pham et al., 1999). While PKA and ERK inhibition affect CRE-mediated gene expression, the effects of PKA are dependent on ERK phosphorylation (Cancedda et al., 2003). Based on these results, ERK has been pointed out as a molecular sensor for visually driven activity. Interestingly, while ERK activation and CRE-gene expression appear to be strongly correlated, it has been shown that ERK activation and phopshorylated CREB do not always overlap (Suzuki et al., 2004), suggesting that other co-activators of CREB are important transducers of synaptic activity. As with many other molecules involved in neural plasticity, CREB levels also decrease with age (Pham et al., 1999). Of particular interest is the finding that activation of CREB is mediated by visual stimulation in young but not adult rats (Putignano et al., 2007), demonstrating that different intracellular pathways contribute to cortical plasticity at different ages. These pathways may also converge to mediate structural rearrangements induced by MD (Gomez et al., 2002; Hsieh-Wilson et al., 2003; Chierzi et al., 2005; Alonso et al., 2004). Additional classes of molecules are also likely to be important for calcium-dependent cellular processes that may mediate brain plasticity: the calcium sensor cardiac troponin C (Lyckman et al., 2008), and calcineurin (Yang et al., 2005).

Extracellular environment

It is becoming clear that the extracellular environment, and in particular the extracellular matrix (ECM), plays an important part in controlling spine dynamics and visual cortical plasticity. Recent studies have shown a key role in OD plasticity for the major components of brain ECM, the chondroitin-sulfate proteoglycans (CSPGs). During development CSPGs condense at high concentration in lattice-like structures, called perineuronal nets (PNNs), which completely envelop visual cortical neurons, in particular parvalbumin-positive neurons. The time course of PNN condensation in the visual cortex tightly matches the visual cortex CP for the effects of MD (Hartig et al., 1992; Köppe et al., 1997; Brückner et al., 2000; Pizzorusso et al., 2002), and development of CSPGs is regulated by visual activity, since the process of PNN condensation is prolonged by DR (Hockfield et al., 1990). The enzymatic degradation of CSPGs from the adult visual cortex reactivates OD plasticity in monocularly deprived adult animals, suggesting that adult ECM exerts a powerful inhibitory control on OD plasticity (Pizzorusso et al., 2002).

The outcome of the study of ECM influence on OD plasticity led to analyze the role of endogenous extracellular proteases in the visual cortical plasticity during the CP. It has been shown that pharmacological inhibition of tPA hampers visual cortical plasticity (Mataga et al., 1996; Muller and Griesinger, 1998), and MD is ineffective in mice with deletion of the tPA gene both at the functional and structural level. Plasticity can be rescued in tPA knockout mice by the exogenous administration of tPA during the period of MD. Moreover, the link between tPA and experience-dependent plasticity is strengthened by the observation that in wild type animals MD elicits a fast and transient increase of tPA activity during the CP but not in the adult (Mataga et al., 2002, 2004). The released tPA increases extracellular proteolysis directly or by the activation of plasmin. These proteases have a wide spectrum of targets and the available information is not sufficient to dissect which of these targets must be cleaved for plasticity to proceed. However, converging data point to an important role for tPA in "freeing up" the extracellular matrix to promote the structural reorganization of connections during deprivation (Mataga et al., 2004; Oray et al., 2004).

Another extracellular factor that regulates the capacity for OD plasticity is myelin from the surrounding oligodendrocytes, particularly via its interaction with the Nogo receptor (McGee et al., 2005). The CP window for OD plasticity is substantially extended in Nogo receptor (NgR) null mice mice, despite the normal development of other factors that regulate plasticity, such as tPA levels and GABAergic transmission. Interestingly, cortical myelination does not appear to be regulated by visual experience, as DR wild-type mice does not affect the expression level of myelin-related proteins. Furthermore, while visual deprivation alters transcription of a number of plasticity-related molecules, developmental increases in myelin-associated genes remain unchanged (Lyckman et al., 2008).

In addition, in the past few years several studies have investigated the molecular mechanisms of visual cortex plasticity using genetic screens, and have opened the door for examination of new families of molecules in plasticity (e.g proteins related to IGF-I pathway or immune/inflammation system signals). Expression of most of these molecules is developmentally regulated and differentially altered by sensory experience (Ossipow et al., 2004; Majdan and Shatz, 2006; Tropea et al., 2006; Lyckman et al., 2008). It has been demonstrated that MD increases the expression of IGF-1 binding protein and affects several genes in the IGF-1 pathway; exogenous application of IGF-1 prevents the physiological effect of MD on OD plasticity examined in vivo (Tropea et al., 2006). This suggests that IGF-1 could be involved in experience-dependent plasticity in the visual cortex.

2.2 Treatment for amblyopia in childhood

The basic strategy for treating amblyopia is first to provide a clear retinal image, and then correct ocular dominance, as early as possible, during the period of visual plasticity (from birth to eight years) (Worth, 1903). The methods of treatment of human amblyopia, including refractive correction used alone or in combination with occlusion or atropine, are known as "passive methods". Occlusion therapy with patching of the dominant eye has been used for centuries as the primary treatment for amblyopia (Loudon and Simonsz, 2005). The success of patching seems to correlate with the actual number of hours that the eye is patched (Loudon et al., 2002); however, comparing the time of prescribed patching per day, the Pediatric Eye Disease Investigator Group (PEDIG) did not find any difference in treatment of moderate amblyopia, in 2 versus 6 hours patching per day (PEDIG, 2003a), and in treatment of severe amblyopia, in 6 versus 12 hours patching per day (PEDIG, 2003b). Moreover, treatment may be just as effective in older (13–17 years) patients who have not been previously treated as in younger (7–12 years) children (PEDIG, 2003a,b and 2005). The treatment outcome is dependent on the depth of amblyopia, binocular status, fixation pattern, the age at presentation and patient compliance (Loudon et al. 2003; Stewart et al. 2005); nevertheless compliance rates vary widely (Woodruff et al., 2004; Loudon et al., 2002; Oliver et al., 1986; Smith et al., 1995) because of physical, visual, social, and psychological issues that are associated with patching (PEDIG, 2002). Atropine penalization is an alternative to patching for amblyopia therapy (Simons et al., 1997; Foley-Nolan et al., 1997; PEDIG, 2002). Atropine paralyzes accommodation and blurs near vision, encouraging use of the amblyopic eye. Moreover it was found that atropine was as effective as patching, but that patching was initially faster and atropine had a higher compliance (PEDIG, 2002). In atropine penalization vision is binocular in the sense that the image at the fovea of the dominant (non-amblyopic) eye is degraded, while input to the amblyopic eye is not affected; binocularity is instead impaired in patching treatment, except for variations of patching therapy that use lenses or translucent material (i.e. Bargenter Occlusion Foils).

Passive treatments may be coupled with, or perhaps replaced by, more "active" forms of treatment, in which certain tasks are prescribed to be performed by the patient. The rationale of this method is that the patient is likely to be more engaged (attentive) and is encouraged to use the amblyopic eye if actively involved during the treatment, and that he is more likely to be compliant if the treatment is enjoyable. In some cases, active treatments employ stimuli, designed to preferentially stimulate certain elements of the visual pathway,

and in others, some treatments use active tasks. Pleoptics is a method for visual diagnosis and training that employs monocular techniques for the detection and elimination of eccentric fixation and amblyopia: a bright ring of light is flashed around the fovea to temporarily "blind" or saturate the photoreceptors surrounding the fovea, which eliminates vision from the eccentric fixation point and forces fixation to the fovea. However, pleoptic treatments are given several times a week to enhance occlusion therapy. Most practitioners have found pleoptics to be no better than standard occlusion therapy (Fletcher et al., 1969; VerLee and Iacobucci, 1967). Some investigators have suggested active stimulation of the amblyopic eye as a way to improve vision in the amblyopic eye. The CAM treatment (Campbell et al., 1968) consisted in a high contrast square wave grating that rotates slowly, at about one revolution per minute. The treatment was based on the findings that spatial frequency and orientation-specific filters, in the visual system, are activated by rotation. The CAM treatment was found not effective (Crandall et al., 1981; Keith et al., 1980; Tytla et al., 1981). It has been recognized that binocular stimulation may be important for the treatment of amblyopia. Indeed, animal research indicates that binocular stimulation promotes binocular cortical connections during recovery from deprivation amblyopia (Mitchell et al., 2009), offering support for binocular stimulation for the treatment of amblyopia. Experimental models of patching therapy for amblyopia imposed on animals in which amblyopia had been induced by a prior period of early MD, indicate that the benefits of patching therapy can be heightened when combined with critical amounts of binocular visual input each day (Mitchell, 2008). Recent studies (Baker et al., 2007; Mansouri et al., 2008; Vedamurthy et al., 2008) helped to understand the way in which signals from the amblyopic and not amblyopic eyes can impact on each other and on binocular vision. These studies indicate that, while interocular differences are likely to be the only factor limiting binocular co-operation in amblyopia, binocularity may be enhanced by attempts aimed at balancing the inputs to the two eyes in amblyopia. An approach used in the treatment of amblyopia, known as “monocular fixation in a binocular field” (MFBF) technique was introduced with the intention of training the amblyopic visual system to integrate information from both eyes (Cohen, 1981). This technique involves the presentation of peripheral stimuli to both eyes, while only the amblyopic eye is stimulated at the fovea. In the meanwhile, the patients are instructed to complete tasks such as crossword puzzles or placing dots in the “o” letters in a text, using a red pen and wearing red-green glasses, with the red lens in front of the non amblyopic eye (Wick et al., 1992).

2.3 Treatment for amblyopia in adulthood

While amblyopia can often be reversed when treated early (Wu and Hunter, 2006), treatment is not generally undertaken in adults. Recently, several studies in the visual system have shed new light on the mechanisms that limit plasticity to early life, indicating that the adult brain is not "hardwired" with fixed and immutable neural circuits; on the contrary, the adult brain, following specific treatments, can reacquire a certain degree of plasticity even well after the end of the CP. Treatments for amblyopia in adulthood are focused on promoting cortical plasticity by the reducing factors that actively limited adult plasticity, or by exploiting endogenous permissive factors; under this conditions, the circuit rewiring may be facilitated in the mature brain, inducing the recovery from amblyopia. Several pharmacological attempts have been done to enhance adult visual cortical plasticity acting on the factors which are thought to contribute to its time course and which have been previously described.

Inhibition/excitation balance

Early in development, excitation appears to dominate cortical circuits, but accumulating evidence supports a pivotal role for late-developing excitatory and inhibitory (E/I) circuit balance in the initiation of sensitive periods. For example, the onset of visual cortical plasticity is delayed by genetic disruption of GABA synthesis or a slowing down of the maturational state of perisomatic inhibition (Hensch, 2005). Conversely, the application of benzodiazepines or other treatments that accelerate GABA circuit function triggers premature plasticity (Di Cristo et al., 2007; Sugiyama et al., 2008). These manipulations are so powerful that animals of identical chronological age may be at the peak, before, or past their sensitive period, depending on how the maturational state of their GABA circuitry has been altered. The E/I circuit balance points out a possible mechanisms for enhancing learning and recovery of function in adulthood, suggesting that a reduction of GABAergic transmission could be a crucial step for the restoration of plasticity processes in the adulthood (Hensch, 2005; Baroncelli et al., 2011). In agreement with this, a recent study showed that pharmacological reduction of intracortical inhibition obtained through the infusion of either MPA (an inhibitor of GABA synthesis) or picrotoxin (a $GABA_A$ antagonist) directly into the visual cortex reactivates OD plasticity in response to MD in adult rats (Harauzov et al., 2010).

While reducing inhibition levels by direct pharmacological manipulations in humans are theoretically appealing, indiscriminately tampering with brakes on plasticity throughout

the brain may cause more harm than good (Pascual-Leone et al., 2005). The pharmacological treatment in humans must consider the ethical and safety concerns.

Neuromodulators

One action of endogenous neuromodulator release such as norepinephrine, acetylcholine, serotonin, or dopamine may be to adjust a favorable E/I balance (Bear and Singer, 1986; Kasamatsu, 1991; Kilgard and Merzenich, 1998; Bao et al., 2001; Goard and Dan, 2009). In agreement with this, it has been demonstrated that chronic treatment with the fluoxetine reinstates OD plasticity following MD and promotes the recovery of normal visual functions in amblyopic animals, by a pronounced reduction of intracortical inhibition (Maya-Vetencourt et al., 2008). SSRIs, typically used as antidepressants in the treatment of depression and anxiety disorders, are approved by the Food and Drug Administration, thus their use for treating amblyopia can be a promising approach. Further support to the notion that diffuse projecting systems of the brainstem affect plasticity in adulthood has been provided by the demonstration that a genetic manipulation of nicotinic cholinergic transmission promotes visual cortex plasticity after the end of the CP (Morishita et al., 2010).

Epigenetic modifications

Drugs that alter the "epigenome" also hold promise, considering how environmental changes alter brain chromatin status (Zhang and Meaney, 2010). It has been shown that a developmental downregulation of experience-dependent regulation of histone H3 and H4 acetylation is involved in closing the CP (Putignano et al., 2007). Recently, Silingardi et al. (2010) found that a chronic intraperitoneal administration of valproic acid, a histone deacetylases inhibitor, causes a complete recovery of visual acuity deficits induced in adult rats by long-term MD. More generally, the use of drugs that specifically target transcriptional regulatory processes, known to be altered in neurodevelopmental disorders, has proven strikingly efficient at alleviating cognitive deficits even when administered in adulthood (Chahrour and Zoghbi, 2007; Ehninger et al., 2008; Greer and Greenberg, 2008). One suggested mechanism of action of these drugs may be to restore a more normal E/I balance, which is commonly impaired in these neurodevelopmental disorders (Rubenstein and Merzenich, 2003; Polleux and Lauder, 2004; Gogolla et al., 2009).

Action on the extracellular matrix

Following the demonstration that PNNs (Pizzorusso et al., 2002), drastically limited adult plasticity, Pizzorusso et al. (2006) demonstrated that adult chABC treatment coupled with reverse suture (i.e. the deprivation of the previously open eye and opening of the previously deprived eye, RS) in amblyopic rats produces a full recovery of both OD and VA (Pizzorusso et al., 2006). They also found that the decrease in spine density, caused by long-term MD, was recovered by chABC treatment, suggesting that formation of synaptic contacts on the newly formed spines by the inputs from the formerly deprived eye might be the mechanism underlying the recovery from amblyopia observed in chABC-treated animals.

Some of the effects of chABC could be mediated by modifications of intracortical inhibitory circuits occurring after PNN degradation, which brings parvalbumin interneurons back to juvenile-like status (Hensch, 2005).

Non invasive treatments

It would be ideal to endogenously recapitulate brain states that promote plasticity in a noninvasive but targeted manner. One potential route is through the endogenous release of permissive factors in response to altered environments. Oddly, amblyopic rats subjected to complete visual deprivation by dark exposure for 10 days recover significant vision once allowed to see binocularly, reactivating balance between excitation and inhibition towards juvenile-like levels (He et al., 2007). However, translation of this treatment to humans is questionable as the proportional length of dark exposure required is likely to be on the order of months rather than days, which may be too disruptive for most. Arguably, a second, more promising approach for humans is environmental enrichment (EE).

EE is an experimental protocol specifically designated to investigate the influence of the environment on brain and behavior (Rosenzweig and Bennett, 1996; Diamond, 2001; van Praag et al., 2000); indeed, EE is classically defined as “a combination of complex inanimate and social stimulation” (Rosenzweig et al., 1978). “Enriched” animals are reared in large groups in wide cages where a variety of toys, tunnels, nesting material and stairs are present and changed frequently. The most important purpose of the EE is to improve the life quality of the animals by providing them with the opportunity to attain high levels of voluntary physical activity on running wheels and to enhance exploration, cognitive activity and social interaction. EE was first shown to determine a robust increase in cortical thickness and weight (Rosenzweig et al., 1964; Beaulieu et al., 1987), in the size of the cell soma and nucleus, in

dendritic arborization, length and dendritic spines (Holloway, 1966; Kozorovitskiy et al., 2005). More recent studies have reported also an effect of EE on increased hippocampal neurogenesis (Kempermann, 1997) and on the reduction of apoptotic cell death (Young, 1999). Recently, Sale et al. (2007) have shown that EE promotes a complete recovery of visual acuity and OD in adult amblyopic animals (Sale et al., 2007). Recovery of plasticity was associated with a marked reduction of GABAergic inhibition in the visual cortex, as assessed by brain microdialysis. Moreover, a decreased cortical inhibition has been demonstrated also at the synaptic level, using the in vitro paradigm of LTP of layer II–III field potentials induced by theta-burst stimulation from the white matter (WM–LTP). The WM–LTP is normally not present in the adult as a result of the maturation of inhibitory circuits (Kirkwood and Bear 1994; Huang et al. 1999), but it can be restored if GABA-mediated inhibition is reduced (Artola and Singer, 1987; Kirkwood and Bear, 1994). Notably, WM–LTP was fully restored in the visual cortex of EE adult rats (Sale et al., 2007). The reduction of cortical inhibition in EE rats was also paralleled by an increased expression of the neurotrophin BDNF and a lower density of PNNs in the visual cortex contralateral to the recovering (previously amblyopic) eye.

Another non-invasive approach for treating amblyopia in adults could be the regulation of caloric intake. Nutrition plays a key role in visual development, and infant formulas containing long-chain polyunsaturated fatty acids, docosahexaenoic acid and arachidonic acid found in breast milk may protect nonbreast-fed infants against visual development problems (Brémond-Gignac et al., 2011). Recently, Spolidoro et al. (2011) demonstrated that a short period of food restriction in adulthood is able both to reinstate OD plasticity and to promote recovery from amblyopia. These effects are accompanied by a reduction of intracortical inhibition without modulation of BDNF expression or extracellular matrix structure.

Finally, another therapeutic approach to the treatment of amblyopia could be the use of TMS technique. A variant of TMS, repetitive transcranial magnetic stimulation (rTMS), involves the application of a series of pulses over a period of seconds or minutes, with direct effects that last up to an hour (Huang et al., 2008) and clinical improvements that can accumulate over a week (Fitzgerald et al., 2006). A recent study showed that rTMS applied over the visual cortex improved contrast detection for high spatial frequencies in the amblyopic eye, directly after and thirty minutes after stimulation (Thompson et al., 2008). A possible explanation for the mechanisms responsible for the rTMS-based improvements is

that rTMS acts to reduce intracortical inhibition (ICI), an effect that has been demonstrated in the motor cortex for both 1 Hz and 10 Hz stimulations (Pascual-Leone et al.,1994; Modugno et al., 2003). Unfortunately, it is not possible to measure ICI in visual cortex with a subjective phosphene report (Sparing et al., 2005).

2.3.1 Perceptual learning as treatment for amblyopia

Perceptual learning (PL) can be a very promising strategy for treating amblyopia in adulthood. Adults improve their performance on sensory tasks, through repeated practice (e.g., Fine and Jacobs, 2002; Fahle, 2005), and PL, as discussed in the first chapter, is considered to engage form of neural plasticity in the cortex.

PL can remarkably improve visual functions in amblyopia on a wide range of tasks, including: vernier acuity (Levi and Polat, 1996; Levi et al., 1997); positional acuity (Li and Levi, 2004; Li et al., 2005, 2007); contrast sensitivity (Polat et al., 2004; Zhou et al., 2006; Huang et al., 2008); first-order letter identification (Levi, 2005; Chung et al., 2008); and second-order letter identification (Chung et al., 2006). Practising each of these tasks results in improved performance on the practised task: PL with the amblyopic eye shows little or no transfer to untrained orientations, (Levi and Polat, 1996; Levi et al., 1997; Li and Levi, 2004), and there is no transfer of learning from a Vernier acuity task to a detection task (Levi and Polat, 1996; Levi et al., 1997). Interestingly, at least in some subject, there is significant but only partial transfer of learning from the amblyopic to the fellow eye (Levi and Polat, 1996; Levi et al., 1997; Li et al., 2008; Zhou et al., 2006). Since PL is specific to the trained stimulus, its therapeutic value for treating amblyopia can be uncertain; instead, PL of many tasks (vernier acuity, position discrimination and contrast sensitivity) appears to transfer, at least in part, to improvements in visual acuity measured, for example, with the Snellen chart (Levi and Polat, 1996; Levi et al., 1997; Li and Levi, 2004; Polat et al. 2004; Zhou et al. 2006; Huang et al. 2008). In addition to visual acuity improvement, other degraded visual functions such as stereoacuity and visual counting improve as well (Li and Levi 2004; Li et al. 2007).

In adults with normal vision, PL effects are often reported to be long-lasting (e.g., Karni and Sagi, 1993). Indeed, Li et al. (2004) showed that the improvement in visual acuity in the amblyopic eye, resulting from position discrimination training, was essentially stable for a long time period (from three to twelve months). Polat et al. (2004) also reported a very high level of retention of the improved visual acuity as much as twelve months following the

cessation of learning, and Zhou et al. (2006) reported that, in the few cases tested, the improvement in visual acuity showed retention of approximately 90% for at least one year. Clinical studies have shown that the time constant for successful patching is long, with acuity improving approximately 26 per cent for every 120 h of occlusion (Stewart et al. 2004); however the time constant for PL in amblyopia is unknown. Recent studies (Li et al., 2007) have shown that the time constant for PL in amblyopia may be much longer than the 10–15 h of practice, that is typical of most PL studies, and have shown that it depends on the degree of amblyopia.

PL might be effective for several reasons. First, some of the improvements obtained with PL may reflect the effects of patching *per se*, since the preferred eye of amblyopic observers is patched while they perform the perceptual task. Second, PL is a form of ‘‘active’’ treatment, in which observers are engaged in making fine judgments near the limit of their performance using their amblyopic eyes. Third, PL can provide intensive, active, supervised visual experience with feedback, and thus may be more efficient than simply relying on everyday experiences. PL could result in a strengthening of already existing connections, rather than the development of new connections, perhaps by learning to attend to the information from the (normally suppressed) amblyopic eye.

Recent empirical and theoretical advances have given important new insights into the psychophysical mechanisms of PL. Much of the focus of recent work is on the question of whether PL operates via a reduction of internal neural noise or trough a more efficient use of the stimulus information (Dosher and Lu, 1998, 1999, 2004; Gold et al., 1999b; Li et al., 2004; Lu and Dosher, 2004). Most patients with amblyopia suffer from abnormally elevated spatial uncertainty, with the neural representation of the visual image being somewhat distorted at the cortical level (Lagreze and Sireteanu, 1991; Wang et al., 1998). Li and Levi (2004) showed that repetitive practice in positional acuity can lower the noise levels and increase the amblyopic brain’s ability to use relevant information more efficiently. In a recent work by Li et al. (2007) it has been shown that the amblyopic brain is able to recalibrate neuronal connections with response feedback to use the spatial information from lower level visual mechanisms more effectively and appropriately. Finally, Li et al. (2008) have demonstrated that the perceptual template of the amblyopic visual system can indeed be retuned gradually through repetitive practice, and that the retuned template is less noisy and more effective in interpreting visual information. Thus, practicing position discrimination can reduce spatial distortion (internal positional noise) and enhance sampling efficiency (the

ability to extract stimulus information) in amblyopic vision (Li and Levi, 2004; Li et al., 2007, 2008). The improved efficiency is a result of template retuning (Li et al., 2008). In a similar way, practicing identification of low contrast letters in noise (Levi, 2005) improves the contrast threshold for letter recognition primarily through increased efficiency.

Chapter 3

Aim of the thesis and experimental design

It has been known that the improvements in performance obtained by perceptual learning (PL) involve plastic changes at early stages of visual processing, however, whether visual PL is accompanied by synaptic plasticity phenomena in primary visual cortex (V1) is yet unknown. Long-term potentiation (LTP) is a valid model to study mechanisms of synaptic plasticity underlying memory and learning; however, while this LTP-learning relation is well established for other learning processes and underlying structures, such as the amygdala for cued fear conditioning or, more recently, hippocampus for spatial learning or inhibitory avoidance (e.g., Mitsuno et al., 1994; Sacchetti et al., 2002; Whitlock et al., 2006) and M1 for motor learning (Rioult-Pedotti et al., 2000), nothing is known for the relation LTP/perceptual learning in V1.

To assess this issue, I used a combination of behavioral visual PL tasks and electrophysiological recordings in V1 slices to evaluate the hypothesis that LTP, one of the best characterized forms of synaptic plasticity in the brain, can accompany visual PL in the rat at the level of V1 neural circuitries. To relate LTP with visual PL, I used two classical approaches: mimicry and occlusion of LTP. Mimicry can be verified by observing whether the discharge properties of neural structures involved in a previous learning task show changes that are similar to modifications known to be caused by LTP (Rogan et al., 1997; Rioult-Pedotti et al., 1998; Whitlock and al., 2006). After several intensive sessions of learning, LTP occlusion can be tested trying to induce LTP in the neural structure involved. The intent is to demonstrate that LTP is occluded or reduced, because most of the available potentiation had already been used in order to permit learning to take its course (Rioult-Pedotti et al., 2000; Whitlock et al., 2006).

I used a PL procedure consisting in training adult rats in a forced-choice visual discrimination task in distinguishing two vertical gratings, differing only for their spatial frequency (SF). The training continued until the animals reached a plateau in performance. It has been seen that visual PL shows an highly specificity for the features of the stimuli used in

the task (Fiorentini and Berardi, 1997). To test whether the PL task shows selectivity for the stimuli used, I rotated the stimulus orientation in a group of rats: at the end of their visual PL task, the animals had the orientation of their stimuli changed (from vertical to horizontal), and their discrimination ability was reassessed. In addition, to control the possibility that the difference in performance was due to a different discrimination ability for horizontal stimuli, I repeated the experiment in a separate group of rats, which were trained first with horizontal stimuli and then with vertical ones.

To test whether visual PL was associated with LTP-like processes in V1, V1 slices of animals trained with visual PL task were used for *in vitro* recordings of field excitatory post-synaptic potentials (fEPSPs) in layer II/III. I investigated whether visual PL led to an increase of excitability, mimicking a LTP effect (mimicry), and whether this increase in intracortical connectivity gain is paralleled by LTP-like phenomena, caused by the learning process: thus if LTP can be occluded by previous visual PL (occlusion). After several intensive sessions of learning, LTP occlusion can be tested trying to induce LTP in the neural structure involved, in this case V1. The intent is to demonstrate that LTP is occluded or reduced, because most of the available potentiation had been already used to permit learning to take its course

To verify mimicry, I measured the fEPSP amplitudes evoked by progressive increases in the stimulation intensity (input/output curves) in layers II-III of V1 slices by stimulation of either vertical (stimulating electrode placed in layer IV) and horizontal (stimulating electrode placed in layer II/III) connections. In order to verify the occlusion of IV-LTP and III-LTP, V1 slices were also used to test the effect of high frequency stimulation on vertical and horizontal connections.

Finally, in order to verify that PL occurred specifically in V1, I investigated if mimicry and occlusion were present in the primary somatosensory cortex (S1) of rats trained with PL task.

The results show a selective effect of mimicry and occlusion in V1. Thus, PL promotes potentiation of V1 visual connection activated during the learning process. If so, practicing PL with the ambliopic eye could favour potentiation of amblyopic eye connections in V1, thus promoting recovery form amblyopia.

I considered therefore whether the PL procedure developed in my thesis could be used as a therapeutic approach in treatment of experimental amblyopia.

The animals were rendered amblyopic by long-term monocular deprivation (MD), starting since the critical period (CP), and then subjected to reverse suture (RS) at 2 months of

age. The recovery of visual acuity in adult animals trained with the visual PL task was evaluated by two methods: I assessed the visual acuity with an electrophysiological technique, visual evoked potentials (VEPs) and, in a separate group of animals, I used the visual water box task, a behavioural technique. The recovery of binocularity was assessed by recording the contralateral-to-ipsilateral VEP ratio in the visual cortex contralateral to the long-term occluded eye in the same animals in which visual acuity measurements were done.

Although several studies (e.g., Polat et al. 2004; Zhou et al. 2006) found that improvement of PL can transfer, at least in part, to improvements in visual acuity, it has been pointed out that PL procedures are characterized by a narrow specificity of the achievable improvements, which are frequently limited to the selected trained stimulus, condition or task (e.g., stimulus orientation, see Levi and Polat 1996, Levi et al. 1997, Li and Levi 2004). Thus, I tested if the recovery of visual acuity was limited to stimuli of the same orientation than that used during the visual training procedure, or whether it was also present for orthogonal stimuli.

In order to use PL procedures as therapeutic strategy to treat amblyopia, it is important that visual acuity could be restored in the amblyopic eye and that this improvement could be stable for a long time period. Thus, using a behavioral technique in order to assess visual acuity, I evaluated if the improvement of visual acuity, reported after the PL procedure, was long lasting.

Finally, one important point which deserves further investigation is the necessity to understand the molecular mechanisms underlying the beneficial effects of PL on amblyopia recovery, in order that these findings could have a bearing in orienting clinical research in the field of amblyopia therapy. In this work, for the first time, I attempt to associate the recovery of vision in PL rats to a reduced inhibition/excitation (E/I) balance. The E/I circuit balance is well known to be crucially involved in the regulation of plasticity during development (Hensch, 2005), and it can point out a possible mechanisms for enhancing learning and recovery of function in adulthood, suggesting that a reduction of GABAergic transmission could be a crucial step for the restoration of plasticity processes in the adulthood (Bavelier, 2010; Baroncelli et al., 2011). I used brain biochemistry technique, as analysis of neurotransmitter release in synaptosomes, to investigate if the expected recovery of vision in PL rats is linked to a reduced E/I balance.

As already reported, an increasing number of clinical studies, have shown that repetitive visual training eliciting PL processes may be a very useful approach for the

treatment of amblyopia in humans, providing a substantial improvement in a variety of visual tasks (Levi and Li, 2009; Astle et al., 2011). My results could contribute to provide a mechanism of action for these beneficial effects of PL in amblyopic subjects and possibly to design more effective intervention protocols.

Chapter 4

Materials and Methods

4.1 Animal Treatment and Surgical Procedures

Long-Evans hooded rats were used in this study, which has been approved by the Italian Ministry of Public Health and was carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

The animals were housed in a room with a temperature of 21°C and a 12/12 hrs light–dark cycle, and food and water were provided ad libitum. For the mimicry and occlusion of LTP experiments, naïve rats of 2-3 months of age were used. For the experiments of amblyopia recovery, I rendered rats amblyopic by using the gold standard procedure adopted for rodents, i.e. long-term monocular deprivation (MD) (Pizzorusso et al., 2006; He et al., 2007; Maya Vetencourt et al., 2008; Silingardi et al., 2010; Spolidoro et al., 2011). Rats were anesthetized with avertin (1 ml/hg) at P21, when MD was performed through eyelid suturing. Animals were allowed to recover from anesthesia and were returned to their cages. Eyelid closure was inspected daily until complete cicatrisation. Rats showing occasional lid reopening were not included in the experiments. Adult rats (P70) were subjected, under avertin anaesthesia, to reverse suture (RS) consisting in reopening of the long-term deprived eye and in the closure of the other eye. Great care was taken to prevent opacities of the reopened eye by topical application of Aureomicin cream (Wyeth Lederle, Italy) onto the cornea during the first 3 days of RS. After RS, rats were allowed to recover from anesthesia and then returned, for 3 weeks, to their previous cage or transferred to rearing conditions specific for the different components of EE. Subjects showing spontaneous lid reopening or eye anomalies were excluded from the study.

4.2 Behavioral tasks

4.2.1 Perceptual learning task

I used a modified version of the visual water box task (Prusky et al., 2000). The apparatus consisted of a trapezoidal-shaped pool with two computer-controlled monitors (diagonal screen size 40 cm) placed side-by-side at one end of the pool. The pool (140 cm long) was made of 6 mm Plexiglas, with 55 cm high walls, and was wider at one end (80 cm) than the other (25 cm). A midline divider (50 cm long) extended between the monitors into the pool, bisecting its longitudinal axis. The length of the divider set the animal choice point and the visual angle at which the spatial frequency (SF) of the stimulus was that calculated by the computer software that generated the grating. An escape platform was placed below one of the two monitors and the pool was filled with tepid (25°C) water to a depth of 15 cm. White paint rendered the platform invisible from water level. Visual stimuli were presented on the two monitors visible through two glass windows (Fig. 1).

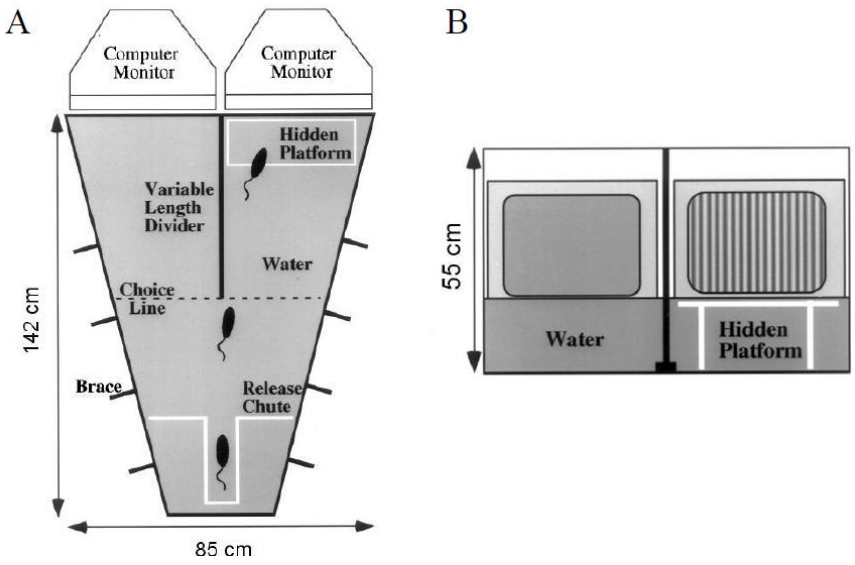

Fig. 1: Schematic diagram and components of the visual water box. (A) View from above showing the major components of the visual water box including pool, midline divider, platform, starting chute and two monitors. (B) Front view showing monitor screens, submerged platform and midline divider. Modified from Prusky et al. 2000.

A group of 10 animals, the perceptual learning group (PL rats), was first trained to distinguish a 0.117 cycles per degree (c/deg) SF grating (reference grating) from a 0.712 c/deg SF grating (test grating). The SF of the reference grating corresponded to 4 cycles on the computer screen. The two gratings had the same luminance (40.06 cd/m^2) and the same

contrast (90%). A custom-made software presented the two stimuli on the monitors, alternating the position of the test and the reference grating in a pseudorandom schedule. The submerged platform was always positioned in correspondence with the reference grating. A group of 10 control animals (associative learning, AL rats) was trained to distinguish the reference grating from a homogeneous grey. The training consisted in three sessions per day of 15 trials each, interleaved by 60 minutes. After animals achieved a level >80% of accuracy in at least three subsequent sessions, the PL protocol was started. The SF of the reference grating was gradually reduced from 0.712 c/deg to 0.127 c/deg in steps consisting in the subtraction of one cycle on the screen (see Table 1). If the animal made a correct choice, the SF of the test grating was decreased by one step and another trial was executed. This procedure continued until an error was made. Once an error occurred, three correct responses in a block of four trials or seven in a block of ten trials were required to decrease the SF by one step. If more than 1/4 or 3/10 errors were made, the SF was increased by one step and another block of trials was run. After trials covering approximately half of the animal's projected threshold were completed, the minimum number of trials in a block was increased to four. For the last three SFs of the test grating, the required performance to decrease the SF was always 7/10 correct choices. For each animal, I measured a daily threshold, calculated as the lowest SF of the test grating that the rat was able to distinguish (70% correct performance) from the reference grating in at least two blocks of trials. The PL task was ended when the animal performance reached a plateau (performance at a given SF of the test grating oscillating around 70% of correct choices for three consecutive days).

C/deg	Criterion
0.712	1 out of 1
0.684	1 out of 1
0.659	1 out of 1
0.636	1 out of 1
0.593	1 out of 1
0.556	1 out of 1
0.523	1 out of 1
0.494	2 out of 2
0.468	2 out of 2
0.434	2 out of 2
0.404	2 out of 2
0.378	2 out of 2
0.356	2 out of 2
0.329	2 out of 2

0.296	3 out of 4
0.269	3 out of 4
0.234	3 out of 4
0.207	3 out of 4
0.178	7 out of 10
0.148	7 out of 10
0.136	7 out of 10
0.127	7 out of 10

Table 1: The scale of SFs utilized during the PL procedure.

One further control group (1st step PL rats, n = 4) learned the PL task but was allowed to only distinguish the reference grating from a test grating whose SF was always maintained at the starting value of 0.712 c/deg (Fig. 2). Control rats were matched to PL animals in terms of overall swim time and training days in the water maze.

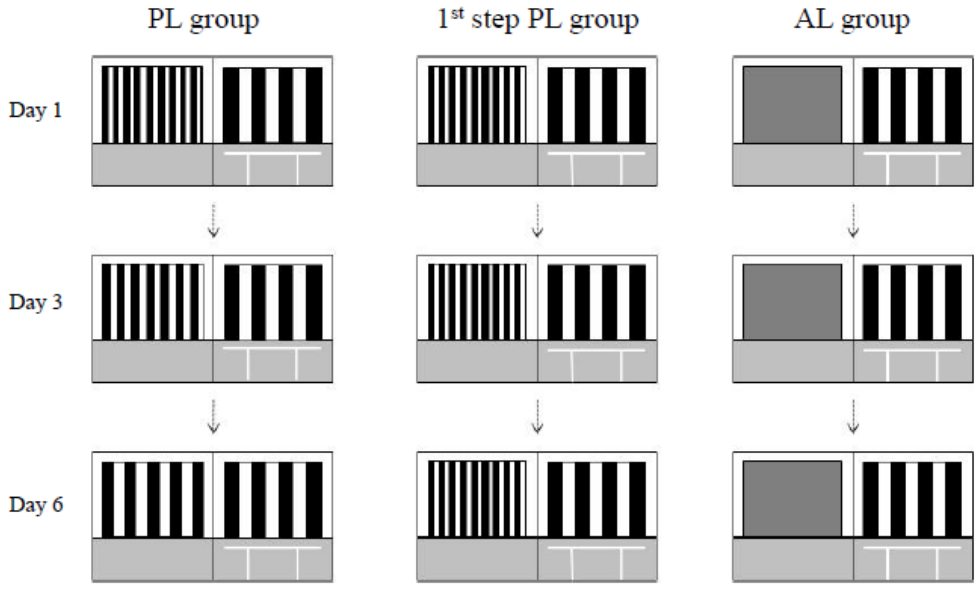

Fig. 2: Schematic diagram for the PL task. Examples of stimuli discrimination for each group are shown (PL group, 1st step PL group and AL group). For the PL group, during the course of the learning sessions, the SF of the test grating was gradually decreased from 0.712 c/deg to 0.127 c/deg, according to the progress of improvement. The 1st step PL group learned the PL task but was allowed to only distinguish the reference grating from a test grating whose SF was always maintained at the starting value of 0,712 c/deg. The AL group learned to discriminate the reference grating from a homogeneous grey. AL and 1st step PL rats were matched to PL animals in terms of overall swim time and training days in the water maze.

In a separate group of rats, I performed an experiment of stimulus orientation rotation. After PL animals (n = 11) reached their performance plateau with vertical gratings, the orientation of stimuli were rotated by 90° and new trials were applied in order to measure the MDSFD (minimum discriminable spatial frequency difference) for horizontal gratings. Then I repeated the experiment in a separate group of rats (n=5), which were trained first with horizontal stimuli and then with vertical ones. When the animal performance reached a plateau the PL task was ended, and then the stimuli were rotated from horizontal to vertical (thus doing the opposite than in the first group of rats). First it was tested whether the animals were able to discriminate the newly oriented stimuli when the SF of the test grating was maintained at the value corresponding to the MDSFD reached before the orientation change. Afterward, to reassess the ability to discriminate two vertically oriented gratings of different SFs, I repeated, starting from this point, the PL procedure, going ahead rendering the SF of the test grating progressively more similar to that of the reference grating.

Perceptual learning task in the amblyopic animals

The PL procedure used for the amblyopic animals follows quite the same procedure as the naïve animals: the amblyopic animals (PL-amb rats) were initially trained to distinguish a low SF grating (reference grating, 0.117 c/deg) from a higher SF grating (test grating, 0.593 c/deg), and, after this training phase, the PL protocol was started by gradually reducing the SF (see Table 2) and ended when the animal performance reached a plateau. For each animal, a daily threshold was calculated as the lowest SF of the test grating that the rat was able to distinguish (70% correct performance) from the reference grating. A group of control animals was trained to distinguish the reference grating from a homogeneous grey (associative learning, AL-amb rats). One further control group (1^{st} step PL-amb rats) learned the PL task but was allowed to only distinguish the reference grating from a test grating whose SF was always maintained at the starting value of 0.593 c/deg. Control rats were matched to PL-amb animals in terms of overall swim time and training days in the water maze.

C/deg	Criterion
0.593	2 out of 2
0.556	2 out of 2
0.523	2 out of 2
0.494	2 out of 2
0.468	2 out of 2
0.434	2 out of 2

0.404	2 out of 2
0.378	3 out of 4
0.356	3 out of 4
0.329	3 out of 4
0.296	3 out of 4
0.269	3 out of 4
0.234	3 out of 4
0.207	3 out of 4
0.178	7 out of 10
0.148	7 out of 10
0.136	7 out of 10
0.127	7 out of 10

Table 2: The scale of SFs utilized during the PL procedure with amlyopic rats.

4.2.2 Behavioral assessment of visual acuity

In a separate group of rats subjected to PL, I also measured visual acuity through the behavioral method of the visual water maze task. Visual acuity (VA) was first measured at P60 through the non amblyopic (not deprived) eye; then, I measured VA through the amblyopic eye three times: immediately after RS, at the end of the PL procedure and after a period of 15 further days.

Behavioral assessment of VA were performed as previously described (Prusky et al., 2000; Sale et al., 2007). I used the visual water task, which trains animals to first distinguish a low (0.117 cycles deg^{-1}) SF vertical grating from grey, and then tests the limit of this ability at higher SFs. The apparatus consists of a trapezoidal-shaped pool with two panels placed side by side at one end. A midline divider is extended from the wide end of the pool into the middle, creating a maze with a stem and two arms. The length of the divider sets the choice point and effective SF. An escape platform is placed below the grating. Animals are released from the centre at the end of the pool opposite the panels. The position of the grating and the platform is alternated in a pseudorandom sequence over training trials while the rats are shaped to swim towards the grating in one of the maze arms. A trial is recorded as incorrect if an animal enters the arm without the platform. Animals are removed from the pool when they find the platform. Once 80% accuracy is achieved, the limit of the discrimination is estimated

by increasing the SF of the grating. VA has been taken as the SF corresponding to 70% of correct choices on the sigmoidal function fitting the psychometric function (Fig. 3).

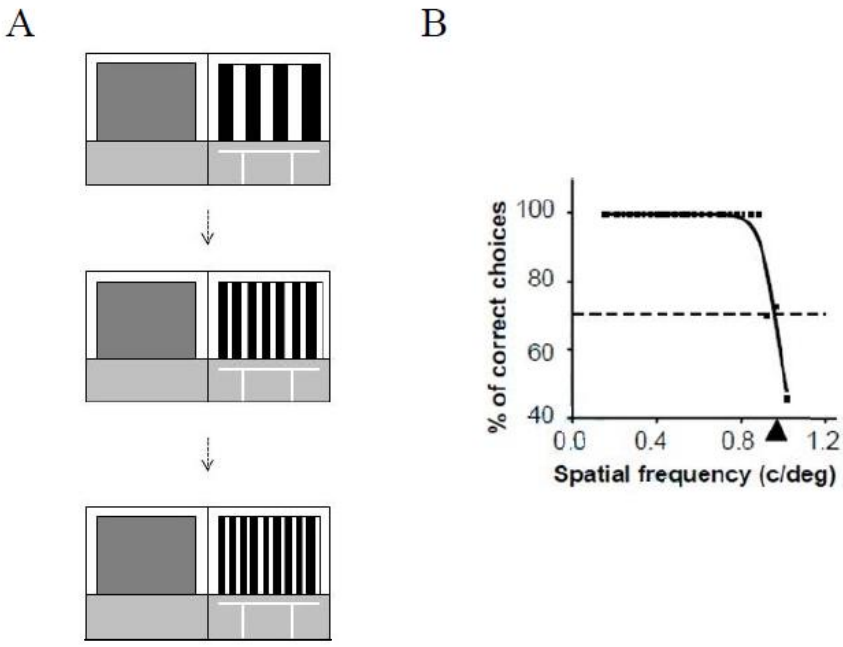

Fig. 3: (A) Examples of steps in the assessment of VA. (B) For the testing phase small incremental changes in the SF of the stimulus are made between successive blocks of trials until the ability of animals to distinguish a grating from grey falls to chance. VA has been taken as the SF corresponding to 70% of correct choices on the sigmoidal function fitting the psychometric function.

4.3 In vitro electrophysiology: LTP

Within 10 min after the last behavioral trial in the maze, brains from 10 PL and 10 AL rats (age between 60 and 75 days) were removed and immersed in ice-cold cutting solution containing (in mM): 220 sucrose, 3.1 KCl, 1.0 K_2HPO_4, 4.0 $NaHCO_3$, 2.0 $MgCl_2$, 1.0 $CaCl_2$, 10 HEPES, 1.0 ascorbic acid, 0.5 myo-Inositol, 2.0 pyruvic acid, and 1.0 kynurenate, pH 7.3. Slices (0.35mm thick) of visual cortex were obtained using a Leica (Nussloch, Germany) vibratome. Slices were perfused at a rate of 2 ml/min with 35°C oxygenated recording solution. The recording solution was composed as the cutting solution with the following differences (in mM): 130 NaCl, 5.0 dextrose, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 0.01 glycine, no kynurenate, no sucrose. Electrical stimulation (100 μsec duration) was delivered with a bipolar concentric stimulating electrode (FHC, St. Bowdoinham, ME). Field potentials in layer II-III were recorded by a micropipette (1–3M) filled with NaCl (3 M). Baseline

responses were obtained every 30 sec with a stimulation intensity that yielded a half-maximal response. After achievement of a 15 min stable baseline (field potential amplitude within 15% of change and with no evident increasing or decreasing trends), theta burst stimulation (TBS) was delivered. The slices were submitted to one of two different stimulation conditions: stimulation of vertical connections (stimulating electrode placed in layer IV: 10 animals, n = 9 slices for controls; 10 animals, n = 8 slices for PL rats; 4 animals, n = 7 slices for 1st step PL rats) or horizontal connections (stimulating electrode placed in layer II/III, in sites displaced horizontally by 0.5 mm from the recording electrode, 10 animals, n = 12 slices for AL rats; 10 animals, n = 8 slices for PL rats). All recordings were made from cortical layer II-III.

In a separate group of PL and AL rats, LTP was elicited by layer IV stimulation in the primary somatosensory cortex (9 animals, n =11 slices for AL; 9 animals, n = 13 slices for PL rats).

4.4. In vivo electrophysiology

Visual evoked potentials (VEPs) were recorded from the binocular portion of the visual cortex (Oc1B) as described in Porciatti et al. 1999. Rats were anesthetized with an intraperitoneal injection of 20% urethane (Sigma, St.Louis, MO, USA; 0.7 ml/hg of body weight) and mounted in a stereotaxic apparatus allowing full viewing of the visual stimulus. Additional doses of urethane were used to keep the anesthesia level stable throughout the experiment. The closed eye was reopened using scissors and both eyes were restrained in a fixed position by means of adjustable metal rings surrounding the external portion of the eye bulb. The pupil was always clearly observable between eyelid margins. Body temperature was continuously monitored with a rectal probe and maintained at 37.0°C with a thermostatic electric blanket during the experiment. An electrocardiogram was monitored and respiration was facilitated by means of an oxygen mask. A portion of the skull (2 x 2 mm) overlying the OcB1 was carefully drilled and the dura madre was removed. A resin-coated microelectrode (Harvard apparatus, Edenbridge, UK) with tip impedance of 2 MΩ filled with NaCl (3M) was inserted into the cortex perpendicularly to the stereotaxic plane, 4.8-5.2 mm lateral to lambda (intersection between sagittal- and lambdoid-sutures). Microelectrodes were advanced 100 or 400 μm within the cortex. At those depths VEPs had their maximal amplitude. Typical visual stimuli were horizontal sinusoidal gratings of different SF generated by a VSG2/2 card

(Cambridge Research System, Cheshire, UK) and presented on the face of a monitor suitably linearized by gamma correction. The display (mean luminance 25 cd/m2, area 24 x 26 cm) was placed 20 cm in front of the animal and centered on the previously determined receptive fields (RFs). Electrical signals were amplified (10000 fold), band-pass filtered (0.1–100 Hz), digitized (12 bit resolution) and averaged (at least 50 events in blocks of 10 events each) in synchrony with the stimulus contrast reversal. Transient VEPs in response to abrupt contrast reversal (0.5 Hz) were evaluated in the time domain by measuring the peak-to-baseline amplitude and peak latency of the major component.

I measured binocularity by calculating the contralateral to ipsilateral VEP ratio (C/I ratio), i.e. the ratio of VEP amplitudes recorded by stimulating the eye contralateral and ipsilateral, respectively, to the visual cortex where the recording is performed. During recording through one eye, the other was covered by a black adhesive tape. To prevent sampling bias for each animal, at least three well-spaced penetrations were performed and at least ten series of responses from each eye were alternatively recorded. Care was taken to equally sample VEPs across the two cortical depths so that all layers contributed to the analysis. VA of each eye was obtained by extrapolation to zero amplitude of the linear regression through the data points in a curve where VEP amplitude is plotted against log SF.

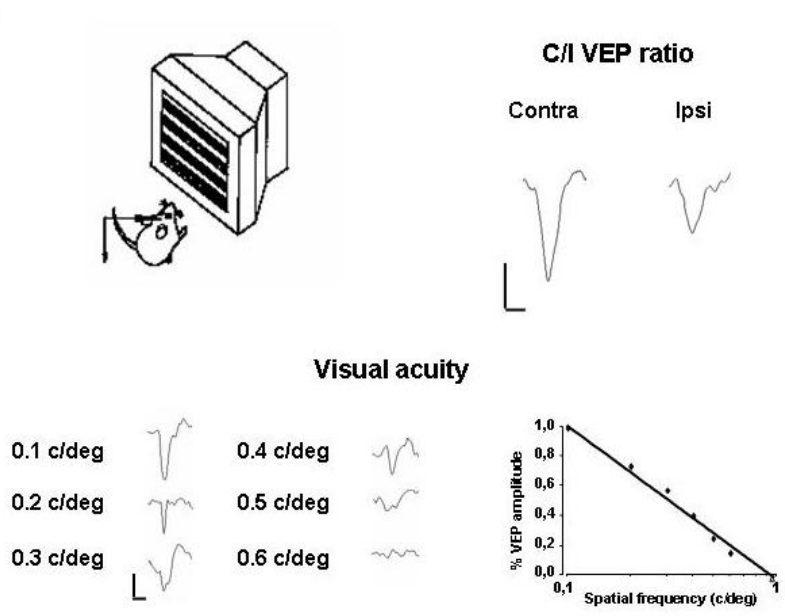

Fig. 4: An illustrative diagram of determination of visual acuity with VEPs. For VEP recordings visual stimuli were horizontal or vertical sinusoidal gratings of different SFs and contrast. C/I VEP ratio was calculated as the averaged ratio of VEP amplitudes recorded by stimulating the eye contralateral and ipsilateral, respectively, to

the visual cortex where the recording is performed. VA 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 SF. Calibration bars: 50 μV, 100 ms.

4.5. Analysis of neurotransmitter release in V1 synaptosomes

Animals were sacrificed and the cortical area corresponding to the V1 was removed. Synaptosomes were prepared essentially as previously described (Stigliani et al., 2006). The tissue was homogenized at 4°C, utilizing a homogenizer Teflon/glass (clearance 0.25 mm), in 10 volumes of sucrose 0.32M, buffered with Tris-HCl at pH 7.4. The homogenized tissue was centrifuged (5 min, 1000 x g a 4°C) in order to remove all nuclei and cellular fragments. Then, the supernatant was gently stratified on a discontinuous Percoll gradient (2, 6, 10, 20% v/v in tris HCl/sucrose) and again centrifuged (33500 x g per 5 min a 4°C). After centrifugation, the stratified fraction of synaptosomes, leaning between 10% and 20% Percoll, was collected, washed by centrifugation (20200 x g per 15 min a 4°C) and then resuspended in a physiologic medium, containing: NaCl 140mM; KCl 3mM; $MgCl_2$ 1.2mM; $CaCl_2$ 1.2mM; NaH_2PO4 1.2mM; HEPES 10mM; glucose 10mM; pH 7.4.

Synaptosomes were incubated at 37°C for 15 min with the radioactive tracers (3H)D-Asp (a widely used non-metabolizable tracer labelling endogenous glutamatergic pool of synaptic vescicles supporting GLU release) or (^{3}H)GABA, at a final concentration of 0.05μM. (^{3}H)GABA labeling was performed in the presence of 50 μM of the GABA transaminase inhibitor amino-oxyacetic acid, to minimize GABA catabolism. Aliquots of the synaptosomal suspensions were layered on microporous filters at the bottom of a set of parallel superfusion chambers (Superfusion System, Ugo Basile, Comerio, Varese, Italy) (Raiteri et al., 1984) maintained at 37°C. Superfusion was started at a rate of 0.5 ml/min with standard medium, supplemented with 50 μM amino-oxyacetic acid in the case of (^{3}H)GABA release. After 36 min of superfusion, to equilibrate the system, samples were collected according to the following scheme: one sample collected for 3-min (t = 36-39 min; basal outflow); one sample collected for 6-min (t = 39-45 min; stimulus-evoked release); one sample collected for 3-min (t = 45-48 min; basal outflow after stimulus-evoked release). A 90-sec period of stimulation was applied at t = 39 min, after the first sample has been collected. Lower-intensity stimulation (i.e. 15 mM KCl, substituting for equimolar concentration of NaCl) was applied in the case of (^{3}H)D-Asp, since augmentation of the release rate was eventually expected.

Higher-intensity stimulation (i.e. 25 mM KCl) was applied in the case of (^{3}H)GABA. Radioactivity was determined in each sample collected and superfused filters by liquid scintillation counting. Tritium released in each sample was calculated as percentage of the total synaptosomal tritium content at the beginning of the respective sample collection (fractional rate x 100). The stimulus-evoked overflow was estimated by subtracting transmitter content of the two 3-min samples (basal outflow) from release evoked in the 6-min sample collected during and after the depolarization pulse (stimulus evoked release).

Chapter 5

Results

5.1 Visual perceptual learning improves visual discrimination abilities in adult rats

A group of adult rats (visual perceptual learning group, PL) practiced in a forced-choice visual discrimination task in distinguishing between two vertical gratings differing only for their spatial frequency (SF). The SF of the test grating was made progressively more similar to that of the reference grating [0.117 cycles/deg (c/deg)], starting from a SF of the test grating of 0.712 c/deg. Thus, visual PL consisted in the improvement of visual discrimination abilities allowing VD animals to distinguish the two gratings when they became more and more similar to each other. The task continued until the animals reached a plateau. A separate group of control rats (associative learning group, AL) was required to discriminate the reference grating from a homogeneous grey. One further control group (1^{st} step PL group) learned the PL task but was allowed to only distinguish the reference grating from a test grating whose SF was always maintained at the starting value of 0.712 c/deg. AL and 1^{st} step PL rats were matched to PL animals in terms of overall swim time and training days in the water maze.

PL rats displayed robust visual PL, as evidenced by the progressive reduction in the minimum discriminable SF difference (MDSFD) between the reference and the test grating across the days: while, on the first day, the mean MDSFD was 0.064 c/deg, this value reached 0.03 c/deg at the end of the testing (Fig. 5A). A clear PL was also revealed by the increase in the percentage of correct choices for a given SF of the test grating (see Fig. 5B for an example)

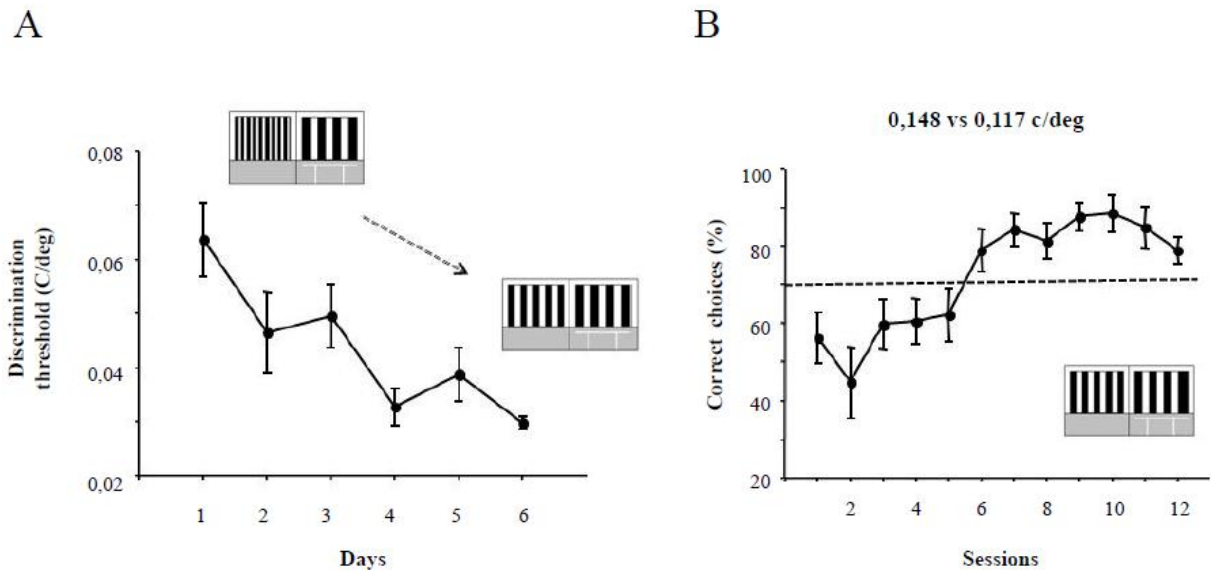

Fig. 5: Visual PL in adult rats. (A) Improvement of discrimination threshold in rats (PL rats, n=10) involved in the visual discrimination task. The threshold, calculated as the minimum spatial frequency difference between the reference and the test gratings discriminated (MDSFD, data represented as mean ± s.e.), decreases significantly with the training days (One way Repeated Measures ANOVA, $p<0.001$). The MDSFD obtained in the sixth day of the PL task was statistically different from that obtained in the 1st day (Holm-Sidak method, $p<0.05$). (B) Mean PL animal performance in distinguishing a test grating of 0.148 c/deg from the reference grating (0.117 c/deg) across the training sessions. The increase in the percentage of correct choices with sessions is significant (Friedman Repeated Measures ANOVA on Ranks $p<0.001$).

5.2 Visual perceptual learning task is selective for stimuli orientation

To test the direct involvement of V1 circuitries in the PL process, I performed an experiment of stimulus orientation rotation in a separate group of rats (n=11). After PL animals reached their performance plateau with vertical gratings, the orientation of stimuli were rotated by 90° and new trials were applied in order to measure the MDSFD for horizontal gratings. I found that PL was selective for the orientation of the gratings, as demonstrated by the marked impairment in the discrimination abilities displayed by PL animals trained with vertical gratings after the stimuli were turned horizontal (MDSFD before rotation: 0.040 ± 0.005 c/deg; MDSFD after rotation: 0.104 ± 0.018 c/deg; Paired t-test: $p<0.05$) (Fig. 6A). Then, to control the possibility that this impairment was not specifically due to a lack of learning effect transfer following the change in orientation but rather to worse discrimination abilities for horizontal stimuli, I repeated the experiment in a separate group of rats (n=5), which were trained first with horizontal stimuli and then with vertical ones. I found that in this group of animals both the PL curve and the final threshold abilities were not different from those

previously reported for vertically-trained rats (Fig. 6B; Two way repeated measures ANOVA, p=0.131; final MDSFD for vertical stimuli: 0.030 ± 0.001 c/deg; final MDSFD for horizontal stimuli: 0.024 ± 0.003 c/deg; t-test: p=0.137). Then, I rotated the stimuli from horizontal to vertical (thus doing the opposite than in the first group of rats) and I reassessed the animal discrimination abilities.

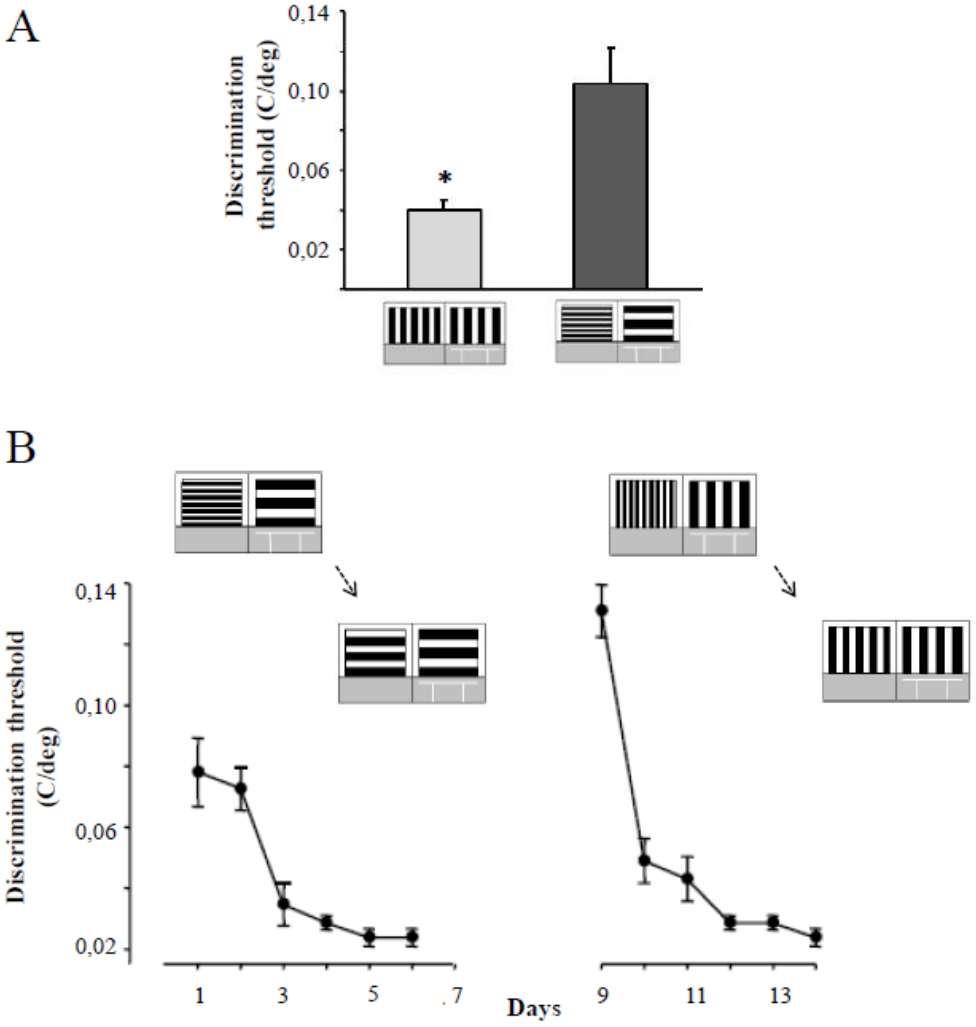

Fig. 6: Visual PL is specific for stimulus orientation. (A) MDSFD obtained in PL animals trained in discriminating first vertical gratings and then tested with horizontal. After the orientation change, the animals displayed a marked impairment in their discrimination abilities (paired t-test $p<0.05$). (B) MDSFD obtained in a separate group of PL animals trained first in horizontal grating discrimination and then, after rotating the stimuli by 90°, in vertical grating discrimination. MDSFD for horizontal gratings decreases significantly with the training days (One way Repeated Measures ANOVA, $p<0.001$). Immediately after the orientation change, there is a marked and statistically significant increase in MDSFD (Paired t-test: $p<0.05$). With practice, a new PL takes place (MDSFD decreases significantly with the training days, (One way Repeated Measures ANOVA, $p<0.001$) and each animal eventually reaches a final MDSFD with vertical gratings equal to that previously reached with horizontal gratings. * = statistical significance; error bars = s.e.m.

In agreement with the previous results, I found that the rats were totally unable to discriminate the newly oriented stimuli when the SF of the test grating was maintained at the value corresponding to the MDSFD reached before the orientation change; indeed, the percent of correct responses fell below 70% correct. This impairment persisted for at least three consecutive training sessions (data not shown). I then assessed the MDSFD which turned out to be 0.131 ± 0.009 c/deg (Fig. 6B), that is, much higher than the value reached with horizontal gratings before the orientation turning (Paired t-test: $p<0.05$). These results replicate those shown in Fig. 6A for the change between vertical and horizontal gratings, confirming that PL effects do not transfer between stimuli of orthogonal orientation. Finally, starting from this point, I repeated the PL procedure, going ahead rendering the SF of the test grating progressively more similar to that of the reference grating (Fig. 6B). This allowed us to obtain a second PL curve (decrease of MDSFD with sessions) and to calculate the new final MDSFD for vertical gratings achievable by the animals after training. Importantly, this threshold was exactly the same found in this group after completing the training with horizontal gratings before the orientation was changed to vertical (Fig. 6B) and did not statistically differ from that originally recorded in the first group of rats, which were trained with vertical gratings before being tested with horizontal ones (Fig. 5A) (t-test: $p=0.137$). Taken together these results confirm a marked lack of PL transfer effect following a change of 90° in stimulus orientation, independently on the orientation chosen to perform the initial PL procedure.

5.3 Visual perceptual learning causes LTP-like changes in primary visual cortex

I tested whether visual PL was associated with LTP-like processes in primary visual cortex (V1). Within 1 h from the end of the PL task, brain slices of visual cortex were obtained from PL, AL and 1st step PL animals to perform *in vitro* electrophysiological experiments. Recording electrode was placed in layer II/III.I tested synaptic efficacy and plasticity of both vertical (stimulating electrode placed in layer IV; 10 animals, n=9 slices for AL rats; 10 animals, n=8 slices for PL rats; 4 animals, n=7 slices for 1st step PL rats) and horizontal (stimulating electrode placed in layer II/III; 10 animals, n=12 slices for AL rats; 10 animals, n=8 slices for PL rats) connections. I measured the f-EPSP amplitudes evoked by progressive

increases in the stimulation intensity (input/output curves, I/O curves). I found that, while the maximum f-EPSP amplitudes did not change between PL and AL slices under both stimulating conditions (mean ± standard error, respectively for layer IV and layer II–III stimulation: 1042.5 ± 110.826 µV for PL rats and 1097.158 ± 209.779 µV for AL rats, t-test p=0.842; 972.555 ± 145.491 µV for PL rats and 890.870 ± 83.133 µV for AL rats, t-test p=0.644), I/O curves recorded from PL animal slices were shifted leftward compared to controls (Fig. 7). These results suggest that PL promoted a synaptic strengthening of V1 connections to layer II–III.

To further demonstrate that the field effects were specifically elicited by PL, I performed a control, using the vertical connection paradigm (stimulating electrode placed in layer IV). First, I checked for any sign of mimicry or occlusion in V1 slices of a separate group of rats (1st step PL rats) that learned the PL task but was allowed to only distinguish the reference grating from a test grating whose SF was always maintained at the starting value of 0.712 c/deg. I found that I/O curves recorded from 1st step PL rats were shifted rightward compared to PL animals and, for some input values, even comparable to controls (Fig. 7, left, see figure legend for statistical details). Thus, no synaptic strengthening of V1 connections was detected in 1st step PL.

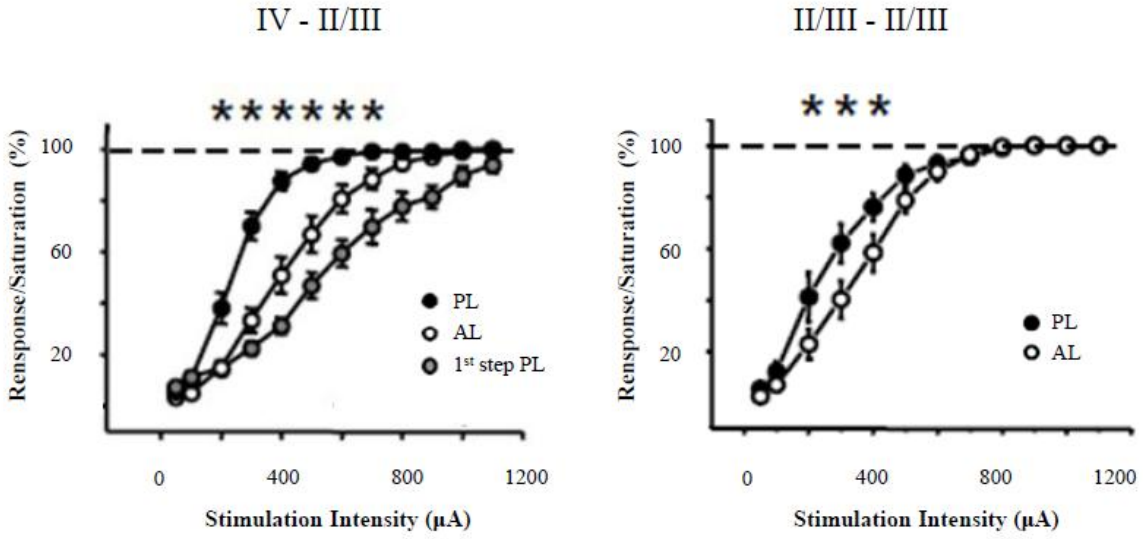

Fig. 7: Amplitude of field excitatory post-synaptic potentials (f-EPSPs) normalised to saturation level as a function of stimulation intensity in PL (black), AL (white) and 1st step PL (gray) animals (left: stimulation from layer IV; right: stimulation from layer II-III). For both layer IV and layer II-III stimulation, two way repeated

measures ANOVA revealed a statistically significant interaction between treatment and intensity of stimulation ($p<0.001$). PL slices exhibited higher f-EPSP amplitudes in the middle part of the input/output curves (Holm-Sidak method, $p<0.05$). For layer IV stimulation, two way repeated measures ANOVA revealed that PL slices exhibited higher f-EPSP amplitudes in the 300-800 μA range of stimulation compared to AL animals and in the 300-900 μA range of stimulation compared to 1st step PL animals (Holm-Sidak method, $p<0.05$). * = statistical significance; error bars = s.e.m.

5.4 Visual perceptual learning occludes LTP in primary visual cortex

I investigated whether visual PL can occlude LTP in V1. The f-EPSP with an amplitude nearly half the saturation level was monitored for a baseline period of 15 minutes, before the first TBS was delivered. The entire LTP protocol consisted of three successive TBS. After each of them the f-EPSP was monitored for 30 minutes before the following one to be triggered. There were two kinds of LTP recordings according to the stimulation site: IV-LTP, in case of layer IV stimulation site and II/III-LTP, in case of layer II/III stimulation site. I found that, under both stimulating conditions, significant LTP was induced in AL slices already after the first TBS induction (Two ways RM ANOVA, $p<0.05$). Level of LTP increased further after the second TBS, but it did not change over during the third post-TBS period (Fig. 8; Fig. 9). Strikingly, for both IV layer induced and III layer-induced LTP, in PL animal slices significant LTP was not achieved even after the third TBS (Fig. 8; Fig. 9), even if a trend toward potentiation was present. A direct comparison between PL and AL slices showed that levels of LTP were statistically different, respectively, for the first and second (IV layer-induced LTP, see Fig. 8 for details) or for the second and third (III layer-induced LTP, see Fig. 9 for details) post-TBS periods (Fig. 8; Fig. 9). Thus, the synaptic reinforcement induced by PL was paralleled by occlusion of further LTP in V1. These results demonstrate that practice with specific visual stimuli induces LTP in V1.

Accordingly to the lack of strengthening of V1 connections in 1st PL animals, neither the occlusion criterion was satisfied in the same group: indeed, LTP levels in V1 slices from 1st step PL rats were totally comparable with AL rats and significantly higher compared to PL animal slices in the first and second post-TBS period (Fig. 8).

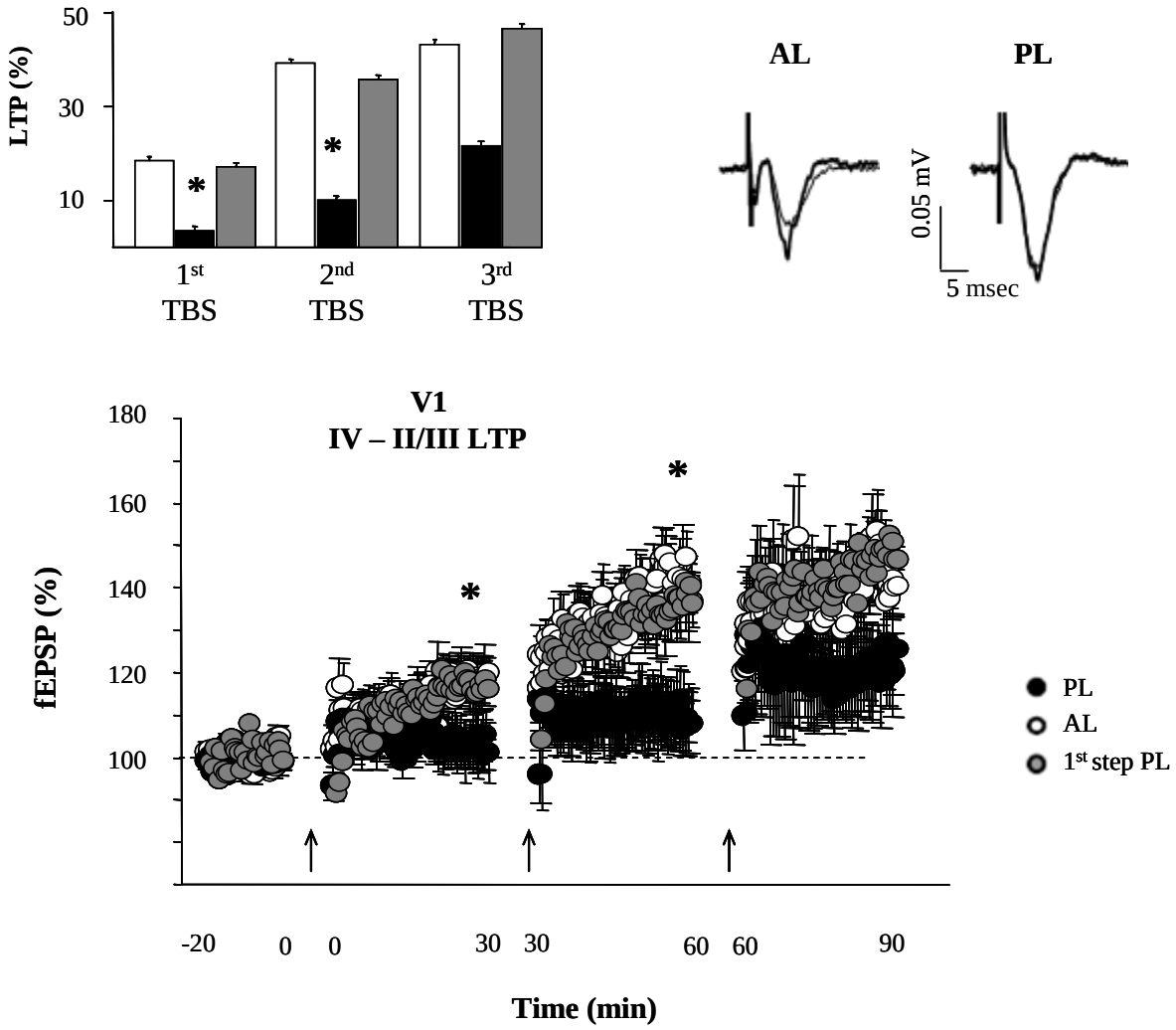

Fig. 8: Visual PL occludes LTP in V1 in vertical connections. LTP evoked in layer II-III of primary visual cortical slices in PL (black), AL (white) and 1st step PL (gray) rats by theta-burst stimulation (TBS) applied to layer IV. Two way Repeated Measures ANOVA revealed that only AL rats and 1st step PL rats exhibited significant LTP already after the first TBS ($p<0.05$), while no LTP was present in PL slices even after the third TBS ($p = 0.11$). After both the first and the second TBS, LTP levels were statistically higher in AL rats than in PL rats and in 1st step PL animals than in PL rats (Two way Repeated Measures ANOVA, $p<0.05$), but they did not differ between AL and 1st step PL rats ($p = 0.84$ and 0.73, respectively). LTP levels in the three groups did not differ among each other after the third TBS (Two way Repeated Measures ANOVA, $p>0.05$). Arrows indicate TBS. Sample traces from PL and AL slices 5 min before (thin line) and 25 min after (thick line) the second TBS are also reported. The inset histogram reports the mean level of LTP recorded during the last 10 minutes of each post- TBS period. * = statistical significance; error bars = s.e.m.

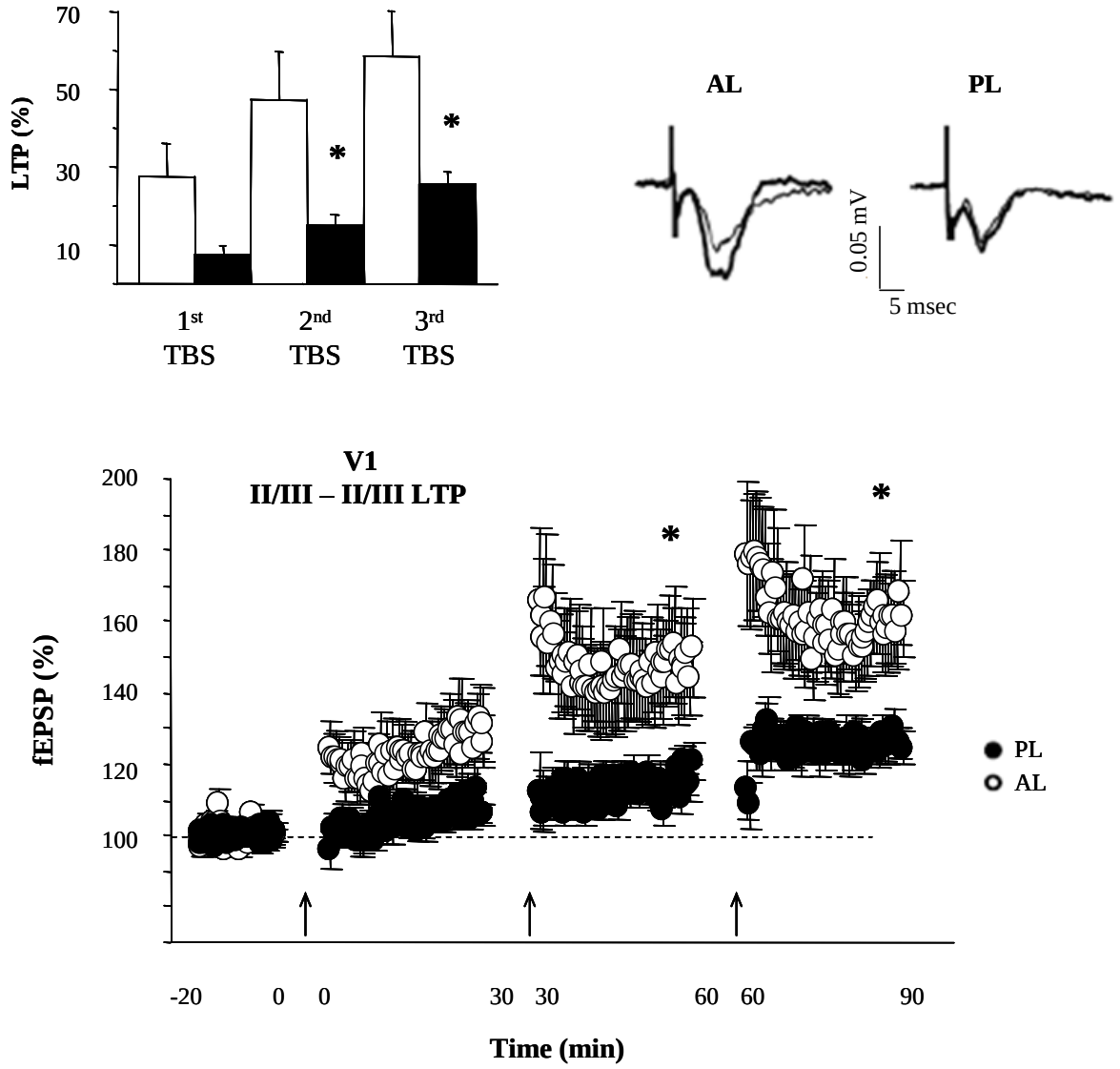

Fig. 9: Visual PL occludes LTP in V1 in horizontal connections. LTP evoked in layer II-III of primary visual cortical slices in PL (black) or AL (white) rats by TBS applied to the same layer. Two way Repeated Measures ANOVA revealed an effect of both treatment ($p<0.05$) and time ($p<0.001$), and a significant interaction between them ($p<0.05$). An all pairwise multiple comparison procedures (Holm-Sidak method) revealed that the LTP level is significantly different between PL and AL rats after the second and the third TBS ($p<0.05$). Arrows indicate TBS. Sample traces from PL and AL slices 5 min before (thin line) and 25 min after (thick line) the second TBS are also reported. The inset histogram reports the mean level of LTP recorded during the last 10 minutes of each post- TBS period. * = statistical significance; error bars = s.e.m.

5.5 Visual perceptual learning does not involve changes in primary somatosensory cortex

I also examined whether the mimicry and occlusion effects detected in PL rats were specific to visual cortex, by examining field potentials in the primary somatosensory cortex (S1). In a

separate group of PL and AL rats, brain slices of the primary somatosensory cortex were used for in vitro recordings of f-EPSPs in layer II/III (9 animals, n=11 slices for AL rats; 9 animals, n=13 slices for PL rats). I tested synaptic efficacy and plasticity of vertical connections only (stimulating electrode placed in layer IV). First I measured the fEPSP amplitudes evoked by progressive increases in the stimulation intensity (I/O curves); then, I monitored susceptibility of f-EPSPs to LTP in a multiple step procedure in which three TBS were applied, interleaved by 30 min. No difference was present either in I/O curves or LTP values between PL and AL S1 slices (Fig. 10A, B).

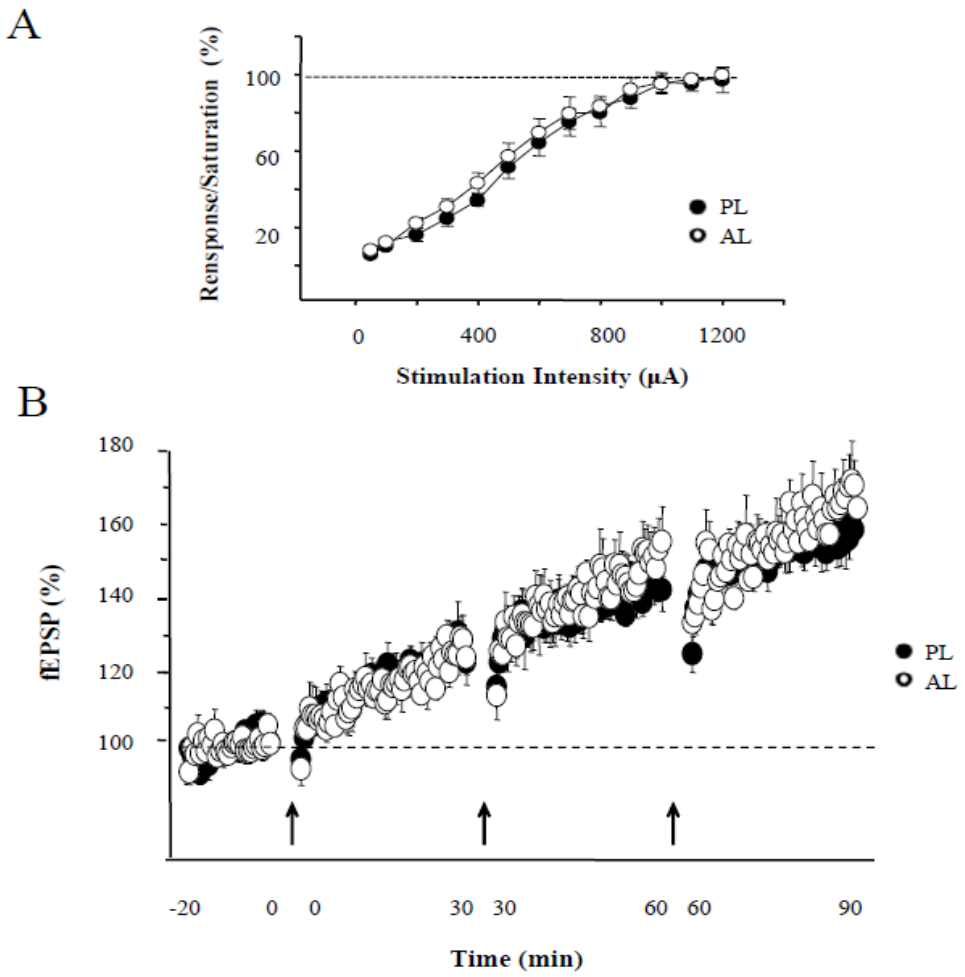

Fig. 10: Mimicry and LTP occlusion are not present in primary somatosensory cortex (A) Amplitude of field excitatory post-synaptic potentials (f-EPSPs) normalised to saturation level as a function of stimulation intensity in the primary somatosensory cortex of PL (black) and AL (white) animals. Two way Repeated Measures ANOVA revealed no difference between the two groups ($p = 0.61$). (B) LTP evoked in layer II-III of primary somatosensory cortical slices in PL (black) and AL (white) rats by theta-burst stimulation (TBS) applied to layer IV. Two way ANOVA revealed no difference between the two groups ($p = 0.63$). Arrows indicate TBS. Error bars = s.e.m. * = statistical significance; error bars = s.e.m.

5.6 Visual perceptual learning task restores visual functions in amblyopic adult rats

PL can be a very promising strategy for treating amblyopia in adulthood. An increasing number of clinical studies have reported that repetitive visual training eliciting PL processes may be a very useful approach for the treatment of amblyopia in humans, providing a substantial improvement in a variety of visual tasks (Levi and Li, 2009).

A group of reverse-sutured amblyopic rats (PL-amb rats, n=7) practiced in a forced-choice visual discrimination task in distinguishing between two vertical gratings differing only for their SF. The SF of the test grating was made progressively more similar to that of the reference grating (0.117 c/deg), starting from a SF of the test grating of 0.593 c/deg. Thus, visual PL consisted in the improvement of visual discrimination abilities allowing PL animals to distinguish the two gratings when they became more and more similar to each other. The task continued until the animals reached a plateau. PL-amb rats displayed robust visual PL, as evidenced by the progressive reduction in the MDSFD between the reference and the test grating across the days: while, on the first day, the mean MDSFD was 0.203 ± 0.022 c/deg, this value reached 0.005 ± 0.009 c/deg at the end of the test (One Way RM ANOVA, $p < 0.001$, Fig. 11).

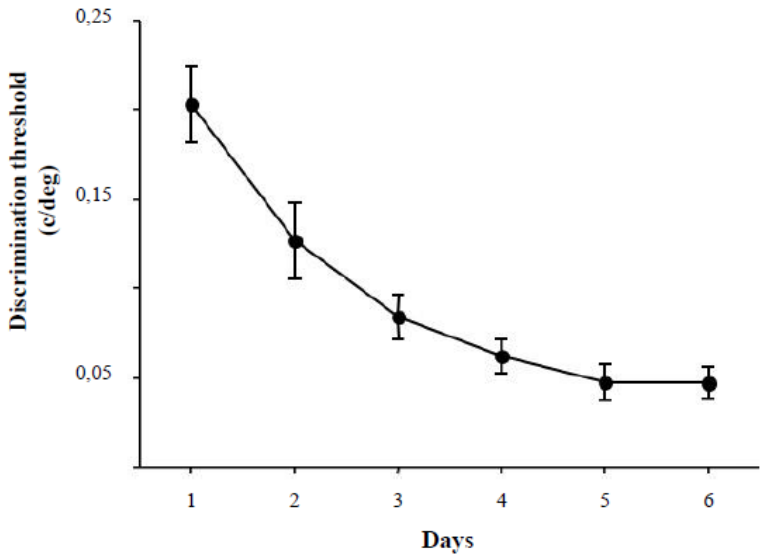

Fig. 11: PL in adult amblyopic rats. Improvement of discrimination threshold in adult amblyopic rats performing the PL task. The threshold, calculated as the minimum spatial frequency difference between the reference and the test gratings discriminated (MDSFD), decreased significantly with the training days (One way RM ANOVA,

p <0.001). The MDSFD obtained in the sixth day of the PL task was statistically different from that obtained in the first day (Holm-Sidak method, p = 0.003). Error bars= s.e.m.

5.6.1 Recovery of visual function measured with electrophysiological technique

After the PL training, visual acuity (VA) and ocular dominance (OD) were first evaluated electrophysiologically by recording visual evoked-potentials (VEPs). In this study, VEPs were recorded from the binocular portion of the primary visual cortex (Oc1B) contralateral to the deprived eye to measure the response of cortical neurons to monocular deprivation (MD). VEPs are routinely used to assess both VA and OD (binocularity) alterations in the visual cortex of rodents (Porciatti et al., 1999). In particular, OD distribution is evaluated calculating the contralateral-to-ipsilateral (C/I) VEP ratio, that is the ratio of VEP amplitudes recorded by stimulating the eye contralateral and ipsilateral, respectively, to the visual cortex where the recording is performed. The C/I VEP ratio is in the 2-3 range for adult normal rats, reflecting the predominance of crossed fibers in rodent retinal projections (Porciatti et al., 1999).

I tested the VA and the C/I VEP ratio in PL-amb and AL-amb group. In PL-amb animals, the VA of the long-term MD eye (0.88 ± 0.04 c/deg) did not statistically differ from that of the normal eye (1.01 ± 0.06 paired t-test, p=0.062; Fig. 12A) and from that recorded in adult naïve animals (Kruskal-Wallis One Way ANOVA on Ranks, post hoc Dunn's Method, p = 0.399), and it was instead statistically different from the VA of the long-term MD eye recorded in AL-amb animals (0.61 ± 0.06 c/deg) (Kruskal-Wallis One Way ANOVA on Ranks, Dunn's Method; Fig. 12A). One relevant limitation of PL is that the achievable effects are often markedly selective for the training conditions (e.g. stimulus orientation,). Therefore, I tested whether recovery of VA in PL-amb rats was also detectable in response to horizontal visual stimuli, i.e. visual gratings orthogonal to those used during the PL training with the same animals. I found a complete recovery of VA also in response to horizontal stimuli (VA of amblyopic eye: 0.95 ± 0.02 c/deg; VA of normal eye: 1.08 ± 0.06 c/deg; paired t-test, p=0.107). In addition, the VA of amblyopic eye in response to horizontal stimuli was different from that recorded in AL-amb animals (0.63 ± 0.06 c/deg; One-way ANOVA, Holm-Sidak method, p=0.001). I also evaluated OD in PL-amb rats: the C/I VEP ratio of these animals (2.45 ± 0.26) was not statistically different from that recorded in adult naïve animals (One-way ANOVA, post hoc Holm-Sidak method, p = 0.863) and it was instead statistically

different from that recorded in AL-amb animals (1.51 ± 0.19) (One-way ANOVA, Holm-Sidak method, p=0.016; Fig. 12B).

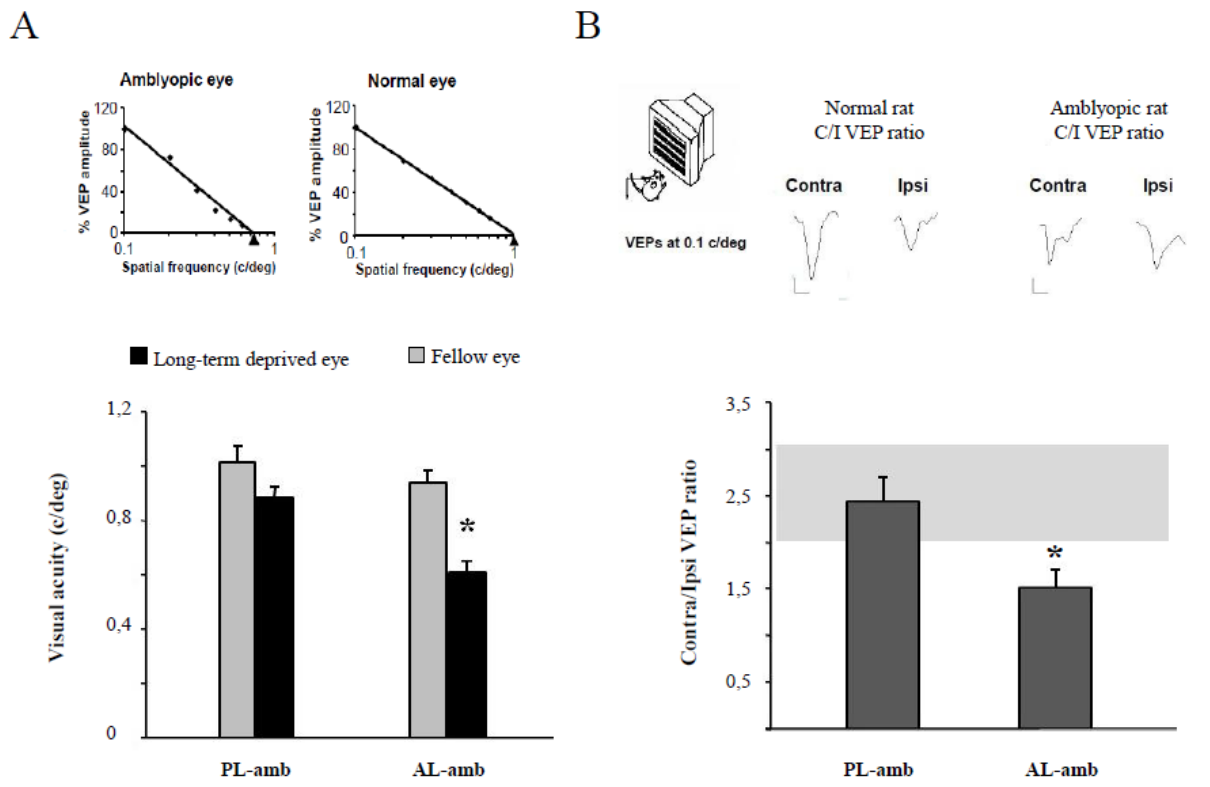

Fig. 12: PL induces a full recovery of VA and OD in adult amblyopic rats. (A) Electrophysiological assessment (by VEPs) revealed that the VA of the formerly deprived eye was not statistically different with respect to that of the fellow eye in rats subjected to PL (PL-amb group: paired t-test, p = 0.062). In contrast, no recovery of VA was observed in AL-amb animals (AL-amb group: paired t-test, p = 0.007). Representative examples of electrophysiological VA assessment for an amblyopic and a normal eye are reported on the upper part: VA is obtained by extrapolation to zero amplitude of the linear regression through the data points in a curve where VEP amplitude is plotted against log SF. * = statistical significance; error bars = s.e.m. (B) The graph shows the contralateral to ipsilateral eye (C/I) VEP ratio mean values for PL-amb and AL-amb groups. The grey box denotes the C/I VEP ratio range in naïve adult animals. One way ANOVA revealed a statistical difference in the mean values among the two groups (p = 0.016). In the upper part, typical VEPs recorded in response to the stimulation of either the contralateral or the ipsilateral eye in amblyopic and normal animals are reported. Calibration bars: 25 μV, 100 ms. * = statistical significance; error bars = s.e.m.

5.6.2 Recovery of visual function measured with behavioural technique

I repeated VA assessments in a separate group of long-term monocularly deprived and reverse-sutured animals subjected to PL training (n=4) by using a standard behavioral method, the visual water-box task. Behavioral assessment of VA were performed as previously described (Prusky et al., 2000) The rationale of this task is to use the animal's

ability to associate a grating with escape from water, as a strategy to measure its VA. Before the test of VA begins, animals are conditioned to distinguish between a low SF square-wave grating and homogeneous grey. Subsequently small incremental changes in the SF of the stimulus are made between successive blocks of trials until the ability of animals to distinguish a grating from grey fails. The highest SF achieved consistently is recorded as the acuity threshold. The VA was first measured at P60 through the non amblyopic (not deprived) eye; then, I measured VA through the amblyopic eye three times: immediately after RS, at the end of the PL procedure and after a period of 15 further days. Behavioral data completely confirmed the electrophysiological outcome: a full VA recovery was evident in the amblyopic eye of rats subjected to PL (VA of the not deprived eye: 0.89 ± 0.04 c/deg, VA of the previously deprived eye: 0.91 ± 0.05 c/deg; paired t-test, p=0.657; Fig. 13). Moreover, VA of the previously deprived eye after PL was statistically higher than that measured before the beginning of the training (0.62 ± 0.02 c/deg). The beneficial effect elicited by PL was long-lasting: indeed, VA recovery in the formerly deprived eye persisted unaltered 2 weeks after the end of the PL procedure (VA of amblyopic eye 2 weeks after PL: 0.90 ± 0.03 c/deg; One Way RM ANOVA, Holm-Sidak method, $p < 0.05$; Fig. 13).

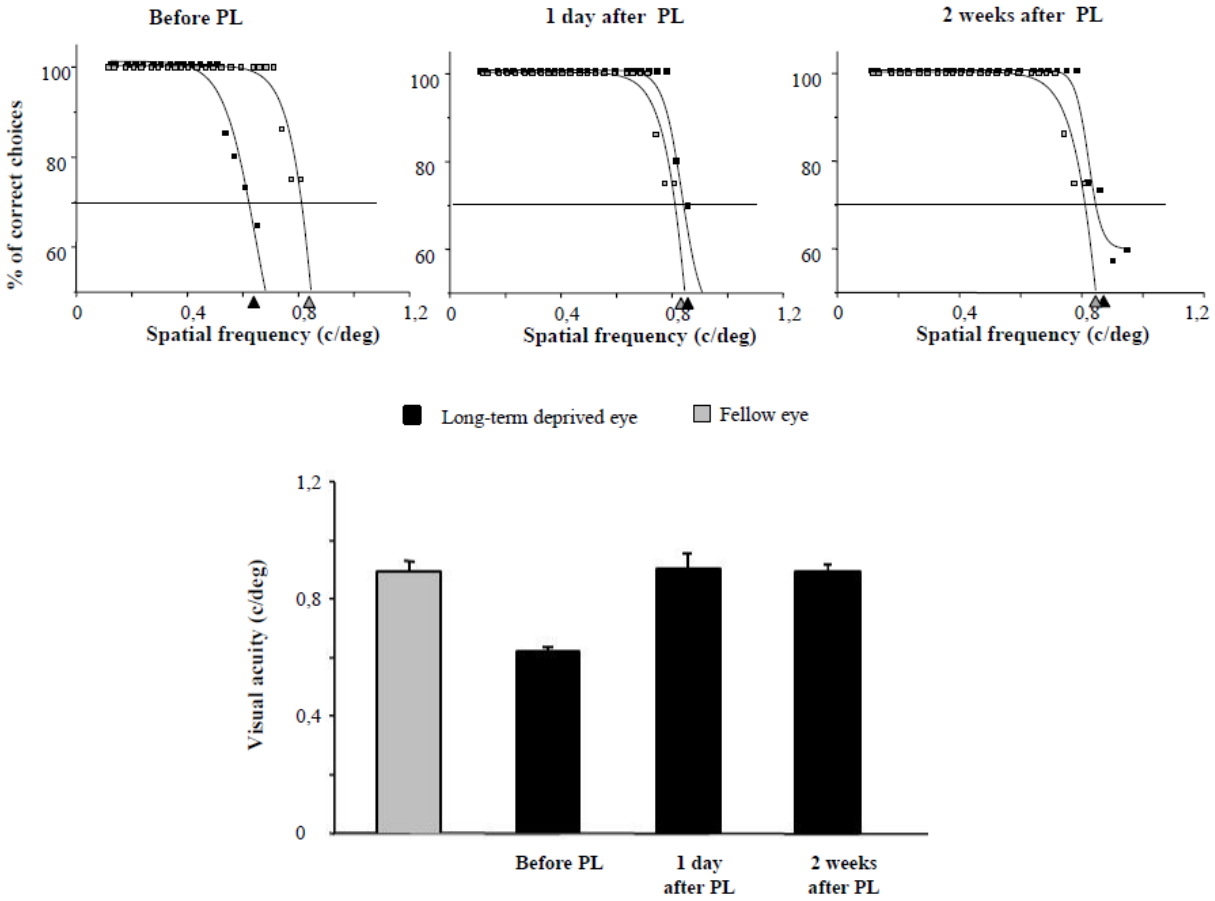

Fig. 13: Behavioral measure of VA recovery in rats subjected to visual PL. VA of both the long-term deprived and the open eye was measured using the visual water box task. At the end of the PL procedure, VA of the previously deprived eye was not different from that of the other eye (paired t-test, p = 0.657). One Way RM ANOVA with Holm-Sidak method revealed that the VA of the previously deprived eye measured after PL was significant increased with respect to that measured before visual training and remained unaltered 2 weeks after the end of PL. In the upper part, examples of the sigmoidal fitting curves for one animal had been shown. VA is obtained by extrapolation to 70% of correct choices on the sigmoidal function fitting the psychometric function in which the percentage of correct choices is plotted against SF. * = statistical significance; error bars = s.e.m.

To demonstrate that VA recovery was specifically elicited by visual PL, I repeated the behavioral assessment of VA in a separate group of rats that learned the PL task but were allowed to only distinguish the reference grating from a test grating whose SF was always maintained at the starting value of 0.593 c/deg (1st step PL-amb rats, n=4). No VA recovery was detected in this control group (VA of the not deprived eye: 0.90 ± 0.03 c/deg; VA of the previously deprived eye: 0.60 ± 0.02 c/deg; paired t-test, p< 0.01; Fig. 14). Furthermore, to rule out the possibility that the recovery effects in PL-amb rats were due to the imposed swimming activity instead than to their practice in discriminating visual stimuli, I also performed VA analysis in a group of control animals that were trained to distinguish the

reference grating from a homogeneous grey, matching this control group (AL-amb animals, n=4) to PL-amb rats in terms of overall swim time and training days in the water maze. I found that VA of the long-term deprived eye (0.61 ± 0.02 c/deg) remained significantly lower with respect to that of the fellow eye (0.91 ± 0.06 c/deg; paired t-test, $p < 0.05$; Fig. 14).

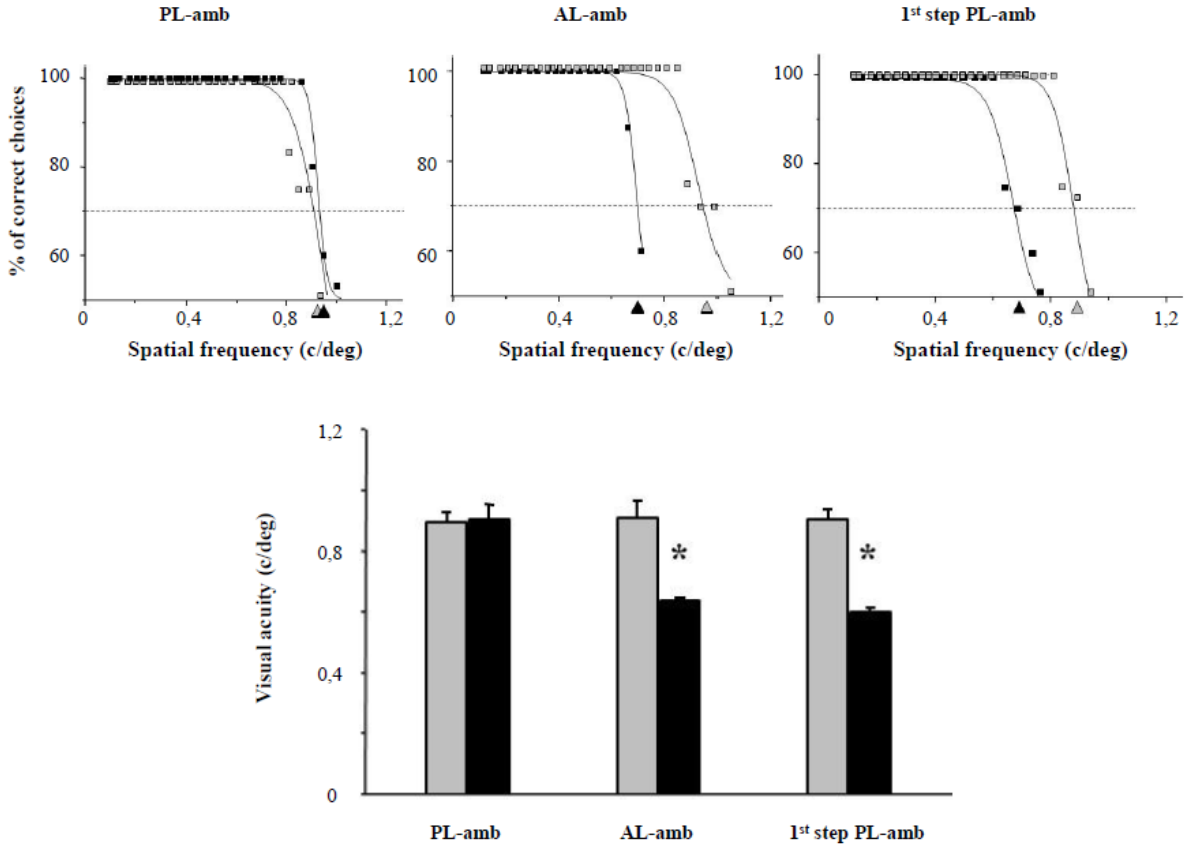

Fig. 14: VA recovery in PL-amb rats is dependent on visual training. The histogram shows behavioral VA of both eyes measured in animals subjected to PL (PL-amb rats), in AL-amb rats and in animals trained only in the first step of PL training (1st step PL-amb). Visual acuity of the previously deprived eye was different from that of the other eye in AL-amb and 1st step PL-amb animals, but not in the PL-amb group (One way ANOVA, Holm-Sidak method). In the upper part, examples of the sigmoidal fitting curves for each group had been shown. VA is obtained by extrapolation to 70% of correct choices on the sigmoidal function fitting the psychometric function in which the percentage of correct choices is plotted against SF. * = statistical significance; error bars = s.e.m.

5.7 Recovery from amblyopia elicited by visual perceptual learning is associated with reduced inhibition/excitation balance in the primary visual cortex

Because there is evidence that the maturation of cortical inhibitory circuits ends plasticity in the visual system (Hensch, 2005), I investigated whether recovery of VA and OD elicited by

the PL task, was accompanied by a change in the intracortical inhibition/excitation balance. For this purpose, I quantified (via synaptosome analysis) the release of GABA and glutamate in Oc1B of PL-amb and AL-amb animals. These measurements have been done in collaboration with the Pharmacology and Toxicology Section of the Department of Experimental Medicine (University of Genoa).

I found that the depolarization-evoked [^{3}H]GABA release was markedly reduced, compared to AL-amb rats (n=7: 5.41 ± 0.55 νmol/mg), in the visual cortex of in PL-amb (n=8: 3.82 ± 0.42 νmol/mg) (t-test p=0.038; Fig.15A). I did not detect any difference in the depolarization-evoked release of [^{3}H]D-Asp, mimicking glutamate release, between the different groups of animals (AL-amb, n=7: 1.75 ± 0.32 νmol/mg; PL rats, n=8: 2.01 ± 0.35 νmol/mg) (t-test p=0.600; Fig.15B). Thus, visual function recovery from amblyopia was related to a reduced inhibitory tone in the visual cortex.

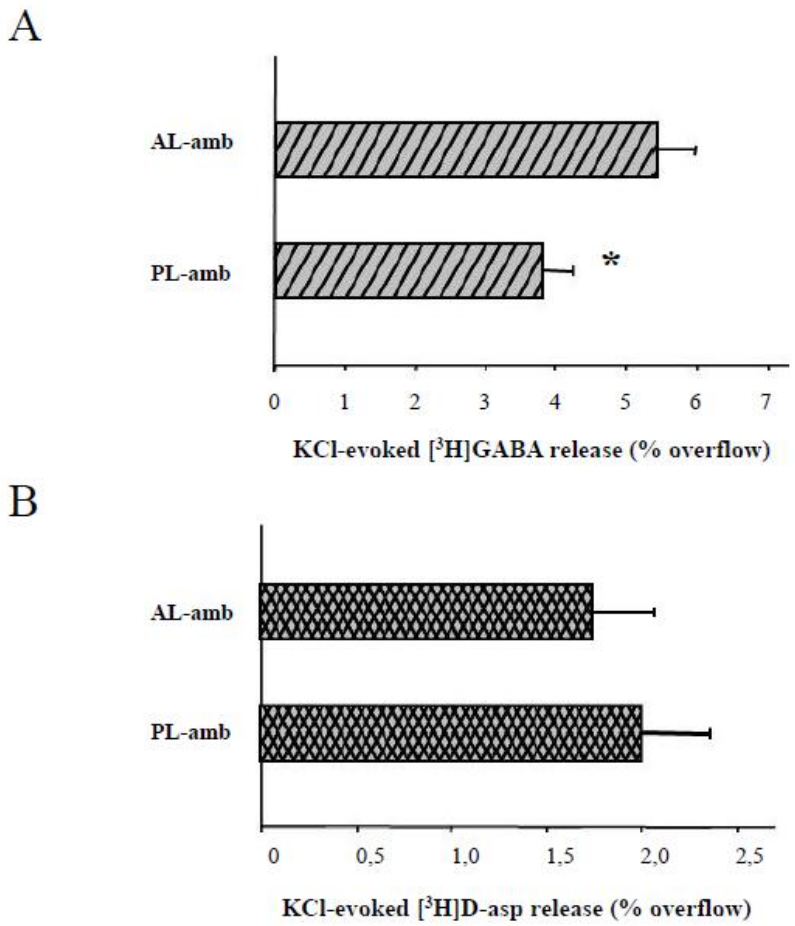

Fig. 15: Excitation-inhibition balance regulates plasticity in adult amblyopic rats. (A) Depolarization-evoked release of [^{3}H]GABA from synaptosomes. 25 mM KCl evoked GABA release from visual cortex synaptosomes. T-test showed a significant difference among the PL-amb and the AL-amb animals (p = 0.038). (B)

Depolarization evoked release of [^{3}H]D-Asp from synaptosomes. 15 mM KCl evoked [^{3}H]D-Asp release from visual cortex synaptosomes of the PL-amb and the AL-amb groups. T-test did not show a significant difference among the group levels ($p = 0.600$). * = statistical significance; error bars = s.e.m.

Chapter 6

Discussion

6.1 Visual perceptual learning induces LTP in the visual cortex

In the first part of the present work I tested the hypothesis that visual perceptual learning (PL) induces LTP-like changes in rat V1. I used two of the most commonly accepted criteria used to relate LTP with learning: mimicry and occlusion. Mimicry can be verified by assessing whether neuronal properties in the brain structures involved in a specific learning task display changes similar to those associated with LTP (Rogan et al., 1997; Rioult-Pedotti et al., 1998; Whitlock et al., 2006). Occlusion is tested by trying to induce LTP in a selected neural structure after the learning process has occurred; the rationale of this approach is that LTP is reduced or occluded when most of the available potentiation had already been used for the learning process to take place (Rioult-Pedotti et al., 2000; Sacchetti et al., 2004; Whitlock et al., 2006).

The data fulfill both of these criteria, showing that visual PL mimicked the effects of a LTP-inducing stimulus, determining a potentiation of synaptic transmission in V1 connections, and that PL occluded LTP induction in V1 vertical and horizontal connections. Thus, I conclude that the reported improvement displayed by PL rats in discriminating visual gratings of progressively closer spatial frequencies (SFs) can be explained in terms of LTP of synaptic efficacy in V1, the same cortical area at work during perception. The involvement of a LTP-like mechanism in visual PL is further supported by the demonstration that neither mimicry nor occlusion were present in V1 slices of 1st step PL rats, which practiced, for a number of trials equal to that performed by PL rats, but only in comparing the reference grating with a test grating whose SF was fixed on the initial value of 0.712 c/d, and which did not improve their discrimination abilities. Moreover, given that PL effects were specific to the visual cortex, being absent in S1, mimicry and occlusion were likely due to PL and not to a generalized stress response possibly induced by the behavioral procedure. A critical role for LTP in mediating learning processes has been previously reported for the amygdala, the

hippocampus and M1 following, respectively, fear conditioning, spatial learning and motor skill learning (Rogan et al., 1997, Rioult-Pedotti et al., 2000; Whitlock et al., 2006). The results, albeit correlative, suggest the possibility to extend these previous findings to include also visual PL and highlight the notion that learning can occur at sensory processing stages as early as the primary sensory cortices.

Synaptic plasticity of horizontal projections have been characterized in various cortical areas in the rat (e.g., Hess and Donoghue, 1994; Bilkey, 1996; Rioult-Pedotti et al., 1998; Yun et al., 2000). Within the visual cortex, reorganization of visual receptive fields in response to sensory perturbations is known to be mediated by horizontal connections (Gilbert, 1993), but a functional change of these connections in association with visual PL has never been reported. Here I showed for the first time that LTP can be elicited by tetanus in layer II/III horizontal projections of V1 and that this long-lasting changes of synaptic strength is involved in visual PL. This strengthens the notion that plasticity of synapses formed by horizontal pathways is an important contributor to learning-related processes throughout the cerebral cortex (Gilbert et al., 2009).

PL improvements for the task stimulus are characterized by both specificity and generalization. In adults with normal vision, visual PL shows an highly specificity for the features of the stimuli used in the task. In many studies it has been found that visual performances are typically improved on test trials using the same stimuli as those used during training, but these performances often return to baseline levels when test trials use stimuli mildly different from training stimuli. As expected, I demonstrated that, in adults naïve rats, training on vertical or horizontal gratings did not transfer to orthogonal stimuli, a result in agreement with human data showing that trained performance on a horizontal discrimination task does not transfer to a vertical version of the same task (Fiorentini and Berardi, 1980; Poggio et al., 1992; Fahle and Edelman, 1993). From the behavioral data, one might expect that LTP would be restricted to the V1 neurons that respond to the trained orientation, while it would be absent from those that do not respond to it, thus making it possible, in principle, to still elicit LTP in the untrained group of V1 neural connections. Accordingly, even if I found a pronounced reduction of LTP in response to visual PL, there was a tendency towards potentiation after the third TBS in V1 slices of PL animals and I showed that the animals were clearly able to learn new discriminations after the orientation change. On the other hand, while most visual neurons in rat V1 exhibit some degree of tuning for orientation, the tuning

sharpness is in general not high, with the vast majority of cells displaying a bandwidth comprised between 45 and 90 deg, with a peak at 60 deg (Maffei et al., 1992; Girman et al., 1999). This means that a stimulus of a given orientation actually evokes the discharge of a large population of cells tuned for different orientation degrees. Thus, it is likely that PL involved not only the neurons selective for vertical gratings, but also a larger group of cells. Despite its specificity for stimulus characteristics, learning has already been shown to induce a large occlusion of LTP in other systems; for instance, Rioult-Pedotti et al. (2000) reported that LTP was markedly reduced in rat M1 after training in a selective motor learning task with the forelimb.

I cannot rule out the possibility that, in parallel with LTP, a process of long-term depression (LTD) of synaptic strength might have been involved in V1 of PL rats to mediate PL. Like LTP, LTD has also been largely investigated to relate synaptic plasticity with various forms of memory and learning (Massey and Bashir, 2007). Visual PL, however, implies an improvement in the detection of subtle differences between cortical representations of similar visual stimuli. Thus, it is likely that a LTP-like mechanism is more suitable to mediate visual PL then a depression-like process, since a synaptic potentiation of the circuitries involved in the elaboration of the trained stimulus could amplify these differences, making them more detectable. This view is supported by a recent investigation in which repeated exposure to grating stimuli of a single orientation has been shown to result in a long-lasting increase of visual evoked potential amplitudes in response to the test stimulus in V1 (Frenkel et al., 2006; Cooke and Bear, 2010).

In my Thesis, I showed that a LTP-like increase of synaptic efficacy in V1 connections occurs in parallel with PL. This result, combined with the specificity for stimulus orientation, indicated V1 as location of the changes underlying PL. However, PL seems to rely on changes on a relatively early level of cortical information processing, such as the V1, under the influence of top-down selection and shaping influences (Ahissar and Hochstein, 2004; Gilbert et al., 2009). Attention is known to exert a significant influence on PL: some studies found that a conscious effort to direct focused attention plays a fundamental part in gating visual plasticity (Shoups et al., 2001; Shiu and Pashler, 1992; Herzog and Fahle, 1998; Gilbert et al., 2001; Ahissar and Hochstein, 1993). These findings can be used to depict a general theoretic model concerning the cellular processes underlying visual PL in V1. Such a model requires taking into account the strategy employed by the trained rats, which improved

their performance by practicing with visual gratings' comparisons in a behavioral task in which they were strongly motivated to find the hidden platform. In this process, an involvement of extra-V1 projections is very likely to take place. An interaction between the appropriate V1 intrinsic connections and the top-down feedback signals associated with the expectations of the behavioural task is a possible explanation for the induction of a potentiation process. V1, indeed, receives strong excitatory projections from higher order areas like V2, the secondary motor cortex, the temporal association cortex and the perirhinal cortex (Coogan and Burkhalter, 1993; Bai et al., 2004). These projections carry information about the animal behavioral and motivational state, setting the early visual areas in a specific working mode that allows the comparison of already stored representations with new bottom-up information concerning the stimulus characteristics (Gilbert et al., 2009). This loop may have a fundamental role in PL. It is likely that the event represented by the finding of the submerged platform is associated with a given SF value and that this association forms the basis for further comparisons performed during subsequent expositions to the new SFs of the test grating. It is admissible that a simultaneous firing of higher centres' projections carrying top-down signals and intrinsic V1 neurons selective for the stimulus parameter may lead to the induction of a synaptic potentiation process of V1 connections which eventually underlies the improvement in sensory discrimination.

6.2 Visual perceptual learning promotes recovery from amblyopia in adult rats

Amblyopia is one of the most common forms of visual impairment, arising from an early functional imbalance between the two eyes (Holmes and Clarke, 2006). It is currently accepted that, due to a lack of sufficient residual plasticity within the brain, amblyopia is untreatable in adulthood, however, recent experimental results obtained both in animal models and in clinical trials have challenged this traditional view. A number of studies over the last decade suggests that PL may be an important strategy for treating amblyopia: the improvements obtained by PL are considered to be a form of neural plasticity that also involved changes in the visual cortex. Neural plasticity is not a prerogative of normal visual system, but exists in the visual system of adults affected by amblyopia, thus PL can promote neural plasticity in the adult visual cortex, in order to restore the visual deficits. Several

studies showed that practicing a visual task improves performance in an amblyopic eye in adults, and, importantly, they also reported that practicing on a variety of different tasks and stimuli seems to transfer to improved visual acuity (Li and Levi, 2004; Polat et al., 2004; Zhou et al., 2006). The mechanisms underlying PL effects on visual acuity improvement in ambyopic subjects are not clear.

My results show that the behavioural protocol which elicited an increase of synaptic efficacy in V1 connections and promoted their potentiation was the protocol that promoted recovery from amblyopia. Indeed, I found that PL, but not 1st step PL or AL animals showed a marked recovery from amblyopia. The classic hallmarks of amblyopia in animal models are a permanent loss of visual acuity (VA) in the affected eye and a pronounced ocular dominance (OD) shift of visual cortical neurons in favor of the normal eye (Mitchell et al., 1984; Kiorpes, 1998; Timney, 1983). Electrophysiological data showed a restored OD, and electrophysiological and behavioral data concordantly documented a full recovery of VA.

Thus I showed that visual PL is accompanied by LTP of thalamo-cortical and cortical-cortical synaptic responses in the rat V1, and this strengthening in synaptic connections can account for the visual function recovery in amblyopia. Moreover, it has been demonstrated that cortical responses to passive stimulation strengthen over time, a phenomenon, expressed by the same core mechanisms as LTP, that is dependent on NMDA receptor activation and AMPA receptor trafficking (Frenkel et al. 2006; Cooke and Bear, 2010). Thus, the PL learning procedure set up in the present study might induce recovery of visual functions via potentiation of synaptic transmission in the visual connections subserving the long-term deprived eye. Accordingly, no recovery from amblyopia was evident in two control groups in which the treatment does not induce LTP in V1, i.e. in swimming rats (AL-amb group) and in animals that were trained with the PL task only until the first step of the discrimination procedure (1st step PL-amb).

In order to use of PL as strategy to treat amblyopia, one important point which needs to be considered is the longevity of the recovery. Several studies have examined the longevity of PL: the improvement in visual acuity was tested for a long time period and resulted long-lasting until 12-18 months (Polat et al., 2004; Zhou et al., 2006). I measured VA at the behavioral level, using the visual water box test, and I found that the recovery outlasted the end of the treatment, persisting for at least 15 days. This effect is remarkable, if one considers

that, in the timescale of human life, it is as the functional improvement lasted for 20 months or more.

One caveat to the therapeutic value of PL procedures in the treatment of amblyopia is the specificity of the achievable improvements, which are frequently limited to the selected trained stimulus, condition or task. Although PL of many tasks appears to transfer, at least in part, to improvements in VA, practising in a PL results in improved performance on the practised task, for example, PL with the amblyopic eye shows little or no transfer to untrained orientations (Levi and Polat, 1996; Levi et al., 1997; Li and Levi, 2004). I reported a generalization of PL in adults amblyopic animals for the recovery in VA. The results showed a transfer effect in two distinct manners: first, the recovery of VA, measured by VEPs, was not limited to stimuli of the same orientation than that used during the PL procedure, but was also present for orthogonal stimuli; second, even if rats practiced in discriminating visual gratings in the 0.1-0.6 c/deg range, they displayed a discrimination improvement in a range of higher SFs, with final VA values in the 0.9-1.0 c/deg.

I demonstrated that VA recovery was specifically elicited by visual PL, in fact no recovery was found in rats who only learned the association stimulus-escape platform (AL-amb group) and in animals that were trained only until the first step of the discrimination procedure, but did not proceed with the task of incrementally finer discrimination leading to improvement in performance (1^{st} PL-amb group). The lack of recovery in these groups could seem controversial since the two control groups were matched to animals trained with PL protocol, in terms of overall swim time and training days in the water maze. Although these groups did not perform a PL, they are subjected to a substantial physical activity which could promote neural plasticity. A great number of studies have investigated the positive effects of motor activity enhancement on brain plasticity, through the voluntary access to running wheels or through forced running on treadmills. Physical exercise increases BDNF levels (Neeper et al., 1995; Farmer et al., 2004), promotes angiogenesis (Swain et al., 2003) and hippocampal neurogenesis (van Praag, 2000), and induces the generation of new microglia in the cortex (Ehninger et al., 2003). The structural changes associated with exercise are also reflected in improvements in synaptic plasticity. LTP is enhanced in hippocampal slices in the dentate gyrus of running versus sedentary mice (van Praag et al., 1999) and, in vivo, in rats that had been housed with a running wheel (Farmer et al., 2004) or given forced treadmill exercise (O'Callaghan et al., 2007).

However, the different effect in the recovery found between PL and control groups could be associated to the difference between voluntary and forced movement: the rats trained in PL task could perform voluntary movement oriented towards the visual gratings' discrimination, instead the physical activity performed by control groups animals could resemble forced movement. The kind of exercise (i.e. voluntary or forced) may be particularly important, since several lines of evidence suggest that forced exercise and voluntary exercise exert different effects on the brain and behavior. For example, forced and voluntary exercise differentially affect monoamine neurotransmitters (Dishman, 1997), hippocampal parvalbumin expression (Arida et al., 2004), hippocampal brain-derived neurotrophic factor and synapsin-1 expression (Ploughman et al., 2005), longevity and body composition (Narath et al., 2001), taste aversion learning (Masaki and Nakajima, 2006) and open-field behavior (Burghardt et al., 2004).

My results provide the first evidence that PL is associated with reduced inhibition/excitation balance in V1. The excitatory-inhibitory balance is well known to be crucially involved in the regulation of plasticity during development and in adulthood (Hensch, 2005; Spolidoro et al., 2009; Morishita and Hensch, 2008; Sale et al., 2010; Harauzov et al., 2010; Baroncelli et al., 2011). It has been proposed that the inhibitory tone surpasses two functional thresholds in the visual cortex: the first one corresponds to the point after which the level of inhibition is sufficient to allow OD plasticity to be expressed; subsequently, as development proceeds further, the inhibitory tone continues to increase until it overcomes a second threshold, which determines the reduced levels of plasticity typically observed in adults. According to this conceptual frame, I reported that the recovery of VA and OD detected in adult amblyopic animals subjected to PL was associated with a marked reduction of GABAergic intracortical inhibition, as revealed by reduced GABA release in synaptosome analysis. No decrease of intracortical inhibition was present for control group which did not induce recovery from amblyopia.

Moreover, the balance between excitation and inhibition has been suggested to be impaired during development in amblyopic human subjects and cortical over-inhibition could underlie the degradation of spatial vision abilities (Polat, 1999; Levi et al., 2002; Wong et al., 2005). Repetitive transcranial magnetic stimulation, which increases cortical excitability, transiently improves contrast sensitivity in adult amblyopes, likely acting on the excitation/inhibition balance (Thompson et al., 2008).

In conclusion, my results indicate that the efficacy of PL in promoting recovery from amblyopia in human subjects (Levi and Li, 2009; Astle et al., 2011) might be related to the effects that PL exerts on the intracortical inhibition/excitation balance, enhancing cortical plasticity and promoting the long-term strengthening of practiced intracortical visual connections. It should be noted that the effects reported in the present work in a rodent model of amblyopia may not apply in the same measure to other species with much higher visual acuities, as monkeys and humans, in which an impairment of early visual experience has more pronounced effects on visual abilities. However, the data encourage efforts in the application of paradigms based on visual experience, such as PL, videogames (Li et al., 2011) or virtual reality, for the therapy of amblyopia in adulthood and offers an insight into the underlying mechanism of action.

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Appendix:
System consolidation of spatial memories in mice: effects of enriched environment

The experiments of Rosenzweig and colleagues (1978) introduced environmental enrichment (EE) as an experimental protocol specifically designated to investigate the influence of the environment on brain and behavior; indeed, EE is classically defined as "a combination of complex inanimate and social stimulation" (Rosenzweig et al., 1978). Animals reared under enriched conditions are housed in large groups, in wide cages where a variety of different and colorful objects (e.g. running wheels, platforms, boxes, toys, tunnels, shelters, stairs and nesting materials) are placed and changed frequently. The most important purpose of the EE is to improve the life quality of the animals by providing them with the opportunity to attain high levels of voluntary physical activity on running wheels and to enhance exploration, cognitive activity and social interaction.

A large number of studies highlighted the fact that EE modifies the behaviour of animals, leading to a marked improvement in complex cognitive functions, particularly learning and memory (van Praag et al., 1999 and 2000; Duffy et al., 2001; Bennett et al., 2006), and positively affecting the animal's emotional and stress reactivity (Chapillon et al., 2002). Rodents living in EE conditions display increased levels of hippocampal long-term potentiation (LTP), a physiological model of synaptic plasticity related to learning and memory (van Praag et al., 2000). This functional improvement is accompanied by prominent changes at the anatomical level, with robust increments in cortical thickness and weight (Rosenzweig et al., 1964; Beaulieu et al., 1987) and modifications of neuronal morphology, in terms of increased dendritic arborization, number of dendritic spines, synaptic density and postsynaptic thickening, occurring in several regions of the brain (Holloway, 1966; Kozorovitskiy, 2005; Mohammed et al., 2002). Moreover, studies have reported also an effect of EE on the reduction of apoptotic cell death (Young, 1999), and on increased hippocampal neurogenesis (Kempermann, 1997). At the molecular level, EE causes a significant change in the expression of a large set of genes involved in neuronal structure, excitability, synaptic transmission and plasticity (Rampon et al., 2000), modulating the synthesis and secretion of

neurotrophic factors throughout the brain (Ickes et al., 2000; Pham et al., 2002) and affecting the cholinergic, serotoninergic and noradrenergic systems (Rosenzweig et al., 1967; Rasmuson et al., 1998; Naka et al., 2002).

EE can be used to modulate the development of the central nervous system in a totally non-invasive manner: it has been shown that EE accelerates the development of the visual system (Sale et al., 2004; Landi et al., 2007; Sale et al., 2007a). EE can also be used as a strategy to reopen plasticity windows in the adult cortex and to reduce the cognitive decline typically associated with ageing: exposing adult rats to EE enhances visual-cortex plasticity and promotes recovery from amblyopia (Sale et al., 2007b); studies of environmental effects on age-related changes in cognitive function widely indicated that living in complex, stimulating environments (both from youth or only during old age) rescues cognitive deficits or, at least, ameliorates the performance on tests of learning and memory in aged animals (Doty, 1972; Cummins et al., 1973; Warren et al., 1982; Winocur, 1998; Soffié et al., 1999; Kempermann et al., 2002; Kobayashi et al., 2002; Frick and Fernandez, 2003; Bennett et al., 2006; Leal-Galicia et al., 2008). Finally, rearing animal models of nervous system disorders, including neurodegenerative diseases (Nithianantharajah and Hannan, 2006; Berardi et al., 2007; Baroncelli et al., 2010) and brain injuries (Johansson, 1996; Comeau et al., 2008), in an enriched environment, leads to beneficial effects.

Despite all the evidence showing that EE acts on complex cognitive functions, enhancing learning and memory, there are no evidence on whether and how EE can be engaged in the process of time-dependent memory reorganization, also known as system consolidation. Declarative memories depend initially on the medial temporal lobe system, including the hippocampus, but, as these memories mature, they become increasingly dependent on other brain regions such as the neocortex. This system consolidation is a prolonged process and involves gradual reorganization of the brain regions that support memory. According to the standard consolidation model (Squire et al., 1995; McClelland et al., 1995), the memory trace is initially encoded in parallel in hippocampal and cortical networks, and subsequent reactivation of the hippocampal network reinstates activity in different cortical networks. This coordinated replay across hippocampal–cortical networks leads to gradual strengthening of cortico-cortical connections, which eventually allows new memories to become independent of the hippocampus and to be gradually integrated with pre-existing cortical memories. In an alternative view, the multiple trace theory proposes that,

although experience is initially encoded in distributed hippocampal–cortical networks, the hippocampus is always required for rich contextual or spatial detail (Nadel and Moscovitch, 1997; Moscovitch et al., 2005). The assumption that reactivation of memories initiates a process of reorganization is present in both models, however: standard models predict that reorganization occurs in cortical networks, multiple trace theory predicts instead that reactivation should also lead to the generation of new traces within the hippocampus.

Spatial memory is a declarative type of memory. Experiments using lesion, electrophysiological, genetic, and neuroimaging approaches have established that the hippocampus plays an essential role in the formation of spatial memories (O'Keefe and Nadel, 1978; Burgess et al., 2002; Morris et al., 2003; Nakazawa et al., 2004; Leutgeb et al., 2005). However, as spatial memories mature, they may become additionally dependent on a broadly distributed cortical network. Many studies point out that the medial prefrontal cortex (mPFC) might have a privileged role in processing this remote memory (Bontempi et al., 1999; Maviel et al., 2004; Frankland et al., 2004; Teixeira et al., 2006). The mPFC consists of several highly interconnected regions, including the anterior cingulate cortex, and prelimbic and infralimbic cortices, and these regions are reciprocally connected to sensory, motor, and limbic cortices (Uylings et al., 2003). In addition, several experiments have provided evidence that inactivation or lesions of the prelimbic or anterior cingulate cortices block recall of remote memory, but not recent memories (Maviel et al., 2004; Frankland et al., 2004), even in the presence of an intact hippocampus. It has been demonstrated that reactivation of hippocampal-cortical connections are crucial for consolidation and recall of declarative memory; for example, using mouse genetic approach, it has been shown that the suppression of N-methyl-D-aspartate receptors function in the week immediately after learning, but not later, blocked the formation of remote memories (Shimizu et al., 2000). During consolidation, the strengthening of cortico-cortical connections is thought to be critical in allowing memories to migrate from the hippocampus to the cortex; for instance, studies of genetically mutant mice for α-CaMKII (Frankland et al., 2001) and p-21 activated kinase (Hayashi et al., 2004) showed that these mice, with abnormal cortical function, were unable to form enduring, hippocampus-independent memories. Moreover it has been demonstrated that cortical consolidation involve rewiring through expression of proteins involved in axonal growth and sprouting (Frankland et al., 2004; Maviel et al., 2004) and time-dependent changes in spine density in hippocampal and cortical networks during the formation of recent and remote memory (Restivo et al., 2009; Vetere et al., 2011).

Although formation and stabilization of long-lasting associative memories require time-dependent coordinated hippocampal-cortical interactions, the underlying mechanisms remain unclear. One possibility is the involvement of synaptic tagging in the hippocampal-cortical interactions in order to ensure progressive stabilization of cortical-cortical connections. It has been proposed that new neurons generated in the dentate gyrus become functionally integrated into existing neural circuits (Deng et al., 2010); in fact the spatial training when new neurons are more receptive to surrounding neuronal activity, favored their subsequent recruitment upon remote memory retrieval (Trouche et al., 2009; Arruda-Carvalho et al., 2011). Thus, these adult-generated neurons become tagged in order that, once mature, they are preferentially recruited into hippocampal networks underlying remote spatial memory representation, and they could act as time clusters for the storage of long-term episodic memories. Therefore tampering with the level of hippocampal neurogenesis could interfere in the hippocampus-dependent period of memory. It has been demonstrated that a decreased neurogenesis is accompanied by a prolonged hippocampus-dependent period of associative memory, and conversely, enhanced neurogenesis by voluntary running-wheel exercise sped up the decay rate of this dependency (Kitamura et al., 2009).

In this study, I tested whether EE could affect the system consolidation process of memory traces, involving the progressive migration with time of the traces from the hippocampus to their final storage site in the cortex. I investigated whether EE could accelerate the time course of the activation of neocortical areas, possibly through a strengthening of the hippocampal-cortical and the cortico-cortical connections. To do so, I characterized the time course of hippocampal and cortical activation following spatial learning; I investigated the possible activation of cortical areas in a brain-wide manner, not restricting myself to mPFC.

To address these hypothesis, wild type mice, housed in standard or in enriched condition, were subjected to a spatial learning (Morris water maze) and then tested 1, 10 and 20 days after learning to evaluate consolidation process. Mice were sacrificed following the probe tests and the regional expression of the inducible immediate early gene c-fos was mapped, as an indicator of neuronal activity (Hall et al., 2001; Fleischmann et al., 2003).

I found that EE not only induces an early recruitment of mPFC, the suggested final storage site in the cortex, but also induces the progressive activation of a distributed cortical

network, that is not activated following memory recall in standard housed mice, at least after 20 days of retention interval.

Materials and Methods

Animal treatment

Male and female C57BL/6 mice of 2 months of age were used in this study. All the procedures were approved by the Italian Ministry of Health. The animals were housed in an animal room with a 12 h/12 h light/dark cycle, with food and water available ad libitum. At 2 months of age, the animals were assigned to one of the following rearing conditions for 40 days: environmental enrichment (EE, n=15) or standard condition (SC, n=15). The SC rearing condition consisted of a 26x18x18 cm cage housing 3 animals. The EE rearing condition was achieved using a large cage (44x62x28 cm) containing several food hoppers, one running wheel for voluntary physical exercise, and differently shaped objects (tunnels, toys, shelters, stairs) that were repositioned twice a week and completely substituted with others once a week.

Two additional groups of control animals, age and gender matched to SC and EE groups who performed the spatial learning task, were housed in SC (home cage standard condition, HC-SC, n=5) or in EE (home cage enriched condition, HC-EE, n=4) but these animals did not perform any behavioural task.

Morris water maze (MWM)

The hidden platform version of the MWM test was performed (Morris et al., 1982). A large water tank of 120 cm of diameter was filled with white opaque water at 21 °C and divided into four quadrants of equal area arbitrarily named Northeast (NE), Southeast (SE), Southwest (SW) and Northwest (NW). The pool was surrounded by curtains, on which two- and three-dimensional visual cues are placed, as spatial cues for navigation. An escape platform of 11 cm of side was submerged 1 cm below the water surface and placed in the center of the SW quadrant. The platform was maintained in this position for all the swim trials through the test. Mice were trained to swim to the platform in 4 daily trials, with a 30 min interval, during 7 consecutive days. Each trial was initiated at one of four different starting positions at the outer edge of the pool, with mice placed into the pool facing the wall. The order of these start

locations was pseudorandomly varied throughout training. The trial was complete once the mouse found the platform or 60 s had elapsed. If the mouse failed to find the platform on a given trial, the experimenter guided the mouse onto the platform. Upon reaching the platform, each mouse was allowed to rest for 20 s on it. After each trial each mouse was returned to its home cage where it rested until the next trial. After the completion of training, spatial memory was assessed in a probe test: a recall probe trial was performed after 1, 10 and 20 days after the end of learning.

Behavioural data from training and probe tests were recorded and analyzed using an automated tracking system (Noldus Ethovision XT). For each trial I measured the latency to reach the platform, in sec, the total distance swam to the platform, in cm, and the average swim speed, in cm/s. For each probe trial I measured the total amount of time spent in each quadrant, in sec, and, to evaluate the precision of search patterns (Moser et al., 1993; Teixeira et al., 2006), the exploration time spent in three concentric circles (radii: 16 cm, 23 cm, and 28 cm) centered on the platform location.

Immunohistochemistry

Mice were anaesthetized and perfused via intracardial infusion with 0.1 M PBS and then 4% paraformaldehyde (PFA, dissolved in 0.1 M phosphate buffer, pH 7.4) 90 min after the completion of behavioral testing. Brains were removed, fixed overnight in PFA, and then transferred to 30% sucrose solution and stored at 4°C. Coronal sections were cut at 40 μm thickness on a freezing microtome (Sliding Leica microtome SM2010R, Leica Microsystems), and free-floating sections were prepared for immunohistochemistry. After a blocking step in 10% NGS and 0.5% Triton X-100 in PBS, sections were incubated in a solution containing 1% NGS, 0.3% Triton X-100, and anti-c-Fos primary rabbit polyclonal antibody (1:3000 rabbit anti c-Fos polyclonal antibody, Calbiochem, USA) for 36 h at 4°C. Subsequently, sections were transferred in a solution containing 1% NGS, 0.1% Triton X-100 and 1:200 anti-rabbit biotinylated secondary antibody (Vector Labs) in PBS. This was followed by incubation in ABC kit (Vector Labs) and final detection with DAB reaction kit (Vector Labs). Sections were finally mounted on gelatinized slides, dehydrated and sealed with DPX mounting medium (VWR International, UK).

Analysis of C-fos positive cells

Countings of c-fos positive cells in different brain areas were performed using a CCD camera (MBF Bioscience, Germany) mounted on a Zeiss Axioskop (Zeiss, Germany) microscope and the Stereo-Investigator software (MBF Bioscience). Brain structures were anatomically defined according to a mouse brain atlas (Franklin and Paxinos, 1997; Fig. 1). The number of c-Fos-positive cells was counted at 20× magnification, following a "blind procedure", in 10-40 fields (50 x 50 µm or 100x100 µm) per section according to the size of brain structure and their density calculated (cells/mm^2), using at least 5 sections for each structure. The mean count in each structure was divided by the mean count in that region for the respective control group (HC and HC-EE) to generate a normalized count for each animal.

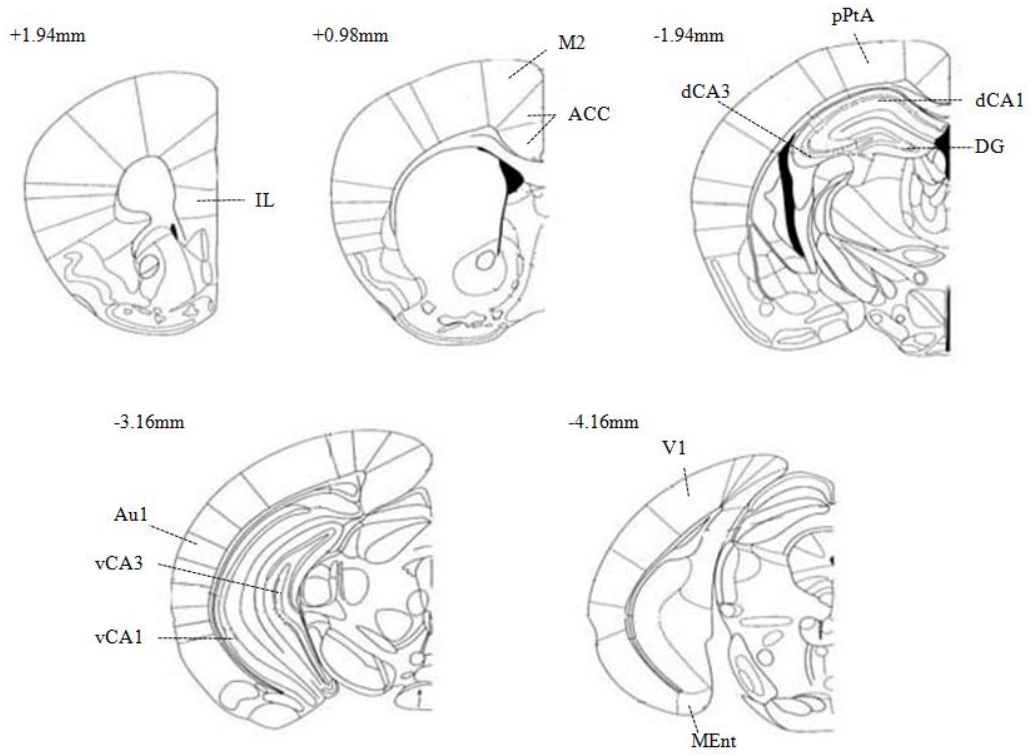

Figure 16: Schematic drawings of mouse brain coronal sections (adapted from Franklin and Paxinos, 1997) showing the regions of interest selected for measurement of cFos-positive nuclei. Numbers indicate the distance (in millimeters) of the sections from bregma. IL: infralimbic cortex; M2: secondary motor cortex; aCC: anterior cingulate cortex, area 1 and area 2; DG: dentate gyrus; dCA1: CA1 field of dorsal hippocampus; dCA3: CA3 field of dorsal hippocampus; pPtA: posterior parietal association cortex; Au1: primary auditory cortex; vCA1: CA1 field of ventral hippocampus; vCA3: CA3 field of ventral hippocampus; V1: primary visual cortex; MEnt: medial entorhinal cortex.

Results

In a section of my master thesis research project, I attempted to replicate the data that pointed out that mPFC was the final storage site for remote spatial memory (Bontempi et al.,

1999; Maviel et al., 2004; Frankland et al., 2004; Teixeira et al., 2006). In a group of standard housed (SC) animals, probe tests were performed 1 day (Recent, rec, n=6) or 30 days (Remote, rem, n=6) after learning. As expected, I found that there was greater activation of the cortical sites in mPFC in the remote group (One way ANOVA,aCC rem vs aCC home cage and aCC rec $p<0.01$, aCC rec v aCC home cage $p=0.262$; data not shown), while the involvement of dorsal hippocampus (dHPC) was present in both groups, with greater c-Fos protein expression on remote than recent memory retrieval, at least for dCA3 and DG (One way ANOVA, for dCA1: dCA1 rec vs dCA1 home cage and dCA1 rem vs dCA1 home cage $p<0.05$, dCA1 rec vs dCA1 rem $p=0.228$; for dCA3: dCA3 rec vs dCA3 rem and dCA3 rec vs dCA3 home cage $p>0.05$, dCA3 rem vs dCA3 home cage $p<0.05$; for DG: DG rec vs DG home cage and DG rem vs DG home cage $p<0.01$, DG rec vs DG rem $p<0.05$; data not shown)

In the present experiment, to test whether EE can affect the system consolidation process, I trained C57BL/6 mice, housed in different conditions (SC n=15 or environmental enrichment EE n=15), in a spatial learning task, using the Morris water maze, and I analyzed the following parameters referred to the average of 4 daily trials, during 7 consecutive days: latency to find the platform, in sec, total distance swam, in cm, and mean swim speed, in cm/s. For the distance swam and the latency to reach the platform during acquisition, a significant learning effect for both housing conditions were found (Two way RM ANOVA, for latency $p=0.016$; for distance swan $p<0.001$), but not a significant difference between the two groups (Fig. 2). For the latency parameter only, I found a housing condition×day interaction: multiple comparisons showed that the main differences resided on days 4 and 5 (Two way RM ANOVA, post hoc analysis Holm-Sidak method, for day 4 $p=0.005$; for day 5 $p=0.001$). I also measured the mean swim velocity throughout the test, in order to exclude differences in navigation speed (data not shown); I observed a significant decrease in the mean swim velocity through the test (Two way RM ANOVA, $p<0.001$), but neither a difference between housing condition ($p=0.167$) nor a housing condition×day interaction ($p=0.389$).

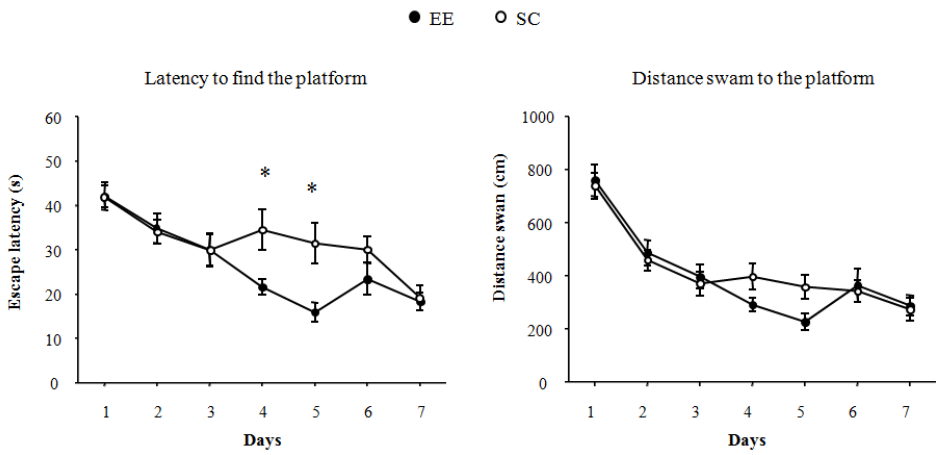

Figure 2: Performances of SC (n=15) and EE (n=15) mice in the MWM. I did not observed differences for any of the recorded parameters in mice subjected to the diverse probe tests within the housing conditions (Two way RM ANOVA for latency within SC p=0.821; for latency within EE p=0.629; for distance swan within SC p=0.761; for distance swan within EE p=0.687; for distance mean swim speed within SC p=0.473; for distance mean swim speed within EE p=0.253). For latency, I found a significant learning effect (Two way RM ANOVA, p=0.016), and a housing condition×day interaction (Two way RM ANOVA post hoc analysis Holm-Sidak method, for day 4 p=0.005; for day 5 p=0.001). For the distance swam, I found a significant learning effect (Two way RM ANOVA, p<0.001) but neither a difference between housing condition (p=0.514) nor a housing condition×day interaction (p=0.301). * = statistical significance; error bars = s.e.m.

Spatial memory was evaluated in a probe test in which the hidden platform was removed. I performed recall probe tests at 1, 10 and 20 days, and I analyzed the time spent in the quadrants NE, NW, SE and SW (quadrant where the platform was placed), and in three concentric circles centered on the platform location, for SC (1 day n=5; 10 days n=5; 20 days n=5) and EE (1 day n=5; 10 days n=5; 20 days n=5) mice (Fig. 3A and B). For probe tests at 1 and 10 days, I found a significant difference between target quadrant SW and the other quadrants in both EE and SC, but a not significant effect for housing condition (Probe test 1 day: two way ANOVA, quadrant factor p=0.011, housing condition factor p=0.371, housing condition×quadrant p=0.829; Probe test 10 days: two way ANOVA, quadrant factor p=0.001, housing condition factor p=0,999, housing condition×quadrant p=0.837; Fig. 3A); instead, for probe test at 20 days, a significant difference between target quadrant SW and the others was found only in the SC group (One way ANOVA, p=0.004; Fig.3A). To evaluate precision of search patterns (Moser et al., 1993; Teixeira et al., 2006), I quantified exploration in three concentric circles (inner: radius 16 cm; middle: radius 23 cm; outer: radius 28 cm) centered on the platform location. The time spent in the inner circle was greater than time spent in the other circles for both SC and EE groups in each probe test, except for probe test at 1 day in SC group in which there was not a significant difference (Probe test 1 day: one way RM ANOVA, circle factor EE group p=0.007, SC group p=0.742; Probe test 10 days: two way

RM ANOVA, circle factor p=0.014, housing condition factor p=0.968, housing condition×circle p=0.776; Probe test 20 days: two way RM ANOVA, circle factor p<0.001, housing condition factor p=0.095, housing condition×circle p=0.284; Fig. 3B).

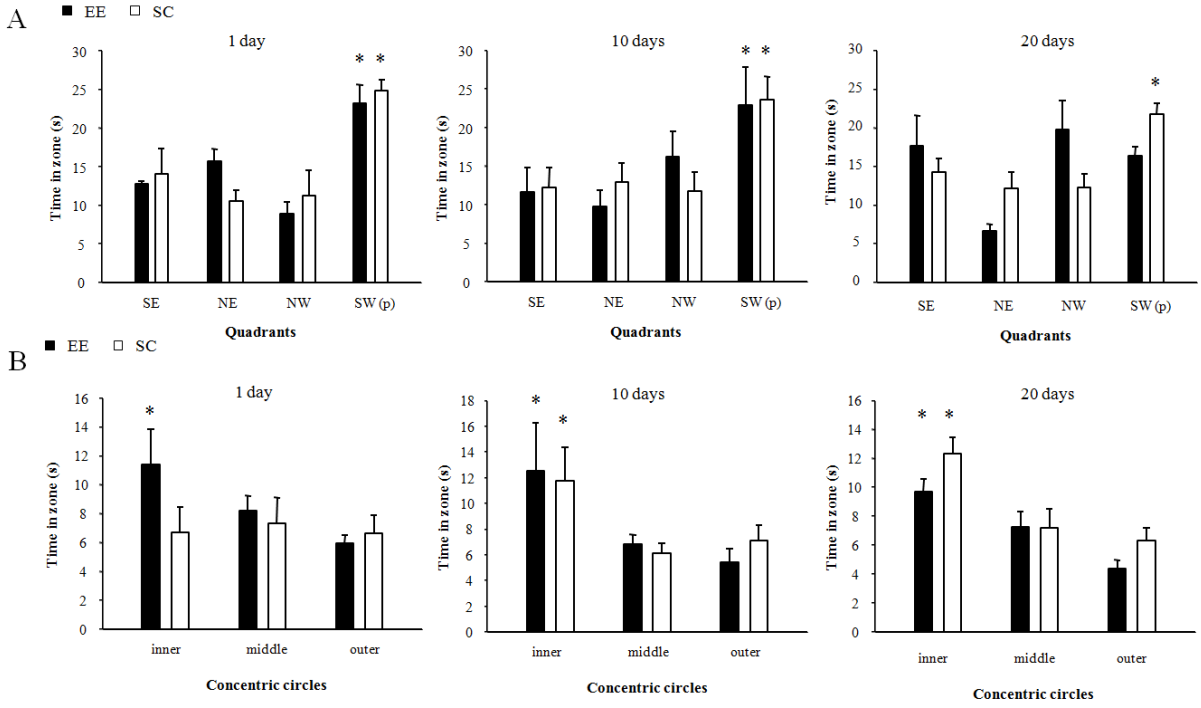

Figure 3: Evaluation of spatial memory. (A) Time spent in the quadrants NE, NW, SE and SW, where the platform was placed, for recall probe tests at 1, 10 and 20 days. 1 day probe test: two way ANOVA, post hoc analysis Holm-Sidak method, target quadrant SW vs other quadrants p<0.05. 10 days probe test: two way ANOVA, post hoc analysis Holm-Sidak method, target quadrant SW vs other quadrants p<0.05. 20 days probe test in SC group: one way ANOVA, post hoc analysis Holm-Sidak method, target quadrant SW vs other quadrants p<0.05. 20 days probe test in EE group: one way ANOVA, post hoc analysis Holm-Sidak method, SW vs SE p=0.395; SW vs NW p=0.026; SW vs NE p=0.736. * = statistical significance; error bars = s.e.m. (B) Precision of search patterns evaluated with time spent in inner (radius: 16cm), middle (radius: 23cm) and outer (radius: 28) circles centered on the platform location. 1 day probe test in EE group: one way RM ANOVA, post hoc analysis Holm-Sidak method, inner circle vs other circles p<0.05. 1 day probe test in SC group: one way RM ANOVA, post hoc analysis Holm-Sidak method, inner vs middle p=0.587, inner vs outer p=0.984. 10 days probe test: two way RM ANOVA, post hoc analysis Holm-Sidak method, inner circle vs other circles p<0.05. 20 days probe test: two way RM ANOVA, post hoc analysis Holm-Sidak method, inner circle vs other circles p<0.05. * = statistical significance; error bars = s.e.m.

After probe test, mice were sacrificed and the protein c-Fos was immuno-labeled as an indicator of neuronal activity. c-Fos expression was calculated as the density of number of c-Fos-positive cells in mm^2. First I investigated c-Fos expression in the hippocampus (dorsal hippocampus, dHPC and ventral hippocampus, vHPC), the structure that is responsible for the formation of long term spatial memory (O'Keefe and Nadel, 1978; Burgess et al., 2002; Morris et al., 2003; Nakazawa et al., 2004; Leutgeb et al., 2005).

Levels of c-Fos expression in HC-EE and HC-SC mice did not differ while levels for EE and SC mice were significantly greater than those in their home-cage controls at all retention intervals for each hippocampal subregion, (two way ANOVA, post hoc analysis Holm-Sidak method, factors: housing condition and day of probe, EE vs SC all p values < 0.05), suggesting that performance, as well as mnemonic, aspects of water-maze testing contribute to activation of the hippocampus. There was not a significant difference between activation at different days, with the exception of dCA1, where activation increased with increasing retention interval for both groups and DG, where this increase was evident at 20 days for EE animals only (Fig. 4).

In each of the hippocampal subregions, c-Fos expression was similar in the EE and SC mice. I found a greater activation for EE mice only in DG at day 20 (Fig. 4).

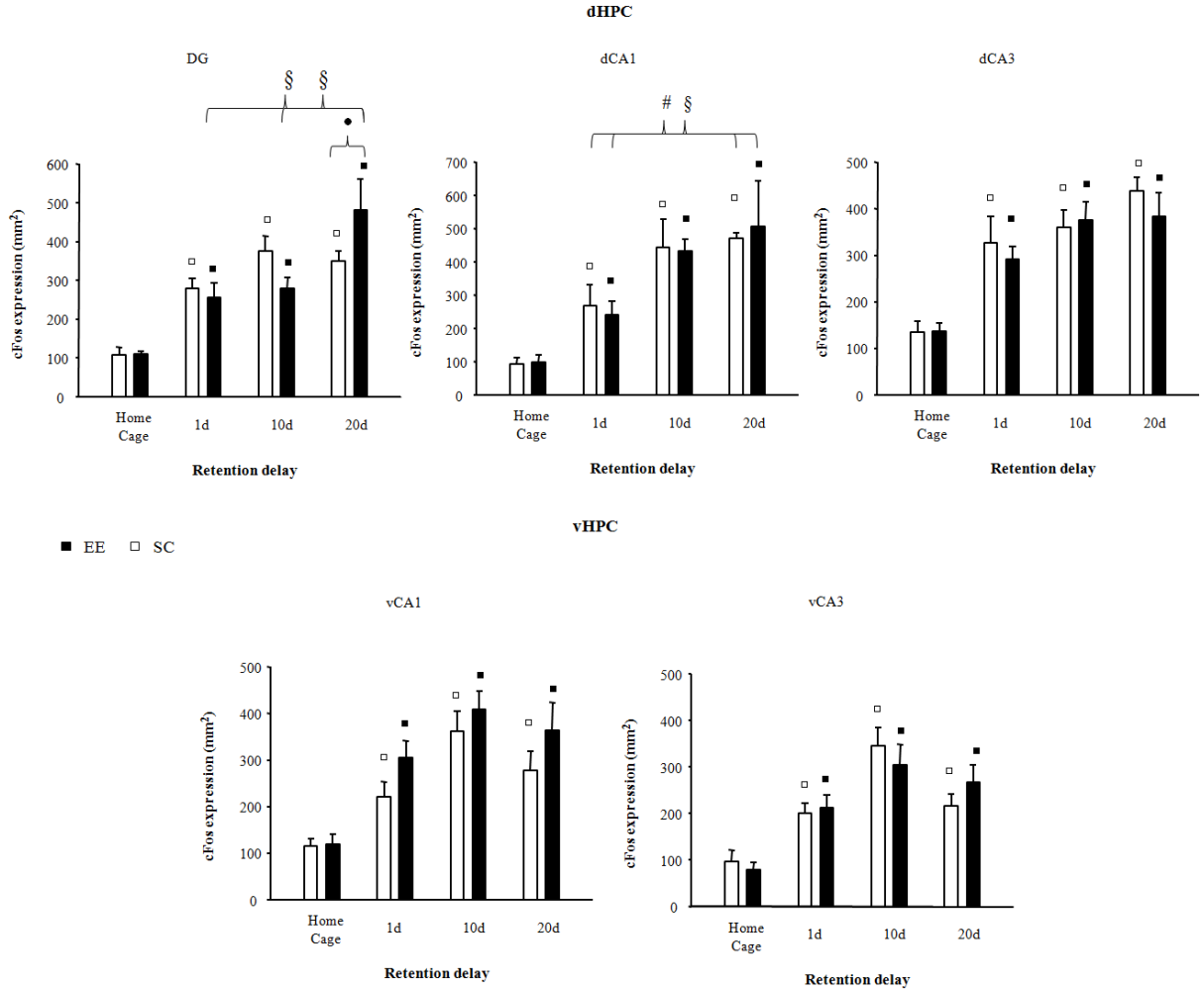

Figure 4: c-Fos expression in subregions of dHPC and vHPC for EE and SC mice subjected to recall probe tests at 1, 10 and 20 days. DG: two way ANOVA post hoc analysis Holm-Sidak method, SC vs EE in probe test at 1 day p=0.627; SC vs EE in probe test at 10 days p=0.054; HC-SC vs HC-EE do not differ; SC versus HC-SC and EE versus HC-EE p<0.05 for all retention intervals; SC vs EE in probe test at 20 days p=0.008. Statistical

difference within EE group were found between probe test at 20 days and the other probe tests p<0.001. dCA1: two way ANOVA post hoc analysis Holm-Sidak method, SC vs EE p=0.998; dCA3: two way ANOVA post hoc analysis Holm-Sidak method, SC vs EE p=0.348. vCA1: two way ANOVA post hoc analysis Holm-Sidak method, SC vs EE p=0.051. vCA3: two way ANOVA post hoc analysis Holm-Sidak method, SC vs EE p=0.752. ▪ statistical significance between EE and HC-EE; ▫ statistical significance between SC and HC-SC; • statistical significance for housing factor within a given level of factor day (comparison between EE and SC); # statistical significance within SC group (comparison for factor day of probe test within SC group); § statistical significance within EE group (comparison for factor day of probe test within EE group); error bars = s.e.m..

The results for c-Fos expression in the mPFC, the final storage site in the cortex, show that both the aCC and the IL followed the same c-Fos expression pattern: c-fos expression at 1 day did not differ from that in home cage animals, both for EE and SC mice and there was no difference between EE and SC or HC-EE and HC-SC mice (Two way ANOVA, post hoc analysis Holm-Sidak method, all p values > 0.05); instead, starting from probe test at 10 days, c-Fos expression was greater in EE group than HC-EE and SC groups, with a further increase at 20 days, when SC mice were also statistically different from their HC controls, but still different from the EE group (see Fig. 5 for details).

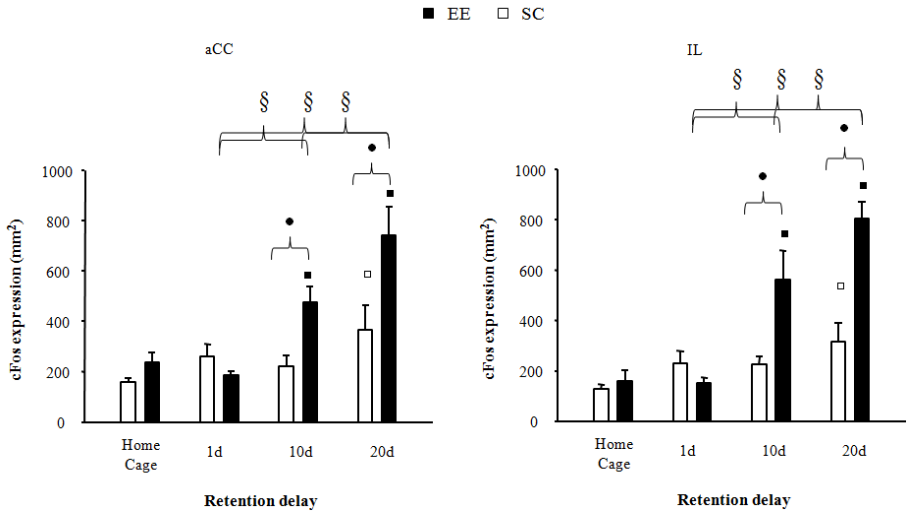

Figure 5: c-Fos expression in aCC and IL. Probe test at 1 day: two way ANOVA post hoc analysis Holm-Sidak method, all p value > 0.05 both for for aCC and IL. Probe test at 10 days: two way ANOVA post hoc analysis Holm-Sidak method, for aCC EE vs HC-EE p=0.006; EE vs SC p=0.002; SC vs HC-SC p=0.435; for IL EE vs HC-EE and vs SC p<0.01; SC vs HC-SC p=0.185. Probe test at 20 days: two way ANOVA post hoc analysis Holm-Sidak method, for aCC, EE vs HC-EE and vs SC p<0.001; SC vs HC-SC p=0.011; for IL, EE vs HC-EE and vs SC p<0.01; SC vs HC-SC p=0.015. Statistical difference within EE group were found in all days of probe tests in both aCC and IL (two way ANOVA post hoc analysis Holm-Sidak method, all p value < 0.01). ▪ statistical significance between EE and HC-EE; ▫ statistical significance between SC and HC-SC; • = statistical significance for housing factor; § = statistical significance for factor day within EE group; error bars = s.e.m.

To examine the time-dependent reorganization of neuronal activation in a brain-wide manner, I observed c-Fos expression in other cortical areas that are important for the

construction of spatial maps. Using several techniques, Burkhalter et al. (2011) determined that distinct areas of extrastriate visual cortex are gateways for ventral and dorsal streams in the mouse. The dorsal stream includes hippocampal circuit - medial entorhinal cortex (Haftig et al., 2005) - posterior parietal cortex (Whitlock et al., 2008) network for spatial navigation; in addition, the dorsal stream is connected to auditory cortex and to frontal areas, such as cingulate cortex, infralimbic cortex and motor areas (Burkhalter et al., 2011). First I investigated c-Fos expression in MEnt and in pPta, and I found that activation at 1 day in both areas was similar in EE mice, SC mice, and in their home-cage controls (Two way ANOVA, post hoc analysis Holm-Sidak method, all p values > 0.05). In probe tests at 10 and 20 days, significant differences between EE and SC mice and between EE and HC-EE mice were found (see Fig. 6 for details). Then, I observed c-Fos expression in V1 and M2, as they are connected to the dorsal stream and Di Garbo et al. (2011) identified a direct monosynaptic connection between motor and visual cortices in the mouse brain. I found that EE group was statistically different from SC and HC-EE groups, only in probe test performed at 20 days (see Fig. 7 for details). Finally, I investigated c-Fos expression in Au1, a sensory cortex not involved in spatial learning, and I demonstrated that activation in this area was similar in all groups (see Fig. 8 for details).

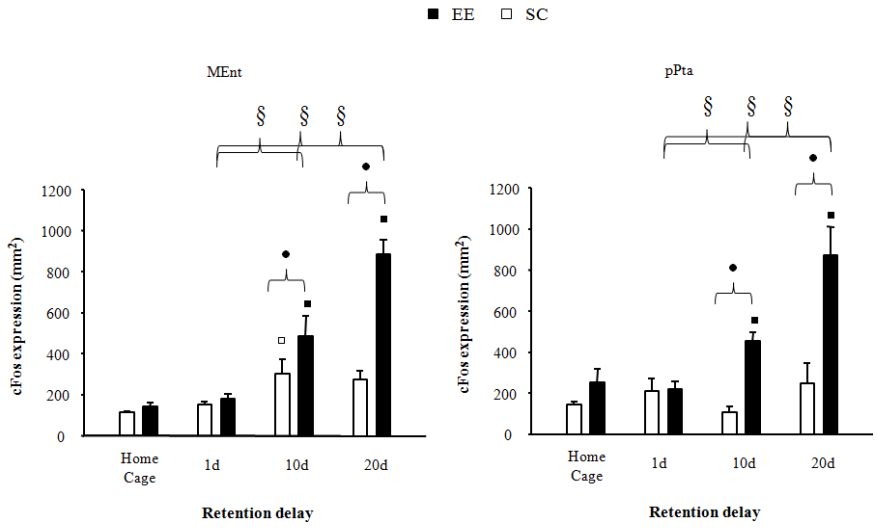

Figure 6: c-Fos expression in MEnt and pPta: Probe test at 1 day: two way ANOVA post hoc analysis Holm-Sidak method, all p value > 0.05 for MEnt and pPta. Probe test at 10 days: two way ANOVA post hoc analysis Holm-Sidak method, for MEnt EE vs HC-EE $p<0.01$; EE vs SC $p=0.008$; SC vs HC-SC $p=0.006$; for pPta EE vs HC-EE $p=0.032$; EE vs SC $p<0.001$; SC vs HC-SC $p=0.861$. Probe test at 20 days: two way ANOVA post hoc analysis Holm-Sidak method, for MEnt, EE vs HC-EE and vs SC $p<0.01$; SC vs HC-SC $p=0.02$; for pPta, EE vs HC-EE and vs SC $p<0.01$; SC vs HC-SC $p=0.185$. Statistical difference within EE group were found in all days of probe tests in both MEnt and pPta (two way ANOVA post hoc analysis Holm-Sidak method, all p value <

0.01). ▪ statistical significance between EE and HC-EE; ▫ statistical significance between SC and HC-SC; • = statistical significance for housing factor; § = statistical significance within EE group; error bars = s.e.m.

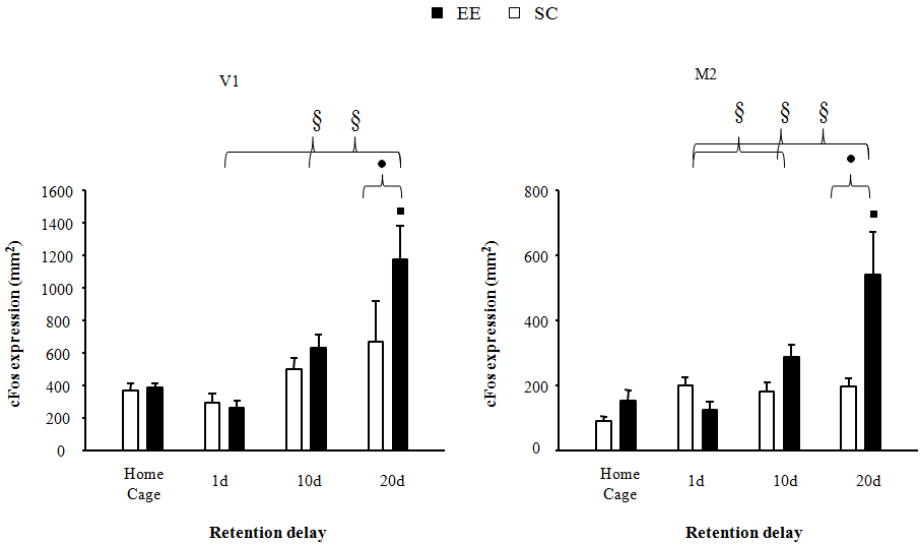

Figure 7: c-Fos expression in V1 and M2: Probe test at 1 day: two way ANOVA post hoc analysis Holm-Sidak method, all p value > 0.05 for V1 and M2. Probe test at 10 days: two way ANOVA post hoc analysis Holm-Sidak method, for V1 EE vs HC-EE p=0.155; EE vs SC p=0.423; SC vs HC-SC p=0.396; for M2 EE vs HC-EE p=0.059; EE vs SC p=0.115; SC vs HC-SC p=0.169. Probe test at 20 days: two way ANOVA post hoc analysis Holm-Sidak method, for V1, EE vs HC-EE and vs SC p<0.01; SC vs HC-SC p=0.061; for M2, EE vs HC-EE and vs SC p<0.01; SC vs HC-SC p=0.111. Statistical difference within EE group were found in all days of probe tests in both V1 and M2 (two way ANOVA post hoc analysis Holm-Sidak method, all p value < 0.05). ▪ statistical significance between EE and HC-EE; ▫ statistical significance between SC and HC-SC; • = statistical significance for housing factor; § = statistical significance within EE group; error bars = s.e.m.

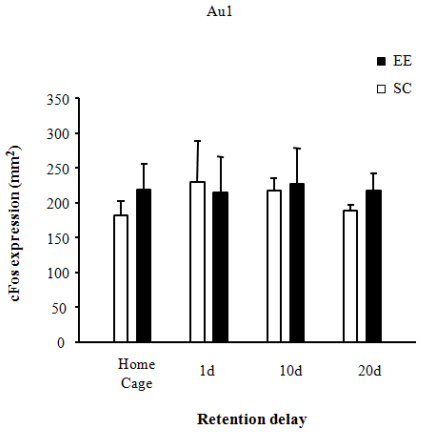

Figure 8: c-Fos-expression in Au1. The data showed no difference between groups (Two way ANOVA, housing condition factor p=0.538, day of probe test factor p=0.927, housing condition×day of probe test interaction p=0.998). In all probe tests, EE and SC groups did not differ from their home cage controls.

Discussion

In this work, I provide the first evidence that EE can affect the time-dependent spatial memory system consolidation. C57BL/6 mice, housed in standard or in enriched condition,

were subjected to a spatial learning and then tested 1, 10 and 20 days after learning to evaluate consolidation process. The behavioural data showed that EE mice were faster, considering latency parameter, in learning the position of the target platform in comparison to SC mice, confirming that young rodents in EE exhibit a better performance in MWM (van Praag et al., 2000). However, evaluating the precision of search patterns in probe test, no significant difference was found between groups. This again is typical of the results in EE rodents when intensive learning protocol for the MWM tasks are found (van Praag et al., 1999).

After memory recall, I mapped the regional expression of inducible immediate early gene c-fos, as an indicator of neuronal activity. First, I demonstrated that hippocampus (dHPC and vHPC) is involved in spatial memory recall in all temporal points tested, and that some hippocampal subregions showed greater activation in remote recalls (20 and 30 days) than recent recalls (1 and 10 days). These results are consistent with the idea that hippocampus is responsible for encoding spatial memory (O'Keefe and Nadel, 1978; Burgess et al., 2002; Morris et al., 2003; Nakazawa et al., 2004; Leutgeb et al., 2005), however its activation in remote spatial memory is not in agreement with studies that showed a progressive independence of this kind of memory from hippocampus (Bontempi et al., 1999; Maviel et al., 2004; Frankland et al., 2004), though it is in line with the hypothesis that remote memory never become totally independent from the hippocampus (Nadel and Moscovitch, 1997). In a more recent study, indeed, Lopez et al. (2011) demonstrated that hippocampus recruitment was influenced by the environmental conditions, such as cue saliency and complexity of the task in which memories are initially formed and subsequently recalled, thus the rich spatial details and the complexity of the training in MWM can account for the greater activation in some hippocampal subregions. Moreover, it has been found that that precise real-time inhibition of the dorsal CA1 region, using optogenetic method, was sufficient to impair remote recall (Goshen et al., 2011).

I also found that EE induces an early recruitment of aCC and IL; these areas were involved in recall of spatial memory in the EE group since probe test at 10 days. Moreover, in agreement with some studies (Maviel et al., 2004; Teixeira et al., 2006) there was a progressive increasing in c-Fos expression in both areas, as consolidation process proceeded. However, the final storage site in the cortex could be the aCC, while the IL could correlated with motivational aspects of performance, encoding other significant aspects of the

environment, such as salient landmarks or preferred locations (Hok et al., 2005). The aCC was activated after remote memory recall in a number of tasks (Bontempi et al., 1999; Frankland et al., 2004; Maviel et al., 2004; Teixeira et al., 2006), and, conversely, inactivation of the ACC disrupted recall of remote five-arm discrimination (Maviel et al., 2004), contextual fear (Frankland et al., 2004) and MWM (Teixeira et al., 2006) memories. The aCC is highly interconnected to other prefrontal regions, and is reciprocally connected to sensory, motor, and limbic cortices (Uylings et al., 2003; Jones et al., 2005), therefore this connectivity places the ACC in favorable position, raising the possibility that this region coordinates retrieval of remote memories stored in distributed cortical networks.

Finally, I demonstrated that EE induces the involvement of distributed cortical network in supporting remote spatial memory. I observed that MEnt and pPta were activated following memory recall at 10 days in EE group. Both areas were included in the dorsal network for spatial navigation (Burkhalter et al., 2011): the entorhinal cortex contains a spatial representation of environment and plays an interface role between the hippocampus and neocortex (Moser et al., 2008), instead the parietal cortex, specifically the multisensory posterior region, translates coordinate information from spatial maps in the entorhinal cortex and hippocampus into egocentric representations (Moscovitch et al., 2005; Whitlock et al., 2008). I also investigated the c-Fos expression in V1 and M2, and I found that they followed the same c-Fos expression pattern: EE group showed a greater activation than SC group, only in probe test performed at 20 days. This is not surprising since they are connected to the dorsal stream (Burkhalter et al., 2011), and a direct monosynaptic connection between motor and visual cortices was identified (Di Garbo et al., 2011).

One mechanism underlying time-dependent reorganization through hippocampal-cortical and cortico-cortical interactions, could be the adult hippocampal neurogenesis. Kitamura et al. (2009) showed that new hippocampal neurons were recruited into neuronal networks supporting retrieval of remote spatial memory and that the enhanced neurogenesis by voluntary running-wheel exercise sped up the disengaging from hippocampus. Since EE was found to increase hippocampal neurogenesis and promote the survival of newly generated neurons (van Praag et al., 2000), it is plausible that EE may accelerate the recruitment of extrahippocampal areas. Moreover, EE increases the production of several factors, including neurotrophins, such as brain-derived neurotrophic factor (BDNF), or insulin growth factor-l (IGF-1) (Ickes et al., 2000; Pham et al., 2002), which affect hippocampal neurogenesis and

hippocampal and cortical plasticity (van Praag et al., 2000; Aberg et al., 2006; Waterhouse and Xu, 2009). IGF-1 plays an important role in cell growth and development, and it also upregulates neurogenesis in the adult hippocampus (D'Ercole et al., 1996; D'Ercole et al., 2002; Lee and Son, 2009). In the adult brain, IGF-1 has been shown to mediate both the neuroprotective effects of physical exercise and the enhancement caused by exercise in hippocampal plasticity and in learning and memory (Aberg et al., 2006). Moreover IGF-1 mediates the increased expression of BDNF subsequent to EE and physical exercise (Carro et al., 2000; Ding et al., 2006; Landi et al., 2009). BDNF has been shown to regulate adult hippocampal neurogenesis, to modulate plasticity during learning by activating signaling pathways that modify local synaptic targets and have long-term effects on transcription, and to mediate the expression of hippocampal LTP, in both the early and late phases (Rossi et al., 2006 ;Lee and Son, 2009; Waterhouse and Xu, 2009; Cowansage et al., 2010). Thus, BDNF plays a critical role in synaptic plasticity and could represent a potential link between learning and synaptic, structural, and behavioral plasticity. In conclusion, it is possible that the increased hippocampal neurogenesis and neuroplasticity could account for the effect on time-dependent memory reorganization induced by EE.

Further investigation will be necessary, in order to verify if distributed cortical network (e.g the dorsal stream areas) that supports memory recall in EE animals is activated specifically for this group, or could also be found in SC group, possibly in probe test later than 20 days, such as 30 days as in the Bontempi and Maviel papers. The presence of an activation in these areas in SC group would suggest that these areas partake to memory system consolidation, and EE might speed up their recruitment, in the same way as it does for the mPFC; on the other hand, if these areas were not to be activated, this would suggest that they are not involved in remote memory recall, and that that their involvement might be specific for the EE group.

Another open issue is whether early activation of several cortical areas in EE animals correlates with their early recruitment in the spatial memory recall; to investigate this issue, it could be tested whether pharmacological inactivation of cortical areas precociously activated in EE animals, such as the mPFC, pPta or MEnt, disrupts recall of spatial memories in EE group.

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