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EXPERIENCE-DEPENDENT EXPRESSION OF miR132 REGULATES OCULAR DOMINANCE PLASTICITY

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Introduction

1. Experience-dependent plasticity

One of the most fascinating properties of the mammalian brain is its plasticity; the capacity of the neural activity generated by an experience to modify neural circuit function and thereby modify subsequent thoughts, feelings, and behaviours. Synaptic plasticity specifically refers to the activity-dependent modification of the strength or efficacy of synaptic transmission at pre-existing synapses, and for over a century has been proposed to play a central role in the capacity of the brain to incorporate transient experiences into persistent memory traces. Synaptic plasticity is also thought to play key roles in the early development of neural circuitry and evidence is accumulating that impairments in synaptic plasticity mechanisms contribute to several prominent neuropsychiatric disorders. Thus, elucidating the detailed molecular mechanisms underlying synaptic plasticity in any number of different brain regions is crucial for understanding the neural basis of many aspects of normal and pathological brain function (Citri and Malenka, 2008).

Experience-dependent regulation of gene expression is a key step in brain plasticity, and long lasting alterations in neuronal connections require complex gene expression programmes and fine regulation of protein synthesis (Greer and Greenberg, 2008).

During the early postnatal development, the brain undertakes intense morphological and functional rearrangement in response to sensory experience alterations, with visual, auditory and somatosensory systems displaying sensitive phases of enhanced plasticity that have been called “sensitive or critical periods” (Berardi, et al., 2000). Critical periods are specific windows of opportunity during

which experience provides information that is essential for normal development and permanently alters performance (Hensch, 2005). Critical periods are known to occur also during early human development, including such behaviours as language acquisition (Doupe and Kuhl, 1999) and visual function development (Levi, 2005).

Although plasticity is enhanced in malleable young brain, adult neural circuits are not completely stable and can undertake some plastic phenomena such as learning and memory. A proper understanding of the molecular mechanism regulating experience-dependent neural plasticity is a crucial point, since dysregulation of neocortical plasticity during development is a leading cause of many brain diseases from mental retardation to amblyopia (lazy eye), from psychiatric disorders to epilepsy. Therefore, investigating the molecular events underling the activation and regulation of critical period's plasticity is of great interest in the field of Neuroscience. Manipulation of such mechanisms may potentially allow reactivation of neural circuit plasticity for clinical purposes during times when the adult brain is normally less plastic.

2. The rodent visual system

In the mammalian visual system, visual information are processed in the retina and sent to different structures of the central nervous system (CNS) through retinal ganglion cells (RGCs) axons, which represent the output of the retina. RGCs project to the visual centres of the brain that are located in the midbrain and in the thalamus. The pattern of retinal projections varies from species to species. In rodents, the vast majority of RGCs project to the superior colliculus (SC) and the pretectal nuclei, with about 30% of them sending collaterals to the dorsal-lateral geniculate nucleus (dLGN) in the thalamus (Dreher, et al., 1985). RGC axons from

each eye project to both sides of the brain, however the major afferents to the SC and dLGN arise from the contralateral eye and only 5% of optic axons project ipsilaterally. Within the dLGN ganglion cell axons are not intermixed; in cats, ferrets and primates they terminate in a set of separate, alternate eye-specific layers (three layers in cats and six layers in primates) that are strictly monocular (Hickey and Guillery, 1974). In rodents there is not a proper lamination of the dLGN; however, ipsilateral and contralateral retinal fibers are segregated in a patchy fashion originating two eye-specific territories in the dLGN: the ipsilateral portion or inner core and the contralateral portion or outer shell. The primary visual cortex (V1), located occipitally in the brain, consists of six layers of cells between the pial surface and the underlying white matter. The dLGN projects to the visual cortex via thalamo-cortical connections that terminate in the layer IV of V1. The neurons of layer IV form synapses in the layer 2/3, that is the first site within the visual system where visual input from the two eyes converges onto single neurons. In carnivores and primates, afferents from the dLGN segregate by eye within the cortical layer IV into alternating, equal-sized stripes called ocular dominance columns (Hubel and Wiesel, 1963, Shatz and Stryker, 1978). The binocular zone is smaller in rodents than in cat and primates and it is not organized in ocular dominance columns. Finally, It is important to remember that the neurons of the rodent visual cortex are predominantly responsive to the inputs deriving from the contralateral eye, due to the high percentage of RGCs fibers crossing at the optic chiasm (**Fig.1**).

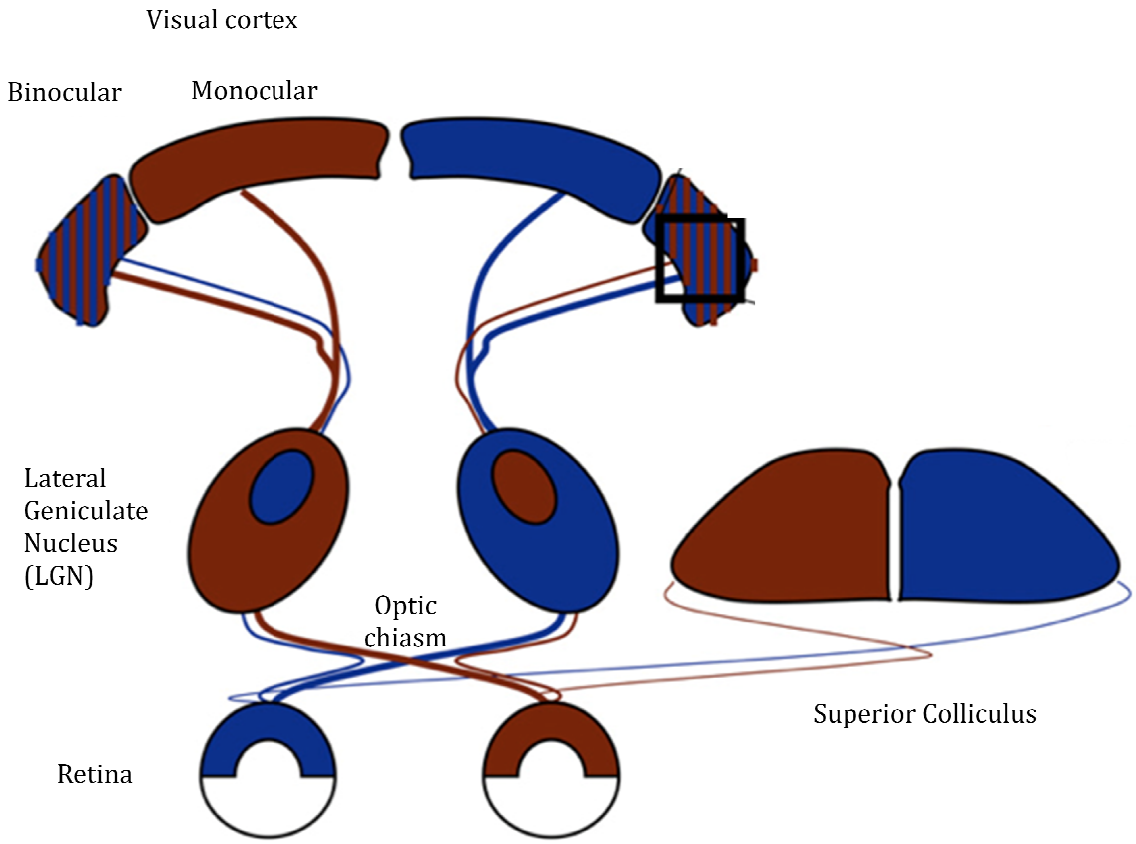


Figure 1. Schematic representation of the mammalian visual system. Retina feeds forward via the optic chiasm to LGN and superior colliculus. Contrasting colors indicate regions receiving input from each eye. LGN eye-specific regions shown as in rodent. The LGN then projects to visual cortex. The binocular zone is smaller and more lateral in rodents than in cat and primate, whose visual cortex shows more overlap of inputs from competing eyes. (Adapted from Hooks and Chen, 2007).

3. Ocular dominance plasticity and critical period in the visual cortex

Visual cortex development is strongly influenced by visual experience during a short period of postnatal development, the so called critical period (Berardi, et al., 2003). The expression "critical period" in the context of mammalian visual system was introduced for the first time by Wiesel and Hubel (1963) in their studies in the cat. They described the physiological shift in responsiveness of neurons in the visual cortex to light stimulation when one eye was deprived of vision early in life. This form of plasticity is strongly robust during a specific developmental age and diminishes once the cat becomes older (Hubel and Wiesel, 1970). From these

experiments, Wiesel and Hubel proposed that there was a period of development when changes in the external visual environment can alter pre-existing neuronal connections. Proper sensory experience during this critical period is essential for shaping neuronal circuits and for the maintenance of appropriate synaptic connections. Neuronal activity evocated by sensory experience allows maturation of important properties of visual system, such as visual acuity (the spatial resolution of the visual system) and orientation preference (Fagiolini, et al., 1994, Wang, et al., 2010). Manipulations of visual experience such as dark rearing (DR) and monocular deprivation (MD) cause structural, functional and molecular changes in the neuronal network of visual cortex (Berardi, et al., 2004). MD consists in the closure of one eye resulting in an imbalance of the inputs from the two eyes with the consequent synaptic reorganization of V1 circuitry. This form of plasticity is called ocular dominance (OD) plasticity, and it represents a classic paradigm to study how experience-dependent activity models neuronal connections. MD causes a loss of visual acuity in the deprived eye resulting from a decrease in the visual inputs of the closed eye which causes an irreversible reduction of the ability of that eye to drive neuronal responses in the cortex; therefore neurons in the binocular zone of the contralateral V1, previously dominated by the deprived eye, shift their responsiveness toward the ipsilateral open eye. Behaviorally, animals MD during development lose visual acuity in the deprived eye and any subsequent experiences or visual stimulations cannot completely reverse the effects of early deprivation after the closure of critical period (**Fig.2**) (Berardi, et al., 2000).

Plasticity in the visual cortex declines with age. Adult visual cortex still responds to experience with plastic changes, as shown by perceptual learning (Schoups, et al., 2001), however the extent of plasticity is reduced. Markedly in

contrast to the profound effects in young animals, prolonged eye closure in adult has little or no effect (Hubel and Wiesel, 1970), furthermore recovery from amblyopia is very limited once the critical period is terminated.

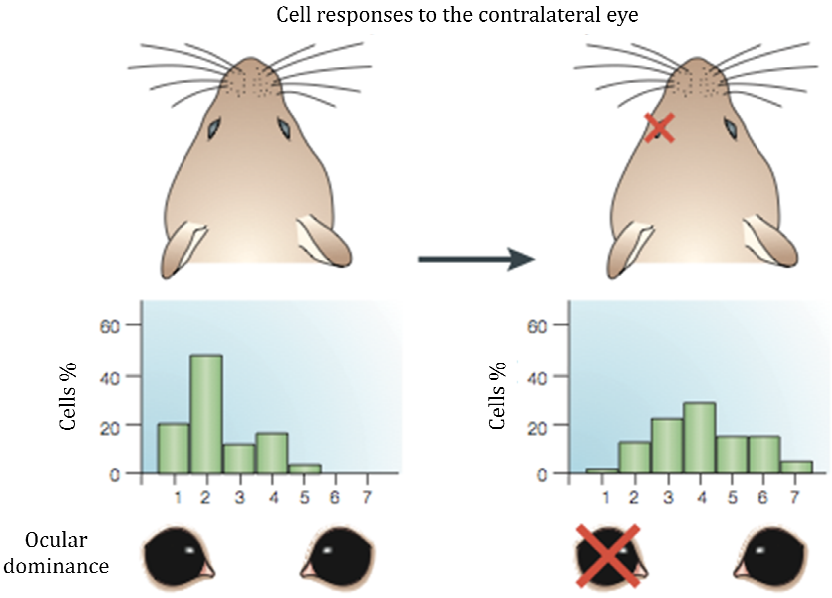


Figure 2. Effect of monocular deprivation during the critical period. MD produces a loss of response to the deprived eye and a gain of open-eye input, as measured by the neuronal discharge of single units from the mouse visual cortex. The ocular dominance of cells, rated on a seven-point scale of neuronal responsiveness, indicates a typical bias toward the contralateral eye (1–3) in the rodent (left). After 3 or more days of monocular deprivation, the distribution shifts toward the open, ipsilateral eye (4–7; right). (Adapted from Hensch 2005).

Anatomically, in higher animals, such as cats and primates, MD causes a retraction of thalamocortical axons conveying the inputs from the deprived eye and the strengthening of projections from the open eye, which results in the expansion of OD columns guided by the non- deprived eye, at the cost of those serving the closed eye (Hubel, et al., 1977, Stryker and Harris, 1986). Thalamocortical projections carrying inputs of the closed eye become less branched and shorter. An opposite effect is observed in axons representing the open eye (Tieman, 1984).

In rodents, two/three days of MD cause an initial decrease in deprived eye responses, due to the weakening of closed eye connections and a reorganization of intracortical horizontal connections in the superficial layers (II/III) of the binocular portion of V1 (Trachtenberg and Stryker, 2001). Prolonged MD (five/seven days) shows an increase in the neuronal responses to the inputs from the ipsilateral open eye (Frenkel and Bear, 2004). Architectural changes in thalamocortical arborization, terminating in layer IV, are evident only much later, more than a month in the mouse (Antonini, et al., 1999).

The anatomical changes observed in rodent visual cortex reflect those of higher mammals, although with some differences, indeed the major effect of deprivation on the contralateral projection is an arrest of growth rather than a prompt retraction of branches, as seen in the cat (Antonini and Stryker, 1996).

Furthermore, prolonged deprivation promoted the growth of the open eye's geniculocortical connections in mouse (Antonini, et al., 1999).

4. The mouse as animal model for visual sytem studies

Studies of cortical visual processing have typically used carnivores or primates, wich are considered to have a more refined visual system, including a much larger cortical region for visual processing, higher acuity, extensive visual behaviors, and orientation, ocular dominance and spatial frequencie columns. Despite the low visual acuity and relatively small region of cortex devoted to visual processing, neurons in mouse V1 show selectivity for stimulus parameters and typical response properties that are near to that found in other species (Niell and Stryker, 2008).

In contrast to the classic notion of a critical period for experience-dependent plasticity, several studies have recently reported that OD shifts in mice can also be induced in adulthood (Hofer, et al., 2006, Tagawa, et al., 2005). Nevertheless, the binocular cortical representation is still more sensitive in juvenile mice, as in adults OD shifts require longer MD durations and are generally smaller. It remains unknown whether substantial structural rearrangements that accompany functional OD shifts in juvenile animals also occur in the mature cortex during MD (Hofer, et al., 2006). Furthermore, OD shift in adult mice depends on the age of the animals and on the length of MD period. OD plasticity after 7 days of MD is present in young adult mice (90–100 days) but significantly weaker already in 109–158 days old mice. In animals older than 208 days, OD plasticity is absent even after 14 days of MD. Therefore, OD plasticity in binocular visual cortex is most pronounced in young animals, reduced but present in adolescence and absent in fully mature animals older than 110 days of age (Lehmann and Lowel, 2008). Mice are thus not basically different in OD plasticity from cats and monkeys which is an absolutely essential prerequisite for their use as valid model for visual system studies.

5. Dark rearing

DR is a form of visual manipulation consisting in the complete absence of sensory input. In animals reared in a completely dark environment from birth visual connections do not consolidate, remaining plastic well after the closure of normal critical period and visual acuity does not reach the adult level. Cortical neurons display immature properties, such as reduced orientation and direction selectivity, larger receptive field sizes and lower visual acuity. DR causes a downregulation in BDNF expression with subsequent delay in intracortical inhibition maturation.

Furthermore, neurons in V1 show change in size and density of dendritic spines (Hooks and Chen, 2007).

6. Exploring the molecular mechanisms of OD plasticity

To date, OD plasticity remains the best studied experimental model for experience-dependent refinement of neuronal circuits because of the ease of manipulating visual experience independently in the two eyes. However, a complete understanding of critical period plasticity requires linking the systems-level change in circuit function with the molecular mechanisms that make circuit changes possible. The cellular and molecular mechanisms that control the developmental plasticity of visual cortical connections and restrict experience-dependent plasticity to short critical periods are still little known, though intensely investigated.

In this paragraph I will overview recent discoveries in this field.

- ***6.1 Glutamate receptors: the NR2B/NR2A switch***

The first modifications induced by experience in visual cortical circuits are likely to be changes in synaptic efficacy. Plasticity is gated by the activation of N-methyl-D-aspartate (NMDA) receptors, which respond to excitatory synaptic transmission by enabling calcium (Ca2+) influx into target synapse and its neuron. NMDA receptors are both transmitter- and voltage-dependent, and their coupling via Ca2+ influx to plasticity-related intracellular signalling, has led to the notion that they might be a neural implementation of Hebbian synapses.

Involvement of NMDA receptors in developmental visual cortical plasticity has been initially suggested by the observation that block of NMDA receptors inhibits the effects of MD (Bear, et al., 1990). A difficulty with pharmacological block of NMDA

receptors can be that it significantly affects visually driven activity, but the use of different NMDA receptor antagonists (Daw, et al., 1999) or antisense oligonucleotides to reduce expression of the NMDAR1 subunit has overcome this problem, showing that it is possible to block the effects of monocular deprivation without affecting visual responses (Roberts, et al., 1998) and confirming NMDA receptor involvement in visual cortical plasticity (Berardi, et al., 2003). However, the receptor's capacity to drive plasticity depends on its subunit composition: some receptors are built from "NR2B" subunits, which enable a high Ca2+ permeability and thus enhanced plasticity, and some are composed by "NR2A" subunits, that have a reduced Ca2+ flux, thus resulting in shortening of NMDA current. A crucial determinant of plasticity is itself regulated by the activity level of the circuit. NMDA receptors are developmentally regulated: their subunit composition varies in the visual cortex, from a dominant presence of receptors containing the subunit 2B to a high presence of receptors containing the subunit 2A, with a time course paralleling that of functional visual cortical development and the critical period. DR, which retards critical-period closure and impairs development of functional properties of the visual cortex and of visual acuity, delays the developmental shortening of NMDA-receptor currents and of subunit 2A expression; as animals are exposed to visual experience, the NR2B/NR2A ratio declines (Quinlan, et al., 1999), thus reducing the capacity for further plasticity and suggesting that the 2B-to-2A switch is related to visual cortical development (Berardi, et al., 2000). Furthermore, placing adult animals in DR for extended periods recovers the NR2B/NR2A ratio, thereby restoring the capacity for plasticity (Chen and Bear, 2007). However, the initial suggestion that developmental shortening of NMDAR currents by a subunit change from NR2B to NR2A closes the critical period (Carmignoto and Vicini, 1992) needs

revision, as animals lacking NR2A show normal sensitivity to MD during critical period, thus suggesting that expression of the 2A subunit is not essential to delineate the time course of the critical period for OD plasticity (Fagiolini, et al., 2003, Lu, et al., 2001) and might be related to other features of visual cortical plasticity.

- ***6.2 Inhibitory circuits maturation***

Recently it has been demonstrated that the development of inhibitory circuitry in the visual cortex plays a crucial role in controlling the onset and time course of critical periods (Hensch, 2005). Mice lacking the synaptic isoform of GABA producing enzyme, glutamic acid decarboxylase (GAD65) show no OD plasticity. This impairment can be rescued by intracortical infusion of the GABA-A receptor agonist diazepam, demonstrating that a decrease in inhibition effectively abolished critical period plasticity (Hensch, et al., 1998). Furthermore, an early enhancement of GABA-mediated inhibition by benzodiazepin treatment triggers the precocious onset of OD plasticity (Fagiolini and Hensch, 2000). Therefore, inhibition not only is a 'brake' for excitation but also has an important role in sculpting the pattern of electrical activity. This action contributes to the detection of imbalance of activity between the afferents to a cortical neuron. A failure of the postsynaptic neuron to evaluate the timing of arrival of its synaptic inputs is bound to be a failure in plasticity (Berardi, et al., 2003). Cortical GABAergic neurons show a high heterogeneity in morphology, protein expression and electrical properties (Huang, et al., 2007). Different interneurons subtypes play specific roles in cortical development, function and plasticity. For instance, it was demonstrated that GABA transmission mediated by the GABA-A receptor containing the α1 subunit is required for the induction of critical period plasticity (Fagiolini, et al., 2004). More recent data suggest a fundamental role

for parvalbumin positive basket interneurons maturation in the onset of critical period OD plasticity, due to the optimization of GABA-A receptors number on the soma-proximal dendritic compartment of pyramidal cells (Katagiri, et al., 2007).

The inhibitory circuits maturation strongly depends on sensory experience, since sensory deprivation significantly retards the morphological and functional development of GABAergic synapses (Chattopadhyaya, et al., 2004). Brain derived neurotrophic factor (BDNF), an activity dependent molecule, is implicated in GABAergic synapses formation. In transgenic mice with precocious BDNF expression, a marked increase in perisomatic inhibitory innervation in the visual cortex is correlated with a premature onset and closure of OD plasticity (Huang, et al., 1999). Furthermore, in adult rats OD plasticity is greatly reduced but can be enhanced by the administration of picrotoxin, an antagonist of GABA-A receptors, and mercaptopropionic acid, an inhibitor of GABA synthesis, at doses that do not disrupt cortical activity (Harauzov, et al., 2010). Furthermore, the administration of the antidepressant fluoxetine reduces GABA content in the adult visual cortex and enhances OD plasticity (Maya Vetencourt, et al., 2008). OD plasticity enhancement by fluoxetine was blocked by the administration of the GABA agonist benzodiazepines suggesting that fluoxetine effect was mediated by a reduction of GABA transmission.

Sugiyama et al (2008) proposed a novel mechanism explaining how visual input is coupled to the onset of OD plasticity. They demonstrated that a homeoprotein produced by the retina, OTX2, is transferred in the visual cortex in an activity- dependent fashion. Once in the cortex, OTX2 triggers the maturation of GABAergic inhibition and promotes critical period plasticity (Sugiyama, et al., 2008).

The correct timing of GABAergic innervation maturation is fine-tuned by inhibitory molecular mechanism that set the appropriate patterns of interneuron's

connection and the starting of critical period plasticity. Polysialic acid (PSA), linked to the neural cell adhesion molecule (NCAM), acts as a negative signal to suppress the formation of inhibitory synapses and the onset of OD plasticity in the developing visual cortex. In the visual cortex PSA expression is developmentally and activity regulated and inversely correlated with the maturation of GABAergic circuitry. Indeed, premature enzymatic removal of PSA in the developing visual cortex results in precocious maturation of perisomatic innervation by basket interneurons, enhances inhibitory synaptic transmission and causes an earlier onset of critical period plasticity (Di Cristo, 2007).

A role for immature GABAergic innervation in promoting OD plasticity is also suggested by a study showing that transplantation of embryonic inhibitory interneurons in the visual cortex of adult mice induces OD plasticity after the closure of the critical period. These findings suggest that OD plasticity is regulated by the execution of a maturational program intrinsic to inhibitory neurons (Southwell, et al., 2010).

- ***6.3 Neuroactive released proteins***

Neurotrophins are a family of neurothophic factors consisting of nerve growth factor (NGF), BDNF, neurotrophin-3 (NT3) and neurotrophin-4 (NT4). Neurotrophins support neuronal survival and differentiation by binding to activating tyrosine kinase receptor of the trk family. Neuronal activity increases the synthesis and secretion of neurotrophins, indeed they are also implicated in activity-dependent neuronal plasticity (Huang and Reichardt, 2003). It was shown that exogenous supply of neurotrophins in the visual cortex strongly affects the OD plasticity induced by MD (McAllister, et al., 1999) and that these factors are crucial

regulator of normal visual cortical development and plasticity (Berardi, et al., 1994). The expression of BDNF and its receptor trkB is widespread in the visual cortex. BDNF levels are activity-dependent and regulated by visual stimulation through retinal activity (Lein, et al., 2000). BDNF increasing after eye opening during post-natal development is prevented by DR (Capsoni, et al., 1999) and MD (Bozzi, et al., 1995, Lein and Shatz, 2000). Infusion of BDNF into the rats' visual cortex blocks the physiologiacal effect of MD (Lodovichi, et al., 2000); conversely, intracortical administration of BDNF restores OD plasticity in adult rats (Maya Vetencourt, et al., 2008). Mutant mice overexpressing BDNF in the visual cortex are characterized by a precocious maturation of the visual system and an accelerated time course of critical period plasticity (Huang, et al., 1999). These effects are mediated by a precocious maturation of intracortical inhibition. These observations suggest that BDNF may regulate the onset of plasticity influencing GABAergic interneuron circuits formation (Berardi, et al., 2000, Huang, et al., 1999).

Several studies on neurotrophin receptors expression and on the effects of neurotrophins on visual cortical neurons or afferents to the visual cortex have indicated that different neurotrophins act on different neuronal targets. Therefore, the synergy between neurotrophins and activity has to be considered to be specific for each neurotrophic factor and the neuronal populations that are its targets (Berardi, et al., 2003).

Astrocytes are capable of releasing neuroactive molecules, such as tumor necrosis factor alpha (TNFα), and thus have the potential to be not only supportive but also signaling cells in the brain. A report shows that TNFα-mediated synaptic scaling is involved in OD plasticity. After MD, neurons in the binocular region of the visual cortex decrease their response to the closed eye and increase their

responsiveness to the open eye. Using transgenic mice, this study suggests that the increase in the open eye response is a homeostatic process mediated by TNFα (Kaneko, et al., 2008). Given that astrocytes are a major source of TNFα, that they respond to visual stimulation, and that they are able to secrete permissive factors for OD plasticity, these results suggest that astrocytes have the potential to be key elements in the control of neuronal network function and plasticity in vivo (Fellin, 2009), and add TNFα to the repertoire of released proteins involved in OD plasticity regulation.

- ***6.4 Extracellular influences.***

Extracellular environment, and in particular the extracellular matrix (ECM), plays an important role in controlling spine dynamics and visual cortical plasticity. Past studies have shown an important role for key components of the brain ECM, the chondroitin-sulfate proteo-glycans (CSPGs), in OD plasticity of the visual cortex. During development, CSPGs condense at high concentration in lattice-like structures, called perineuronal nets (PNNs), which completely ensheath visual cortical neurons. The process of condensation of CSPGs into PNNs begins during late development and is completed after the end of the critical period (Berardi, et al., 2004). The degradation of PNN, trough intracortical injection of chondroitinase ABC, reactivates OD plasticity in the adult rat visual cortex (Pizzorusso, et al., 2002); furthermore chondroitinase ABC treatment allows a complete recovery of visual acuity and OD in adult amblyopic rats (Pizzorusso, et al., 2006). Therefore, PNNs, that condensate and entangle cortical neurons and synapses, represent an obstacle to structural and functional plasticity. The formation of PNNs is triggered by neuronal production of cartilage link protein Crtl1. Mice lacking Crtl1 have attenuated PNNs, but the overall

levels of CSPGs and their pattern of glycan sulphation are unchanged. Interestingly, Crtl1 KO animals retain juvenile levels of OD plasticity and their visual acuity remains sensitive to visual deprivation (Carulli, et al., 2010).

PNNs preferentially enwrap inhibitory interneurons, albeit CSPG-containing nets were found also around pyramidal neurons and their spines (Berardi, et al., 2004). During development, dendritic spines are highly dynamic, appear and disappear at a rapid rate. As the brain matures, spines turnover strongly decreases and MD is totally ineffective in reduce spine density in adult mice (Holtmaat and Svoboda, 2009). The inhibitory nature of the mature ECM could be one of the factors at the basis of spines remarkable stability (Berardi, et al., 2004). The extracellular protease tissue-type plasminogen activator (tPA) has been shown to be highly expressed at periods of maximal plasticity (Mataga, et al., 2004) and plays a crucial permissive role in enabling circuit remodeling during OD plasticity (Mataga, et al., 2002, Oray, et al., 2004). The released tPA increases extracellular proteolysis directly or by the activation of plasmin from the zymogen plasminogen. These proteases have a wide spectrum of targets, including CSPGs, growth factors, neurotrophins etc. Past results demonstrated tPA proteolitic activity as a key regulator of dendritic spines dynamics in the visual cortex, trough the generation of a permissive plastic environment enabling spines motility, protrusion or pruning (Berardi, et al., 2004).

Similarly, it has been demonstrated that fear memories in adult mice can be made susceptible to erasure via degradation of PNNs (Gogolla, et al., 2009).

In conclusion, ECM provides a form of “hard wiring” that can be dissolved to allow structural plasticity and to modulate circuits remodeling in the mature cortex.

In favor of this hypothesis, a recent study showed that matrix metalloproteinases (MMPs), a family of activity-dependent zinc- dependent

extracellular endopeptidases mediating extracellular matrix remodeling, play an important role in visual cortical plasticity. Indeed, the inhibition of MMPs acitivity selectively prevented the increase in the response to the nondeprived-eye stimulation after 7 days of MD, whereas no effect was present on the depression of the responses to the deprived eye both after 3 or 7 days of MD. Moreover, MMP inhibition also affects the rise in the number of spines. Therefore, the authors propose that MMPs could be essential molecular mediators of the experience-dependent potentiation process and could influence structural remodeling influencing the consolidation of plasticity events (Spolidoro, et al., 2012)

A similar restrictive role of the extracellular environment in limiting OD plasticity following the critical period has been shown using knockout animals for the Nogo-66 receptor (NgR) and Nogo-A/B. Knockout animals showed OD plasticity at adult ages (after P40 and P120) when wild-type mice do not respond to MD (McGee, et al., 2005). Therefore, NgR and Nogo-A/B are required for maturation-dependent restrictions of OD plasticity in the visual cortex. This study provides genetic evidence for the hypothesis that myelination consolidates neural circuitry by suppressing plasticity in the mature brain.

Surprisingly, a major histocompatibility complex class I (MHCI) receptor, paired-immunoglobulin–like receptor B (PirB), that is expressed in subsets of neurons at synaptic levels, is involved in the control of critical period plasticity. In mutant mice lacking functional PirB, cortical OD plasticity is more robust at all ages. Therefore, an MHCI receptor functions to limit the extent of experience-dependent plasticity in the visual cortex possibly affecting the ability of activated integrins to engage the neuronal cytoskeleton (Syken, et al., 2006).

Recently, it was discovered that cholinergic signals play a role in the regulation of critical periods plasticity. Lynx1, which is an endogenous prototoxin similar to α-bungarotoxin in snake venom and binds to the nicotinic acetylcholine receptor (nAChR), increases in V1 after the closure of the critical period, acting as a molecular "brake" for visual cortical plasticity. Adult Lynx1 knockout mice exhibites a robust OD shift during short MD. Lynx1 protein directly binds to nAChRs, to reduce their sensitivity to acetylcholine. Thus, mecamylamine, a broad- spectrum antagonist of nAChR, is sufficient to prevent OD plasticity in adult KO mice. The authors propose that modulation of cholinergic activity could affect the local excitatory-inhibitory balance, allowing maturation of cortical circuits and the reduction of juvenile brain plasticity (Morishita, et al., 2010).

- ***6.5 Environmental influences***

Experience is a strong determinant for the duration of critical periods: total lack of experience usually prolongs critical periods and delays development of sensory functions. The clearest example of this come from studies showing that DR prolongs the critical period for OD plasticity. Another approach to investigate the influence of experience on the brain is to manipulate the pattern of environmental stimulation to which animals are exposed. Environmental enrichment (EE) is an experimental protocol specifically devoted to investigate the influence of environment on brain and behaviour, showing that the morphology, chemistry and physiology of the brain can be artificially altered by modifying the quality and intensity of environmental stimulation (Rosenzweig and Bennett, 1969). Many studies have been performed showing that EE can elicit various plastic responses in

the brain, ranging from molecular to anatomical and functional changes (Nithianantharajah and Hannan, 2006).

Rearing animals in EE has profound effects on the development of the nervous system, leading to an acceleration of visual system development at the behavioral, electrophysiological and molecular level (Cancedda, et al., 2004, Ciucci, et al., 2007, Sale, et al., 2004). It has been shown that EE promotes a complete recovery of visual acuity and OD in adult amblyopic animals (Sale, et al., 2007). During my PhD, I have conducted some investigation trying to use EE to promote functional recovery from amblyopia in adult rats modeling a pathological condition in which amblyopia becomes extremely detrimental for patients because of the loss of the non amblyopic eye. Briefly, I studied the effect of EE on the recovery of visual acuity in adult amblyopic rats in which the normal eye had been disconnected from the brain by optic nerve section. I will show you the results of this study, recently published in PloS One, in the appendix.

- ***6.6 Signalling pathways***

Modification of synaptic strength is the outcome of plasticity phenomena. Electrophysiological changes are the results of intacellular molecular pathways activation that determines and gates the "dynamic status" of synapses, cells and circuits.

Recent experiments identified protein kinases implicated in OD plasticity.

ERK/MAPK pathway. Electrical activity and neurotrophins are among the strongest activators of extracellular signal–regulated kinase 1,2 (ERK) (also called p42/44 mitogen- activated protein kinase) (Grewal, et al., 1999, Pizzorusso, et al., 2000). The activation of ERK is required for white matter LTP and for OD plasticity in

the visual cortex during the critical period (Di Cristo, et al., 2001). ERK is also implicated in activity-dependent plasticity as demonstrated by studies of learning and memory (Adams and Sweatt, 2002). ERK downstream targets include important plasticity triggers such as CREB (Impey, et al., 1998), Arc (Ying, et al., 2002), and transcription factors that regulate the expression of immediate early genes (Xia, et al., 1996). Therefore, ERK signaling cascade activation leads to the modulation of activity of crucial plasticity molecules such as synaptic proteins and ion channels, thus promoting coherent integration of inputs between single neuron and networks.

ERK phosphorylation is induced by activation of different signalling cascade. A well known upstream regulator of ERK is the protein Ras, that results extremely interesting for OD plasticity. Indeed, a constitutively active form of H-ras (H-rasG12V), expressed presynaptically at excitatory synapses in mice, accelerates and enhances multiple, mechanistically distinct forms of plasticity in the developing visual cortex. In vivo, H-rasG12V increases the rate of OD change in response to MD and accelerates the recovery from deprivation by reverse occlusion (Kaneko, et al., 2010).

cAMP-dependent protein kinase cascade. Pharmacological block of cAMP-dependent protein kinase (PKA) inhibits the OD shift induced by MD during the critical period (Beaver, et al., 2001). Investigations exploring the connection of PKA in LTD and OD have also been used to study a possible role of LTD in OD plasticity. Loss of one PKA regulatory subunit disrupts LTD, but not OD (Hensch, et al., 1998), while loss of a different subunit leaves LTD intact but disrupts OD plasticity (Rao, et al., 2004). Alternatively, a study of the predominant cortical regulatory subunit of PKA indicates that the subunit RII beta is required for OD plasticity and LTD, though LTP is not disrupted (Fischer, et al., 2004). The disparity in the cited results could be

explained by the fact that different PKA regulatory subunits are known to localize this enzyme to distinct subcellular domains and that the expression of these subunits may vary among the different types of cortical neurons (Hooks and Chen, 2007).

Calcium-Calmodulin kinase II signaling. Calcium entry at synaptic sites leads to the activation of calcium-calmodulin kinase II (CaMKII). This kinase is spatially positioned at synaptic spines to directly capture NMDA mediated calcium fluxes (Bayer, et al., 2001) and responds by favoring the localization of AMPA receptors to synapse (Hayashi, et al., 2000). α-CaMKII has the interesting properties of autophosphorylation, which allows it to undergo long-term modification and activation. The process of autophosphotylation maintains α-CaMKII activation independently of intracellular Ca2+ concentration. In this way, the transient activation produced by the coincidence detection operated by NMDA receptors is converted into a longer-lasting molecular signal (Berardi, et al., 2003). Genetic suppression of α-CaMKII autophosphorylation, blocks the OD shift that normally follows MD (Taha, et al., 2002). Intriguingly, α-CaMKII seems to be crticial for consolidation of synaptic plasticity without impacting the architecture of sensory cortex (Gordon, et al., 1996).

Interfering with PKA, ERK or CaMKII pathways causes the same outcome: the suppression of OD plasticity during the critical period. This result is not surprising because of the crosstalk and complex overlapping interactions of these three signaling cascades, so that the blockade of a single kinase reverberate on the entire network.

- ***6.7 Gene expression control***

Long-lasting changes in neuronal circuits require changes in gene expression and protein synthesis. This is true also for OD plasticity in the visual cortex. Thus, several groups have attempted to identify set of genes that are regulated in response to visual experience or deprivation. High-throughput analysis of mRNA expression is now being used to explore plasticity mechanisms in the cortex. Ossipow and collaborators implicated kinase-signaling pathways as key regulators of plasticity in rodent visual cortex, and this has been confirmed in subsequent studies (Ossipow, et al., 2004). Genes whose expression could be altered during the height of the critical period are good candidates for plasticity regulators, and two recent extensive studies in mouse have provided new insight: the Sur laboratory (Tropea, et al., 2006) identified the involvement of the IGF1 receptor pathway in OD plasticity using a microarray screen. Another independent screen identified five genes expressed during the height of the critical period; other visual cortical genes, such as BDNF and Fos, are regulated by visual experience at all times of development (Majdan and Shatz, 2006).

It is important noting that the pattern of kinase activation has to be translated into a pattern of gene expression, probably through the activation of transcription factors. The intensity of induction of the immediate early gene Arc in layer 4 of visual cortex has been proposed as a molecular marker for OD shifts in visual cortex (Tagawa, et al., 2005). Arc is regulated by visual experience, and can be manipulated by 4 days of visual deprivation not simply during the critical period, but also as early as P17 and as late as 13 weeks. An additional role for Arc in regulating visual cortical function was proposed by the Tonegawa group (Wang, et al., 2006), where replacement of the Arc gene with GFP restricted development of orientation selectivity in visual cortex. A recent article demonstrated that Arc knockout renders

the visual cortex impervious to the effect of sensory experience or deprivation. $Arc^{-/-}$ mice did not exhibit depression of deprived-eye responses or a shift in OD after MD, suggesting that Arc is required for the experience-dependent processes that normally establish and modify synaptic connections in visual cortex (McCurry, et al., 2010).

An important hint leading to the molecular identity of the transcription factors necessary for plasticity is offered by studies demonstrating that the activation of CREB is necessary for OD plasticity (Mower, et al., 2002, Pham, et al., 1999). Activated kinases translocate to the nucleus where they phosphorylate CREB, which enhances the expression of genes under the cAMP-response-element (CRE) promoter, with the consequent production of transcripts essential for establishment and maintenance of plastic changes. Both PKA and ERK are well-characterized activators of CREB (Berardi, et al., 2003). Indeed, ERK is required for visually stimulated transcription mediated by CREB (Cancedda, et al., 2003).

Activation of specific transcription factors might be only one among many possible ways by which visual experience can regulate gene expression. Increasing evidences show neural activity is able to induce posttranslational modifications of histones that, by rendering chromatin more or less accessible to transcription machinery, resulted in changes in gene expression (Crosio, et al., 2003, Korzus, et al., 2004). Considering that over the past decade, research into the regulation of transcriptional potential through modifications of chromatin structure has exploded, I will summarize the major findings that have linked epigenetic mechanisms to neuronal plasticity.

7. Epigenetic chromatin remodelling and neuronal plasticity

Most DNA in eukaryotic cells is densely packed into chromatin, where 147 base pairs (bp) are wrapped around a nucleosome core in ~1.7 super-helical turns. Nucleosomes are composed of octamers that contain four histone homodimers, one each of histones H2A, H2B, H3 and H4, with H1 binding to spans of non-nucleosomal DNA. Numerous types of post-translational modifications (PTMs) of the amino (N)-terminal tails of histones alter chromatin structure to create more "open" states (euchromatin, which is transcriptionally permissive) versus "closed" states (heterochromatin, which is transcriptionally repressive)(Robison and Nestler 2011). Structural studies indicate that the N-terminal tails of histones protrude beyond the chromosomes. The current hypothesis is that these histone tails serve as signal integration "platforms", whereby post-translational modifications are combined in a "histone code" that ultimately directs the activity of numerous transcription factors, co-factors and the transcriptional machinery in general (**Fig.3**)(Levenson and Sweatt, 2005).

Histones, particularly histones H3 and H4, are subject to extensive covalent post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitylation, SUMOylation, biotinylation, ADP ribosylation and proline isomerization, and probably others that have yet to be discovered, each occurring at specific sites and amino-acid residues. Most modifications localize to the N- and carboxy (C) -terminal histone tails, and a few localize to the histone globular domains (Berger, 2007). Some histone modifications act in *cis* to alter the local chromatin structure directly, whereas others act in *trans* to influence the recruitment of chromatin-modifying factors. In *trans* histone modifications enable specific binding partners to dock, often as part of larger multimolecular complexes that induce further chromatin remodeling (Dulac, 2010).

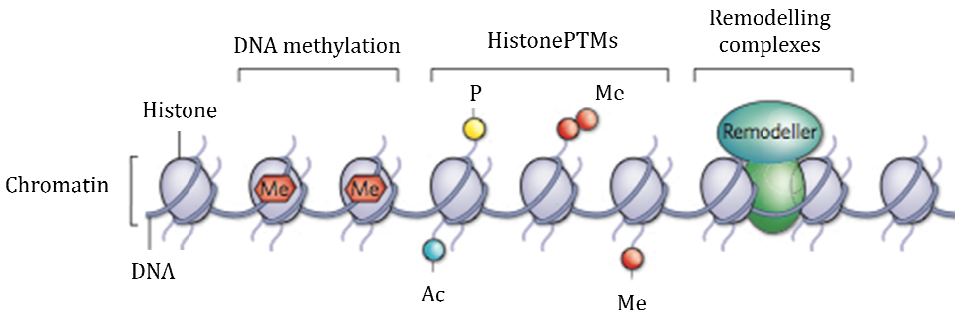


Figure 3. Mechanisms involved in chromatin modification. Schematic representation of some epigenetics mechanisms implicated in causing changes in chromatin structure. All of these processes are important for development and cell-fate determination of tissues, including those of the CNS. (Adapted from Dulac, 2010).

- ***7.1 Epigenetic tagging of histones***

Acetylation is the best characterized of the post- translational modifications on histones. Acetylation of lysine (Lys) residues occurs on the amino group in their side chain, which effectively neutralizes their positive charge, changing nucleosome structure by weakening the interaction between the positively charged histone tails and the negatively charged DNA. The reaction is catalyzed by histone acetyltransferases (HATs), which transfer an acetyl group from acetyl- coenzyme A to the ε-NH+ group of a Lys residue within a histone. The process is reversible, and the enzymes that catalyze the reversal of histone acetylation are known as histone deacetylases (HDACs).

Histone methylation is another histone-directed epigenetic tag. Similar to acetylation, methylation of histones occurs on ε-NH+ groups of Lys residues, and is mediated by histone methyltransferases. Unlike acetylation, methylation of Lys preserves their positive charge. In addition, Lys can accept up to three methyl groups. Arginine residues within histones can also be mono- or dimethylated on their guanidine nitrogen.

Phosphorylation of histones H1 and H3 was first observed more than 30 years ago in the context of chromosome condensation during mitosis. H3 was the first histone whose phosphorylation was characterized in response to the activation of mitogenic signalling pathways and this PTM is correlated with active gene transcrtiption. Phosphorylation of serine 10 on H3 is mediated for example by ribosomal protein S6 kinase 2 (RSK2) and mitogen- and stress-activated protein kinase 1 (MSK1), which are both downstream of extracellular signal-regulated kinase (ERK). In order to reverse these phosphorylation events, phosphatases remove phosphate groups from histones. So far, the phosphatases PP1 and PP2A have been shown to regulate levels of phorphorylation on H3 (Levenson and Sweatt, 2005).

Acetylation, methylation and phosphorylation involve small chemical groups, whereas ubiquitylation and SUMOylation add large moieties, two-thirds the size of the histone proteins themselves,which may lead to more profound changes in chromatin structure. Another degree of complexity is that methylation can occur several times (mono-, di- or trimethylation) on one lysine side chain, and each level of modification can have different biological outcomes. Some of the functional outcomes of these modifications are clear. For example, there is abundant evidence that acetylation is activating, whereas SUMOylation seems to be repressing for gene expression, and these two types of modification may mutually interfere. By con-trast, methylation and ubiquitylation have variable effects, depending on the precise residues and contexts. For example, silenced chromatin typically has low levels of histone acetylation, together with high levels of H4K20me3 and H3K27me3, whereas hyperacetylation, H3K4me3 and H3K36me3 are recognizable marks of active transcription. Two ubiquitylation sites in the C termini of H2B and H2A correlate with active and repressed transcription, respectively (Berger, 2007).

- ***7.2 DNA methylation***

The methylation of cytosine nucleotides in DNA forms 5-methyl- cytosine, which in mammalian cells is mainly confined to CpG dinucleotides. Methylation of DNA is catalysed by a class of enzymes known as DNA methyltransferases (DNMTs) and it is known to have a role in the constitutive silencing of chromatin regions, the inactivation of one of the X chromosomes in females, the imprinting of parental alleles, and the silencing of retroviral genes and other individual genes. The precise mechanisms by which DNA methylation marks are set, maintained and erased, however, are the subject of much debate. DNA methylation leads to marked changes in the structure of chromatin that ultimately result in significant downregulation of transcription and it can directly interfere with the ability of transcription factors to bind to regulatory elements (Dulac, 2010).

Moreover, evidence indicates that DNA methylation may serve as a contributing mechanism in memory formation and storage (Day and Sweatt, 2010). Very recent studies demonstrated that neuronal activity modifies DNA methylation pattern of adult mouse dentate granule neurons in vivo (Guo, et al., 2011) and that 5-hydroxil-methyl cytosine, a DNA base that is derived from 5-methylcytosine and has been implicated in DNA methylation–related plasticity, is dynamically regulated during postnatal development through adulthood and ageing in mouse hippocampus and cerebellum (Szulwach, et al., 2011).

The importance of DNA methylation in assisting essential gene regulatory events that are associated with brain function is revealed by neurological disorders caused by disregulation in DNA methyation processes, such as Rett Syndrome and fragile X Syndrome.

- ***7.3 Chromatin remodelling influences synaptic plasticity***

Long-term modifications of chromatin may underlie some of the changes in gene expression that lead to neural plasticity. Repeated patterns of synaptic transmission lead to diverse forms of synaptic plasticity at excitatory and inhibitory synapses: long-term potentiation (LTP) or long-term depression (LTD), whereby the efficacy of synaptic transmission is up- or downre- gulated, respectively. Certain forms of LTP and LTD are long lasting and depend on changes in gene expression. Based on the critical role that chromatin remodeling plays in creating a transcription permissive or silencing state of the genome (Felsenfeld and Groudine, 2003), growing evidence suggests that histone PTMs may be involved in these processes. For example, H4 acetylation at specific promoters in Aplysia is altered after LTP and LTD (Guan, et al., 2002). Plasticity-induced epigenetic changes are also observed in mammalian models of synaptic plasticity. Several forms of LTP require the activation of NMDA receptors and engagement of the MEK–ERK/MAPK signalling cascade (English and Sweatt, 1997, Morris, et al., 1986). Direct activation of NMDA receptors in the hippocampus leads to an increase in acetylation of histone H3, which can be blocked by inhibition of the MEK–ERK/MAPK cascade. In addition, activation of dopaminergic, cholinergic and glutamatergic signalling pathways in the hippocampus induces ERK- dependent increases in the phosphorylation of histone H3. These results suggest that the induction of mammalian synaptic plasticity leads to ERK-dependent increases in histone acetylation and phosphorylation in the hippocampus (Levenson and Sweatt, 2005).

As previously mentioned, the transcription factor CREB is essential for activity- induced gene expression. At the mechanistic level, CREB-associated

transcriptional regulation has been shown to involve the recruitment of multicomponent regulator complexes, as well as the initiation of chromatin-remodelling events. Activated CREB recruits CREB-binding protein (CBP; also known as CREBBP) or its paralogue p300 (also known as EP300), which functions as both a scaffolding protein and a HAT. CBP recruitment, in turn, stimulates histone acetylation and transcriptional-complex formation at the promoters, leading to transcriptional activation of many CREB-target genes. Mutations in the gene encoding CBP are responsible for the mental-retardation syndrome Rubinstein–Taybi, the phenotype of which may result from impairment of either or both of the CREB-dependent and CREB-independent functions of CBP. The essential role of HAT activity in CBP-mediated neuronal plasticity has been genetically demonstrated by the selective long-term memory defects of a transgenic mouse line carrying a dominant-negative CBP that blocks the HAT activity of the endogenous protein (Korzus, et al., 2004). In another study, the induction of early-phase LTP and LTD — forms of plasticity that do not require transcription — was not affected in CBP+/– animals. However, the induction of late-phase LTP, which requires transcription, was significantly impaired in CBP+/– mice. Treatment of hippocampal slices from CBP+/– animals with an HDAC inhibitor significantly improved late-phase LTP induction, which indicates that inhibition of HDACs had compensated for HAT haploinsufficiency (Alarcon, et al., 2004). In other studies using hippocampal slices, induction of LTP through high-frequency stimulation was significantly enhanced by two HDAC inhibitors, trichostatin A (TSA) and sodium butyrate (Levenson, et al., 2004). In addition, LTP in the amygdala that was induced by forskolin was also enhanced by the HDAC inhibitor TSA (Yeh, et al., 2004). These discoveries indicate

that the epigenetic state of the genome affects the induction of long-term forms of mammalian synaptic plasticity.

Recent studies implicated histone PTMs in memory formation and storage. Guan et al (2009) demonstrated that neuron-specific overexpression of HDAC2, but not that of HDAC1, decreased dendritic spine density, synapse number, synaptic plasticity and memory formation. Conversely, HDAC2 deficiency resulted in increased synapse number and memory facilitation, similar to chronic treatment with HDAC inhibitors in mice. These results suggest that HDAC2 functions in modulating synaptic plasticity and long-lasting changes of neural circuits, which in turn negatively regulates learning and memory (Guan, et al., 2009). Moreover, histone methylation is actively regulated in the hippocampus and facilitates long-term memory formation. Trimethylation of histone H3 at lysine 4 (H3K4), an active mark for transcription, is upregulated in hippocampus 1 h following contextual fear conditioning, while dimethylation of histone H3 at lysine 9 (H3K9), a molecular mark associated with transcriptional silencing, is increased 1 h after fear conditioning and decreased 24 h after context exposure alone and contextual fear conditioning. Furthermore, mice deficient in the H3K4-specific histone methyltransferase, Mll, displayed deficits in contextual fear conditioning relative to wild-type animals. This suggests that histone methylation is required for proper long-term consolidation of contextual fear memories (Gupta, et al., 2010).

Drug addiction can be viewed as a form of drug-induced neural plasticity, whereby repeated exposure to drugs of abuse leads to long-lasting changes in the brain's natural reward centers and associated memory circuits, which underlie the addiction phenotype. Multiple drugs of abuse induce changes in histone acetylation in the brain, and evidence has begun to accumulate that these modifications underlie

some of the functional abnormalities found in addiction models. First, global levels of H3 and H4 acetylation are increased in the NAc after acute or chronic exposure to cocaine (Kumar, et al., 2005, Renthal and Nestler, 2008), and gene promoters that show increased H3 or H4 acetylation have been mapped genome-wide (Renthal, et al., 2009). Despite these global increases, many genes show decreased histone acetylation after chronic cocaine, raising a key question as to what governs gene-specific acetylation changes in the face of global modifications.

Histone methylation is also directly regulated by drugs of abuse: global levels of histone 3 lysine 9 dimethylation (H3K9me2) are reduced in the NAc after chronic cocaine exposure (Maze, et al., 2010), and a genome-wide screen revealed alterations in H3K9me2 binding on the promoters of numerous genes in this brain region (Renthal, et al., 2009); both increases and decreases were observed, indicating again that epigenetic modifications at individual genes often defy global changes. The global decrease in H3K9me2 in the NAc is probably mediated by cocaine-induced downregulation of two histones methyl transferases, G9a and G9a-like protein (GLP), which catalyse the dimethylation of H3K9me2. These adaptations mediate enhanced responsiveness to cocaine, as selective knockout or pharmacological inhibition of G9a in the NAc promotes cocaine-induced behaviours, whereas G9a overexpression has the opposite effect (Maze, et al., 2010).

Accumulating data show that chromatin remodeling events may be critical for providing specificity and plasticityin circadian clock regulation. Although our under-standing of the mechanisms that direct circadian epigenetic control is still limited, emerging evidence implicates histone modifications and some chromatin remodeling proteins as particularly important in directing circadian clock entrainment and resetting (Masri and Sassone-Corsi, 2010).

Given the involvement of epigenetic mechanisms in nervous system function, it is not surprising that a growing number of disorders, in particular mental retardation and autism spectrum syndromes, have been linked to chromatin remodeling defects. The most well-studied ‘‘epigenetic disease’’ associated with altered neurological function, is Rett’s syndrome, an X-linked postnatal autism spectrum disorder characterized by stereotypical motor, learning, and social abnormalities that generally worsen over time (Moretti and Zoghbi, 2006). Candidate gene analyses identified MeCP2 as the causative gene (Amir, et al., 1999). MeCP2 was identified on the basis of binding selectively to methylated CpG dinucleotides in heterochromatic regions and it acts in a methylation-dependent repressive fashion. In Rubinstein-Taybi syndrome (RSTS), characterized by mental retardation and developmental abnormalities, the DNA-binding hook is provided by CREB. Phosphorylation of CREB leads to CBP recruitment and activation of target promoters. In RSTS, mutations in the CBP gene result in impairment of HAT activity. Mice haploinsufficient for CBP display impaired cognitive function, altered neuronal plasticity, and aberrant histone acetylation at target gene promoters. Interestingly, the behavioral symptoms can beameliorated by administration of HDAC inhibitors (Vo and Goodman, 2001).

Fragile X syndrome, the most commonly inherited form of mental retardation, is brought about by an abnormal expansion of repeated trinucleotide sequences within one of two Fragile X genes: FMR1 and FMR2 (Ashley, et al., 1993). FMR1 and FMR2 each contain a polymorphic trinucleotide repeat (CGG and CCG, respectively) in their 5’-untranslated regions that are responsible for the loss of gene expression. Expansion of these repeats results in hypermethylation of these regions and flanking

CpG islands, leading to transcriptional silencing of the FMR and surrounding genes (Levenson and Sweatt, 2005).

A striking example of developmental disruption caused by mutations in a chromatin factor gene is alpha-thalassaemia/mental retardation, X-linked syndrome, the gene for which is a helicase (spinocerebellar ataxia-7) involved in chromatin remodeling, by regulating several HAT complexes. Mutations lead to defects in psychomotor, urogenital and haematopoietic development, with maturational defects in erythroid precursors resembling those of alpha-thalassaemia (Feinberg, 2007).

These pathologies are just some examples of disorders involving alteration in epigenetic mechanisms (for a review see (Portela and Esteller, 2010).

All these observations indicate that dysfunction of the normal epigenetic status of the genome can have marked consequences on synaptic plasticity processes and normal cognitive function, indicating that drugs that target the epigenome might represent viable therapies for treating various diseases that affect cognition.

- ***7.4 Histone PTMs and visual cortical plasticity***

Several studies have demonstrated that regulation of gene expression through posttranslational modifications of histones is present in neuronal cells in vivo. Indeed, stimuli that reset the circadian rhythms induce phosphorylation of H3 in the suprachiasmatic nucleus (Crosio, et al., 2000) and acetylation of H3 and H4 during the transcriptional activation phase of the circadian rhythm has been described (Etchegaray, et al., 2003). Recent evidences demonstrated that experience-dependent regulation of chromatin structure could be an important mechanism of regulation of gene expression also in the developing visual cortex. Visual experience

activates mechanisms regulating gene transcription at different levels: an episode of light stimulation in juvenile mice activates CREB-mediated gene expression and induces phosphorylation of the transcription factor CREB; in parallel, visual experience also induces histone phosphorylation and acetylation, and ERK mediates visually stimulated biochemical modifications of CREB and H3 (Putignano, et al., 2007). Visual experience induces histone acetylation during the critical period for ocular dominance plasticity, but its action becomes downregulated in adult animals, in correlation with the lower levels of adult cortical plasticity: this suggests that, as it does with plasticity of visual cortical connections, visual experience progressively reduces its own effectiveness in regulating gene transcription and/or modifies the ensemble of regulated genes (Majdan and Shatz, 2006). This epigenetic mechanism might be important for plasticity in the visual cortex during the critical period, and its downregulation could be involved in the closure of this sensitive period. Therefore, increasing histone acetylation in the visual cortex through TSA treatment, promotes ocular dominance plasticity in adult animals (Putignano, et al., 2007). Furthermore, pharmacological epigenetic treatments increasing histone acetylation reverse visual acuity deficits induced by long-term MD initiated during the critical period in rodents. Indeed, visual acuity of the amblyopic eye recovers to normal values in adult rats treated with HDAC inhibitors, tested both electrophysiologically and behaviorally (Silingardi, et al., 2010).

Thus, multiple molecular mechanisms acting at different levels, from the cytoplasm to the nucleus, might contribute to the developmental downregulation of plasticity occurring in coincidence with the closure of the critical period (Putignano, et al., 2007).One of these mechanisms involves the kinase ERK, that acts through the transcription factor CREB and the induction of epigenetic histone marks correlated

with active gene expression. Indeed, ERK is required for visually stimulated transcription mediated by CREB (Cancedda, et al., 2003), and CREB plays a pivotal role in various forms of plasticity in visual cortex (Mower, et al., 2002, Pham, et al., 1999) and other brain structures (Lonze and Ginty, 2002).

However, it is still to be determined which specific CREB-dependent genes regulate OD plasticity and are target of experience-dependent epigenetic remodeling. To answer this question, in my thesis I studied the role of miR132, an activity and CREB- dependent microRNA, in the development and plasticity of the visual cortex.

8. MicroRNAs

- ***8.1 Biogenesis and function***

MicroRNAs (miRNAs) are endogenous evolutionarily conserved short non-coding RNAs that interact with specific target mRNAs based on sequence imperfect complementarity in the 3' untranslated region (UTR) of the target mRNAs, resulting in translational repression or mRNA deadenylation and degradation.

MiRNAs are processed from RNA polymerase II (RNAPII)-specific transcripts of independent genes or from introns of protein-coding genes. In the canonical pathway, primary precursor (pri-miRNA) processing occurs in two steps, catalysed by two membersof the RNase III family of enzymes, Drosha and Dicer, operating in complexes with dsRNA-binding proteins, for example DGCR8 and transactivation-responsive (TAR) RNA-binding protein (TRBP) in mammals.

In the first nuclear step, the Drosha–DGCR8 complex processes the pri-miRNA into an ~70-nucleotide precursor hairpin (pre-miRNA), which is exported to the cytoplasm. Some pre-miRNAs are produced from very short introns (mirtrons) as a result of splicing and debranching, thereby bypassing the Drosha–DGCR8 step. In

either case, cleavage by Dicer, assisted by TRBP, in the cytoplasm yields an ~20-bp miRNA/miRNA* duplex. In mammals, argonaute 2 (AGO2), which has an RNaseH-like endonuclease activity, can support Dicer processing by cleaving the 3′ arm of some pre-miRNAs, thus forming an additional processing intermediate called AGO2-cleaved precursor miRNA (ac-pre-miRNA). Following processing, one strand of the miRNA/miRNA* duplex (the guide strand) is preferentially incorporated into a miRNA-induced silencing complex (miRISC), whereas the other strand (passenger or miRNA*) is released and degraded. Generally, the retained strand is the one that has the less stably base-paired 5′ end in the miRNA/miRNA* duplex. miRNA* strands are not always by-products of miRNA biogenesis and can also be loaded into miRISC to function as miRNAs. Argonaute (AGO) proteins, which directly interact with miRNAs, and glycine-tryptophan protein of 182 kDa (GW182) proteins, which act as downstream effectors in the repression, are key factors in the assembly and function of miRISCs. As part of miRISC, miRNAs base- pair to target mRNAs and induce their translational repression or deadenylation and degradation (Krol, et al., 2010).

- ***8.2 MiRNAs in the central nervous system***

Among their varied roles, many miRNAs are essential for the correct function of the nervous system, which has the broadest spectrum of miRNA expression of all human tissues. Around 70% of miRNAs are expressed in brain, and many of them are specific to neurons. They are involved in neurodevelopment, dendritic spine formation and neurite outgrowth, and their dysregulation has been described in almost all neurological diseases studied (Esteller, 2011).

It is important to note that miRNAs are emerging as key modulators of post-transcriptional gene regulation in a plethora of tissues. Recent evidence points to a

widespread role for neural miRNAs at various stages of synaptic development, including dendritogenesis, synapse formation and synapse maturation.

Experience-dependent plasticity, which occurs at the level of dendrites and synapses, underlies the brain's ability to adapt to changes in the environment and to store information. At the molecular level, plasticity is orchestrated by sophisticated gene expression programmes that ensure that environmental stimuli are converted into long-lasting alterations in synapse structure and function. Among these mechanisms, the local control of mRNA translation in neuronal dendrites can account for the tight spatial regulation of plasticity at the level of individual dendrites or spines. Another feature of interest is that many miRNAs do not act as on–off switches, but rather fine-tune gene expression profiles. MiRNA-mediated fine-tuning usually occurs in the absence of mRNA degradation, a scenario that fits well with the local regulation of dendritic mRNAs during storage and/or activation at the synapse (Schratt, 2009).

In vertebrate species, a central role for miRNAs in neurogenesis was primarily shown through studies in which the enzyme Dicer was ablated (globally or targeted deletion) thus blocking mature miRNA biogenesis and leading to various abnormalities and/or death of the organism (for a review see (Saba and Schratt, 2010). MiRNA are also involved in regulation of dendritic plasticity in mature neurons. Transcription of miRNAs is regulated by neuronal activity, indeed several promoters of neural miRNAs are occupied by classical activity-regulated transcription factors, such as CREB and MeF2, which couple Ca2+-regulated signalling cascades to the transcriptional machinery (Fiore, et al., 2009, Vo, et al., 2005). Therefore, many neuronal miRNAs target proteins important for neuronal plasticity and they are involved in dendritic arborization outgrowth, spines

morphology regulation, synapse maturation, maintenance and plasticity (for reviews see (Schratt, 2009, Vo, et al., 2010).

Recently, a flurry of papers reported important physiological roles of miR212/132 family. Thus, I'm going to summarize studies about discovery and functions of these two miRNAs.

- ***8.3 MicroRNA212/132 family: molecular transducer of neuronal function and plasticity.***

Structure

The miR212/132 family is highly conserved in vertebrates. MiR212 and miR132 were identified for the first time using a genome wide screen to discover miRNAs target of cAMP response element (CRE)-binding protein (CREB). In rats and mice, miR132 and miR212 are processed as a policystronic primary transcript (pri-miR212/132) from the stable intron of a 2-exon cryptic non-coding RNA (Vo, et al., 2005)(**Fig.4**). Recently, a new transcript variant with different exon-intron structure was identified in mice. This variant encodes both microRNAs in its second exon (Ucar, et al., 2010). The miR212/132 gene has an equivalent structure in all species wherein the two miRNAs genes are arrayed in tandem on chromosome 10 in rat, 11 in mouse and 17 in humans. The miR212/132 gene cluster produces four mature miRNAs: miR132, miR132 star (*), miR212 and miR212*. In humans miR212* is not found possibly because it is quickly degraded. MiRNA and miRNA* sequences represent the two strands of the duplex deriving by the enzymatic cleavage of the precursor miRNA. They are produced in equal amounts by transcription, but their accumulation is asymmetric depending on the thermodynamic stability of the 5' end of each strand of this duplex. Mature miR132 and miR132* are composed by 22

nucleotides and derived from the cleavage of the two opposite arms of the same precursor stem-loop of 66 nucleotides. Mature 22 nucleotides long miR212, and 23 nucleotides long miR212* derive from a precursor stem-loop of 91 nucleotides. In primary cortical neurons miR132 is expressed at much higher levels than miR132*, whereas miR212 and miR212* have similar expression levels, albeit much lower than miR132 (Remenyi, et al., 2010).

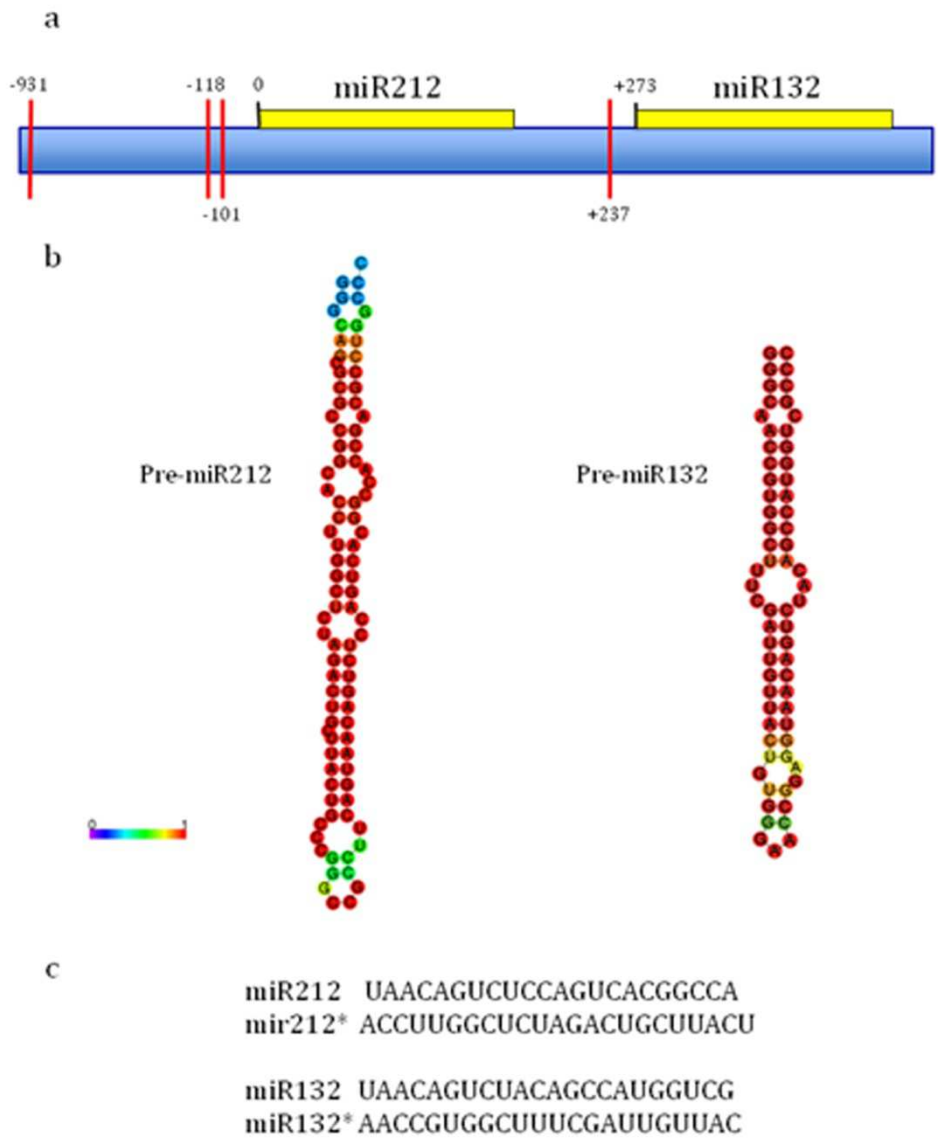


Figure 4. MiR212/132 gene cluster structure and precursor and mature miRNAs products. (a) Schematic representation of miR212/132 genomic locus. MiR212 and miR132 coding sequences are in yellow, red bars represented CRE sequences localization. **(b)** Precursor stem loop (pre-miRNA) of miR212 (miRBase entry MI0000696) and miR132 (miRBase entry MI0000158). Structures drawn

using the Vienna RNA Package, a software predicting the minimum free energy structures and base pair probabilities from single RNA sequences. **(c)** Mature mouse miRNA and miRNA* sequences (miRBase entries MIMAT0000144 for miR132, MIMAT0016984 for miR132*, MIMAT0017053 for miR212, and MIMAT0000659 for miR212*). (modified from Tognini and Pizzorusso, 2011)

MiRNA family members have similar target specificity because they share common "seed regions", the 2-7 or 2-8 bases positioned at the 5' end of the miRNA, representing the primary contributors to mRNAs target recognition. However, the interactions of the 3' end of the miRNAs are also important determinants of target specificity within miRNA families. Thus, members of a miRNA family may have common targets as well as distinct targets. Therefore, miR212 and miR132, that belong to the same family, may be functionally redundant in regulation of some targets but not for others (Remenyi etal., 2010).

Expression, activation and turnover

MiR132 expression is enriched in the brain, while miR212 expression is very low in all tissues tested (Klein, et al., 2007, Remenyi, et al., 2010, Vo, et al., 2005). In the miR212/132 gene cluster four different CRE lociare present (**Fig.4**), one located in the 500 bp region upstream to the exon 1, two consensus CRE are 5' to the miR212 transcript and one CRE site closer to miR132 transcript, all implicated in the control of miR212/132 transcription (Remenyi, et al., 2010, Vo, et al., 2005). Studies performed in neurons demonstrated that miR132 and miR212 are CREB regulated miRNAs (Hollander, et al., 2010, Remenyi, et al., 2010, Vo, et al., 2005). Neuronal culture treatment with CREB-activating stimuli such as forskolin and depolarizing agents strongly increased miR132 levels (Klein, et al., 2007, Nudelman, et al., 2010, Vo, et al., 2005, Wayman, et al., 2008). MiR132 and miR212 were increased by BDNF treatment in primary cortical neurons. Inhibition of MEK1/2 and the downstream

effector MSK1 interfered with this induction, suggesting that ERK1/2 pathway regulates miR212/132 expression (Remenyi, et al., 2010). Further more, glucocorticoid receptor activation suppressed BDNF-dependent miR132 increase in cultured cortical neurons (Kawashima, et al., 2010). Avery recent study demonstrated that alsothe basal fibroblast growth factor (bFGF) upregulated levels of miR132 in cortical cultures. Conversely, insulin- like growth factor 1 (IGF-1), glial cell line- derived neurotrophic factors (GDNF), and epidermal growth factor (EGF) did not have a positive influence on miR132 and glutamate receptors in neuronal cultures. Furthermore, bFGF significantly upregulated miR132 in cultured astroglial cells, while other growth factors failed to elicit such a response (Numakawa, et al., 2011).

Mature miR212 and miR132 follow the canonical pathway of miRNA biogenesis. Into the nucleus, the pri-miRNA is cleaved by the RNAse III family enzyme Drosha in a ≈ 70 nucleotides precursor hairpin RNA (pre-miRNA) and is exported to the cytoplasm. Here, Dicer (a RNAse III enzyme) processes the pre-miRNA in a ≈ 20 bpmiRNA duplex. One strand of the duplex, the mature miRNA, is loaded into the miRNA induced silencing complex (RISC), whereas the star strand (also called passenger strand) is degraded (Krol, et al., 2010).

MiR132 seems to have a peculiar turnover regulation in neuronal cultures. In contrast to a substantial number of miRNAs showing enhanced degradation in response to transcription inhibitors and spiking activity blockers, mature miR132 levels did not change in response to these stimuli. Furthermore, blocking glutamate receptors activates the decay of miR132, whereas glutamate treatment did not have effect (Krol, et al., 2010). These findings suggest a fine local regulation of miR132

stability at specific synaptic compartments and its involvement in synaptic plasticity modulation.

Biological functions

Overexpression of miR132 in neuronal cultures induced a marked increase in primary neurite outgrowth (Vo, et al., 2005). Synaptic activity rapidly induced CREB-dependent miR132 expression and its induction was required for activity-dependent dendritic growth *in vitro*. These effects on dendrite morphology were mediated by miR132 translational inhibition of its target protein p250GAP, a Rho family GTPase activating protein, resulting in increased Rac signalling cascade activity (Wayman, et al., 2008). Furthermore, activity-dependent spine formation in hippocampal neurons was regulated by miR132 through suppression of p250GAP translation resulting in activation of the Rac1-PAK actin-remodelling pathway (Impey, et al., 2010). Conditional knock down of miR212/132 locus *in vivo* caused a significant decrease in total dendritic length, arborization and spine density in newborn hippocampal neurons of adult mice (Magill, et al., 2010). Considering the much lower expression of miR212 with respect to miR132, the authors suggested that miR132 is the predominant regulator of this morphological plasticity. Transgenic mice overexpressing miR132 in forebrain neurons showed a marked increase in dendritic spine density and impairments in a novel object recognition memory test (Hansen, et al., 2010). MiR212/132 role in synaptic plasticity is also suggested by two electrophysiological studies (Lambert, et al., 2010, Wibrand, et al., 2010). The induction of long-term potentiation (LTP) in the dentate gyrus of adult rats was accompanied by a strong upregulation of mature miR212 and miR132. Surprisingly, the pharmacological blockade of N-methyl-D-aspartate receptors enhanced the LTP-

dependent induction of these miRNAs whereas the selective blockade of mGlur1 receptor inhibited the enhancement of mature miRNAs expression in response to LTP-inducing stimuli (Wibrand, et al., 2010). These data suggest that different signalling pathways might specifically regulate miR132 and miR212 expression by acting at transcriptional and possibly at biogenesis or decay level. Another study showed that miR132 overexpression by means of viral transduction modulated short-term synaptic plasticity without affecting basal synaptic transmission in hippocampal cultures (Lambert, et al., 2010).

A series of *in vivo* studies (**Fig. 5**) revealed the role of miR132 in modulating the circadian-clock (Alvarez-Saavedra, et al., 2011, Cheng, et al., 2007). Indeed, miR132 was induced by photic entrainment cues via a MAPK/CREB-dependent mechanism, modulated clock-gene expression, and attenuated the entraining effects of light (Cheng, et al., 2007). MiR132 regulated target genes involved in chromatin remodelling (MeCP2, Ep300, Jarid1a) and translational control (Btg2, Paip2a) in the mouse suprachiasmatic nucleus (SNC). Coordinated regulation of these targets underlined miR132-dependent modulation of clock entrainment and its roleas an orchestrator of chromatin remodelling and protein translation within the SCN clock (Alvarez-Saavedra, et al., 2011).

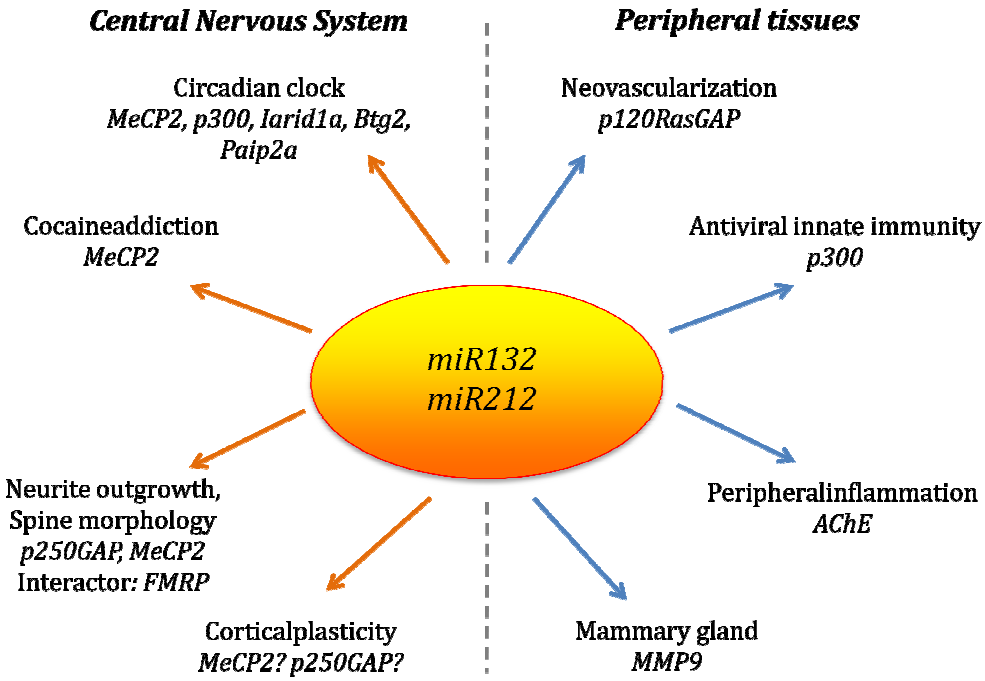


Figure 5. MiR132 and miR212 biological functions. Schematic representation of processes involving miR132 and/or mir212 and implicated proteins. (Modified by Tognini and Pizzorusso, 2011)

Striatal miR212 was found to be an essential component of the mechanism underlying cocaine addiction (Hollander, et al., 2010, Im, et al., 2010). MiR212 is induced by cocaine and controls drug intake by activating CREB, an important negative regulator of cocaine reward. Therefore, miR212 protects against developing of compulsive drug taking (Hollander, et al., 2010). Furthermore, miR212 controls vulnerability to cocaine addiction through a negative homeostatic balance resulting in MeCP2-miR212 reciprocal expression regulation. The interaction between miR212 and MeCP2 was proposed to regulate sensitization to the motivational effects of cocaine (Im, et al., 2010). Considering the overlapping miR212 and miR132 seed regions, the data showing that cocaine induces miR132, and the homeostatic relationship between miR132 and MeCP2 observed in cortical neurons (Klein, et al., 2007), it is possible that also miR132 is involved in regulating cocaine addiction.

MiR212 and miR132 are not expressed exclusively in neurons. Mir132 is among the most highly induced miRNAs in macrophages treated with lipopolysaccharide. MiR132 has an anti-inflammatory effect via the targeting of the mRNA encoding acetylcholinesterase, a well-known inhibitor of peripheral inflammation and a mediator of the neuroimmune axis (Shaked, et al., 2009). Furthermore, miR132 regulated antiviral innate immunity by inhibiting the expression of the p-300 transcriptional co-activator (Lagos, et al., 2010).

MiR132 plays a role in tumour proliferation acting as an angiogenic switch by targeting p120RasGAP, a negative regulator of vascular development and remodelling, in the endothelium. MiR132-mediated suppression of p120RasGAP lead to Ras activation and induction of pathological neovascularisation (Anand, et al., 2010). Further studies have provided evidence for miR212/132 family requirement in the development of mammary glands in mice, by targeting the matrix metalloproteinase MMP9. Indeed, miR212/132 null female mice have difficult in feeding their pups (Ucar, et al., 2010). Moreover, miR132 expression increases in human preadipocytes and in differentiated adipocytes *in vitro* in response to serum deprivation, downregulating silent information regulator 1 (SirT1) expression. Thus, SirT1 repression results in p65 acetylation, leading to activation of NfKβ signalling pathway and transcription of IL-8 and MCP-1, cytokines implicated in inflammatory response (Strum, et al., 2009).

MiR132/212 in preclinical models and disease

Mutations and gross rearrangements of the MeCP2 gene account for a large proportion of cases of Rett syndrome (RTT). Intriguingly, duplications of MeCP2 also result in RTT symptoms in patients and in preclinical models, suggesting that MeCP2

levels are precisely controlled in normal subjects (Na and Monteggia, 2011). It has been proposed a homeostatic mechanism in which MeCP2 increases BDNF expression that in turn activates miR132 transcription to maintain MeCP2 levels by translational repression (Klein, et al., 2007). This model is supported by a recent genome-wide analysis of miRNAs in a mouse model of RTT revealing that miR132 and miR212 are downregulated in the cerebellum. BDNF is decreased in MeCP2 mutants thereby forming a regulatory loop between MeCP2 and BDNF-induced miRNAs (Wu, et al., 2010). Thus, miR132 could be crucially involved in the fine-tuning of MeCP2 levels.

MiR132 could also be involved in Fragile X syndrome (FXS). Indeed, miR132 effects on spine morphology in neuronal cultures were mediated by its interaction with FMRP (Fragile X mental retardation protein). In cells with knockdown of FMRP the effects of miR132 were completely abolished (Edbauer, et al., 2010). These data suggest a possible role of miR132 in modulating the spine phenotype of FXS.

Clinical investigations showed altered levels of miR132 and miR212 in cancer, psychiatric disorders, Alzheimer's disease, Huntington's disease and autism spectrum disorders (Abu-Elneel, et al., 2008, Cogswell, et al., 2008, Kim, et al., 2010, Packer, et al., 2008, Talebizadeh, et al., 2008). Psychiatric, neurodegenerative and autism spectrum disorders are associated with alteration in neuronal connectivity and synaptic plasticity, therefore misregulated expression of miR132 and miR212 may explain some pathological tracts of these diseases and help to find new therapeutic approaches for these severe disorders.

Aims of this thesis

In the last years, several studies showed that CREB-mediated gene transcription together with epigenetic mechanisms controlling histone post-translational modifications are crucial for synaptic plasticity (Fischer, et al., 2007, Guan, et al., 2009, Levenson and Sweatt, 2005). CREB-mediated gene transcription and histone phosphoacetylation are activated in the visual cortex by visual experience with a peak during the critical period (Cancedda, et al., 2003, Pham, et al., 1999, Putignano, et al., 2007). Interfering with CREB-mediated transcription dramatically affects ocular dominance plasticity (Liao, et al., 2002, Mower, et al., 2002, Pham, et al., 2004). Furthermore, pharmacological activation of histone acetylation in adult rodents enhances visual cortical plasticity (Putignano, et al., 2007, Silingardi, et al., 2010). Taken together, these evidences suggest that CREB regulated genes could be pivotal in the mechanisms underlying visual cortical plasticity. However, the identity of these genes is still largely unknown.

In my thesis, I investigated the role of a CREB-regulated microRNA, miR132 in visual cortical plasticity. In spite of relative abundance of information about cellular roles of miR132, no data are available about its role in brain plasticity. To answer this question I analyzed the effects of visual stimulation and visual deprivation on epigenetic transcriptional control of the miR132/212 cluster during the critical period. Moreover, I investigated whether the primary transcript (pri-miR132) and the mature form of miR132 are activtity-dependent in the visual cortex, by using the paradigm of DR and MD. Finally, I assessed if experience-dependent regulation of miR132 is necessary for visual cortical plasticity in juvenile mice.

Materials and methods

1. Chromatin immunoprecipitation (ChIP)

For chromatin immunoprecipitation experiment, P27 wild type C57BL/6J mice were sacrificed in darkness or at 20 minutes upon light exposure after 3 days of DR. Visual cortices were removed and the fresh tissue was processed immediately. Monocular deprivation was performed in critical period mice (P25) anesthetized with avertin (1ml/50mg) by suturing the eyelids of the right eye. Mice were sacrificed and the binocular part of the visual cortex was removed. The binocular right and left cortices of three different animals were pooled together and processed.

Fresh tissue was chopped and cross-linked using 1% formaldehyde in PBS, shaking it at room temperature for 15 minutes. The cross linking reaction was stopped by adding 0,125 M glycine. Samples were centrifuged (1000 x g; 5 minutes; 4°C). Supernatant was discarded and the pellet was washed once with ice- cold PBS plus protease inhibitors cocktail 10 µl/ml (Cat. n. P8340 SIGMA-Aldrich) and NaBu 20 mM by centrifugation (1000 x g; 5 minutes; 4°C). Pellets were re- suspended in new PBS plus protease inhibitors and NaBu and homogenized to obtain a single cells suspension. The suspension was centrifuged (1000 x g; 5 minutes; 4°C) and the volume of the pellet was estimated. The pellet was re-suspended in 6 X volumes of cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0,5% Igepal, plus protease inhibitors and NaBu). The samples were incubated on ice for 10 minutes and centrifuged (1000 x g; 5 minutes; 4°C). Supernatant was discarded and the pellet was re-suspended by vortexing in Nuclear lysis buffer (50 mM TrisHCl, 10 mM EDTA, 1% SDS plus protease inhibitors and NaBu). Samples were incubated on ice for 5 minutes and sonicated to shear the chromatin using the BioruptorTM (Diagenode) for 30

minutes (30 seconds “ON”, 30 seconds “OFF”). The sheared chromatin was diluted with the dilution buffer (0,01% SDS, 1,1% Triton X100, 1.2 mM EDTA, 16,7 mM Tris-Cl pH 8.1, 167 mM NaCl). 100 µl of sheared chromatin was incubated with protein A coated magnetic beads (Dynabeads protein A Cat. n. 100.02D, Invitrogen) previously incubated with 1µg of specific antibody: anti-AcH3 (lys 9-14) (Cat. n. 06-599, Upstate), anti-phospho(Ser10)Acetyl(Lys14)H3 (Cat. n. 07-081, Upstate), anti- Me2K4H3 (Cat. n. 17-677, Upstate); or 1 µg of control antibodies: normal rabbit IgG (Cat. n. 17- 614, Upstate) and normal mouse IgG (Cat. n. 17-677, Upstate). Samples were incubated overnight at 4°C in constant rotation (40 rpm).

The magnetic beads were washed for two times with the dilution buffer and 6 times with the washing buffer (100 mM Tris-Cl, 500 mM LiCl, 1% NP40, 1% Deoxycholic Acid). At the end of the washing beads were incubated with 10% chelex (Chelex 100 Resin, Cat. n. 143- 2832, Biorad) in boiled water for 10 minutes. Samples were treated with Proteinase K (Cat. n. P4850, SIGMA-Aldrich) for 30 minutes at 55°C with shacking (1000 rpm). The samples were incubated in boiled water for 10 minutes and after that centrifuged (14000 x g; 1 minutes; 4°C). Part of the supernatant (DNA) was recovered. 100 µl of ChIP grade water was added and the samples were centrifuged again (14000 x g; 1 minutes; 4°C). The supernatant, that contains DNA, was recovered and DNA was ready for PCR reaction.

2. Quantitation of Chromatin immunoprecipitation by Real time PCR

Levels of specific histone modifications at gene promoters of interest: CREmiR132, CREmiR212 and CREc-fos, were determined by measuring the amount of that promoter in chromatin immunoprecipitates by use of Real time PCR (Step one machine Applied Biosystems, Foster City, CA). Input DNA (non-immunoprecipitated

DNA) and immunoprecipitated DNA were PCR amplified in duplicate in the presence of Taqman probes. The relative quantities of immunoprecipitated DNA fragments were calculated by using the threshold cycle number (Ct) obtained during the exponential growth of the PCR products and normalized to the Input Ct. Primer and probe sequences used for Real time PCR were as follows: CREc-fos forward GAGACCCCCTAAGATCCCAAA, reverse CCCCCGTCTTGGCATACA, MGB probe TGAACACTCATAGGTGAAAG; CREmiR132 forward GGCCCCGCAGACACT, reverse CGGTTGCCCTGGAGACG, MGB probe CAGGCTGACGTCAGCAC; CREmiR212 forward CCACCGCCGGAAATGC, reverse CGACCGTGACGTCAGAGT, MGB probe CTGTGACGTCAAAGATG.

3. Western blotting

P27 mice were sacrificed in darkness or at 20 minutes upon light exposure after 3 days of DR. Visual cortices were removed and frozen on dry ice. The tissue samples were homogenized in cell disruption buffer (Ambion, Italy). Histones were extracted as previously described in Putignano et al. 2007. Briefly, histones proteins were extracted from the nuclear fraction by the addiction of five volumes of 0.2 M HCl and 10% glycerol and pelletted by centrifugation (18,000 x g; 30 min; 4°C). Histones were precipitated from the acid supernatant by the addiction of ten volume of ice- cold acetone followed by centrifugation (18,000 x g; 30 min; 4°C). The obtained pellet was re- suspended in Urea 9 M. Proteins concentration was determined by Bradford assay (Biorad, Italy). For the running, each sample was boiled and 15μg of histones were loaded in each lane of a 12% acrylammide gels, using the Precast gel System (Biorad, Italy). The samples were blotted onto nitrocellulose membrane (Biorad, Italy) and blocked in 4% nonfat dry milk or 4%

BSA in Tris-buffered saline (TBS) for 2 hours. The nitrocellulose membrane was incubated with the following antibodies: H3 (Cat. N. 05-499 Upstate, NY) and Me2K4H3 (Cat. N. 17-677 Upstate, NY). Anti-H3 was diluted (1:300) in TTBS and 2% BSA and anti-Me2K4H3 was diluted (1:1000) in TTBS 2% milk, and both incubated overnight at 4°C. Blots were than washed for 3 times in TTBS for twenty minutes, incubated in HRP- conjugated anti-mouse (Biorad, Italy) diluted (1:3000) in 2% BSA or 2% milk in TTBS for two hours at RT. The membranes were than rinsed three times in TTBS and incubated in enhanced chemiluminescent substrate (Biorad, Italy) and exposed to film (hyperfilm, Amersham Biosciences, Europe). Films were scanned and densitometry was analyzed through ImaJ software. To minimize variability, each sample was loaded in parallel in two lanes and two gels were run simultaneously on the same apparatus. For each gel, the corresponding blotted filters were cut in two in order to obtain in each filter a complete series of samples. One of the filters was reacted with the antibody against the modified histone (Me2K4H3) and the other with the antibody insensitive to the target protein modification (H3). The densitometric quantification of the band corresponding to the methylated histone was normalized to the value obtained for the total amount of histone (H3) from the same gel. For MeCP2 Western blots, Right and left cortex were homogenized in cell disruption buffer (Ambion, Italy) and samples were centrifuged (14, 000 x g; 2 minutes; 4°C). The supernatant was used for the experiment. 25µg of proteins were loaded in each lane of a 10% acrylammide gels, using the Precast gel System (Biorad, Italy). The samples were blotted onto nitrocellulose membrane (Biorad, Italy) and blocked in 4% nonfat dry milk or 4% BSA in Tris-buffered saline (TBS) for 2 hours. The nitrocellulose membrane was incubated with the following antibodies: β-tubulin

1:5000 (Sigma Aldrich) and MeCP2 1:1000 (cat. n. M 9317 Sigma Aldrich). Other steps as in the histone western blot protocol.

4. Thricostatin A (TSA) treatment

TSA (5 mg/ml in DMSO) was administered by means of daily i.p. injection at a dose of 2,5 mg/kg. Adult wild type C57BL/6J mice (P100-120) received four injection of either TSA or the same volume of vehicle beginning from the first day of dark rearing (DR) until the day of light re-exposure (4th day). The last day all the mice received the i.p. injection and after one hour a group of mice were ri-exposed to light. A group of mice was sacrificed in darkness and a second group after 105 minutes of visual stimulations. Visual cortices were removed and frozen on dry ice.

5. RNA extraction, reverse transcription and Real time PCR

For RNA extraction and western blotting, P27 mice were killed by decapitation in darkness or at different times after visual stimulation (20 minutes, 105 minutes, 7 hours). P7-P15-P20-P25-P30-P35 standard (STR) mice were sacrificed by decapitation. A group of mice were reared in completely darkness (DRB) from birth and at P7-P15-P20-P25-P30-P35 were decapitated in darkness. The effects of monocular deprivation were assessed after three days of deprivation (MD3d mice). Mice were sacrificed and the binocular visual cortices were removed and frozen on dry ice. The contralateral and the ipsilateral visual cortices were processed separately. Visual cortices were removed and frozen on dry ice. Tissue samples were homogenized in cell disruption buffer (Ambion, Italy). RNA was extracted by the addiction of Phenol/guanidine-based QIAzol Lysis Reagent (Qiagen, italy). Chloroform was added and the samples were shacked for 15 seconds. The

samples were left at RT for 3 minutes and then centrifuged (12000 x g, 15 min, 4°C). The upper phase aqueous solution, containing RNA, was collected in a fresh tube and the RNA was precipitated by the addiction of isopropanol. Samples were mixed by vortexing, left at RT for 10 minutes and then centrifuged (12000 x g, 10 min, 4°C). Supernatant was discarded and the RNA pellet was washed in 75% Ethanol by centrifugation (7500 x g, 5 min, 4°C). Supernatant was discarded and the pellet was re-suspended in Depc (Diethyl pyrocarbonate Cat. N. D5758, Sigma-Aldrich, Germany) water. Total RNA concentrations were determined by measuring absorbance at 260 nm with a SmartSpec Plus Spectrophotometer (Bio-Rad). RNA quality was analyzed through a gel running (1% agarose). Total RNA was reverse transcribed using QuantiTech Reverse Transcription Kit (Cat. n. 205311, Quiagen). MicroRNAs were reverse transcribed with the TaqMan microRNA reverse transcription Kit (Part n. 4366596 Applied Biosystems, CA) using miRNA specific primers for mature miR132 (Cat. n. 457 Applied Biosystems, CA) and SNO234 (small nucleolar 234 RNA cat. n. 1234 Applied Biosystems, CA). Pri-microRNA and microRNA expression was analyzed by Real time PCR (Step one, Applied Biosystems, Foster City, CA). Taqman inventoried assays were used for pri-miR132 (Cat. n. Mm 03306275), miR132 (Cat. n. 457 Applied Biosytems) and SNO234 (Cat. n. 1234 Applied Biosytems). Taqman assay was used for Glyceraldehyde phosphate dehydrogenase (GAPDH), GADPHprobe ATCCCAGAGCTGAACGG, GADPHforward CAAGGCTGTGGGCAAGGT, GADPHreverse GGCCATGCCAGTGAGCTT. Quantitative values for cDNA amplification were obtained from the threshold cycle number (Ct) obtained during the exponential growth of the PCR products. Threshold was set automatically by the Stepone software. Data were analyzed using the ΔΔCt methods

using GAPDH and SNO234 to normalize primiRNA and mature miRNA cDNA levels, respectively.

6. In situ hybridization

MD3d critical period mice and age matched nondeprived control mice were transcardially perfused with ice-cold 4% paraformaldehyde in phosphate buffer and brains were post-fixed for two hours before being placed in 30% sucrose in PBS overnight. 40 m coronal sections of the brains were cut on microtome. In situ hybridization was executed as described in (Decembrini, et al., 2008) briefly:

1st day: free-floating sections were treated with 4% paraformaldehyde at RT for 10 minutes. Sections were washed with PBS and treated with 1: 40000 proteinase K (20 mg/ml) in PBS 0,1% tween-20 (PBT) for 10 minutes at RT. The reaction was blocked by washing two times with glycine 2mg/ml in PBT at RT. Slices were re-fixed with 4% paraformaldehyde for 15 minutes at RT and washed three times in PBT. Slices were prehybridised 30 minutes at 48°C in hybridization buffer [HB, 50% formammide (Sigma Cat. N. F9037), 5X SSC (20X SSC: 3 M NaCl, 0,3 M trisodium citrate in H2O), 0,1% tween-20, 50 ug/ml Heparin (Sigma Cat. N. H3393), 500 microg/ml torula yeast RNA]. Sections were hybridised at 48°C overnight (O/N) whit 0,3ul/ 150ul of mercury LNA 3'DIG labelled mmu-miR132 probe (Exiqon, Cat. N. 39033-05).

2nd day: Slices were washed twice with 2X SSC and once with 0,2X SSC at RT for 10 minutes and blocked 30 minutes at RT in blocking solution [1% blocking reagent (Roche Cat. N. 11096176001), 1% sheep serum in MABT (100 mM maleic acid, 150 mM NaCl at pH 7,5, 0,1% tween-20]. Sections were incubated with Anti-

Dig-AP Fab fragments Ab 1:2000 (Roche Cat. N. 11093274910) in blocking solution O/N at 4°C.

3rd day: Slices were washed five times with PBT and three times with NMNT (100 mM NaCl, 100 mM Tris- HCl ph 9,5, 50 mM MgCl2, 0,1 % tween- 20, 2 mM tetramisole. Sections were incubated in darkness with Fast Red tablets (1 tablet in 2 ml 0,1 M Tris- HCl pH 8,2, Roche Cat. N. 11496549001) and the staining reaction was controlled every 10-15 minutes. Slices were washed three times in PBS and mounted on glass slides. Images acquisition was performed by using a Confocal microscope (LEICA DM6000).

7. MiRNAs mimic treatment

Critical period mice (P25) were anesthetized with avertin (1ml/50mg) and monocularly deprived. Monocular deprivation was achieved by suturing the eyelids of the right eye. The mice were implanted in the cortex contralateral to the deprived eye with a cannula (gauge 30) connected via a PVC tubing to an osmotic minipump (Model 1007D; Alzet, Palo Alto, CA, reservoir volume 100 µl, flux 0,5 µl/hr). The cannula was inserted at a location 4 mm anterior and 3 mm lateral to lambda. Minipumps were filled with 25 µM double strands miR132 mimic (Thermo Scientific Dharmacon) or 25 µM double strands control miRNA mimic (Thermo Scientific Dharmacon) in Depc saline. Double strands miRNAs mimic had the following modification on the sense strand: all ribonucleotides were 2'-O – methyl modified, six phosphorothioate backbone modifications were also included with two phosphorothioates located at the 5' –end and four at the 3'-end and a cholesterol moiety at the 3'-end (Kuhn, et al., 2010). The oligonucleotides had been converted 2'-hydroxyl, purified, annealed and desalted duplex. The miRNAs mimic used had the

following sequences: miR132 mimic sense UAACAGUCUACAGCCAUGGUCG, antisense CGACCAUGGCUGUAGACUGUUA; control miRNA mimic sense UUGUACUACACAAAAGUACUG, antisense CAGUACUUUUGUGUAGUACAA.

To visualize miR132 mimic infusion in the visual cortex a group of MD3d animals was treated with 5'- biotinylated miR132 mimic. Transcardial perfusion was executed with ice-cold 4% paraformaldehyde in phosphate buffer and brains were post-fixed for two hours before being placed in 30% sucrose in Depc-PBS overnight. Brains were frozen in isobutene and 40 μm coronal sections were cut on cryostat. Free-floating sections were subjected to a 2 hr block (Depc-PBS containing 10% BSA and 0.3% Triton X-100) followed by incubation with ABC kit (Vector laboratories, Burlingame, CA) for 1 hour. The reaction was completed using DAB (Vector Laboratories, Burlingame, CA) as a chromogen.

8. Spine morphology assessment

To study spine morphology, the visual cortex of critical period Thy-1 GFP transgenic mice (line M, (Feng, et al., 2000) were infused with miR132 mimic or the control miRNA for three days. At the end of the treatment mice were perfused with 4% paraformaldehyde and brains were post-fixed for two hours and incubated in 30% sucrose in PBS O/N. 200 micron coronal sections were cut on vibratome. Slices were mounted on glass slides and stacks of images (1/0.2 micron) were acquired by using a confocal microscope (LEICA DM6000)] using a 100x (NA 1.4) objective (0.09 micron/pixel). Spines were manually counted using ImageJ.

9. In vitro miRNAs mimic treatment

The NIH-3T3 cell line was cultured in modified Dulbecco's medium supplemented with 10% new born calf serum, 1% L-glutamine and antibiotics (100 units/ml penicillin- streptomycin). The cells were plated on glass disks that were 6 cm in diameter at 60-70% confluence, and transfected using Lipofectamine 2000 (Invitrogen) with 100 nM miR132 mimic or control miRNA. 48 hours after transfections, cells were treated with RIPA buffer (50 mM Tris-Cl, 150 mM NaCl, 1% NP40, 0,5% Sodium Deoxycholate, 0,1% SDS plus protease inhibitors cocktail 10 µl/ml (Cat. n. P8340 SIGMA-Aldrich)) and scraped from the dish to obtain a whole cells protein extract. Proteins concentration was determined by Bradford assay (Biorad, Italy). For the running, each sample was boiled and 35 µg of proteins were loaded in each lane of a 10% acrylammide gels, using the Precast gel System (Biorad, Italy). The samples were blotted onto nitrocellulose membrane (Biorad, Italy) and blocked in 4% BSA in TBS for 2 hours. The nitrocellulose membrane was incubated with the following antibodies: β-tubulin (Sigma Aldrich) and p120RasGAP (Santa Cruz biotechnology). Anti-β-tubulin was diluted 1:3000 and anti- p120RasGAP was diluted 1:500 in TTBS and 2% BSA, and both incubated overnight at 4°C. Blots were than washed for 3 times in TTBS for twenty minutes, incubated in HRP- conjugated anti- mouse (Biorad, Italy) diluted (1:3000) in 2% BSA in TTBS for two hours at RT. The membranes were than rinsed three times in TTBS and incubated in enhanced chemiluminescent substrate (Biorad, Italy) and exposed to film (hyperfilm, Amersham Biosciences, Europe). Films were scanned and densitometry was analyzed through ImaJ software. The densitometric quantification of the band corresponding to p120RasGAP was normalized to the value obtained for β-tubulin from the same gel.

10. Single unit recordings

We analyzed 6 mice untreated mice (nondeprived), 5 MD3d mice, 5 MD3d mice infused with miR132 mimic (MD3d miR132 mimic), 5 MD3d mice infused with control miRNA mimic (MD3d control miR). At the end of the treatments, spiking activity of cortical neurons was recorded under urethane anesthesia (0,6ml/100g I.p., 20% solution in saline) at P28. For each animal, eight-ten cells were recorded in each of at least three tracks spaced evenly (>100 um) across the binocular primary visual cortex, to avoid sampling bias. None of the treatments apparently altered the number of visually responsive cells in that visually driven cells were recorded at an interdistance of 60 microns from each other in control animals as well as in all treatment groups. The position of receptive fields of single units were mapped and only cells with receptive fields within 20° of the vertical meridian were included in our sample. Spontaneous activity, peak response and receptive field (RF) size were determined from peristimulus time histograms (PSTHs) recorded in response to computer-generated bars, averaged over at least 15 stimulus presentations as described in Lodovichi et al. 2000. Binocularity was expressed as the normalized OD score of single neurons (Rittenhouse, et al., 1999)and plotted the cumulative distribution for each experimental group. OD score was computed on cells with complete PSTH analysis of peak and baseline spiking activity after closure of either eye. OD score was defined as {[Peak(ipsi)-baseline(ipsi)] – Peak(contra)-baseline(contra)]} / {[Peak(ipsi)-baseline(ipsi)] + [Peak(contra)-baseline(contra)]}. To express the contralateral bias of the response of each animal we used the contralateral bias index (CBI). This index is defined as {[N(cells 1)-N(cells 7)] + 1/2[N(cells 2-3)- N(cells 5-6)] + Ntot} / 2Ntot.

11. VEP recordings

Visual Evoked Potentials (VEPs) recordings were performed using a micropipette inserted into the binocular part of the primary visual cortex (depth 400 μm) in urethane-anesthetized mice as described (Rossi, et al., 2001). Eyes were not restrained in a fixed position, but their position remained stable throughout the experiment and was similar between different mice (Schuett, et al., 2002). Stimuli consisted of full-field computer-generated horizontal gratings (0.08 c/deg, mean luminance 15 cd/m2, 100% contrast, 90 x 72 deg) alternating in time (1 Hz) presented at 20 cm from the animal. To measure VEP receptive field, grating width was 20°, its position was changed by 20° steps along the horizontal meridian, and VEP amplitude was recorded. Contra/Ipsi ratio was assessed by acquiring VEP responses to interleaved stimulation of each eye in electrode tracks with receptive field centered on the vertical meridian.

12. Statistical analysis

Parametric tests such as t-test, paired t-test, and ANOVA were used as first choice. However, if the assumptions of normality of data distribution and omoschedasticity required by these tests were not satisfied, we adopted non parametric tests such as Mann-Whitney (to compare two groups), signed rank test (to compare two repeated measures on the same subjects), or Kruskall-Wallis ANOVA. Cumulative distributions were compared using the nonparametric Kolmogorov-Smirnov test. P=0.05 was assumed as significance level. Statistical analysis was performed using the Sigma Stat (Systat, USA) software. Data are expressed as mean ± standard error of mean (SEM).

Results

1. Visual stimulation regulates histone acetylation, phosphorylation and methylation on a CRE sequence important for transcription of the miR132/212 cluster.

To investigate miR132 action in visual cortical plasticity, we first analysed histone posttranslational modifications on CRE loci contained in the miR132/212 cluster. Previous data showed that visual experience causes an increase in the overall levels of two epigenetic marks generally associated with active gene transcription, namely acetylation (Lys9) and phosphorylation (Ser10) of H3 (Putignano et al., 2007). Recent studies revealed that histone methylation is also dynamically regulated in neurons (Shi and Whetstine, 2007) and it is involved in different forms of neuronal plasticity (Gupta, et al., 2010, Maze, et al., 2010). In particular, methylation and dimethylation of H3 Lys4 is linked to transcriptional activation (Ruthenburg, et al., 2007), therefore we analysed whether dimethylation of Lys4 in H3 [Me2(Lys4)H3] is induced in the visual cortex of mice reared in complete darkness for 3 days (DR3d) (from P24 to P27) and exposed to light for 20 minutes (LR20m). Western blot analysis showed a dramatic increase in Me2(Lys4) in histone H3 as a consequence of visual stimulation (**Fig. 1**). Thus, we performed chromatin immunoprecipitation (ChIP) using antibodies specific for histone H3 acetylation (Lys9-14), phospho (Ser10) acetylation (Lys14), and methylation [Me2(Lys4)] to analyze whether visual stimulation could enhance the amount of histones carrying these posttranslational modification on the CRE loci located within the miR132/212 cluster. The CRE locus in the *Fos* promoter was used as a positive control considering

the strong induction of Fos expression observed with our protocol of visual stimulation (**Fig. 2**).

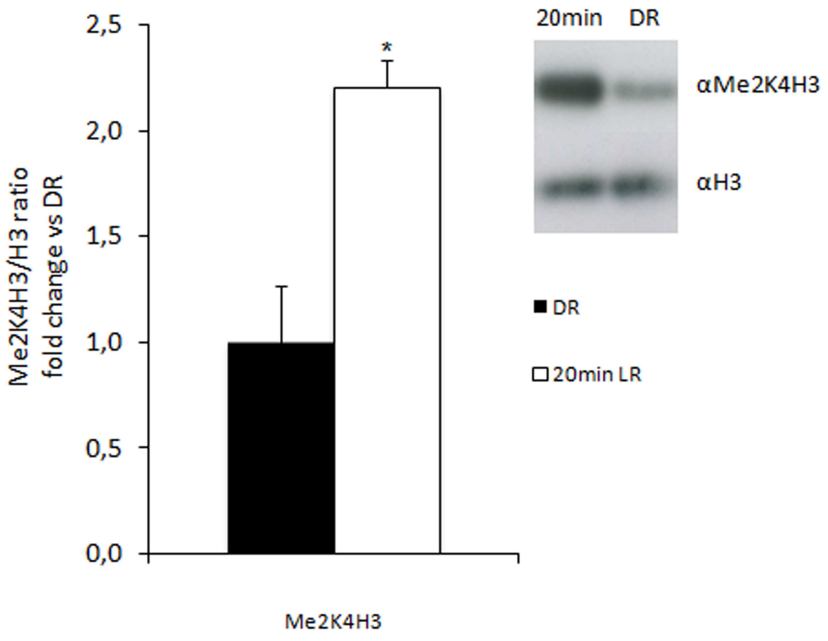


Figure 1. Visual stimulation increases Lys4 dimethylation of Histone H3 in critical period mice. Western blot of visual cortex samples of three days dark reared (DR3d) and 20 minutes visually stimulated (LR20m) juvenile mice. The ratio between the intensity of the band of Me2K4H3 and total H3 was taken as an index of the amount of dimethylated H3. Visual stimulation strongly increased H3 Lys4 dimethylation. (DR3d n=4 vs LR20m n= 4, t test p=0.007). Error bars represent SEM.

We found that visual stimulation increased the presence of all the analyzed epigenetic marks on the CRE sequence located close to miR132 transcript [Ac(Lys9-14)H3 DR3d n=16 vs LR20m n=16, t test p=0.035; p(Ser10) Ac(Lys14)H3 n=10 DR3d vs n=10 LR20m, t test p=0.018; Me2(lys4)H3, DR3d n=7 vs LR20m n=9, t test p=0.017] and in the promoter region of *Fos* gene[Ac(Lys9-14)H3 DR3d n=16 vs 20min LR20m n=16, Mann-Withney rank sum test p=0.018; p(Ser10) Ac(Lys14)H3, DR3d n=10 vs LR20m n=10, t test p=0.05; Me2(lys4)H3, DR3d n=10 vs LR20m n=12, t test p=0.020](**Fig. 3a-c**). By contrast, a significant increase of H3 acetylation, but not in phosphoacetylation and dimethylation, was present on the two consensus CREs located immediately 5' to the miR212 transcript (**Fig. 3a-c**) [Ac(Lys9-14)H3,

DR3d n=13 vs LR20m n=14, Mann- Withney rank sum test p=0.04;p(Ser10)Ac(Lys14)H3, DR3d n=10 vs LR20m n=10, Mann- Withney rank sum test p=0.88; Me2(lys4)H3, DR3d n=9 vs LR20m n=9, t test p= 0.379].

Specificity of ChIP experiments was controlled using normal rabbit and normal mouse IgG (**Fig. 4**).

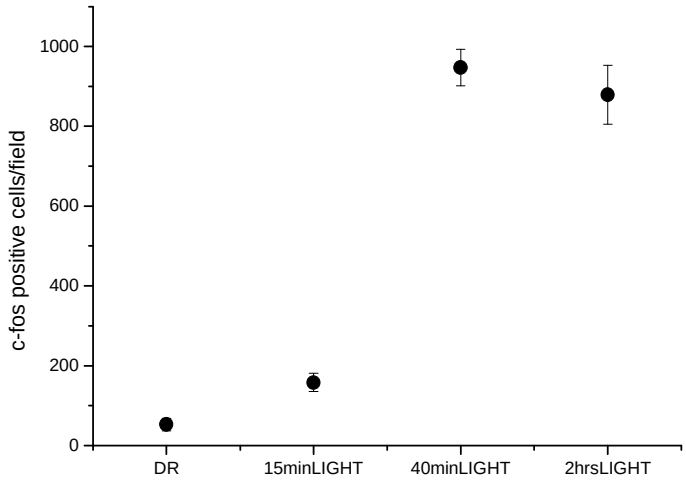


Figure 2. Fos staining is increased by visual stimulation in the visual cortex of P27 mice.

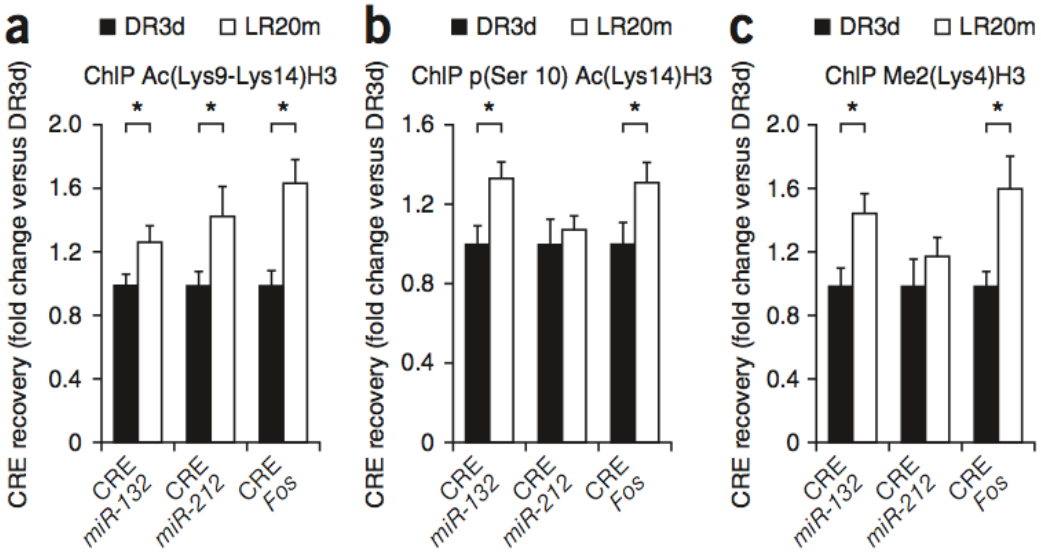


Figure 3 Visual stimulation induces histone mark modifications on specific CRE loci close to the miR132 coding sequence. (a) Ac(Lys9-Lys14)H3 ChIP revealed visually induced H3 acetylation at

the CRE loci upstream of miR132, miR212 and Fos. *P < 0.05. **(b)** p(Ser10) Ac(Lys14)H3 ChIP revealed visually induced H3 phosphoacetylation at the CRE sequences upstream of miR132 and Fos, but not miR212. **(c)** Me2(Lys4)H3 ChIP revealed visually induced H3 dimethylation at the CRE sequence upstream of miR132 and Fos, but not miR212. Error bars represent SEM.

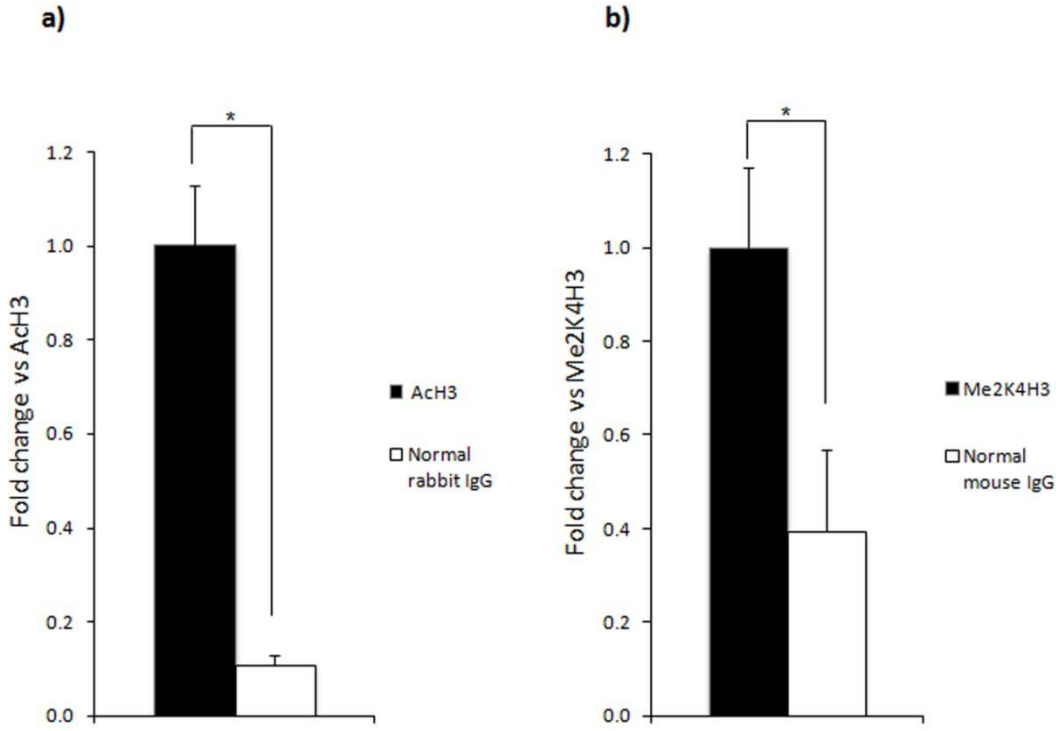


Figure 4. Specificity of ChIP experiments. Normal rabbit IgG or normal mouse IgG caused a statistically significant less recovery of chromatin respect to specific posttranslationally modified histone antibodies. CREmiR132 sequence abundance was measured using RT-PCR in cortical samples immunoprecipitated with antibodies specific for AcH3 or Normal rabbit IgG **(a)** or Me2K4H3 antibodies and Normal mouse IgG **(b)**. Data were normalized to the average recovery obtained with the antibody for the posttranslationally modified histone. a) AcH3 n=7 vs Normal rabbit IgG n=7,Mann-Withney rank sum test p<0,001. b) Me2KH3 n=4 vs Normal mouse IgG n=4, t test p=0,047. Error bars represent SEM.

2. MiR132 levels are dynamically and bidirectionally regulated by visual experience.

Our ChIP study demonstrated that visual stimulation promotes changes in epigenetic marks associated with gene expression. Thus, we evaluated miR132 expression in visual cortex during development and in response to manipulations of

visual experience. Primary transcript of *miR132/212* cluster (pri-miR132), mirroring transcriptional regulation, and mature miR132 expression levels were assessed using RT-PCR at different ages in mice reared from birth in a standard environment (STR) or in darkness (DRB). In standard rearing conditions, both pri- and mature miR132 levels progressively augmented after eye opening with a dramatic increase of expression between P20 and P30. This developmental time course was completely blocked in mice reared in complete darkness from birth. Indeed, pri- and mature miR132 levels of DRB mice remained very low at all ages analyzed (**Fig. 5 (a)**; Pri-miR132: two way ANOVA, only the age×rearing interaction was significant p=0.012. Post hoc Holm-Sidak comparisons showed that DRB significantly reduced pri-miR132 expression at P20 (n=3), P25 (n=4), P30 (n=3) and P35 (n=3) with respect to STR. Within the factor age, P15 (n=6) differed from P25 (n=6), and P7 (n=6) differed from P20 (n=6), P25, P30 (n=6) and P35 (n=6)in STR mice only. **(b)** MiR132: two way ANOVA, only the age×rearing interaction was significant p=0.003. Post hoc Holm-Sidak comparisons showed that DRB significantly reduced miR132 expression at P25, P30 and P35 with respect to ST. Within the factor age P7 differed from P25, P30, P35; P15 was different from P30 and P35; P20 was different from P30 and P35 in STR mice only).

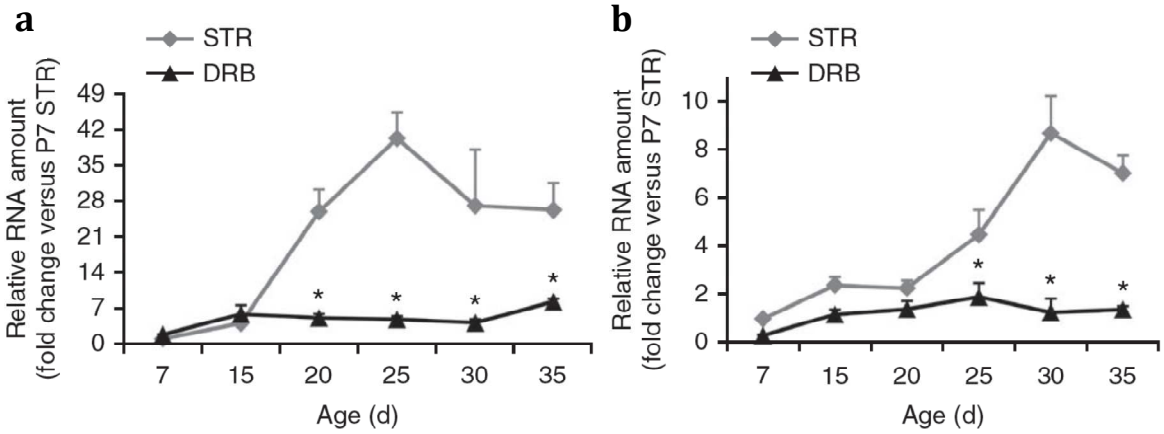


Figure 5. Developmental curves of pri- and mature miR132. Developmental expression of pri-miR132 and miR132 in the visual cortex of STR and DRB mice. Pri-miR132 **(a)** and mature miR132 **(b)** expression significantly increased with age in STR mice, but not in DRB mice. Error bars respresent SEM.

Next, we studied the dynamic experience-dependent regulation of pri-miR132 and mature miR132 using the same manipulation of visual stimulation used to assess experience-dependent regulation of epigenetic marks. We found that dark rearing for 3 days from P24 was sufficient to downregulate pri-miR132 and miR132 visual cortical levels (pri-miR132: DR3d, n = 4; P27 STR, n = 4; t test, P < 0.001; miR132: DR3d, n = 4; P27 STR, n = 4; Mann-Whitney rank-sum test, P = 0.029; **Fig. 6**).

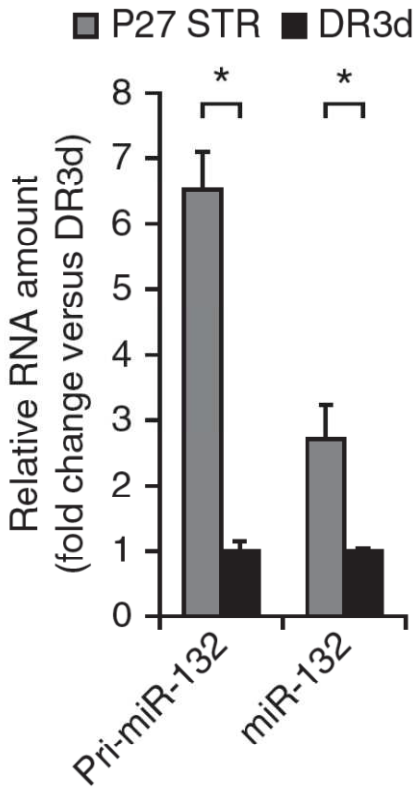


Figure 6. DR3d decreased pri-miR132 (left) and miR132 (right) expression in visual cortex compared with age-matched control mice (P27 STR). Error bars represent SEM.

Light exposure of DR3d (n=6) mice for 105 minutes (LR105m; n=6) induced a pronounced increase in primary and mature miR132 declining 7 hours (LR7h; n=4) after stimulation (**Fig.7 (a)**: pri-miR132: one-way ANOVA, P < 0.001; post hoc Holm-

Sidak test, LR105m versus DR3d, P < 0.05; LR105m versus LR7h, P < 0.05; other comparisons were not significantly different, P > 0.05; **(b)**: miR132: one-way ANOVA, P = 0.01; post hoc Holm-Sidak test, LR105m versus DR3d, P < 0.05; LR105m versus LR7h, P < 0.05; other comparisons were not significantly different, P > 0.05).

These results indicate that visual experience quickly regulates miR132 levels and suggest a possible involvement of miR132 in experience-dependent synaptic maturation and plasticity of the visual cortex during the critical period.

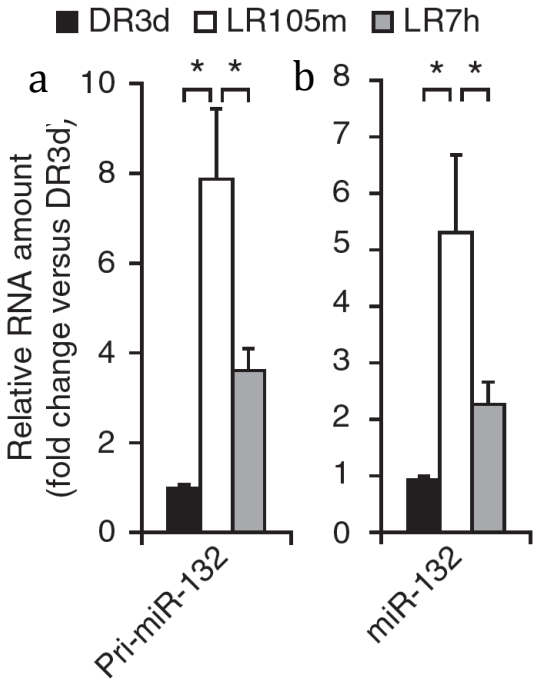


Figure 7. Light exposure induces miR132 expression. 105 minutes of visual stimulation after three days of dark rearing increased pri-miR132 **(a)** and miR132 **(b)** levels in the visual cortex of juvenile mice. Error bars represent SEM.

In adult mice, visual induction of pri-miR132 levels was reduced (**Fig. 8**). 105 minutes of light exposure (ADLR105m) after 3 days of dark rearing in adult control mice (ADCON, P100-120) resulted in a significant induction of pri-miR132 expression with respect to adult DR3d mice (ADDR3d n=5 vs ADLR105m n=5 Mann-Withney rank sum test p<0.05). However, the induction levels present in ADCON

mice were significant reduced with respect to juvenile critical period mice (CP, P27, data reproduced from **Fig. 7a**). Treatment of adult mice with trichostatin A (ADTSA), during the 3 days of dark rearing, significantly enhanced light induced pri-miR132 expression (One way ANOVA p=0.026, post hoc Bonferroni t test: ADCON n=5 vs ADTSA n=6 p<0,05; ADCON vs CP P<0.05; ADTSA vs CP p=0.078, **Fig. 8**). Therefore, enhancement of histone acetylation levels by means of TSA treatment increased pri-miR132 visual induction in adults, indicating that histone marks are involved in experience-dependent pri-miR132 transcription.

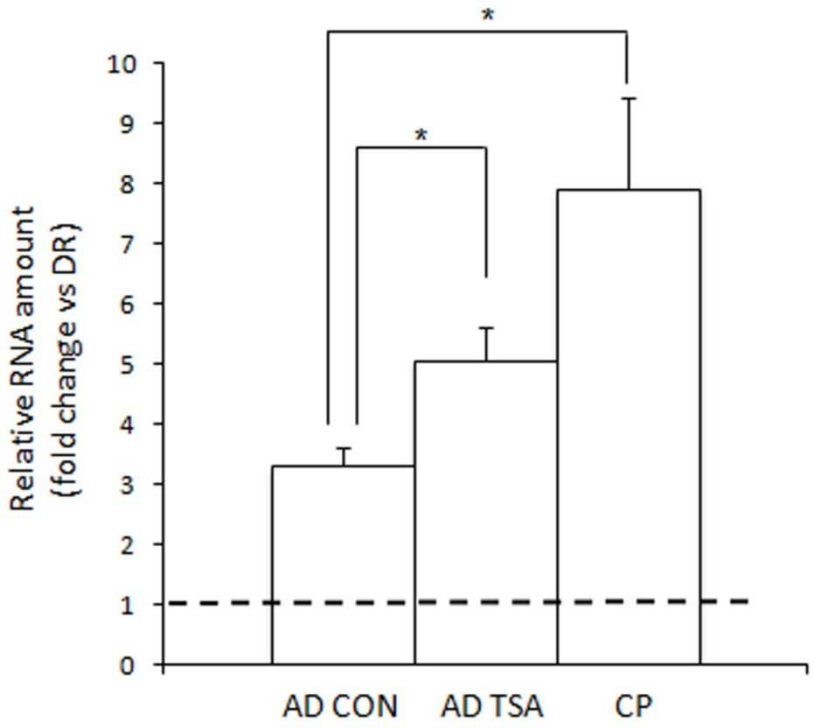
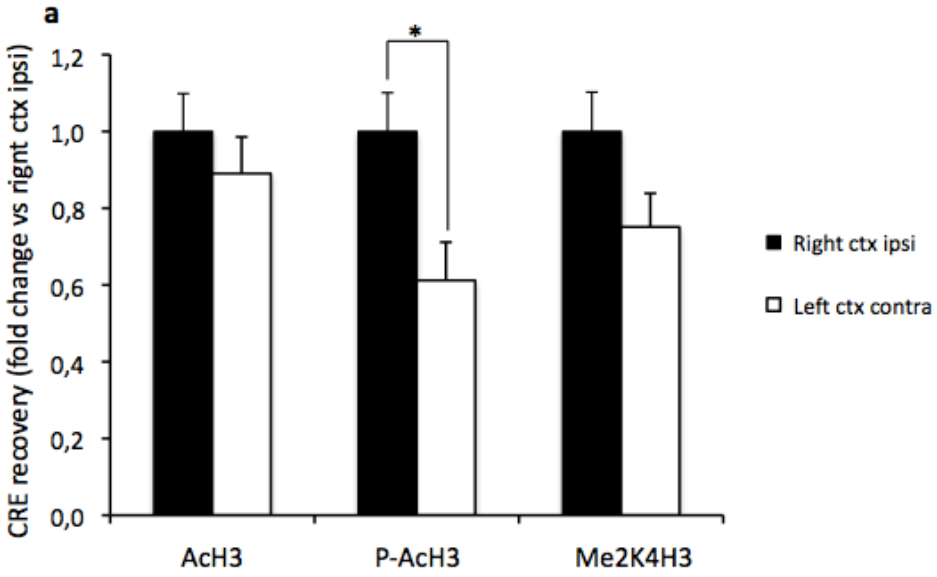


Figure. 8. Enhancement of pri-miR132 light induced expression by TSA treatment in adult mice. 105 minutes of light exposureafter 3 days of dark rearing in ADCON mice resulted in a significant induction of pri-miR132 expression respect to adult DR3d mice, but the induction level in ADCON mice were significant lower with respect CP mice. TSA treated adult mice (ADTSA) showed a significant enhancement of pri-miR132 induction by visual stimulation. Dashed line represents pri-miR132 DR level of each group. Error bars represent SEM.

Experience-dependent plasticity of the visual cortex is classically tested using monocular deprivation. To investigate a possible role of miR132 in ocular dominance (OD) plasticity, we analyzed the effect of monocular deprivation (3 days from P25, MD3d) on histone marks. MD3d induced a significant decrease in H3 phosphoacetylation, an epigenetic mark correlated with transcription and plasticity (Day and Sweatt, 2011), at all of the CRE sites tested, and a trend for decreased H3 dimethylation (P = 0.07) at the miR132 CRE site in the visual cortex contralateral to the deprived eye (**Fig. 9, (a)** CREmiR132: p(Ser10) Ac(Lys14) H3 n=13, right ctx ipsi vs left ctx contra paired t test p=0.015; Ac(Lys9-14) H3 n=13, right ctx ipsi vs left ctx contra paired t test p=0.3; Me2(Lys4) H3 n=16, right ctx ipsi vs left ctx contra paired t test p=0.07. **(b)** CREmiR212: p(Ser10) Ac(Lys14) H3 n=13, right ctx ipsi vs left ctx contra paired t test p=0.036; Ac(Lys9-14) H3 n=13, right ctx ipsi vs left ctx contra paired t test p=0,28; Me2(Lys4) H3 n=13, right ctx ipsi vs left ctx contra paired t test p=0.28. **(c)** CREc-fos: p(Ser10) Ac(Lys14) H3 n=13, right ctx ipsi vs left ctx contra paired t test p=0.048; Ac(Lys9-14) H3 n=13, right ctx ipsi vs left ctx contra paired t test p=0.42; Me2(Lys4) H3 n=13, right ctx ipsi vs left ctx contra paired t test p=0.41). Thus, prolonged deprivation does not merely reverse the histone marks pattern observed with acute visual stimulation (see **fig. 3a-c**).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Right ctx ipsi
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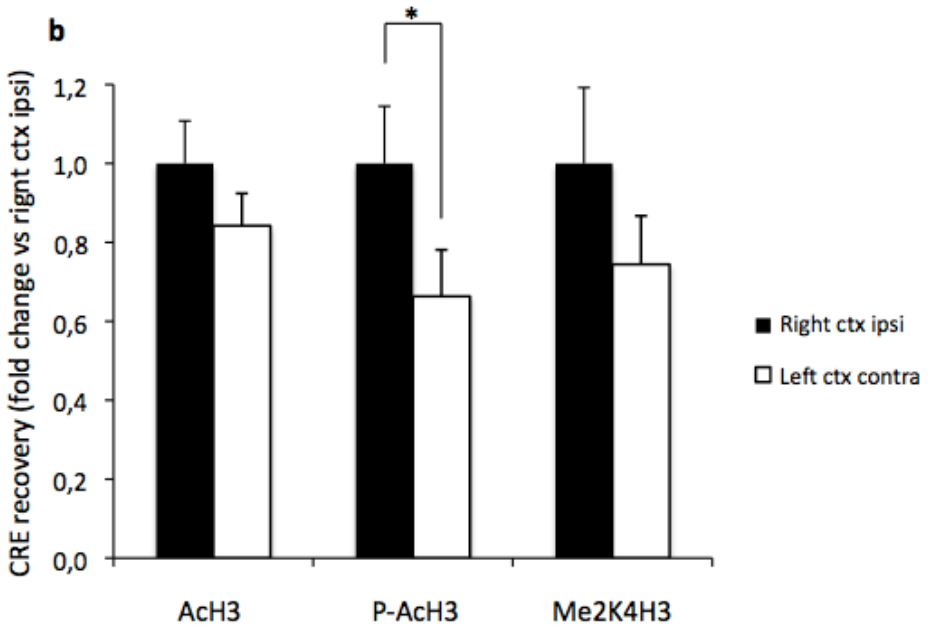
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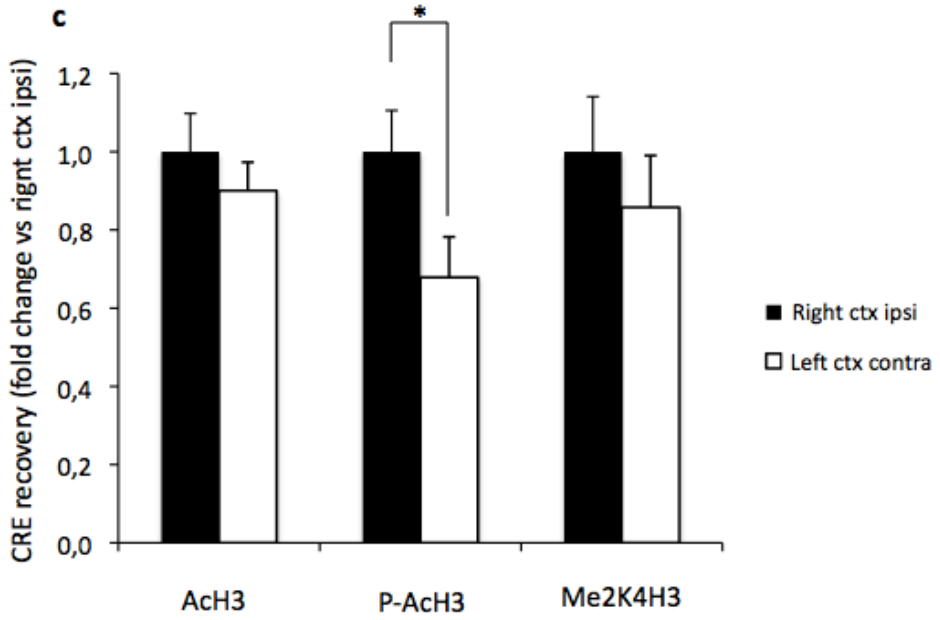


Figure 9. Monocular deprivation causes decrease in p(Ser10) Ac(Lys14) H3 on specific CRE loci in the visual cortex of juvenile mice. MD3d significantly reduced H3 phosphoacetylation, but not Lys9-14 acetylation and Lys4 dimethylation of H3 on CREmir132 **(a)**, CREmiR212 **(b)** and CREc-fos **(c)** sequences in the binocular visual cortex contralateral to the deprived eye (left ctx contra) with respect to the ipsilateral cortex (right ctx ipsi). Error bars represent SEM.

MD3d also reduced pri-miR132 (n = 17, cortex ipsilateral to the deprived eye versus contralateral, paired t test, $P < 0.001$; **Fig. 10a**) and miR132 expression (n = 17, ipsilateral versus contralateral, paired t test, $P = 0.013$; **Fig 10b**). Pri-miR132 and miR132 expression in left and right cortex of nondeprived mice was not different (**Fig. 10a** pri-miR-132: control nondeprived mice, n = 4, right cortex versus left cortex, paired t test, $P = 0.09$; **Fig. 10b** miR-132: control nondeprived mice, n = 4, right cortex versus left cortex, paired t test, $P = 0.70$). In situ hybridization (ISH; **Fig. 11**) revealed a significant reduction of mature miR132 expression in both monocular and binocular visual cortex contralateral to the deprived eye as compared with the ipsilateral cortex (n = 4, one-way repeated-measures ANOVA, $P < 0.001$; post hoc Holm-Sidak test, $P < 0.05$ for both monocular and binocular cortex).

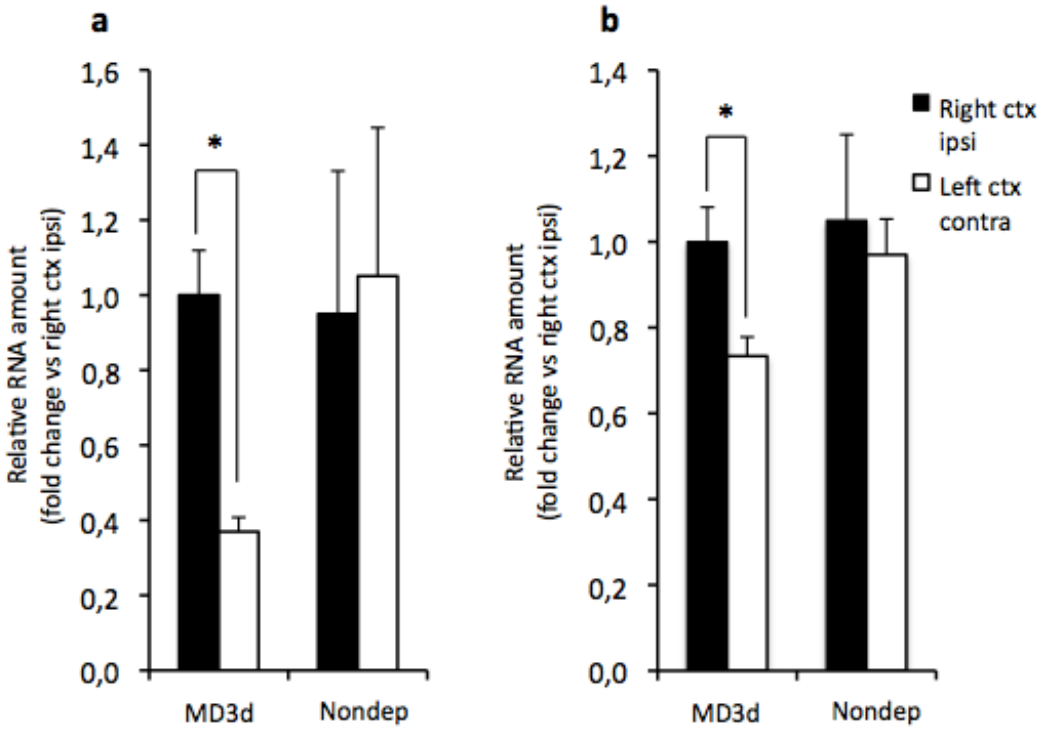
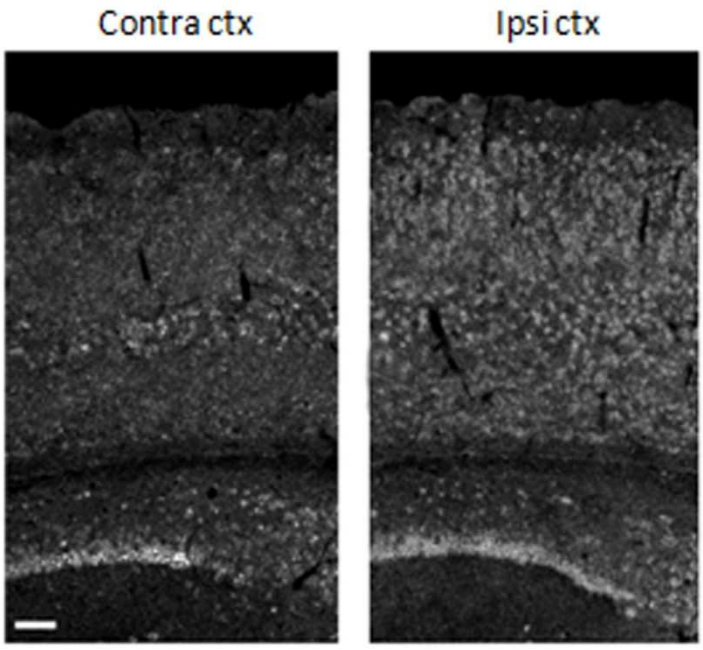


Figure 10. MD3d effects on miR132 levels in the visual cortex of critical period mice. MD3d decreased pri-miR132 **(a)** and miR132 **(b)** expression in the binocular visual cortex contralateral to the deprived eye. No differences in pri-miR132 and miR132 levels were present between left and right cortex of contol nondeprived (Nondep) animals. Error bars represent SEM.



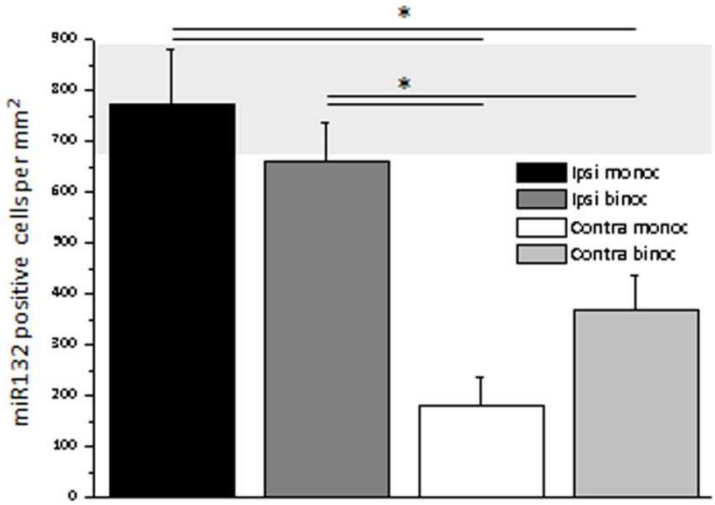


Figure 11. MiR132 ISH in the visual cortex of MD3d juvenile mice. ISH for mature miR132 in MD3d animals revealed a reduction in the cortex contralalteral to the deprived eye. Calibration bar represent 100µm. Grey shaded area represents miR132- positive cell density in control age matched nondeprived mice (mean ± s.e.m., n=4). Error bars represent SEM.

Therefore, reduced levels of miR132 were present in the visual cortex contralateral to the deprived eye of MD3d young mice.

3. Counteracting miR132 dowregulation during monocular deprivation inhibits ocular dominance plasticity.

We then asked whether the downregulation of miR132 levels occurring in MD3d mice was required for OD plasticity. To answer this question we counteracted miR132 downregulation in the deprived cortex of MD3d mice and we assessed the functional effects of monocular deprivation on binocularity of cortical neurons. To increase miR132 levels we administered a chemically modified double-strand miRNA mimic (Kuhn, et al., 2010). MiR132 mimic oligos were able to reduce the expression

of p120rasGAP, a miR132 target protein (Anand, et al., 2010), in cell culture (**Fig. 12**).

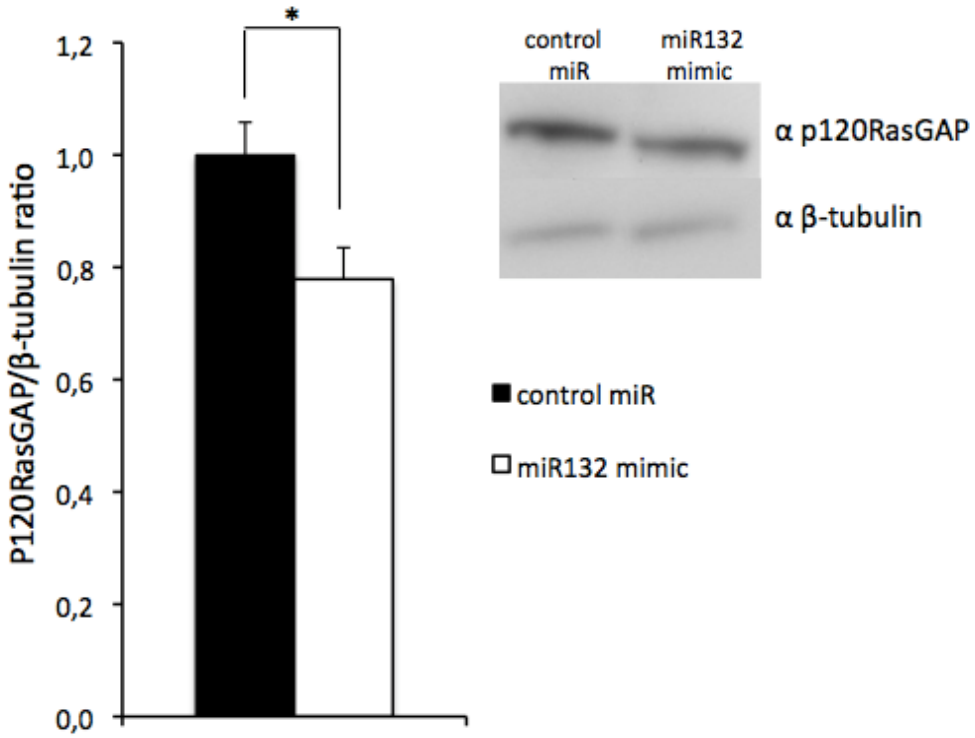


Figure 12. MiR132 mimic treatment *in vitro*. Western blot for p120RasGAP showed a downregulation in NIH-3T3 cultures transfected transfected with 100 nM miR132 mimic with respect to control miRNA (control miR n=3 vs miR132 mimic n=3, t test $p<0.05$). Error bars represent SEM.

MiR132 mimic was infused in the visual cortex contralateral to the deprived eye during the 3 days of MD by means of an osmotic minipump connected to a cannula located 3 mm anterior to the visual cortex. Visualization of biotinylated miRNA mimic showed that the binocular visual cortex is reached by the infused oligo (**Fig. 13a**). Furthermore, the treated cortex showed reduced protein level of MeCP2 (**Fig13c**), another miR132 target (Klein, et al., 2007) that is important for synaptic development (Boggio, et al., 2010).

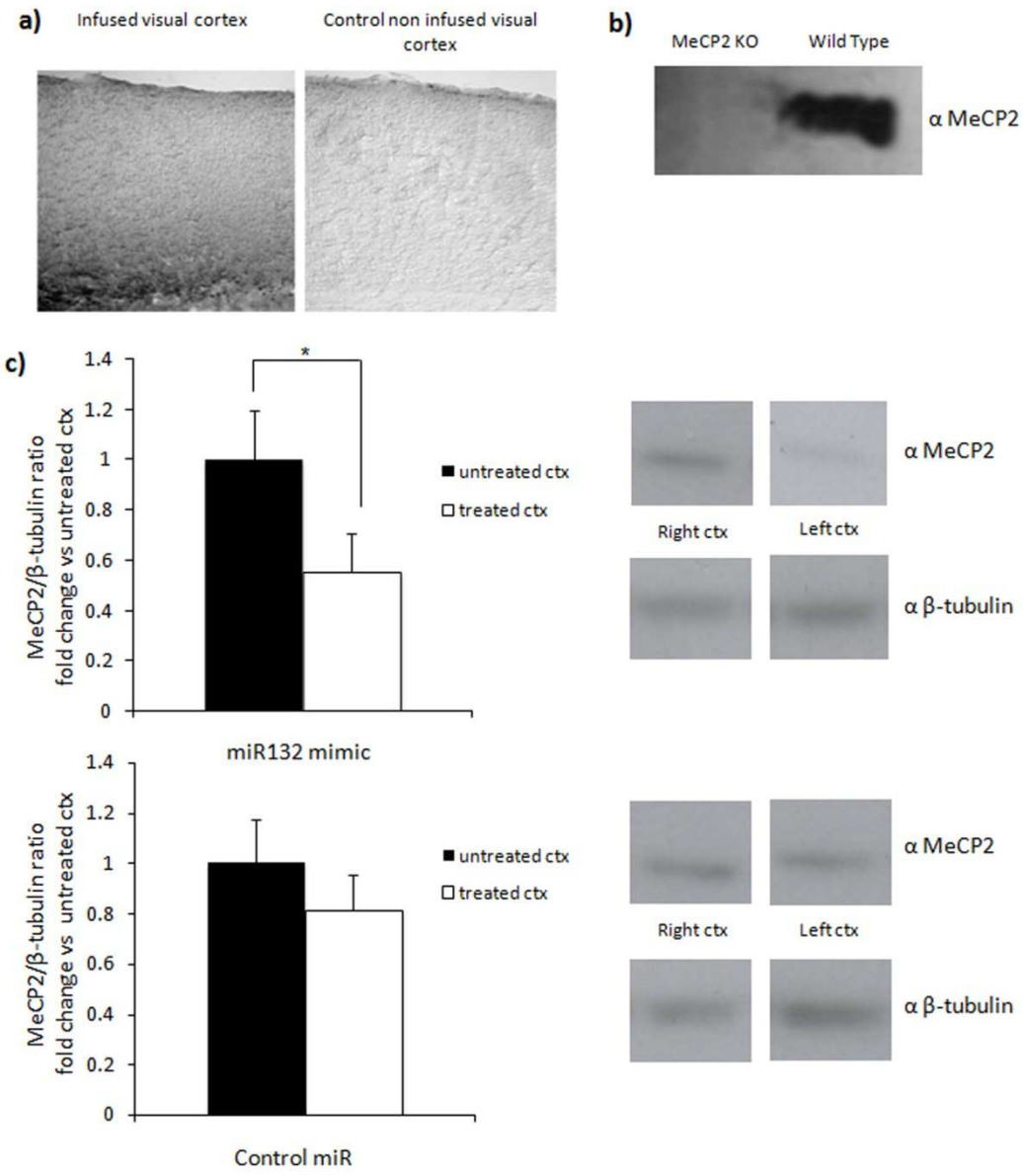


Figure 13. In vivo effect of miR132 mimic infusion in the visual cortex of MD3d critical period mice. (a) DAB staining of biotinylated miR132 mimic infused for 3 days in the visual cortex contralateral to the deprived eye of MD3d mice. Biotinylated miR132 mimic reached the binocular and monocular visual cortex but did not diffuse into the contralateral hemisphere. **(b)** Western blot of cortical extracts of MeCP2 KO and wild type mice showed the specificity of the antibody used in the experiment. **(c)** MiR132 mimic caused a significant decrease of MeCP2 protein expression in the infused cortex (ctx) with respect to the non infused ctx (upper graph, miR132 mimic n=6, untreated ctx vs treated ctx, Signed rank test $p = 0.031$). The infusion of a control miRNA mimic (control miR) did not significantly change MeCP2 protein levels (lower graph, control miR n=6, untreated ctx vs treated ctx, Signed rank test $p = 0.31$). Error bars represent SEM.

To further verify the efficacy of our *in vivo* miRNA mimic treatment, we analyzed dendritic spines morphology. Previous *in vitro* studies demonstrated that hippocampal neurons overexpressing miR132 were characterized by stubby and mushrooms spines and overexpressed miR132 caused an increase in average protrusion width, without affecting the average length. In addition, average protrusion density fell significantly (Edbauer, et al., 2010). To assess the effect of miR132 mimic on spine morphology, we infused miR132 mimic or control miRNA in the visual cortex of line M GFP expressing non deprived juvenile mice, using the same protocol adopted for electrophysiological assessment of OD plasticity. We did not observe any differences in dendritic spine density or length between treated and untreated cortex both in miR132 mimic and control miRNA infused mice; no difference was also present between miR132 mimic and control miRNAtreated cortex (**Fig. 14a-b**). However, the miR132 mimic induced an icrease in the percentage of mushroom/stubby spines (**Fig. 14c**), an action of miR132 that was previously shown *in vitro* (Edbauer, et al., 2010).

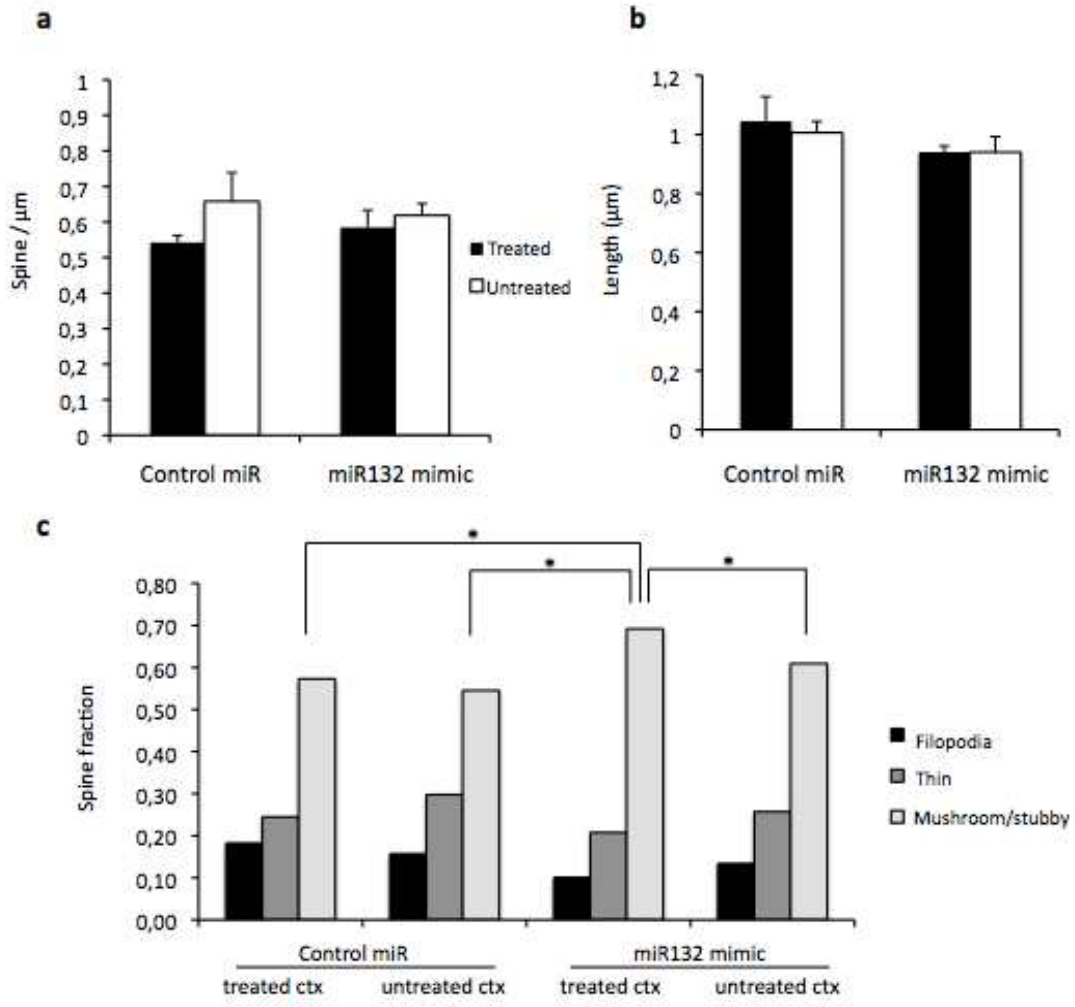


Figure 13. Three days miR132 mimic treatment increases the fraction of mushroom/stubby spines. To assess the effect of miR132 mimic on spine morphology, we infused miR132 mimic or control miRNA in the cortex of line M GFP expressing non deprived critical period mice. **(a)** No difference in spine density was present between treated and untreated cortex both in miR132mimic and control miRNA (control miR) infused mice (control miR n=3, paired t test p=0.22; miR132 mimic n=5, paired t test p=0.41). No difference was also present between miR132 mimic and control miRNA treated cortex (t test p=0.07). **(b)** No difference in spine length was present between treated and untreated cortex both in miR132 mimic and control miRNA infused mice (control miR n=3, paired t test p=0.65; miR132 mimic n=5, paired t test p=0.99). No difference was also present between miR132 mimic and control miRNA treated cortex (t test p=0.17). **(c)** Spines were classified as filopodia (protrusions without an enlargement of the tip), thin (protrusions with a head smaller than spine length), mushroom/stubby (large head spines). We found a significant increase (chi-square test with Bonferroni correction for multiple comparisons) in the fraction of mushroom/stubby spines in the cortex treated with miR132 mimic (miR132 treated ctx n=704 spines vs. miR132 untreated ctx n=650 spines, p=0.006; miR132 treated ctx vs control miR treated ctx n=466 spines, p<0.001; miR132 treated ctx vs. control miR untreated ctx n=598 , p<0.001. Other tests not significantly different, p>0.05).

OD plasticity was analyzed by electrophysiological recording in MD3d critical periods mice that were administered with miR132 mimic or control miRNA in the cortex contralateral to the deprived eye during the period of eyelid suture. Quantitative PCR (qPCR) analysis showed that the treatment with miR132 mimic restored miR132 levels to those seen in non deprived mice (**Fig.14**).

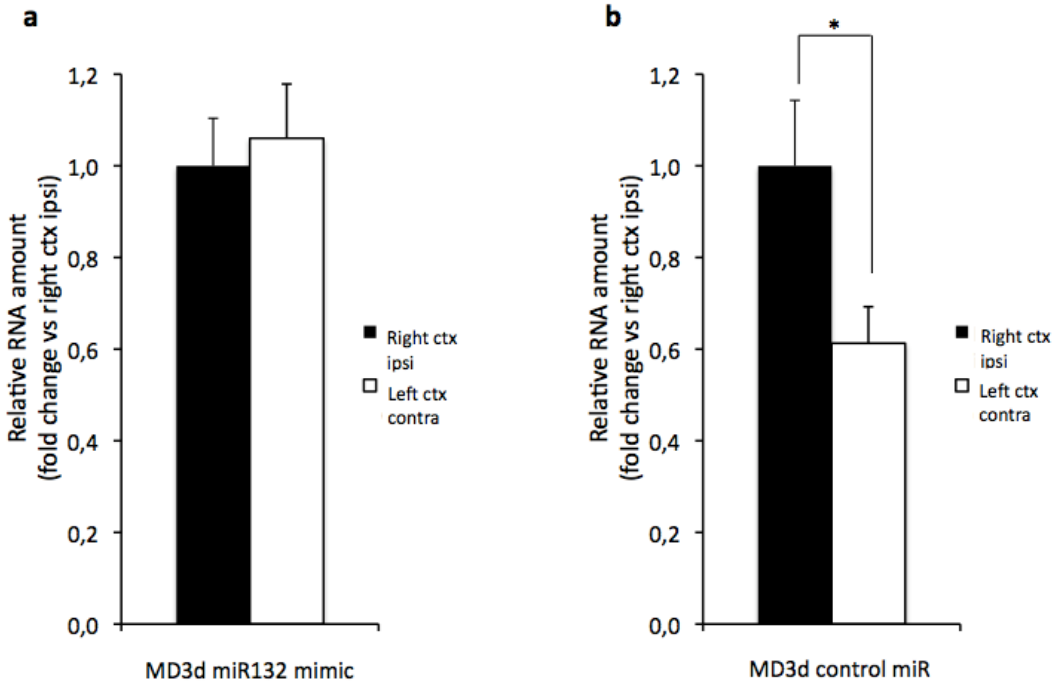


Figure14. Mature miR132 levels after miR132 mimic or control miR infusion in the visual cortex of MD3d critical period mice. MiR132 downregulation in the visual cortex controlateral to the deprived eye in MD3d juvenile mice was not present after miR132 mimic treatment (**a**; MD3d miR132 mimic n=4, right cortex ipsi vs left cortex contra, paired t test p=0.7). Infusion of the visual cortex contralateral to the deprived eye with a control miRNA mimic (control miR) showed the expected decrease of miR132 expression after MD3d (**b**; MD3d control miR n=4, right ctx ipsi vs left ctx contra, paired t test p=0.041). Error bars represent SEM.

OD plasticity was assessed by extracellular recordings of single-unit activity in the infused cortex (contralateral to the deprived eye) using two different indexes:the contralateral bias index (CBI) was used to estimate the strength of the responses to visual stimulation of the contralateral eye for each animal, and the cumulative distribution of the OD score (ODS) of each neuron was used to assess the ocular

dominance distribution of cortical cells (Spolidoro, et al., 2011). As expected, a significant shift of OD distribution toward the ipsilateral nondeprived eye were present in untreated MD3d mice or in MD mice infused with a control miRNA (MD3d control miRNA). By contrast, 3 days of MD were totally ineffective in shifting OD distribution in miR132 mimic treated mice.

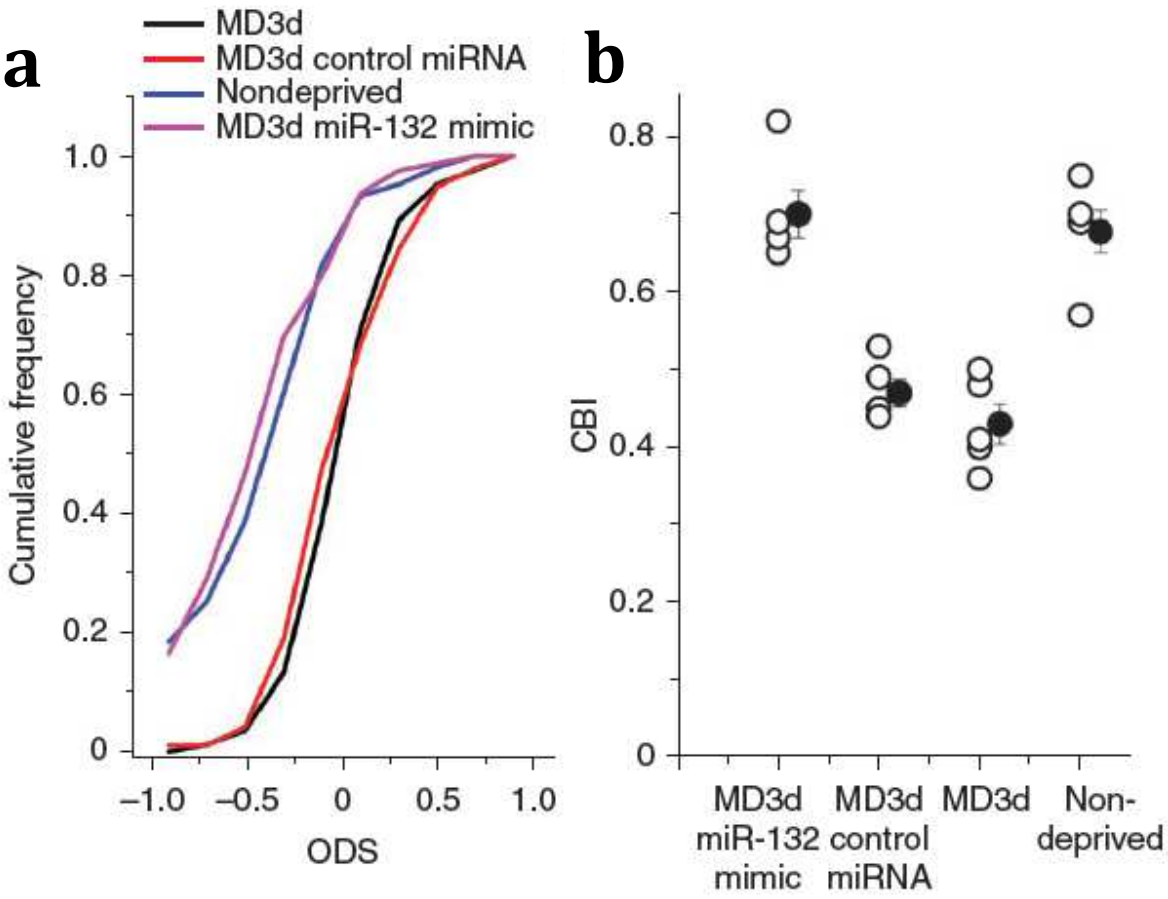


Figure 15. ***In vivo*** **single cells recording of MD3d critical period mice treated with miR132 mimic. (a)** Cumulative distribution of ODS in nondeprived (n = 6 mice and 103 cells), MD3d (n = 5 mice and 83 cells), MD3d treated with miR-132 mimic (n = 5 mice and 79 cells), and MD3d treated with control miRNA (control miRNA) mice (n = 5 mice and 95 cells). **(b)** CBI of nondeprived (n = 6), MD3d (n = 5), MD3d miR-132 mimic (n = 5) and MD3d control miRNA (n = 5) mice. Open circles indicate data points from a single mouse; black circles indicate mean CBI s.e.m.

Indeed, the OD distribution of miR132 mimic treated mice was not different from that of nondeprived mice (**Fig. 15a**, MD3d miR132 mimic versus MD3d control miRNA and MD3d untreated, Kolmogorov-Smirnovtest, $P < 0.05$; nondeprived versus

MD3d control miRNA and MD3duntreated, Kolmogorov-Smirnov test, $P < 0.05$; other comparisons were not significantly different, $P > 0.05$). The analysis of the CBI gave similar results: MD3d mice or MD3d control miRNA showed a significant reduction of CBI with respect to mice infused with miR132 mimic or nondeprived mice. No difference was present between the CBI of mice infused with miR132 mimic and that of nondeprived mice (**Fig. 15b**, one-way ANOVA,$P < 0.001$; post hoc Holm-Sidak test, MD3d miR132 mimic versus MD3d control miRNA and MD3d untreated, $P < 0.05$; nondeprived versus MD3d and MD3d control miRNA, $P < 0.05$; other comparisons were not significantly different, $P > 0.05$). MiR132 mimic infusion did not influence general functional properties of visual cortical neurons (**Fig. 16a-d**).

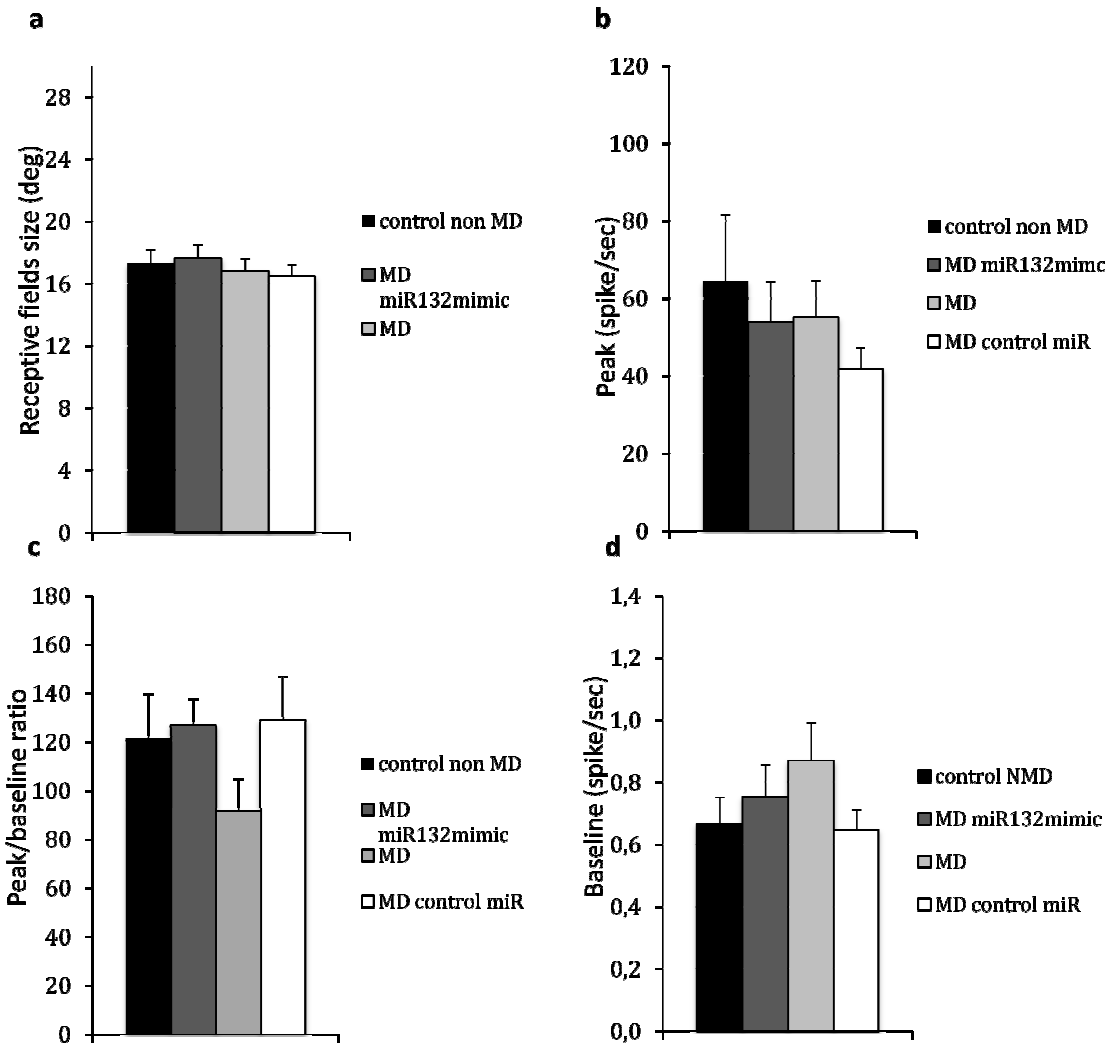


Figure 16. MiRNA mimic treatment does not affect functional properties of visual cortical neurons. **(a)** Receptive field size (RFS) was not altered by miR132 or control miR mimic treatments in MD3d critical periods mice (nondeprived 103 cells; MD3d miR132 mimic 79 cells; MD3d 83 cells; MD3d control miR 95 cells). None of the treatments significantly altered RFS (one way ANOVA p=0.733). Receptive fields were mesured on peri-stimulus time histograms elicited by light bars of optimal orientation drifting in the visual field. The width of the part of visual field eliciting cell discharge greater than spontaneus discharge plus two SDs was taken as RFS. **(b)** Peak was mesured on peri-stimulus time histograms as the maximum response of each cell, (nondeprived 103 cells; MD3d miR132 mimic 79 cells; MD3d 83 cells; MD3d control miR 95 cells. One way ANOVA p=0.16). **(c)** Cell responsiveness for each unit of the different groups (nondeprived 103 cells; MD3d miR132 mimic 79 cells; MD3d 83 cells; MD3d control miR 95 cells) was expressed as ratio between peak response frequency and baseline discharge frequency. No statistical difference is present between all groups (one way ANOVA on ranks p=0.1). **(d)** Baseline activity of each cells of the different groups was not affected by the miRNA mimic treatments (nondeprived 103 cells; MD3d miR132 mimic 79 cells; MD3d 83 cells; MD3d control miR 95 cells. One way ANOVA on ranks p= 0.071). Error bars represent SEM.

The lack of OD plasticity in miR132 mimic treated mice was confirmed also recording visual evoked potentials (VEPs) elicited by stimulation of each eye and recorded in the cortex contralateral to the deprived eye. The contralateral-to-ipsilateralratio of VEP response amplitude was significantly reduced by MD3d in untreated mice or in mice infused with control miRNA. By contrast, MD3d was ineffective at altering the contralateral-to-ipsilateral ratio in mice infused with miR-132 mimic. Indeed, their contralateral-to-ipsilateral ratio was not different from that of nondeprived animals (**Fig. 17**, one-way ANOVA,P = 0.002; post hoc Holm-Sidak test, MD3d miR132 mimic versus MD3d control miRNA and MD3d untreated, $P < 0.05$; nondeprived versus MD3d control miRNA and MD3d untreated, $P < 0.05$; other comparisons were not significantly different, $P > 0.05$).

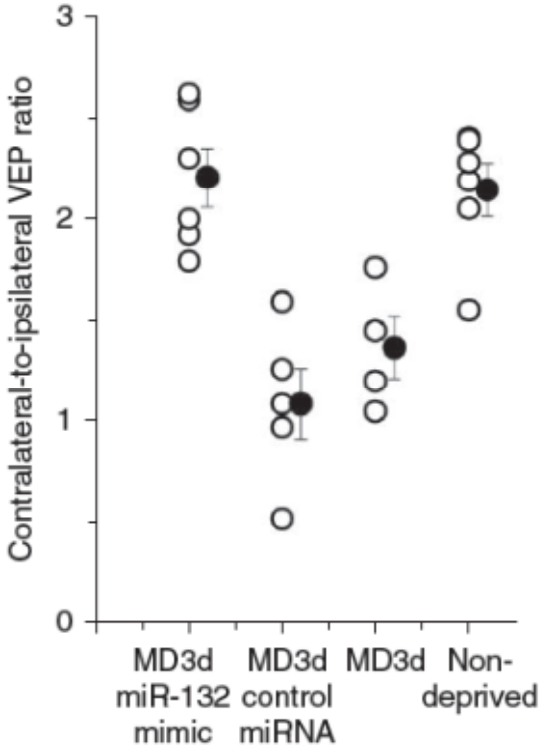


Figure 17. VEP recordings of MD3d juvenile mice treated with miR132 mimic. Contralateral-to-ipsilateral VEP ratio of nondeprived (n = 5), MD3d (n = 4), MD3d miR-132 mimic (n = 5) and MD3d control miRNA (n = 5) mice. Open circles indicate data points from a single mouse; black circles indicate average ratio ± s.e.m.

These data indicate that miR132 is a molecular transducer of theaction of visual experience on developing visual cortical circuits, possibly acting through modulation of dendritic spine plasticity.

Discussion

Recent evidences point to a widespread role for neural miRNAs at various stages of synaptic development, including dendritogenesis, synapse formation and maturation. However, knowledge about the role of the specific miRNAs in synaptic plasticity is still very limited. Our data show that visual experience dynamically regulates miR132 levels in the visual cortex and that the experience-dependent regulation of miR132 levels is required for OD plasticity. These data suggest that miR132 is a crucial molecular transducer of the action of visual experience on plasticity of developing visual cortical circuits.

1. Visual experience during the critical period induces epigenetic modifications on specific CRE loci on miR212/132 gene cluster.

Neurons change their gene expression program in response to electrical activity. These modifications are tightly regulated by molecular mechanism and are critical for experience-dependent plasticity in neuronal circuits. Among these mechanisms, the control of mRNA translation can account for a fine regulation of plasticity, also at the level of individual dendrites or spines. MicroRNAs are a recentely discovered, extensive class of small non-coding RNAs that act as post-transcriptional regulators of genes and represent good candidates in the field of specific and local control of gene expression pattern. MiR132 is an activity-dependent microRNA and *in vitro* studies showed that miR132 is induced by synaptic activation, such as BDNF or bicuculline treatment, through induction of ERK-MSK pathway and CREB (Remenyi, et al., 2010, Vo, et al., 2005, Wayman, et al., 2008).

Furthermore, electrical activity and LTP inducing stimuli increase miR132 expression *in vivo* (Nudelman, et al., 2010, Wibrand, et al., 2010). Experience-dependent molecular mechanisms are involved in mediating the action of visual experience on the development and plasticity of the visual cortex and ERK-CREB pathway is also activated by visual experience in visual cortical cells and its inhibition blocks OD plasticity (Cancedda, et al., 2003, Di Cristo, et al., 2001, Liao, et al., 2002, Mower, et al., 2002, Pham, et al., 2004, Pham, et al., 1999, Putignano, et al., 2007). Visual activation of this pathway was also found to be necessary for visually induced histone phosphoacetylation (Putignano, et al., 2007). Epigenetic modifications of histones are involved in the regulation of gene expression and subsequently they can influence plasticity phenomena. Indeed, it is well known that a specific gene expression pattern is essential for establishment of plasticity. Studies performed in the the visual system showed that the effects on transcription are specific for the different type of manipulation of visual experience and for the age at which they are performed (Majdan and Shatz, 2006). Here, we observed that visual experience results in histone methylation and phosphoacetylation, epigenetic marks correlated with active gene transcription, on a CRE locus important for miR132 transcription (Remenyi, et al., 2010). Indeed, the same protocol of visual manipulation, three days of dark rearing and re-exposure to light, causes a strong increase in pri-miR132 and in its mature form in the visual cortex of critical period mice.

Visual experience could regulate miR132 expression acting at transcriptional level, even if experience-dependent stabilization of miR132 (Krol, et al., 2010) could also contribute to set cortical miR132 levels. Indeed, our data show a stronger alteration in the level of the primary transcript with respect to the mature form of

miR132 in response to all the manipulation of visual experience studied. Moreover, during development the peak in the expression of mature form is at P30, five days after the peak of the pri-miR132. This delayed time may be due to the action of proteins important for miRNAs maturation control. Therefore, It would be very interesting to deeper investigate the regulation of biogenesis of this microRNA and the possible influences of visual experience on the different steps of this molecular pathway.

Previously it was demonstrated a developmental downregulation of the action of visual experience on the control of histone posttranslational modification. Light stimulation is no longer able to activate phosphoacetylation of histones and phosphorylation of CREB in the visual cortex of adult animals. TSA treatment, a deacetylase inhibitor that is known to increase the level of histones acetylation also in the brain (Korzus, et al., 2004, Levenson, et al., 2004), restores OD plasticity in adult mice (Putignano, et al., 2007). Moreover, HDAC inhibitors treatment promotes the recovery of visual acuity in amblyopic adult rats (Silingardi, et al., 2010). In our experiments, we observed a downregulation in the visual induction of pri-miR132 after dark rearing in the adults. Interestingly, TSA administration significantly increase pri-miR132 induction, albeit not at the levels of juvenile mice.Thus, histone acetylation could be an important component involved in the control of activity-dependent expression of miR132, suggesting that its induction could be implicated in critical period plasticity in the visual system. Considering the importance of histone posttranslational modifications in regulating visual cortical plasticity as well as other forms of plasticity, it is tempting to speculate that epigenetic regulation of miR132 expression could be a general feature of many forms of plasticity.

2. Visual experience regulates miR132 expression levels.

To further correlate miR132 expression to visual cortical development, we analyzed dark rearing from birth. This manipulation consists in the total absence of light stimuli from P0 and it dramatically alters visual system maturation causing a strong impairment in visual functions and synaptic plasticity. In standard reared animals both pri-miR132 and mature miR132 levels are strongly regulated during development. At P15 there is an increase in miR132 respect to P7, in coincidence with eye opening in agreement with activity-dependent expression of this miRNA. Interestingly, the maximum expression is at P25-30 during the critical period for OD plasticity. Before the opening of the critical period, synapses are immature and highly dynamic filopodia represent the prevalent form of spines. In this time-window miR132 expression is very low. We could speculate that miR132 increase after P15-P20 is implicated in the processes underlying synaptic stabilization and pruning occurring at maximal level during the critical period. Conversely, in DRB animals the levels of primary and mature miR132 remain very low, without any developmental change in expression possibly contributing to the abnormal maturation of visual cortical circuits in dark reared animals.

Previous findings showed that miR132 is required for activity-dependent dendritic growth and promotes activity-dependent increases in spine density and protrusion size by downregulating p250GAP expression and activating the Rac1-PAK pathway (Impey, et al., 2010, Vo, et al., 2005). Furthermore, overexpression of miR132 in hippocampal neurons increased the formation of stubby and mushrooms spine with high protrusion width and enhanced synaptic strength (Edbauer, et al., 2010). Conversely, loss-of- function of miR132 caused dendritic arbour pruning (Edbauer, et al., 2010), reduced spine formation and excitatory synaptic transmission

(Impey, et al., 2010), and reduced spine volume (Siegel, et al., 2009). *In vivo*, deletion of the miR132/212 locus in newborn neurons of the adult hippocampus decreases dendrite length, arborisation, and spine density (Magill, et al., 2010). Our results show a downregulation of mature miR132 after three days of monocular deprivation in the visual cortex contralateral to the deprived eye that receives the majority of inputs from this eye. Short MD during the critical period decreases dendritic spines stability and density (Mataga, et al., 2004, Oray, et al., 2004) mirroring the effect of miR132 loss-of-function experiments conducted on cultured neurons. We speculate that miR132 downregulation in MD could make dendritic spines less stable and might favour mechanism of synaptic plasticity resulting in depression of deprived eye responses. In situ hybridization experiment confirms the data obtained by qPCR showing a decrease in miR132 expression both in the monocular and the binocular zone of the visual cortex contralateral to the deprived eye. Interestingly, ISH reveals a trend of higher down-regulation in the monocular area that receives input exclusively from the deprived eye respect to the binocular area that has inputs also from the ipsilateral eye, suggesting that the activity of ipsilateral open eye might influence miR132 levels.

In addition, MD3d causes a reduction of H3 phosphoacetylation at all the CRE sites tested, suggesting that H3 acetylation could be an important epigenetic mark for fast, visually induced, upregulation of primary miR132, whereas reduced H3 phosphoacetylation could be associated with the effects of prolonged visual deprivation (three days) on primary miR132 transcription. Acetylation of histone H3 is not significantly changed by MD. Although, MD is not the reverse protocol of the 20 min of visual stimulation: the effects of MD were analyzed 3 days after eyelid suture. By contrast in the visual stimulation paradigm, histone marks are assessed after only

20 min of stimulation. Furthermore, the MD protocol affects differently the binocular cortex neurons dominated by the deprived eye, that reduce their input activity, with respect to the neurons dominated by the nondeprived eye that keep normal levels of input activity. In all these neurons, MD activates complex mechanisms of synapse specific potentiation and depression and homeostatic changes of excitability (Hensch, 2005, Hofer, et al., 2006) that could possibly involve different epigenetic mechanisms (Borrelli, et al., 2008, Graff, et al., 2011), whereas 20 minutes of light exposure cause a homogenous visual activation of all cortical neurons. These important differences between the acute visual stimulation protocol and the MD protocol could justify the differences in the epigenetic marks involved in transcriptional regulation. It is well known that different epigenetic marks can be activated, even at the same DNA sequence and even using the same stimuli, by acute or prolonged (chronic) treatments (Kumar, et al., 2005, Tsankova, et al., 2004). Our data indicate that histone phosphoacetylation is a common histone marks reduced by MD at all the CRE sites tested. Thus, a parsimonious interpretation of our data related to histone posttranslational modifications could be that visual stimulation triggers changes in histone marks consisting in H3 acetylation, and at least for CREmiR132, also by changes in phosphoacetylation and dimethylation. Histone acetylation could be the histone mark more directly correlated with the activation of primary miR132 transcription after this specific stimulus and at this time point. A three days monocular deprivation does not simply reverse this scenario, but specifically induces a downregulation of histone phosphoacetylation at the CRE sites analyzed. It could be intriguing if this specific histone posttranslational change could be a type of latent epigenetic modification, persistent for a long period after the three days of MD. We might speculate that phosphoacetylation of H3 could be a sort of

"molecular scar", in analogy with recent studies on addiction (Robison and Nestler, 2011), that might alter the individual susceptibility to future manipulation of visual experience. Therefore, a juvenile experience of MD could leave a lasting epigenetic trace in the altered cortical connection that could make the cortex more responsive to a second monocular deprivation, later during the adult age (Hofer, et al., 2006).

3. Mir132 downregulation during MD is necessary for OD plasticity.

Monocular deprivation, an easy manipulation of visual experience, is the classic paradigm used to assess ocular dominance plasticity. To deeper investigate the role of miR132 in visual cortical functions, we asked if its downregulation during MD could be necessary for OD plasticity. To answer this question we counteracted miR132 decrease in the cortex contralateral to the deprived eye by infusing chemically modified double strand miR132mimic. The treatment was obtained by using osmotic minipumps that allow a controlled release and a homogenous diffusion of miR132mimic in the visual cortex. Three days of miR132mimic treatment are able to perfectly restore the levels of miR132 in the cortex contralateral to the deprived eye to the levels of the ipsilateral one. Furthermore, miR132mimic is able to downregulate MeCP2 protein expression, an *in vitro* validated target of miR132. MiR132 is involved in regulation of spines morphology both *in vitro* and *in vivo*(Edbauer, et al., 2010, Hansen, et al., 2010). Cultured hippocampal neurons overexpressing miR132 are characterized by stubby and mushroom spines and show increased protrusion width. In addition, average protusion density significantly fell. This effects on spines morphology required miR132 interaction with FMRP (Edbauer, et al., 2010). Transgenic mice overexpressing miR132 show an increase in spine density in CA1 neuronal dendrites,

suggesting miR132 modulates neuronal structures features associated with synaptic communication (Hansen, et al., 2010). Three days of miR132mimic infusion in the visual cortex causes a significant increase in the fraction of mushroom/stubby spines, which represent a type of mature, less plastic spines.

Finally, we assessed visual cortical plasticity by *in vivo* electrophysiology experiments. Strikingly, critical period mice MD for three days and contemporary treated with miR132mimic in the visual cortex contralateral to the deprived eye do not show OD plasticity, indeed CBI and VEP ratio are the same of nondeprived control mice. Therefore, miR132 downregulation during MD plays an important functional role, suggesting this microRNA acts as a molecular transducer of experience-dependent plasticity.

Concluding remarks

Our study, for the first time, provides evidence for the role of miR132 as mediator of plasticity of neuronal responses in a classical *in vivo* model of developmental experience-dependent plasticity (Tognini and Pizzorusso, 2012, Tognini, et al., 2011). Our work is accompanied by a parallel study that confirmed visual experience dynamically regulates miR132 levels in the visual cortex (Mellios, et al., 2011). In this study, miR132 levels in the cortex were reduced by the injection of a miR132competitive inhibitor (sponge)–expressing lentivirus that predominantly infects neurons and results in specific 'sequestering' of endogenous miR132. They measured the expression of p250GAP, a GTPase that is targeted by miR132 and is known to affect spine growth and morphology (Vo, et al., 2005, Wayman, et al., 2008), and examined structural changes in transfected pyramidal neurons. They found increased p250GAP and reduced spine density in pyramidal neurons.

Examination of the changes in spine morphology revealed that miR132 inhibition resulted in a reduction of the percentage of mushroom spines and an increase in the proportion of filopodia. Two- photon calcium imaging demonstrated that *in vivo* miR132 inhibition disrupted the ocular dominance shift that was normally present after a short MD in juvenile mice (Mellios, et al., 2011).

Our data and that from Sur's laboratory show that an optimal concentration of miR132 is necessary for plasticity during the critical period. Interestingly, miR132mimic treatment increased the fraction of mushroom/stubby dendritic spines representing the mature, more stable form of spines (Tognini et al., 2011). Conversely, miR132 inhibition resulted in more immature spine, mainly increasing the percentage of filopodia (Mellios et al., 2011). Taken together, these two results suggest that the miR132 decrease induced by MD could be necessary to make dendritic spines less stable, allowing the occurrence of the structural plasticity mechanisms underlying OD plasticity, whereas the strong miR132 reduction obtained by viral transduction would make spines too unstable to consolidate OD plasticity (**Fig. 18**).

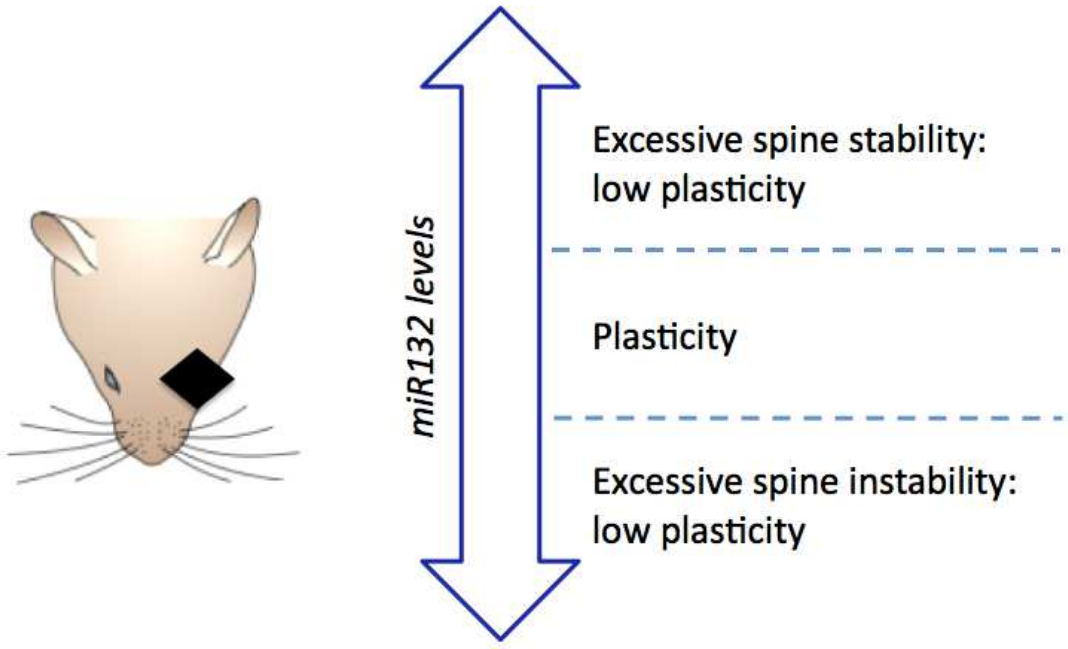


Figure 18. Possible model of miR132 role in visual cortical plasticity. OD plasticity induced by MD requires an optimal miR132concentration allowing dendritic spines plasticity processes (adapted from Tognini and Pizzorusso, 2011)

An important question is: Which target proteins mediate the effect of miR132 on OD plasticity? In the central nervous system the best candidates proteins are MeCP2 and p250GAP. MeCP2 is a DNA methyl binding protein implicated in transcriptional regulation, and its gene mutations cause Rett Syndrome, a form of autism characterized by dendritic spines abnormalities. P250GAP is a Rho family GTPase activating protein. Previously, It was demonstrated that p250GAP is a bona fide miR132 target (Vo, et al., 2005, Wayman, et al., 2008) and it was shown that this protein is enriched in the post-synaptic density (Nakazawa, et al., 2003, Okabe, et al., 2003). P250GAP interacts with multiple synaptic proteins that are effectors of synaptic plasticity including the NMDA NR2B receptor subunit, the scaffold protein PSD-95 (Nakazawa, et al., 2003, Okabe, et al., 2003), the NR2B kinase Fyn (Taniguchi, et al., 2003), and the signaling intermediate beta-catenin (Okabe, et al., 2003). P250GAP activity is also regulated by CaM kinase II phosphorylation and its localization at the post-synaptic density may be regulated by NMDA receptor signaling (Nakazawa, et al., 2003). P250GAP is an inhibitor of Rho family GTPases which play a critical role in the regulation of spine structure via their ability to regulate actin dynamics (Van Aelst and Cline, 2004). It was proposed that miR132 regulates spine formation in hippocampal neurons by downregulating p250GAP and activating the Rac1-PAK actin remodeling pathway (Impey, et al., 2010). Therefore, we can speculate that regulation of p250GAP expression could be a mechanism by which miR132 modulates structural plasticity at the synapse, in particular dynamic changes in spines associated with OD plasticity.

Recently, a genetic cell-type based analysis of microRNAs profiles in the mouse brain demonstrated that miR132 is predominantly expressed in excitatory neurons and in somatostatin interneurons, innervating the more distal dendrites and controlling the input and plasticity of pyramidal neurons (He, et al., 2012). Thus, we can speculate the effect of miR132 on neuronal plasticity could be due not only to an action on dendritic spines dynamic but also to a modulation of molecular pathway important for inhibitory circuits function.

It is worth noting the possible involvment of miR132 in neurodevelopmental disorders. Indeed, a well-known miR132 target protein, MeCP2, is mutated in Rett syndrome, an X-linked neurodevelopmental disease that primarily occurs in female. Furthermore, miR132 levels are altered in models of Rett syndrome (Klein, et al., 2007) and MeCP2 KO mice display abnormalities in dendritic spines density and morphology (Boggio, et al., 2010). Interestingly, miR132 action on spine morphology involves its interaction with FMRP, a protein acting as translational repressor of specific mRNAs (Edbauer, et al., 2010). Transcriptional silencing of FMRP expression causes Fragile X syndrome (FXS), the most common inherited cause of mental retardation. Strykingly, in both human patients and mouse model of FXS, cortical neurons have an excess of dendritic spines, as well as thin, long and immature spines (Ronesi and Huber, 2008). Thus, we are tempting to speculate a possible role of miR132 as molecular transducer of plasticity in neuronal circuits, acting through modulation of dendrtic spines dynamics and maturation. Moreover, our observation that experience-dependent fine tuning of miR132 levels is required for activity-dependent plasticity of brain circuits during critical periods of development raises the possibility that alterations of miR132 biogenesis, function and decay could play an important role in the pathogenesis of these neurodevelopmental disorders.

Clinical investigations showed expression changes in miR132 in psychiatric disorders, Alzheimer's disease (AD) and autism spectrum disorders (ASD) (Abu-Elneel, et al., 2008, Cogswell, et al., 2008, Kim, et al., 2010, Talebizadeh, et al., 2008). MiRNAs expression profiling in the prefrontal cortex of individuals affected with schizophrenia reveals miR212 and miR132 alterations. Interestingly, authors found a negative correlation between the expression of these miRNAs and their predicted gene target, tyrosine hydroxylase and phosphogluconate dehydrogenase (Kim, et al., 2010). Intriguingly, a very recent publication demonstrated that miR132 is significantly down-regulated in the dordolateral prefrontal cortex (PFC) of schizophrenic subjects with a parallel increase in target genes such as p250GAP, GATA2, DNMT3A and PDE7B. Moreover, the authors show that miR132 expression in the murine PFC exhibits significant developmental regulation and overlaps with critical neurodevelopmental processes during adolescence in analogy with our observations in the visual cortex. Consistent with NMDA-mediated hypofunction observed in schizophrenic subjects, adult prefrontal expression of miR132 can be down-regulated by pharmacologic inhibition of NMDA receptor signaling during a brief postnatal period. These data suggest miR132 dysregulation and subsequent abnormal expression of miR132 target genes could contribute to the neurodevelopmental and neuromorphological pathologies present in schizophrenia (Miller, et al., 2012)

The role of miRNAs in neurodegenerative diseases such as AD is little understood and is still at its beginning. A recent clinical study showed decreased levels of miR132 and miR212 in the hippocampus and medial frontal gyrus, two regions primarily affected by AD pathology, in Alzheimer's patients' post-mortem brain. MiR132 misregulated expression in these areas suggests deficits in neuronal

differentiation in the earliest stage of AD (Cogswell, et al., 2008). Another analysis of miRNA expression profiles from sporadic progressive supranuclear palsypatients, a specific form of tauopathy, showed that miR132 is specifically down-regulated in this disease (Smith, et al., 2011).

Dysregulation of miR132 expression was observed in postmortem cerebellar cortex of individuals with ASD (Abu-Elneel, et al., 2008) and in lymphoblastoid cell lines obtained from autistic children (Talebizadeh, et al., 2008).

Psychiatric, neurodegenerative and autism spectrum disorders in humans are associated with alteration in neuronal connectivity and synaptic plasticity, thus misregulated expression of miR132 may be explain some pathological tracts of these diseases. Although the exact functional role of experience-dependent changes in miR132 expression has not been explored, it is possible that miR132 acts as a general regulator of experience-dependent plasticity in multiple brain regions. Therefore, miR132 could become an attractive candidate for uncovering novel molecular pathways implicated in neurological diseases and possibly develop therapeutic strategies.

Appendix

"Environmental enrichment promotes plasticity and visual acuity recovery in adult monocular amblyopic rats."

Abstract

Loss of visual acuity caused by abnormal visual experience during development (amblyopia) is an untreatable pathology in adults. In some occasions, amblyopic patients loose vision in their better eye owing to accidents or illnesses. While this condition is relevant both for its clinical importance and because it represents a case in which binocular interactions in the visual cortex are suppressed, it has scarcely been studied in animal models. We investigated whether exposure to environmental enrichment (EE) is effective in triggering recovery of vision in adult amblyopic rats rendered monocular by optic nerve dissection in their normal eye. By employing both electrophysiological and behavioral assessments, we found a full recovery of visual acuity in enriched rats compared to controls reared in standard conditions. Moreover, we report that EE modulates the expression of GAD67 and BDNF. The non invasive nature of EE renders this paradigm promising for amblyopia therapy in adult monocular people.

Key words: amblyopia; environmental enrichment; optic nerve dissection; GAD67; BDNF.

Introduction

Amblyopia (also called lazy eye) is a pathological reduction in visual perceptual abilities due to a defective sensory experience during development. This condition, which has a prevalence of about 3% of the total world population (Holmes and Clarke, 2006), derives from a functional imbalance between the two eyes, typically caused by unilateral congenital cataract, anisometropia or strabismus, resulting in an ocular dominance shift toward the normal eye in the primary visual cortex and a prominent loss of visual acuity (VA) in the affected eye (Levi and Li, 2009, Lewis and Maurer, 2005). In animal models, amblyopia can be artificially induced by imposing a long-term reduction of inputs from one eye by lid suture (monocular deprivation, MD) (He, et al., 2007, Mitchell and MacKinnon, 2002, Pizzorusso, et al., 2006, Sale, et al., 2007). While VA and ocular dominance can recover to normal if a normal visual experience is restored early in development during the so-called critical period, no recovery is instead observed in the adult, due to a dramatic reduction of plasticity levels which occurs in the visual cortex at the end of the critical period (Hensch, 2005, Sale, et al., 2009).

One case of particular interest is that of amblyopic patients who loose their better eye, thus becoming severely visually impaired. While it has been reported that adult amblyopes can occasionally improve VA following loss of vision in the fellow eye either due to a sudden accident or to progressive development of ocular illnesses (El Mallah, et al., 2000, Fronius, et al., 2005, Hamed, 1991, Vereecken and Brabant, 1984, Wilson, 1992), which factors promote improvement under these circumstances are still totally unknown. Besides its clinical importance, this condition is also highly relevant from a basic research perspective, representing a

useful model to investigate visual cortex plasticity when binocular competition is completely suppressed. Starting from the pioneering work by Hubel and Wiesel and, subsequently, as demonstrated by Stryker and colleagues, it has been shown that visual cortical plasticity operates through a competitive interaction between inputs from the two eyes (Antonini and Stryker, 1998, Chapman, et al., 1986, Gordon and Stryker, 1996, Wiesel and Hubel, 1965). In contrast, much less evidence has been reported in favor of experience-dependent plasticity processes in the total absence of binocular interactions between the two eyes. Experiments conducted over thirty years ago on monocularly deprived cats showed that the effects of MD on cat striate cortex cells can be partially reversed after the critical period by enucleation of the experienced eye (Smith, et al., 1978). However, whether VA recovery is possible under these conditions was never investigated.

We recently reported that environmental enrichment (EE), a milieu characterized by increased social interactions, sensory-motor activity and exploratory behavior, is highly effective in enhancing plasticity in the primary visual cortex of adult amblyopic rats, promoting a full recovery of VA and ocular dominance, accompanied by a marked reduction of intracortical GABAergic inhibition (Sale, et al., 2007). Since EE is a totally non-invasive procedure with a high potential to be applied to human patients, here we asked whether exposure to EE could also be employed to trigger recovery of vision in adult amblyopic rats rendered monocular by means of optic nerve dissection (OND) in their normal eye.

Materials and methods

1. Animal treatment and surgical procedures

All experiments were performed on Long-Hevans hooded rats in accordance with the Italian Ministry of Public Health guidelines for care and use of laboratory animals. Specifically, the experiments described in this study were authorized by the Italian Ministry of Health via decree # 185/2009-B, released on November 4, 2009. All experiments respected the welfare of animals and have been conducted strictly according to the legal and ethical requirements demanded by law. The Institute of Neuroscience CNR has been authorized by the Italian Ministry of Public Health to the use of animals for scientific purposes (authorization # 129/2000-A, December 13, 2000). The lowest degree of neuropsychological sensitivity, pain, suffering and distress have been ensured by the employment of approved methods of anaesthesia and animal handling and by the application of specific anti-pain drugs whenever required. The experimental activity was guided by the principle of the Three R's (Replacement, Reduction and Refinement).

Rats lived in an animal house with a temperature of 21°C, 12/12 light/dark cycle and food and water available ad libitum. To assess the effects of long term monocular deprivation (MD), postnatal day (P) 21 rats were anesthetized with avertin (1 ml/hg) and monocularly deprived through eyelid suturing until adult age (P60). Rats showing occasional lid reopening (observed with a surgical microscope) were not included in the experiments. The long-term deprived eye of adult amblyopic rats was re-opened using thin scissors, under anaesthesia, while the optic nerve of the other eye was dissected mechanically with thin surgical forceps. The crush was performed intracranially, 3 mm from the posterior pole of the eye; special

attention was taken to avoid mechanical damage of the retinal artery. Great care was taken during the first days after surgery to prevent inflammation or infection in the previously deprived eye through topical application of antibiotic and cortisone.

After OND, the rats were divided into two groups. A group was reared in standard laboratory cages (30 x 40 x 20 cm, standard condition SC, OND-SC) housing each 2 rats, and a second group of animals was reared in environmental enrichment (EE, OND-EE). For comparison, a further group of untreated rats reared in SC (naïve animals) was also included in the analysis. Enriched environment consisted of large wire netting cages (60 x 50 x 80 cm) with three floors containing several food hoppers, running wheels to allow physical activity, and differently shaped objects (tunnels, shelters, stairs) that were completely substituted with others once a week. Every cage housed 6-8 rats.

After three weeks of EE or SC, the VA of the re-opened eye was analyzed by *in vivo* electrophysiology.

2. In vivo electrophysiology

The animals (OND-SC, n = 6; OND-EE, n = 6; naïve, n = 8) were anesthetized with urethane (0.7 ml/hg; 20% solution in saline) by i.p. injection and placed in a stereotaxic apparatus. Body temperature was continuously monitored and maintained at ~37°C by a thermostated electric blanket during the experiment. An ECG was continuously monitored. A hole was drilled in the skull, corresponding to the binocular portion of the primary visual cortex (binocular area Oc1B) contralateral to the previously deprived eye. After exposure of the brain surface, the dura was removed, and a micropipette (2 MΩ) filled with NaCl (3 M) was inserted into the cortex 5 mm from λ. The re-opened eye was fixed and kept open by means of adjustable metal rings surrounding the external portion of the eye bulb. We

measured VA using visual evoked potentials (VEPs). To record VEPs, the electrode was advanced at a depth of 100 or 400 μm within the cortex. At these depths, VEPs had their maximal amplitude. Signals were band-pass-filtered (0.1-100 Hz), amplified, and fed to a computer for analysis, as described previously (Huang, et al., 1999). Briefly, at least 128 events were averaged 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 positive or negative component. Visual stimuli were horizontal sinusoidal gratings of different spatial frequencies and contrast, generated by a VSG2/2 card running custom software and presented on a monitor (20 x 22 cm; luminance 15 cd/m2) positioned 20 cm from the rat's eyes and centred on the previously determined receptive fields. Visual acuity was obtained by extrapolation to zero amplitude of the linear regression through the last four to five data points in a curve where VEP amplitude is plotted against log spatial frequency.

3. Behavioral assessment of visual acuity

We measured VA of the fellow (not deprived) eye in long-term monocularly deprived rats at P50-P60, before performing OND. Next, we measured VA of the formerly deprived eye (long-term deprived), after OND and three weeks of SC (n = 3) or EE (n = 4) rearing. Therefore, VA measurement of the formerly deprived eye was completed when the animals were about P90-100. To measure VA, we used the visual water box task (Prusky, et al., 2000), which trains animals to first distinguish a low (0.1 cycles per degree; c/deg) spatial frequency vertical grating from grey, and then tests the limit of this ability at higher spatial frequencies. 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 spatial frequency. 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 spatial frequency of the grating. Visual acuity has been taken as the spatial frequency corresponding to 70% of correct choices on the sigmoidal function fitting the psychometric function. During each session, the experimenter was blind to the experimental group.

4. Immunohistochemistry

Animals (BDNF staining: OND-SC, n = 6, OND-EE, n = 7; GAD67 staining: OND-SC, n = 4, OND-EE, n = 8) were deeply anesthetized with chloral hydrate and perfused transcardially with PBS followed by fixative (4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.4, PB). Brains were gently removed, post-fixed for 4-6 hours before being cryoprotected by immersion in 30% sucrose. Coronal sections (50 µm) from the cortex were cut on a microtome and collected in PBS.

BDNF staining. Free-floating sections were incubated for 1 hour in a blocking solution (10% BSA, 0.3 % Triton X-100, in PBS, pH 7.4). Sections were incubated overnight at 4°C in a solution of chicken polyclonal anti-BDNF antibody (1:400, Promega). Primary antibody was revealed with biotinylated donkey anti-chicken (1:200, Promega) followed by fluorescein-conjugated extravidin (1:300, Sigma).

Sections of the two experimental groups were reacted together within the same immunohistochemistry session. Images were acquired at 20x magnification (NA = 0.7; field 707 x 707 µm acquired at 1024 x 1024 pixels) using a confocal Leica microscope and imported to the image analysis software MetaMorph to analyse the number of BDNF positive cells. To compare different specimens, the parameters of acquisition (laser intensity, gain, offset) were optimized at the start and then held constant throughout image acquisition. Counts were done on the entire thickness of Oc1B and normalized to the Oc1B area (mm2). For each animal, at least five Oc1B sections were analyzed.

GAD67 staining. Free-floating sections were incubated for 1-2 hour in a blocking solution (10% BSA, 0.3 % Triton X-100, in PBS, pH 7.4). Sections were then incubated overnight at 4°C in a solution of mouse monoclonal anti-GAD67 antibody (1:1000, Chemicon MAB5406), 1% BSA, 0,3% Triton X-100 in PBS 1x. Primary antibody was revealed with Alexa 568 antimouse (1:400, Molecular Probes, Invitrogen). Images were acquired and analyzed with the same method described for BDNF staining.

Results

Rats were deprived at P21 and raised under standard conditions until adulthood. At this time, the deprived eye was reopened and rats rendered monocular by OND of their fellow eye. Then, the animals were transferred to either standard conditions (OND-SC) or to EE (OND-EE), for three weeks. At the end of the differential rearing period, VA of the amblyopic eye was assessed using in vivo electrophysiological recordings of VEPs from the visual cortex contralateral to the long-term deprived eye. We found that the VA of OND-EE rats (0.93 ± 0.03 c/deg) was significantly higher than that of OND-SC rats (0.65 ± 0.03 c/deg) and was not different from that of untreated naïve animals (0.90 ± 0.02 c/deg) (**Fig. 1a, b**).

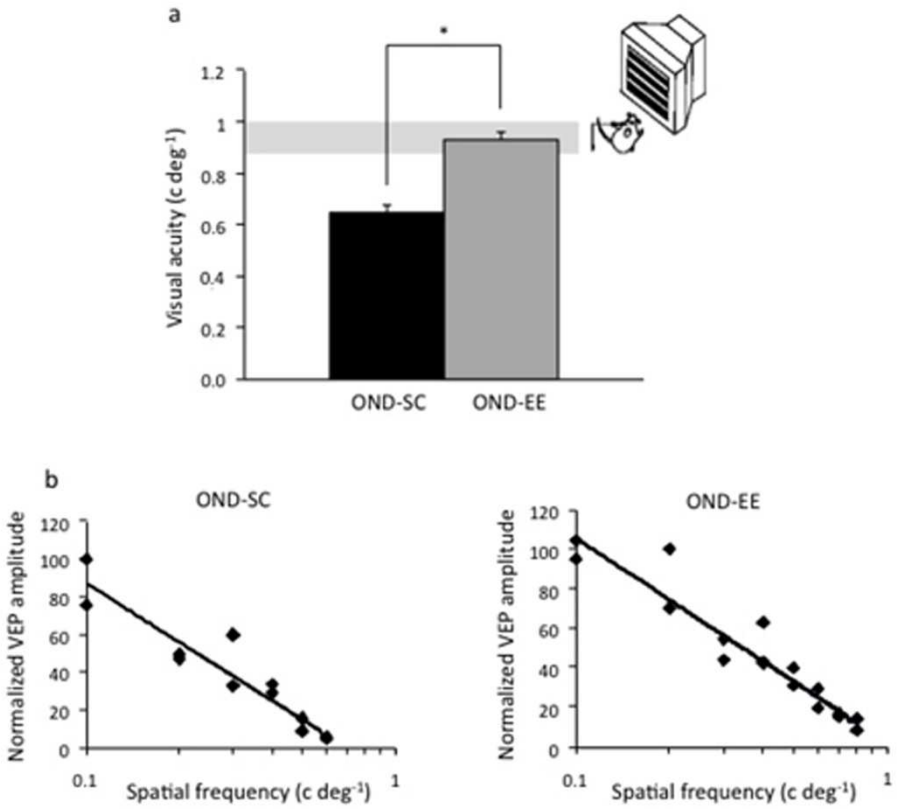


Fig. 1. Environmental enrichment promotes recovery of visual acuity in adult amblyopic monocular rats. a) Electrophysiological assessment of visual acuity (VA) showed a significant difference between OND-EE rats and OND-SC animals and between OND-SC rats and untreated controls (grey shadow in the graph), but not between untreated controls and OND-EE rats (One Way ANOVA $p < 0.001$, post-hoc Holm-Sidak test OND-SC vs. OND-EE and untreated controls $p < 0.05$, other comparisons were not significantly different). Insert shows a schematic representation of the apparatus used for VEP recording. **b)** Representative examples of VA estimates for the previously deprived eye in OND-SC and OND-EE animals. Percentage of normalized VEP amplitude is plotted against log spatial frequency. Visual acuity 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 spatial frequency. *, statistical significance; error bars represent s.e.m.

In amblyopia, recovery of a proper cellular response to visual stimuli in the primary visual cortex is useless if such effect does not transfer to behavioral perceptual abilities. Therefore, we repeated VA assessments also by using a standard behavioral task, the visual water-box task (Cancedda, et al., 2004, Prusky, et al., 2000). The behavioral measure of VA completely confirmed the electrophysiological data: a marked recovery was evident in OND-EE animals (0.87 ± 0.05 c/deg) with respect to OND-SC rats (0.60 ± 0.01 c/deg) (Fig. 2a, b).

Previous studies demonstrated that the maturation of inhibitory circuits (Hensch, 2005) is one of the most important factor responsible for the closure of critical period plasticity in the visual system and that EE can restore plasticity in the adult brain through a reduction of intracortical inhibition (Sale, et al., 2007). To assess whether the restored plasticity detected after three weeks of EE in OND amblyopic rats was accompanied by a change in the GABAergic system, glutamic acid decarboxylase 67 (GAD67) protein levels were measured in the visual cortex of OND-EE and OND-SC animals by using immunohistochemistry. The number of GAD67-positive cells normalized to the area of Oc1B (GAD67+) was significantly decreased in OND-EE animals compared to OND-SC rats (OND-SC GAD67+ = 123.81 ± 4.41 cells/mm2; OND-EE GAD67+ = 109.16 ± 5.09 cells/mm2; Fig. 3a).

Finally, we investigated whether exposure to EE also resulted in an increase of the neurotrophin BDNF, which is known to be involved in the development and plasticity of the visual system (Berardi, et al., 2003) and which has also been linked to visual acuity recovery after deprivation (Kaneko, et al., 2008, Maya Vetencourt, et al., 2008, Sale, et al., 2007). Immunoistochemistry analysis revealed a significant increase in BDNF positive cells normalized to the area of Oc1B (BDNF+) in the visual cortex of OND-EE animals compared to OND-SC rats (OND-SC BDNF+ = 216.2 ± 24.99 cells/mm2; OND-EE BDNF+ = 429.22 ± 70.09 cells/mm2; Fig. 3b).

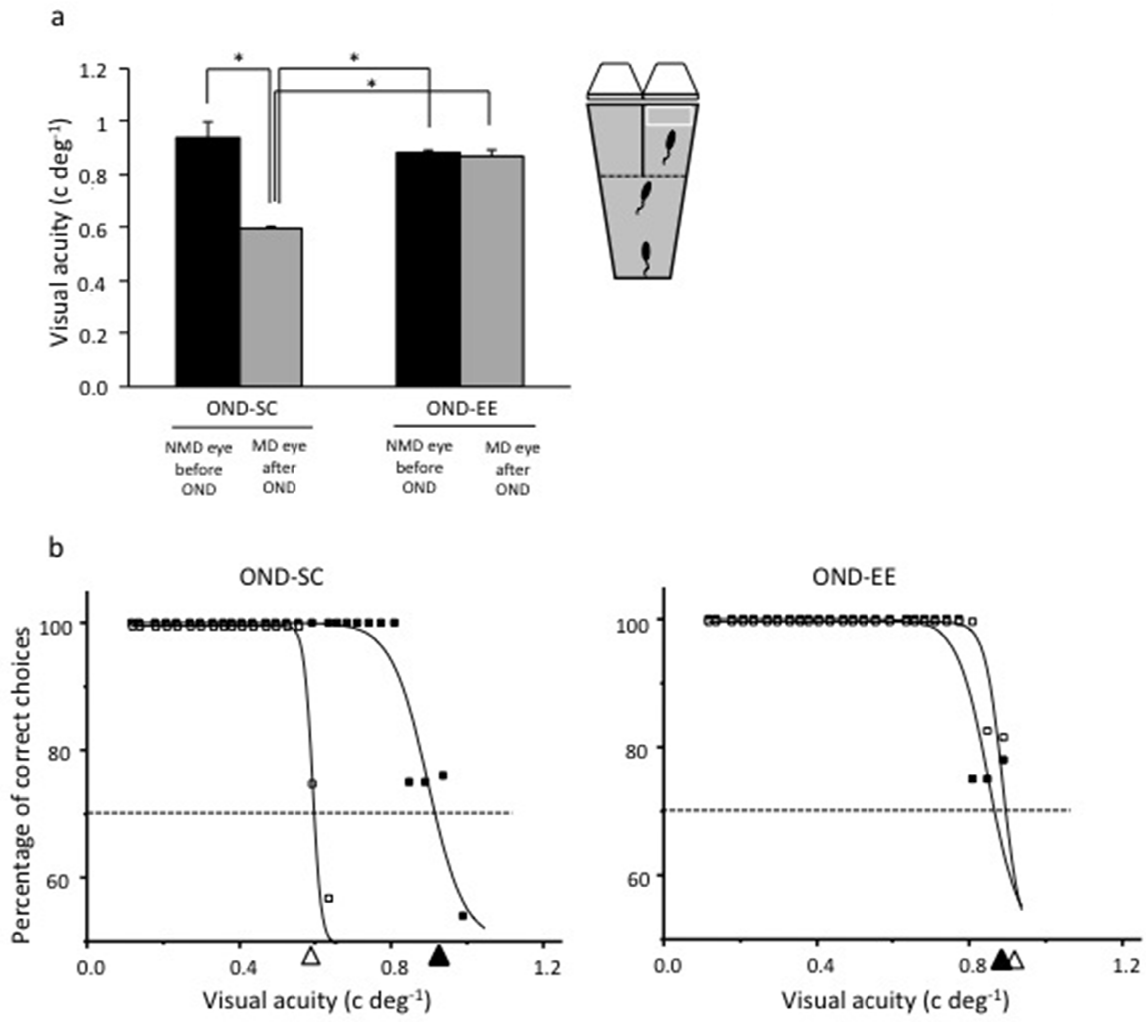


Fig. 2. Environmental enrichment promotes recovery of behavioural perceptual abilities in adult amblyopic monocular animals. a) Statistical analysis showed that the visual acuity (VA) of the previously deprived eye was not different from that of the other eye (fellow eye) in OND-EE rats (paired t-test, p = 0.51); a statistical difference was instead present between the VA of the previously

deprived eye and that of the fellow eye in OND-SC animals (paired t-test, $p < 0.05$). Insert shows a schematic representation of the visual water-box task, the apparatus used for behavioral assessment of visual acuity. **b)** Representative examples of behavioral VA estimates for both the previously deprived and the fellow eye in OND-SC (left) and OND-EE (right) animals. Visual acuity 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 zero spatial frequency. *, statistical significance; error bars represent s.e.m.

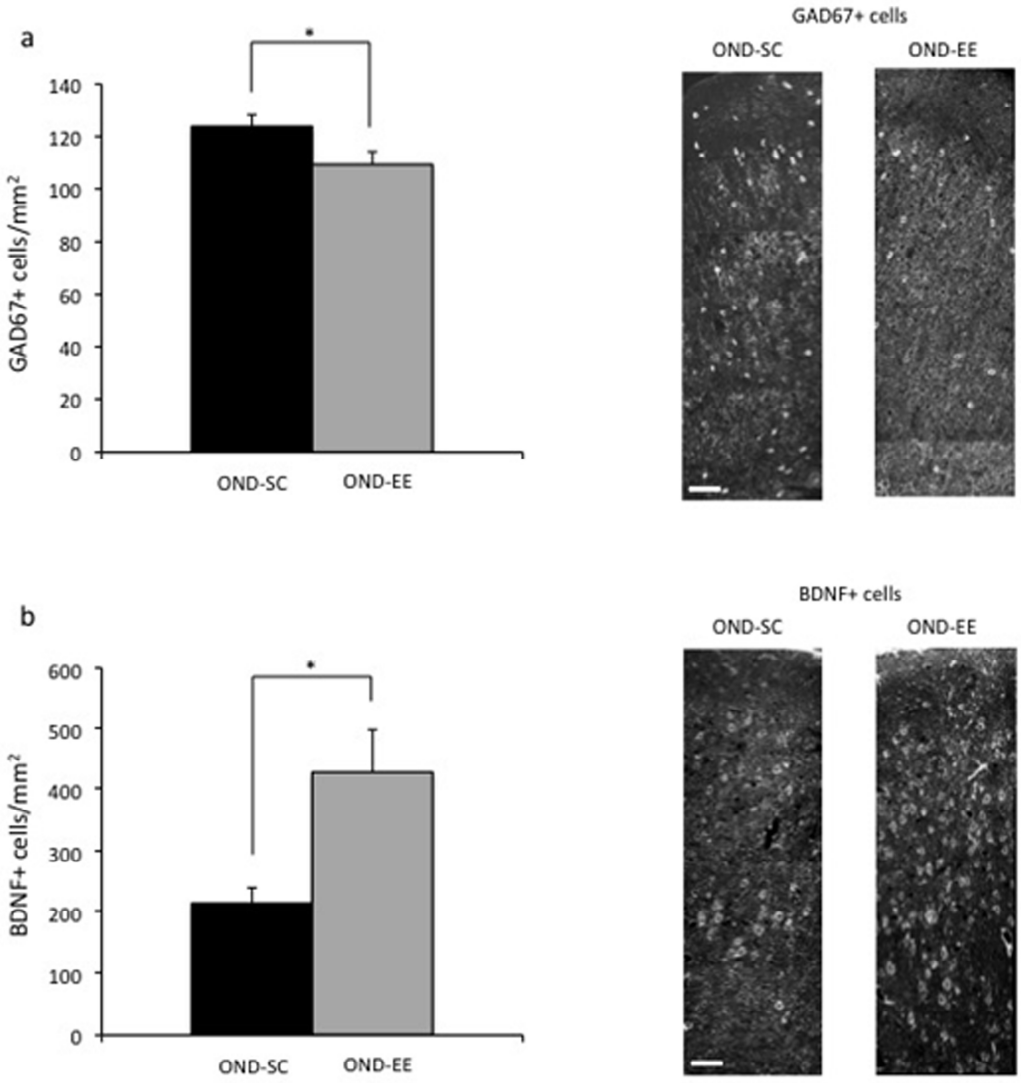


Fig. 3. Environmental enrichment induces changes of plasticity factors in the visual cortex of adult amblyopic monocular rats. a) EE decreases GAD67 expression in the visual cortex contralateral to the previously deprived eye. The number of GAD67+ cells was statistically lower in the visual cortex of OND-EE with respect to OND-SC rats (t-test, $p < 0.001$). **b)** EE increases BDNF expression in the visual cortex contralateral to the previously deprived eye. The number of BDNF+ cells was statistically higher in the visual cortex of OND-EE with respect to OND-SC rats (Mann-Whitney Rank sum test, $p = 0.035$). *, statistical significance; calibration bar = 40 μm; error bars represent s.e.m.

Discussion

In the present work we addressed the possibility to rescue VA in amblyopic adult rats exposed to EE immediately after silencing of retino-thalamic projections of the fellow (non amblyopic) eye due to OND. We observed that, while no spontaneous rescue of the VA impairment was detected in rats reared under standard environmental conditions, a full recovery of VA in the amblyopic eye was achieved in monocular adult rats after three weeks of EE. Moreover, we also showed that this effect is accompanied by modulation of two classic factors critically involved in visual cortex plasticity, BDNF and the GABAergic system. The lower number of GAD67+ cells after exposure to EE indicates an overall reduction of GABAergic inhibition in the visual cortex of OND-EE rats, in agreement with our previous results showing a reduced GABA release in the visual cortex of enriched amblyopic animals (Sale, et al., 2007). It is instead very unlikely that this effect is due to a change in the total number of inhibitory neurons, as this would imply increased cell death rates induced by EE.

The possibility to induce plasticity in the visual cortex of adult amblyopic monocular rats is intriguing since after OND of the fellow eye binocular interactions in the visual cortex are suppressed. According to a competition-based model, the stronger inputs from the fellow eye could mask activity in the weaker connections from the amblyopic eye. Thus, the connections from the long-term deprived eye may be suppressed rather than completely eliminated, making it possible to unmask them after loss of the fellow eye.

Plasticity in monocular amblyopic rats is also consistent with the predictions of BCM theory, proposed by Bienenstock, Cooper and Munro (Bienenstock, et al., 1982). In contrast to competition-based models, the BCM model introduces a

homosynaptic-learning rule. Therefore, a change in synaptic strength in one input upon a cell may occur without alteration in another set. The model also incorporates a sliding "modification threshold" for synaptic modification that varies depending on the average postsynaptic activity over time. Synaptic strengthening occurs when presynaptic activity causes postsynaptic activity to exceed the modification threshold. According to the BCM theory, inputs from the two eyes interact through changes in the average activity of the postsynaptic cell (Mitchell, et al., 2001). In our model, OND of the fellow eye can lead to a strong reduction of postsynaptic cortical activity, which would prevent recovery from amblyopia in monocular animals reared in standard conditions. In contrast, under EE conditions, the complex sensory stimulation exerted by this experimental protocol may cause an increment in the firing rate of the presynaptic thalamo-cortical neurons, leading to enhanced stimulation of postsynaptic cells in V1. Therefore, the modification threshold may be exceeded and, consequently, the strengthening of the synapses may favour recovery of normal visual functions. This may also explain the intriguing report that some adult amblyopic patients spontaneously improve vision in their amblyopic eye after loss of vision in the fellow eye. Humans, indeed, are certainly much more 'enriched' in terms of visual stimulation than rodents, which are nocturnal animals mostly relying to other senses, such as olfaction and touch.

The concept by which an increased sensory stimulation is necessary to trigger vision recovery in the long-term deprived eye is somewhat in contrast with previous findings showing an equal amount of increase in the percent of cells responsive to the deprived eye both in kittens which had the experienced eye enucleated at four months of age while the deprived eye remained closed and in kittens in which the previously deprived eye was opened for an additional three to four months (Smith, et

al., 1978). However, it is possible that visual acuity recovery, differently from the pure number of neurons responsive to the deprived eye, which seems not to be affected by visual experience, is a process that requires the additional amounts of sensory stimulation provided by living in an enriched environment.

Finally, it is worth analysing our results with reference to previous data obtained by dr. M. Caleo and colleagues, which showed a strong contribution of callosal connections to visual cortex plasticity (Restani, et al., 2009). These authors demonstrated that, in rats monocularly deprived during the critical period, a continuous silencing of callosal inputs originating from the visual cortex contralateral to the open eye throughout the period of MD attenuates the OD shift, selectively enhancing deprived eye responses with no effect on the open eye-driven activity. A similar process may also have an effect in the experimental model analysed in the present paper. Indeed, our procedure of OND may lead to a robust reduction in the activation of the visual cortex contralateral to the open eye, leading to enhanced neuronal responses to the stimulation of the long-term deprived eye. This would facilitate recovery in V1, an effect consistent with the partial visual function improvements occasionally observed in amblyopic monocular humans. However, since it has been demonstrated that silencing of callosal inputs increases the response of visual cortical neurons only in a low-spatial frequency range, without any impact on visual acuity (Restani, et al., 2009), the contribution given by reduction of callosal input strength prompted by OND on visual acuity recovery might be negligible.

Conclusions

Amblyopic patients severely visually impaired in their non-amblyopic eye become nearly blind. Our results suggest that increasing levels of environmental stimulation may help trigger sight recovery in this unfortunate condition. The non invasive nature of EE renders this paradigm very promising in the field of amblyopia therapy in adult people. An open issue is to what extent is EE in animal models relevant for humans. EE is a complex paradigm, since an increased stimulation is provided at multiple sensory, motor, cognitive and social levels. Although most humans do experience a high degree of environmental complexity and novelty, levels of stimulation vary greatly among individuals and in different periods of life. Two types of enriched environment extensively studied in humans are perceptual learning and playing video games. Numerous papers have documented various and robust beneficial effects on visual functions elicited by these treatments in adult amblyopes after the end of the critical period (Bavelier, et al., 2010, Levi and Li, 2009). It remains to be investigated whether these paradigms based on active visual stimulation are also effective in triggering plasticity and vision recovery in monocular amblyopic people.

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