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EFFECTS OF VISUAL EXPERIENCE ON FUNCTIONAL AND ANATOMICAL MEASURES OF VISUAL CORTEX PLASTICITY

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1. INTRODUCTION

1.1 OPTICAL SYSTEM

1.1.1 Interplay between genetic and environmental factors

Brain development is determined by molecular and cellular mechanisms ruled genetically, however the whole organization of the nervous system cannot be understood just by genetic factors because it is susceptible also to experience and environmental factors. Once the early connections are formed, which means that tens of billions of neurons with trillions of synaptic connections are set up, the nervous activity itself, conditioned by environment, has the main role in modifying the synaptic circuits. The interactions between the organism and the experience is very effective especially during the growth, in specific temporal windows called "critical periods", when the brain is more sensitive to specific stimuli, and needs those stimuli in order to develop normally. After the end of the maturation, the brain becomes refractory to experience-dependent modification and just a few particular conditions still permit changes in the neuronal wiring.

The primary visual cortex is a successful model for identifying the cellular and molecular mechanisms underlying the interplay of genetic and environmental factors, since, in this paradigm, it is possible to control the visual stimuli supplied to the experimental animals.

1.1.2 Visual system in mice

Much of the recent discovery in identifying the cellular and molecular mechanisms underlying visual cortex plasticity has been made by using the mouse as a model system.

The mouse eye exhibits an axial length of approximately 3mm and a relatively large cornea and lens (Chalupa & Williams, 2008). The retina of the mouse is characterized by 97% of rod photoreceptors with a peak density of 100,000 cells/mm2, whereas retinal cones are found in a considerably lower numbers and show a peak density of 16,000 cells/mm^2, which is a distinctive feature of animals which live in dim light (Chalupa & Williams, 2008).

In contrast with other mammals that possess a pitted cone filled foveal region, the retina in mouse is characterized by an area centralis where rod and cone photoreceptor density is maximum (Chalupa & Williams, 2008).

Rod photoreceptor cells relay light signals via rod bipolar cells (ON and OFF) onto retinal ganglion cells (RGCs). In this pathway horizontal cells and amacrine cells function as interneurons, so they permit lateral interaction. Instead cone photoreceptors transmit light signals directly to RGCs passing through just cone bipolar cells. Bipolar cells may be ON or OFF sensitive depending on different kind of glutamate receptors that produce a selective signal on to retinal ganglion cells.

The binocular region of the cortical retinotopic map receive input from the 2-3% of the ipsilateral RGCs projections corresponding to the central 30-40°of visual field (Drager & Olsen, 1980).

The RGCs axons form the optic nerve that relays the light information to the dorsal lateral geniculate nucleus (dLGN) and in turn onto the primary visual cortex. In contrast with primate and carnivores the mouse dLGN is not organized in eye specific laminae, but it contains a binocular segment, retinotopically organized, where there are both contralateral and ipsilateral eye responsive relay cells. Stimulating the binocular visual space, the mouse dLGN receives a differential degree of input from contralateral and ipsilateral RGCs projections. Therefore a point in binocular visual space excite with a relation 9:1 contra and ipsilateral RGCs. In contrast the volume of the binocular dLGN occupied by contralateral RGC afferents is just 2.4 times more than the volume of the

ipsilateral afferents (Coleman et al., 2009), a similar relation as the degree of contralateral eye dominance detected in the binocular cortex (Gordon & Stryker, 1996; Frenkel & Bear, 2004a; Cang et al., 2005)

The dLGN relay cells transmit visual information to the cortex via their axons which make up the optic radiation.

In the occipital region of the neocortex there are different areas retinotopically organised which receive either direct thalamic visual input or input from other visual cortical areas. The primary visual cortex (V1) is also known as area 17 and it is the most investigated area in mouse visual cortex. It is constituted of a retinotopically organized map of the contralateral visual space. Its size is about $3mm^2$ and in this area the most medial part correspond to the most horizontally peripheral visual field (Drager, 1975; Gordon & Stryker, 1996; Kalatsky & Stryker, 2003).

V1 mainly receives input from the optic radiation originated from the dLGN which primarily makes excitatory synapses onto the dendrites of layer 4 stellate cells, but there is also some thalamocortical input to layer 3 (Liu et al., 2008). In addition the mouse cortex is linked with the contralateral part since it receives the callosal projection via commissural fibers into its own layers 1-3 and 5, and into the lateral part of the retinotopic map –the binocular region of the V1 (Mizuno et al., 2007; Restani et al., 2009). These transcallosal projections may clarify the high degree of binocularity in mouse cortex in the absence of a large number of ipsilateral RGCs innervating the dLGN (Coleman et al., 2009).

1.1.3 Functional organization – ocular dominance

Hubel and Wiesel were the first researchers who successfully investigated V1 organization in the cat. Using circular spots of light, they attempted to stimulate cells in V1, but such stimuli failed to elicit responses in the majority of the neurons even though they were previously shown to be effective in driving neurons in the retina and in the lateral geniculate nucleus, pars dorsalis (dLGN).

By analyzing the discharge properties of singles neurons qualitatively and at length, they found that neurons in V1 responded to light stimuli at a specific angle,

orientation and position in the visual space. Another feature discovered was that most V1 neurons were binocularly driven, different neurons might respond better to stimulation of one eye than to the other and many were excited by stimulating both eyes together. They coined a term to express the degree of responses between the two eyes, by which it is still known, "ocular dominance" (OD).

Hubel and Wiesel also established that the "functional architecture" consisted of radial columns which extends through all the layers of cortex from the surface to white matter (Figure 1.1; Hubel et al., 1976).
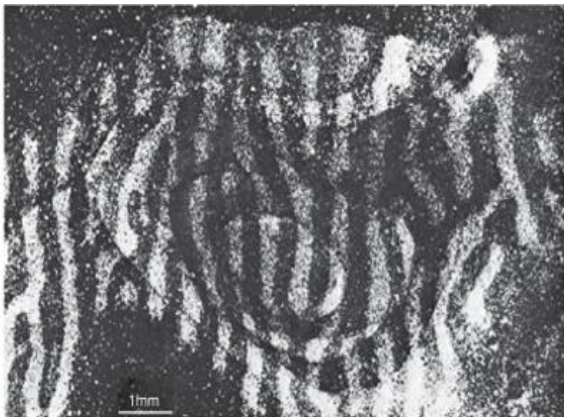
Figure 1.1 The ocular dominance columns in cat visual cortex demonstrated by autoradiography after injection of a radioactive tracer into one eye. The tracer is transported to the lateral geniculate nucleus and then transneurally transported to the striate cortex. The cortex is labeled in bands that alternate with unlabeled bands whose input is from the uninjected eye. (From Hubel DH, Wiesel TN: Proc R Soc Lond B 198:1, 1977)

The functional architecture of V1 is not identical in each species. V1 neurons in carnivores and most primates are arranged in radial columns according to preferred stimulus orientation that progress through a complete cycle of 180 degrees of orientation over about 1 mm of cortex, referred to as an orientation "hypercolumn" (Hubel et al., 1976). But the mouse V1 lacks ocular dominance columns and even if the cells show an orientation selectivity, they are not organized into columns but cells of diverse orientation preferences are intermingled.

1.1.4 *Ocular Dominance (OD) in mouse*

In mouse V1, the visual cortical neurons have many of the receptive field properties observed in other mammals like spatial frequency and orientation selectivity

and simple vs. complex receptive fields (Drager, 1975; Niell & Stryker, 2008; Kerlin et al., 2010). At the beginning a lack in orientation selectivity was claimed (Drager, 1975), however subsequent studies showed that 74% of excitatory visually responsive cells are orientation selective (Niell & Stryker,2008). The layers 2-3 of the visual cortex are the ones where the highest number of orientation selective cells are found, with a median tuning half-width at half maximal response of 20 degrees reported by Niell & Stryker (2008), which is comparable to the 19-25 degrees reported in cat (Van Hooser, 2007).

As has been mentioned above, the cortical maps of many carnivorous mammals and primates have been observed to be organized in columnar clustering specifically responsive to a specific part of the receptive field. In contrast to this kind of organization, in mouse V1 ocular dominance columns have not been observed, but instead a "salt and pepper" model where the cells are randomly selective (Figure1.2)
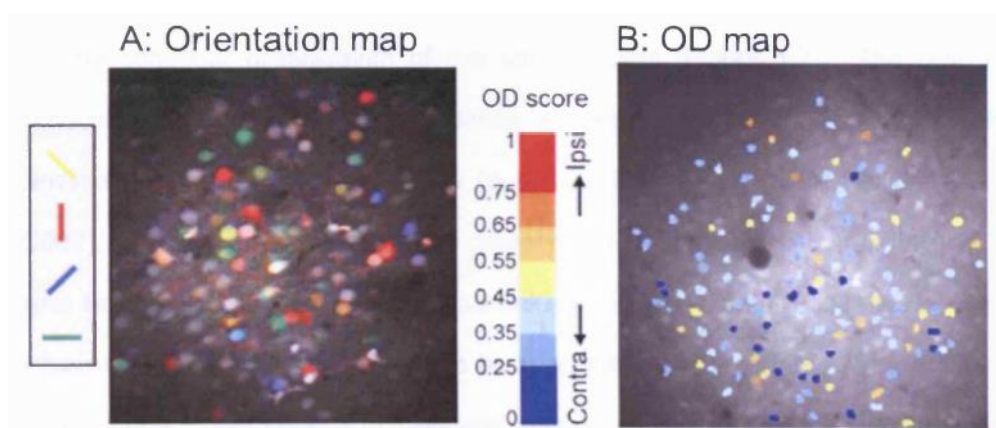
Figure 1.2 Cellular resolution calcium imaging of mouse V1. **A** Orientation map (from Ohki et al.,2005). **B** Ocular dominance map (from Mrsic-Flogel et al.,2007)

Therefore the only super-cellular level of organization of receptive field properties observed in the mouse is the retinotopic map. Nevertheless, the precision of receptive field organization in carnivorous mammals, primates, and also rodents is an indication of a specificity at the level of single cells of the connections made by neurons in V1 (Ko et al., 2011). For this reason, the essential mechanisms which act at the level of single cells are thought to be the same, independently of the presence or absence of columnar organization for other stimulus features. That was investigated at the beginning by

electrophysiological studies and recently with in vivo cellular resolution imaging (Ohki et al., 2005; Mrsic-Flogel et al., 2007).

1.1.5 Binocular region

The central part of the mouse's visual field, which corresponds to 30-40°,can be perceived by both eyes, so into the visual cortex exist an area able to integrate the information coming from both eyes, that area is known as the binocular region (Drager, 1975;Kalatsky & Stryker, 2003; Cang et al., 2005). The binocular region of the visual takes up about one third of the total primary visual cortex area (Figure 1.3) The cells in binocular mouse visual cortex possess a substantial contralateral bias, indeed only around 5% responding exclusively to the ipsilateral eye (Mrsic-Flogel et al., 2007), and this contralateral bias appears with a similar gradation both in layer 2/3 and layer 4. As discussed earlier, the natural contralateral tendency observed in the cortex was already originated in dLGN where it is possible to measure a similar bias even though with a differ ratio. Therefore the 9:1 contralateral to ipsilateral ratio of retinal ganglion cell innervation becomes 2:1 ratio in to the binocular visual cortex (Coleman et al., 2009).

Convergent evidence of this contralateral bias in cortical ocular dominance has been obtained from electrophysiological single unit studies (Drager, 1975; Gordon & Stryker, 1996), cellular resolution calcium imaging (Mrsic-Flogel et al., 2007), layer 4 VEP recordings (Sawtell et al., 2003) and population level intrinsic signal imaging (Cang et al., 2005; Hofer et al., 2006). (Figure 1.3)
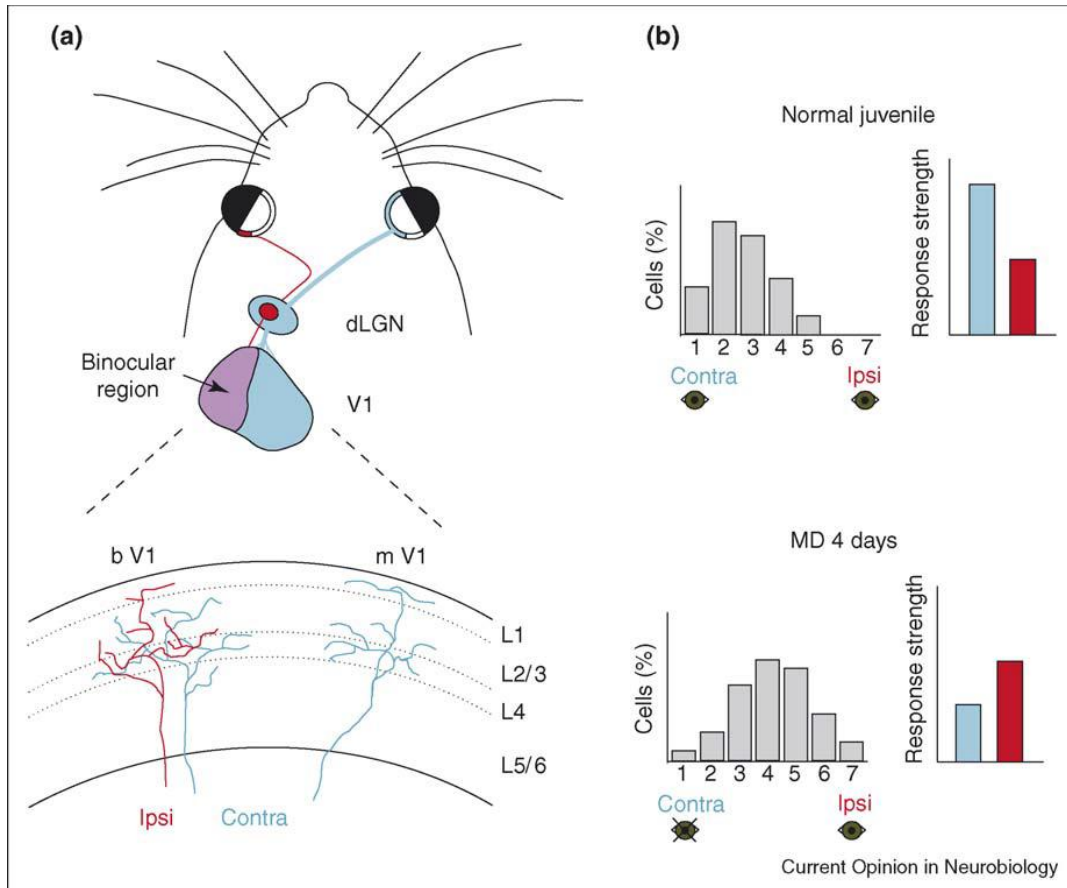
Figure 1.3 Ocular dominance (OD) plasticity in juvenile mouse visual cortex. (a) An illustration of the mouse visual system. The major part of the primary visual cortex (V1) receives input only from the contralateral retina (turquoise projections). The lateral third of V1 is innervated additionally by ipsilateral projections (red). Although neurons in the binocular region are dominated by contralateral eye input, most neurons respond to both eyes. Thalamocortical axons from the dorsal lateral geniculate nucleus (dLGN) arborise not only within layer 4 (L4) but also in superficial layers (L1-3). (b) Four days of MD in juvenile mice (around P28) lead to strong changes in binocular cortical responses. The OD distribution of cortical neurons shifts towards the eye that remained open (ipsilateral eye), as can be seen when comparing the two histograms (grey). Ocular dominance (Hofer et al.,2006)

1.2 OCULAR DOMINACE PLASTICITY

1.2.1 Critical Period

During the development, in many brain regions the neuronal circuits undergo defined phases of re-arrangement when an enhanced plasticity occurs. They are termed critical periods. Studies in mouse visual cortex have led to the comprehension of the cellular and molecular mechanisms regulating the timing of the critical period.

Molecular guidance signals have the most important role in the building of neuronal network during the prenatal phase, whereas neuronal activity and its pattern is the main player during subsequent stages of development. This activity is intrinsically generated by the developing brain, but it can also be driven by external stimuli as soon as the peripheral sensory organs are working.

The effect of sensory experience on nervous system maturation is stronger during a brief and defined period of the early life, the same stimuli have a weak impact before and after such phase. This temporal interval of increased sensitivity to certain types of sensory input is the critical period. The changes in neuronal circuit generated by appropriate stimuli during a critical period produce certain capabilities which otherwise the animal would not have. Several studies have proved that there are variation to the general concept of a critical period, so critical-period timing is not always absolute, in fact the opening and closing of a critical period are correlated to specific characteristics of the sensory stimuli, therefore it may be extended into the adulthood (Levelt & Hubener, 2012).

The mechanisms involved in the critical period regulation are complicated and various. The pioneers in the study of these mechanisms were Wiesel and Hubel, who examined the effects of the temporary visual deprivation in one eye. Monocular deprivation (MD) caused a considerable change in the ocular dominance distribution in kitten visual cortex (Wiesel and Hubel, 1963). For this reason, MD early on became a useful model to analyze the CP regulation. Later they found out that plasticity was maximal between the 4th and 8th postnatal, so such period was the one critical for the visual plasticity. In adulthood the same condition, MD, did not cause the same effects (Hubel & Wiesel, 1970).

Although the first studies were carried out in cats, ferrets and monkeys, nowadays mice are the most appropriate paradigm to improve the knowledge on the molecular mechanisms underlying the control of critical periods. In this context, the mouse shows OD shifts that are comparable to those of higher mammals (Drager,1978; Gordon & Stryker,1996)

1.2.2   Monocular deprivation

Since the 1960s, when Wiesel and Hubel (Wiesel & Hubel, 1963) discovered that the binocular representation in primary visual cortex is particularly susceptible to changes in visual experience transient lid closure of one eye, known as monocular deprivation (MD), has been used as a procedure to demonstrate that fast and strong adaptive changes in neuronal circuit occurred in kitten. A transitory depriving one eye of vision during a critical period in early life stimulate a reorganization of visual responses in primary visual cortex (V1)..

Such monocular deprivation (MD) reduces the strength of neuronal responses driven by the deprived eye while increasing responses driven by the open eye, leading to an overall shift in ocular dominance toward the open eye with a simultaneous imbalance of the binocular input. The consequence of this status entails an extension of the non-deprived cortical field, therefore a properly cortical blindness, rather than the deprived one.

Ocular dominance (OD) has served as an important model system for exploring the cellular and molecular mechanisms underlying the plasticity of cortical circuits. It was coined the term Ocular Dominance Plasticity to describe the capability of cortical circuits being shaped by experience. (Figure1.4)

Unilateral eye-lid closure reproduce the Amblyopia condition, a relatively common human disease which still cause blindness if the underlying reasons, such as strabismus or cataract, are not solved during childhood. Much of the recent progress in identifying the cellular and molecular mechanisms underlying ocular dominance plasticity has been achieved by using the mouse as a model system even though the phenomenology of OD shifts has been described in detail in several species (Wiesel &

Hubel,1965; 1977) In this paradigm, the mouse shows OD shifts that are comparable to those of higher mammals (Drager,1978; Gordon& Stryker,1996, so it could be very useful even to find application in human.
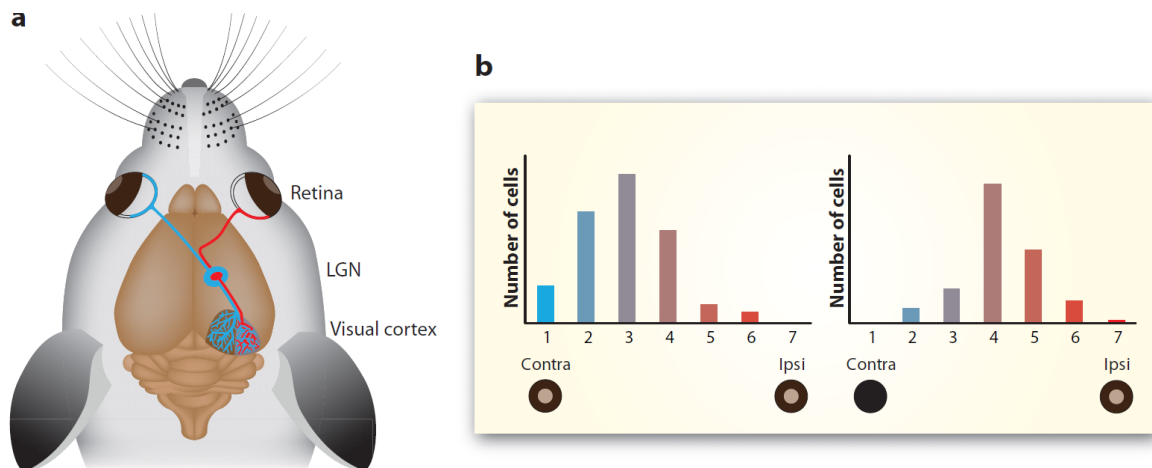
Figure 1.4 Ocular dominance (OD) plasticity in the mouse visual cortex. (a) Schematic of the mouse visual system. Most retinal ganglion cell axons (blue) cross the midline and terminate in the lateral geniculate nucleus (LGN) of the contralateral hemisphere. A very small number of axons from the temporal retina (red) do not cross at the optic chiasm; instead they terminate in the ipsilateral LGN. In the LGN, inputs from both eyes remain fully segregated. The major (medial) part of the primary visual cortex (V1) receives input from only the contralateral retina (blue), whereas the smaller (lateral) third of V1 also receives ipsilateral projections (red). In this binocular region of V1, most neurons respond to visual stimuli presented to either eye, but contralateral eye input is stronger overall. (b) (Left) On the basis of their responses to monocularly presented stimuli, neurons in the binocular part of V1 can be grouped into different OD classes, from complete contralateral (class 1) to complete ipsilateral (class 7) dominance. Neurons in class 4 are driven equally strongly by both eyes. (Levelt and Hubener. 2012)

1.2.3   Synaptic balance

Most studies addressing the mechanisms of OD plasticity have concentrated on changes at excitatory synapses. Initially, long-favoured theories hypothesized that inputs from the eyes concurrently compete for postsynaptic space on, and resources from, target neurons, such as neurotrophic factors (Guillery,1972; Bonhoeffer,1996). However, as yet no evidence has been provided for such direct competition in the binocular cortex. In rodents, after contralateral MD, modifications also in the monocular region of the visual cortex (where no competition occur) similar at those measured in the binocular region have been identified (Heinen et al.,2003; Pham et al.,1999).

In recent times, a number of studies have tried to improve the knowledge on the cellular and molecular mechanisms underlying the strengthening and weakening of inputs from the eyes by comparing (at central synapses) them to the inputs involved in long term potentiation (LTP) and long term depression (LTD). Despite the electrical induction of synaptic plasticity in vitro being impossible to direct compare with the experience-dependent modifications of neuronal circuits in an intact animal, such studies are useful to understand the molecular pathways that probably govern the plasticity in vivo. Indeed, the very early phase of MD-induced plasticity in the visual cortex resembles N-methyl-D-aspartate (NMDA) receptor-dependent LTD or at least employs similar mechanisms (Heinen et al.,2003).

The shift in functional connectivity that takes place during MD leads to a substantial reorganization of thalamocortical axon arbors (Antonini & Striker,1993) However it has been noticed that a higher number of structural changes occur at level of dendritic spine which bear the majority of excitatory synapses in the cortex. Motility of spines is increased after 2 days of MD, while four days of MD, (Gordon & Striker,1996; Hofer et al.,2006), leads to significant spine loss on apical dendrites of layer 2/3 pyramidal neurons, according to the early, strong reduction of cortical responsiveness to deprived eye stimulation (Mataga et al.,2004). Formation of new connections from spared eye inputs occurs only after longer deprivation periods (Mataga et al.,2004), consistent with the subsequent strengthening of the representation of the open eye.

Intrinsic, compensatory mechanisms avoid the total loss of connectivity in monocular neurons subserving the deprived eye. Indeed, in neural preparations with globally manipulated activity levels, there is consistent evidence encouraging the presence of homeostatic processes which alter the overall synaptic strength or intrinsic excitability (Turrigiano & Nelson,2004; Burrone & Murthy,2003), or to modify the threshold for synaptic plasticity (Bienenstock et al.,1982; Bear,1995). In this paradigm, a flexible modification threshold, could modify the strength and direction of plasticity at single synapses during MD, depending on the overall strength of activation of the neuron (Bienenstock et al.,1982; Bear,1995).

Though it is commonly believed that it is the pattern of activity reaching the cortex that determines the extent and direction of cortical plasticity, recent data highlight another potential mechanism based on BDNF levels (Mandolesi et al.,2005). Indeed in rats, the

levels of BDNF decreased because of MD in the deprived retina, and replenishing retinal BDNF by exogenous application counteracted the MD effect in the cortex. Moreover, it was also shown that BDNF is transported from the retina to the LGN. These results suggest that there might be an additional signal that informs the cortex of an altered sensory input, namely the anterograde transport of molecular factors.

Other research works has demonstrated that after a prolonged period of MD ,treating with fluoxetine a rat, an inhibitor of the serotonin reuptake, the vision was rehabilitated better than in control animals (Maya Vetencourt et al., 2008). Indeed it was demonstrated that serotonin is fundamental for plastic capability during the critical period (Gu & Singer, 1995) and its reduction decreases the OD plasticity in visual cortex. In addition, mice deficient for an inhibitor of the nicotinic acetylcholine receptor, such as lynx1, after a brief MD showed an increased degree of plasticity (Morishita et al., 2010). For this reason, it is thought that cholinergic synapses are another important factor capable to stimulate plasticity in adulthood. Therefore it seems that the equilibrium between excitatory and inhibitory inputs has a fundamental importance in this paradigm (Gu & Singer, 1995, Morishita et al., 2010). So the reactivation of OD plasticity could be induced from a change in excitatory-inhibitory balance.

1.2.4 *Signaling pathway*

Moreover, for OD plasticity and for different forms of synaptic plasticity in the visual cortex in vitro the signaling pathway through specific isoforms of PKA -cAMP-dependent protein kinase A is required (Fischer et al.,2004; Rao et al.,2004). Recent data in mice disagree with the view that only homosynaptic LTD at excitatory synapses is the mediator of OD shifts during the first days of deprivation. Alpha calcium calmodulin kinase II (a-CaMKII) activity, which is related to synaptic LTP in vitro, has an important role in short-term OD plasticity (Taha et al.,2002). A number of studies have reported that the activation of kinases and transcriptional regulators, such as Erk and CREB respectively, and changes in gene expression have been showed in OD shifts (Di Cristo et al.,2001; Mower et at.,2002; Taha et al.,2002; Krahe et al.,2005). These results mean

that OD plasticity requires a number of interacting processes that could operate differentially on different kinds of cells and not a single signaling pathway.

1.2.5 Ocular dominance plasticity in juvenile visual cortex

As already mentioned, cats, ferrets and primates, thalamic afferents from eye specific layers in the lateral geniculate nucleus (dLGN) segregate in the visual cortex during the first stage of the maturation, organizing themselves in to OD columns with a bias to the contralateral inputs (Hubel & Wiesel,1977; LeVay et al.,1978). In contrast, the binocular region of the primary visual cortex in mice does not exhibit such organization, but nonetheless it contains mostly binocular cells that have a preference for the contralateral eye. (Figure 1.5) (Gordon & Stryker,1996; Drager,1975; Antonini et al.,1999).
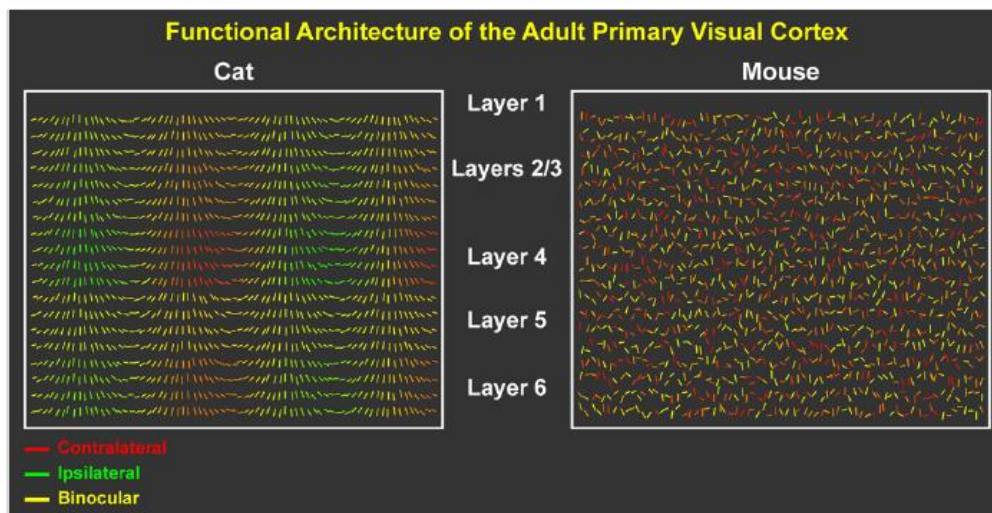
Figure 1.5. Functional Architecture of V1 in Cat and Mouse Both cats and mice contain neurons in V1 that must receive and transform precise inputs from the LGNd. V1 in the adult cat (left) consists of neurons highly selective for specific orientations (denoted by the angle of lines) and dominated to varying degrees by the contralateral (red) or ipsilateral (green) eye, with many cells driven by both eyes (yellow). Both orientation and ocular dominance properties are organized into columns. Preferred orientation columns span all cortical layers, while ODCs are most pronounced in layer 4, where many cells are driven monocularly. Mouse V1 (right) does not have columnar organization of orientation or ocular dominance. However, neurons are still highly orientation selective and display a range of ocular dominance but with a bias toward the contralateral eye (Espinoza &Stryker, 2012)

In juvenile mice, few days of monocular deprivation (MD) are enough to evoke a consistent shift in OD in the visual cortex (Gordon & Stryker 1996). The effect is more evident during a relatively brief phase, precisely between postnatal day 19 and postnatal day 32, it is generally assumed as the critical period for OD plasticity in the mouse visual cortex (Gordon & Stryker 1996). However recent discoveries highlight plasticity in mouse visual cortex during the entire life, but the binocular representation still seems to be most sensitive to experience-dependent changes early in life, at around postnatal day 28 (Gordon & Stryker,1996), during the critical period. This period is not constant, but undergoes the influence of molecular and external signals which could accelerate or delay its onset and closure. OD plasticity may also be activated before this period or after, but with different quantity and quality. Using transgenic mouse models to detect the molecular and cellular mechanisms behind OD plasticity, Hensch et al. (1998) discovered that the development of gamma-aminobutyric acid GABA-ergic innervation is essential for the critical period onset. In mice with a genetic mutation on the DNA sequence which synthetize GAD65 -- GABA-synthesizing enzyme glutamic acid decarboxylase— there is reduced GABA synthesis and that reduction prevents opening of the critical period for OD plasticity till the animals are injected the GABA-A receptor agonist diazepam (Fagiolini & Hensch 2000, Hensch et al. 1998). The induction of earlier critical period was possible by treating them with benzodiazepines (Fagiolini & Hensch 2000). In transgenic mice overexpressing BDNF, brain-derived neurotropic factor, during postnatal development a premature onset of the critical period was observed, probably because BDNF promotes the development of GABAergic synapses in the visual cortex .The opposite effect is caused by dark rearing, indeed the dark exposure of the wild-type mice causes a down-regulation of BDNF expression, therefore the development of inhibitory

innervation and critical-period opening are delayed (Hanover et al. 1999, Huang et al. 1999). (Figure 1.6)
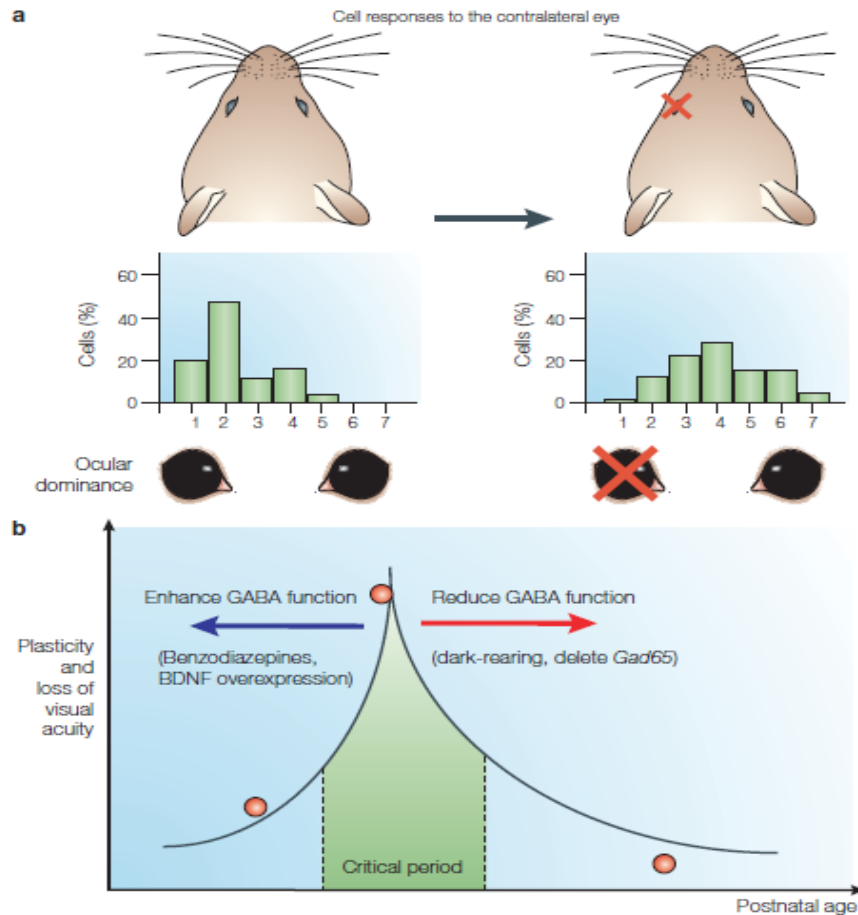
Figure 1.6 a. Monocular deprivation produces a loss of response to the deprived eye and a gain of open-eye input, as measured by the neuronal discharge of single units from the mouse visual cortex. The ocular dominance of cells, rated on a seven-point scale of neuronal responsiveness, indicates a typical bias toward the contralateral eye (1–3) in the rodent (top left). After 3 or more days of monocular deprivation, the distribution shifts toward the open, ipsilateral eye (4–7; top right). **b.** Sensitivity to monocular deprivation is restricted to a critical period that begins, in mice, about 1 week after the eyes open (at postnatal day 13) and peaks 1 month after birth. Monocular deprivation causes amblyopia only during the same critical period. Red circles indicate the onset, peak and end of amblyopia resulting from monocular deprivation. The onset of plasticity can be delayed by directly preventing the maturation of GABA (γ-aminobutyric acid)-mediated transmission by gene-targeted deletion of Gad65, which encodes a GABA-synthetic enzyme, or by dark-rearing from birth (red arrow). Conversely, the critical period can be brought forward by enhancing GABA transmission directly with benzodiazepines just after eye-opening or by promoting the rapid maturation of interneurons through excess brain-derived neurotrophic factor (BDNF) expression (blue arrow). (Takao K. Hensch,2005)

IGF-1 is an important factor able to accelerate the maturation of inhibitory network and the increase of visual acuity, which is considered a distinctive feature of the critical period (Ciucci et al. 2007). Moreover, in pre-critical period mice injecting

intracortically the enzyme which dissolves polysialic acid from the neural cell-adhesion molecule (NCAM) ,has been shown that the inhibitory circuit matures faster and that OD plasticity is earlier than normal (Di et al. 2007). These studies have indicated that several days after eye opening there are all prerequisites for critical-period plasticity in place, except for the appropriate level or type of inhibitory input.

However, it is not completely clear which is the timing necessary for the maturation of the inhibitory network after the eye opening to allow OD plasticity to take place. Maturation of the excitatory system may be another important factor in order to OD plasticity to happen. The development of excitatory circuit could even alter the tuning properties of inhibitory neurons, allowing the onset of the critical period (Kuhlman et al. 2011).

1.2.6 Ocular dominance plasticity in adult visual cortex

Notwithstanding the classic knowledge about experience-dependent plasticity during the critical period (Hubel & Wiesel,1970), there is a lot of research which has highlighted that OD shifts in mice may even take place in adulthood (Hofer et al.,2006; Sawtell et al.,2003; Lickey et al.,2004; Tagawa et al.,2005). A consistent OD plasticity has been shown after MD in adult mice using different methods, such as recording extracellular single-cell responses (Hofer et al.,2006), intrinsic signal imaging (Hofer et al.,2006) and visually evoked potentials (VEPs) (Sawtell et al.,2003) in anesthetized and in awake animals as well (Sawtell et al.,2003). Using the activity reporter gene Arc, it has been observed that MD effects can occur before and after the traditional 'critical' period (Tagawa et al.,2005) .Therefore, the concept of 'critical' period for OD plasticity seems, at least in the mouse, not to be so strict. Nevertheless, the binocular cortex remain still more experience-sensitive in juvenile mice, as in adults OD shifts need more days of MD and are generally less pronounced (Hofer et al.,2006; Sawtell et al.,2003; Lickey et al.,2004). Earlier studies failed to detect adult plasticity because barbiturate anesthesia was used (Fagiolini & Hensch,2000), indeed recently it has demonstrated to be a specific blocker of the OD shift in adult mice (Pham et al.,2004). Since the effects of barbiturates on adult and young mice were different it is likely that the mechanisms of OD plasticity

differ at different ages. Therefore, on one the hand in juvenile mice both early weakening of deprived eye responses and later strengthening of open eye responses are responsible for the shift in OD (Frenkel &Bear,2004), on the other hand, in adulthood the MD effect seems to be caused by NMDA receptor dependent strengthening of open-eye inputs alone (Sawtell et al.,2003).

Lack of a fast loss of input from the contralateral eye subsequent to the deprivation probably explains why a briefer MD period (3 days) is not enough to evoke OD shifts in adult mice, although in juvenile animals strengthening of the open-eye representation is delayed and takes longer to develop (Frenkel &Bear,2004). The ipsilateral eye, less represented in visual cortex, remain experience-sensitive even in adulthood because it has shown that after the ipsilateral closure is notable changes in visual cortex (Hofer et al.,2006; Tagawa et al.,2005). Could be possible that essential structural rearrangements that occur in OD shifts in juvenile animals also take place in the adult cortex during MD. Morphological plasticity of synaptic structures is associated with functional reorganization after retinal lesions in the visual cortex of adult cats (Darian-Smith & Gilbert,1994). Furthermore, in untreated adult mice, formation and pruning of synapses still occur in visual cortex (Holtmaat et al.,2005), and in the somatosensory cortex spine turnover is intensified by sensory deprivation (Trachtenberg et al.,2002). The spine loss in the juvenile visual cortex after 4 days of contralateral eye MD was not seen in adult animals (Mataga et al.,2004), in line with the absence of significant weakening of deprived-eye inputs in the contralateral region at older ages (Hofer et al.,2006; Sawtell et al.,2003).

Interestingly, a weak spine gain has been observed, which could correspond to the strengthening of non-deprived eye inputs (Mataga et al.,2004). Because in adult visual cortex long periods of deprivation are necessary to complete OD shifts, it is likely that longer periods of MD (>6 days) might trigger a significant synaptic remodeling. As structural plasticity is thought to become more limited in scope as the brain matures (Trachtenberg et al.,2002; Grutzendler et al.,2002; Pizzorusso et al.,2002), it is possible that OD plasticity in adulthood depends on other mechanisms, but changes in inhibitory system are also detected, as indicated by the masking of adult OD shifts by barbiturates, which mainly augment inhibitory transmission. Thus the potentiation of the non-deprived, ipsilateral eye responses might actually be produced by a depression of eye-specific

inhibitory activity (i.e. a form of disinhibition). The potentiation of field potentials in adult visual cortex has been attributed entirely to LTD at inhibitory synapses (Yoshimura et al.,2003). The application of barbiturates might, therefore, gain more inhibitory transmission and thereby block the disinhibition of the non-deprived eye inputs.

1.2.7   Recovery from MD

The effect of re-establishing binocular vision after MD is a good way to observe visual cortex plasticity and it could be clinically important in terms of treating amblyopia. The first studies showed that a long term MD during the critical period aroused an irreversible shift through the eye still opened. That happened because the eye was deprived of salient inputs during early postnatal development that permit the correct formation of thalamocortical connections and the integration of deprived eye inputs into the cortical network, thus permanently disrupting the visual processing of this eye. Therefore in models of OD plasticity which the deprived eye is simply re-opened, recovery from MD is not expected to occur. But a completely recovery of binocular responses after a period of binocular vision has been observed in different species (Hofer et al.,2006; Mitchel et al.,1977; Kind et al.,2002; Liao el al.,2004; Faulkner et al.,2005). It is thought that induction of OD shifts and the recovery are probably mediated by basically different processes, as recovery from MD does not require CREB activation or protein synthesis (Krahe et al.,2005; Liao et al.,2002). Moreover, in ferrets, the recovery of deprived eye responses has been showed to occur within hours, thus much faster than the initial OD shift, and this rapid recovery is not restricted to the critical period for MD effects (Krahe et al.,2005; Liao el al.,2004).

1.2.8   Enhancement of plasticity

The plastic capability in adulthood, as already mentioned, in primary visual cortex could not be compared to that in juvenile animals. Adult cats reared in the dark were observed to exhibit strong plasticity explainable by a delay in the maturation of cortical

circuits (Cynader & Mitchell,1980). More recently, it has also been demonstrated that brief complete visual deprivation in adult rats causes the visual cortex to be returned to a more immature state, thus an activation OD plasticity (He et al.,2006), even though these effects are not permanent. Indeed if the adult animal spent a short period again in light rearing it was observed that the capability for plasticity weakened, thus the cortex was returned to the original state (Philpot et al.,2003). It was tried to identify the factors underlying the restriction of the plastic capacity in adulthood. One of these seems to be the extracellular matrix (ECM), indeed pharmacological degradation of the ECM breaking the glycosaminoglycan chains reopens OD plasticity in adult rat visual cortex (Pizzorusso et al.,2002). Moreover, deletion of the gene for the myelin component Nogo-66 or its receptor, NgR, whose activation blocks neurite outgrowth after nerve injury, appears to prolong plasticity indefinitely (McGee et al., 2005). The maturation of the extracellular matrix is clearly an inhibiting factor for the OD plasticity because obstructs the reorganization of synaptic structures.

Is was noted that a second period of MD causes faster and longer lasting OD changes many weeks later in both juvenile and adult cortex (Hofer et al.,2006). According to this discovery, it seems that MD leaves an intrinsic concealed mark on the organization of the cortical network independently of the age that the MD occurs.

These studies show the adult plasticity can be extended not just by removing the plasticity-inhibiting factors but even by prior experience.

1.3   ROLE OF THE INHIBITION

1.3.4   Inhibition and critical period

How visual experience and neuronal activity drive the maturation of inhibitory innervation is not completely clear. BDNF release could be one of the factors regulating the development of GABAergic networks (Gianfranceschi et al. 2003, Hanover et al. 1999, Huang et al. 1999).

Another interesting component is OTX2, a transcription factor is transcribed and translated in the retina, but it is also transported to the visual cortex where it is taken up predominantly by parvalbumin (PV)-expressing interneurons (Sugiyama et al. 2008). Studies demonstrated that OTX2 deficiency prevent the beginning of the critical period, however it can be restored by intracortical infusion of OTX2.

An important still not completely solved question regards how the maturation of inhibitory inputs permits the start of the critical period. An increased degree of inhibition may modulate plasticity at excitatory synapses. Alternatively, plasticity of inhibitory output itself could alter the expression of OD. It is also possible that a dynamic interaction between these two mechanisms exists, causing a selective change in the excitatory/ inhibitory balance that promotes plasticity of excitatory neurons, thereby driving their responsiveness toward the open eye (Gandhi et al. 2008).

The mechanisms underlying the closure of the critical period seem more diverse than those above mentioned for the opening. The first studies hypothesized that an important pathway could involve N-methyl-D-aspartate receptor (NMDAR), but nowadays this hypothesis is discounted. In young animals, NMDARs contain the NR2B subunit, which permits a more efficient strengthening of non-deprived eye responses on MD rather than the NR2A subunit displayed in adulthood (Cho et al. 2009). The shift to the mature NR2A subunit cannot be considered to be directly responsible for the closure of the critical period, however, since this occurs normally in mice deficient for NR2A (Fagiolini et al. 2003).

1.3.4 Parvalbumin-positive interneurons (PV⁺)

Parvalbumin (PV), an EF-hand calcium-binding protein, is expressed in a particular population of GABAergic neurons in different brain regions, like the cerebral cortex (Celio, 1986) and hippocampus (Kosaka et al., 1987). Initial studies have underlined that PV expression in GABAergic neurons during the developmental period could be influential for neuronal maturation (Stichel et al., 1987; Del Rio & DeFelipe, 1994). Recently it has showed that PV-expressing (PV+) neurons are involved into the regulation of neural plasticity and critical period (Chevaleyre & Piskorowski, 2014). Overexpression of BDNF (brain-derived neurotrophic factor) enhances both the expression of PV in GABAergic neurons and the time course of the critical period in the visual cortex (Huang et al., 1999). Development of the PV+ neuron network that controls plasticity onset is regulated by selective re-expression of embryonic orthodenticle homeobox 2 (Otx2) in the visual cortex (Sugiyama et al., 2008).

Observations about a precocious critical period have shown that it can be induced with benzodiazepine treatment in young mice. This effect is blocked when the alpha1 subunit of the pentameric GABA-A receptor is modified genetically, becoming insensitive to benzodiazepines (Fagiolini et al.,2004). In contrast, making the alpha2 subunit insensitive to benzodiazepines, does not affect the critical-period onset.

Several alpha1 subunits are present in inhibitory synapses formed by PV+ basket cells terminating on the soma and proximal dendrites of their target neurons (Klausberger et al., 2002).

The presence of the alpha1 subunit in the GABA-A receptor decreases its decay-time constant (Brussaard et al.,1997), important to ensure the faithful transmission of the high firing rates of PV+ interneurons, which are mostly fast spiking, to postsynaptic conductance changes. Together with the specific uptake of OTX2 by PV+ interneurons, these findings suggest that these interneurons are the dominant subtype regulating critical-period onset.

There are numerous ways through which inhibitory network could regulate plasticity of excitatory connections in the visual cortex. One of the most important mechanisms is by modulating spike-timing-dependent plasticity (STDP). PV+ interneurons principally innervate excitatory neurons and other interneurons on the soma

and proximal dendrites, where they are in the perfect position to alter backpropagation of signals into dendrites (Tsubokawa & Ross, 1996). Indeed, blocking inhibition when pairing presynaptic activity with single postsynaptic action potentials facilitates spike timing- dependent synaptic strengthening.

Moreover, another mechanism may play a role. PV+ interneurons build networks made up of 30–50 cells that are connected through reciprocal chemical and electrical (gap junction) synapses and this arrangement makes them exquisitely sensitive to synchrony in inputs from excitatory neurons, which is translated into the synchronous firing of groups of PV+ interneurons (Galarreta & Hestrin, 1999; 2002). Therefore, PV+ interneurons are able to coordinate the firing of large sets of cortical excitatory neurons.

Inhibitory inputs onto excitatory neurons may change during OD plasticity. This could alter the expression of OD plasticity and/or drive synaptic plasticity in favor of inputs from the non-deprived eye. Inhibition of excitatory neurons may also change in response to the experience dependent plasticity of inhibitory synapses. Indeed MD during the critical period increases miniature inhibitory postsynaptic current frequency in layer 4 star pyramidal neurons in slices of the binocular visual cortex (Maffei et al., 2010). Other studies by in vivo intracellular recordings have observed that blocking inhibitory inputs by intracellular injection of GABA antagonists reinforces the binocularity of neurons in the primary visual cortex (V1), whereas reducing inhibition after MD causes a reversal of the eye bias of excitatory neurons (Yazaki-Sugiyama et al., 2009). This proves that OD plasticity is accompanied by a change in the balance of excitation and inhibition at the single-cell level. Thus, the evidence indicating PV+ basket cells initiate the critical period has been accumulating during the past decade and has become very convincing.

1.3.3 Perineuronal nets (PNNs)

The perineuronal net (PNN) is a reticular structure that enwraps the soma and dendrites of neurons. Previous studies have shown that the PNN is composed to specialized components of the extracellular matrix, which is enriched with CSPGs, chondroitin sulfate proteoglycans, and hyaluronan (Bandtlow & Zimmermann, 2000;

Giamanco & Matthews, 2012). Recently it has been suggested that PNNs play an important role in the regulation of neural plasticity (Wang & Fawcett, 2012). The emergence of the PNN correlates with the end of the critical period in the developing neocortex (Pizzorusso et al., 2002; Dityatev et al., 2007; Makara et al., 2007). News studies have aimed to understand which mechanisms permit the regulation of neural plasticity by the PNN.

Therefore it has been confirmed that the PNN has a decisive role in the regulation of neural plasticity (Hensch et al., 1998; McRae et al., 2007; Nowicka et al., 2009). Indeed, degradation of PNNs by chondroitinase ABC consents the neuronal reorganization, thus the plasticity, in the adult visual cortex (Pizzorusso et al., 2002). Formation of PNNs is a key event in the decrease of visual plasticity (Carulli et al., 2010). Moreover the maturation of PV+ neurons controls plasticity onset in the visual cortex in early postnatal life, which is regulated by selective re-expression of the embryonic Otx2 homeoprotein (Sugiyama et al., 2008).

In addition, several studies have focused on the possible link between PNN and PV. For instance, the number of PV+ neurons is reduced 7 days after chABC injection in the adult visual cortex (Beurdeley et al., 2012). After this evidence it was accepted that one role of the PNN could be to promote the persistent internalization of Otx2 by PV+ neurons to effect critical period closure.

Moreover, the extracellular matrix changes the dendritic spine turnover, so after long-term MD and chondroitinase treatment was discovered that the deprived eye could regain the vision and in this paradigm there is also a gain in spine density in pyramidal neurons (Pizzorusso et al., 2006). In mice, the tissue plasminogen activator (tPA), protein that dissolves the matrix, is increased during MD and it seems necessary for OD plasticity (Mataga et al., 2002). Even though the inhibition of tPA/Plasmin activity in kittens subjected at MD, it does not modify the OD shift, but it prevents recovery of vision in the deprived eye (Muller & Griesinger, 1998). Plasmin activity results in an increase of spine motility (Oray et al., 2004), such that in mice genetically deficient in tPA, the spine loss, usually noted after 3 days of MD during the critical period was reduced (Mataga et al., 2004).

As already mentioned above also the receptor for myelin-associated growth inhibitor, Nogo-66 receptor (McGee et al., 2005) is involved in the closure of the critical

period, and in mice that are lacking this receptor, the OD plasticity may be induced in adulthood.

1.3.4 Dark rearing

Duffy and Mitchell have studied the effects of dark rearing on plasticity and on the recovery of vision in kittens. Their focus was on neurofilament involved in the stabilization of neuronal structure. The NF-L density into V1 was measured in 3 different groups of kittens that had been raised with normal vision until P30 and then placed into the dark room for 5, 10 or 15 days. A significant reduction in the degree of the NF-L density was observed after 10 and 15 days of darkness according to the concept that dark rearing allows the extension of plasticity state into the brain.

Moreover they even used other three groups of kittens, all raised in normal dark/light condition until P30 and then subjected to one week of MD, but exposed to 10 days of DR in 3 different moments (immediately, after 5 weeks and after 8 weeks from the deprived eye reopening) and the level of visual acuity was measured . Into the first group which 10 days of darkness were imposed immediately after 1 week of MD the development of the Amblyopia was prevented, instead an extremely rapid recovery from amblyopia after 10 days of darkness imposed either 5 or 8 weeks after one week of MD was observed in the other 2 groups. No difference were observed in the grating acuity among the 3 different groups after 75 days of recovery in both eyes, although the recovery time was been different. Therefore, the heightened instability of the cytoskeleton induced by darkness likely represents one of many parallel molecular changes that promote visual recovery, possibly by release of the various brakes on cortical plasticity (Duffy & Mitchell, 2013) (Figure 1.7).
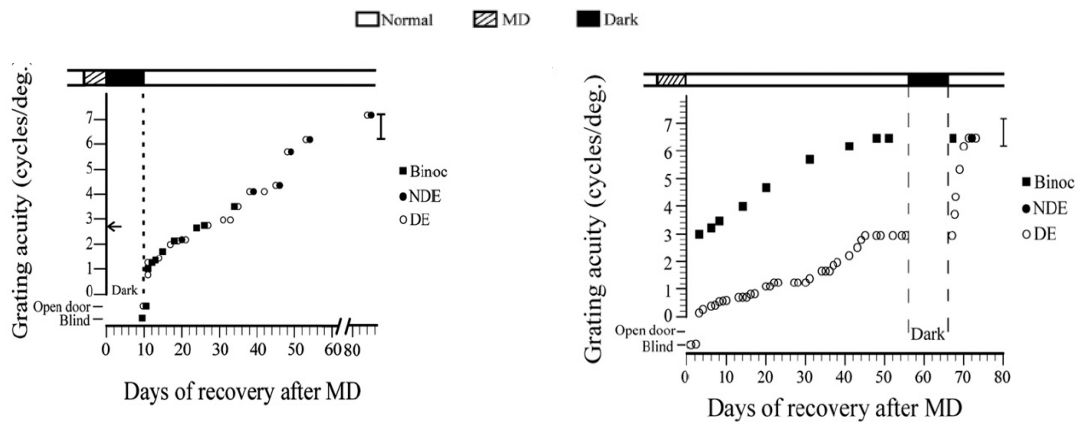
Figure 1.7 a. Ten Days of Darkness Imposed Immediately after 1 Week of MD Prevented Development of Amblyopia. Changes in the visual acuity of the two eyes of one kitten (C153) following termination of 1 week of MD. **b**. Extremely Rapid Recovery from Amblyopia after 10 Days of Darkness Imposed Either 5 or 8 Weeks after 1 Week of MD. Changes in the visual acuity of the two eyes of one kitten following termination of 1 week of MD before and after a 10 day period of darkness. (Duffy and Mitchell,2013)

The effects of dark exposure on ocular dominance plasticity in adult rats were also detected by He groups in 2007. Visually evoked potentials (VEPs), recorded from the binocular region of the primary visual cortex, were used to measure the restoration of the ocular dominance plasticity. An evident decrease in VEP was occurred after 3 or 10 days of dark exposure into adult deprived visual cortex (contra laterally to the deprived eye) subjected to a brief period of MD. Moreover, Chronic MD induced a significant ocular dominance shift, which was restored to normal when DE was followed by binocular vision (BV) or reverse occlusion (RO). Thus they confirmed that a period of darkness promote the recovery of the acuity that otherwise not occur with the solely light rearing (He et al,2007). (Figure 1.8)

Dark exposure in adulthood reverses several molecular correlates of cortical development (Yashiro et al.,2005; He et al.,2006) and is likely to enhance ocular dominance plasticity by lowering the threshold for the induction of long-term depression and long-term potentiation, as predicted by the 'sliding threshold' rule for synaptic modification (Bienenstock et al., 1982). That evidence demonstrates that the adult cortex retains substantial potential for synaptic plasticity (Iny et al.,2006; Frenkel et al.,2006) and that great progresses could be make in that sense.
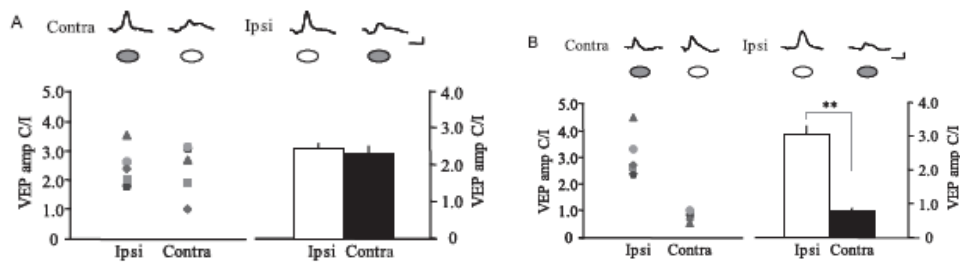
Figure 1.8 Visual deprivation reactivates rapid, juvenile-like ocular dominance plasticity in the adult visual cortex. *A*, Brief (3 d) MD does not induce an ocular dominance shift in lightreared adults. Left, VEP amplitudes (*C/I*) recorded from each subject in the visual cortex ipsilateral and contralateral to the occluded eye. Right, Summary data reveal no significant difference in VEP *C/I* across two hemispheres. Inset, Representative VEPs (average of 100 trials) recorded from each hemisphere in response to stimulation of the occluded (filled circle) and open (open circle) eye. Calibration: 100 ms, 50_V. *B*, Brief MD after 10 d of visual deprivation induces a significant ocular dominance shift. Left, VEP *C/I* from each subject. Right, Summary data reveals a significant difference in VEP *C/I* across two hemispheres, indicating an ocular dominance shift (**p_0.01, paired t test). Inset, Representative VEPs (average of 100 trials). (He et al, 2006)

1.4   AIMS OF THE PROJECT

Clarifying the question about how is possible to manipulate the structure and the function of the neurons could be a great goal for the research which would permit to solve a number of clinical human disease. First of all, amblyopia could become a solvable problem even during adulthood. The 1-5% of the population is affected by the amblyopia, commonly known as lazy eye, it is associated structural problems of the eye (as strabismus or cataract) which prevent the symmetric reception of the visual stimuli causing repercussions on the development of the visual cortex and blindness into the weaker eye. MD method is the best way to reproduce this visual disequilibrium in animals and dark/ light rearing is a successful way in order to understand the effects of different recovery, such as different combinations of dark and light rearing after MD on the neuronal reorganization, therefore to understand the general principles underlying plasticity. According to this concept, the aim of the project is to improve the knowledge about the plasticity processes and to confirm the importance of dark rearing, both at level of the brain activity, observed by optical imaging (ODI), and at cellular level, by immunohistochemistry. Hopefully we could find a correlation between rearing conditions and level of plastic state. Measuring contralateral cortex activity we expected a better recovery in the groups subjected to the darkness, thus an ODI which become similar to the control group, than the groups recovery just in the light. Moreover, by

immunohistochemistry we should find a bigger percent of colocalization of PV and PNN in control and dark born groups, which would confirm both the PNN role in the maturation of cortical circuits and the dark rearing role to maintain a more immature state of the V1 organization. It could be also interesting to detect about the different degree of colocalization of PV and PNN between contra- and ipsi- lateral cortex. VGAT immunostaining, instead, could inform us about the inhibitory state in the mice's brains which are part of different groups of rearing.

This knowledge on the underlying mechanisms of plasticity in visual cortex is not limited to solve Amblyopia, but it could be extended into other systems. Several neuropsychiatric disorders such as schizophrenia, Rett syndrome, and autism have been linked to alterations in cortical organization, particularly in the inhibitory/excitatory balance (Chao et al., 2010; Dani et al., 2005; Kehrer et al., 2008; Rubenstein & Merzenich, 2003; Yizhar et al.,2011).. Finally, recovery of neuronal function after trauma, stroke, or other insults to brain tissue is typically hampered by the adult brain's limited capacity for plasticity. Promoting the circuit reorganization by specific dark/light recovery may eventually restore brain function.

2. MATERIALS AND METHODS

2.1 MOUSE STRAIN

TDPV mice were obtained by the crossing of 2 different strains using Cre/lox system to observe the PV-expressing cells with a red fluorescence, tdTomato protein. Cre/lox system consists of LSL excision into target gene, which includes a stopping signal between 2 LoxP sites, and this excision can occur solely in Cre-expressing cells.

Cre is a site-specific recombinase enzyme which recognizes 34pb DNA sequence loxP and it has the capacity for cutting the DNA part of the target gene between 2 loxP sequences. In order to obtain this paradigm we crossed 2 different transgenic mice:

-        one mouse who has Recombinase Cre under the control of a cell type specific promoter (PV-Cre),

-         one mouse who has the target gene (tdTomato protein) blocked from a stop cassette loxP double ended sequences (LSL) under the control of an ubiquitous promoter.

In this way one can obtain an offspring which expresses the Cre enzyme just in specific cells which are able to activate the expression of the target gene. In this case, it was obtained a TDPV strain that expresses Cre only in PV-expressing cells, since Cre is under the endogenous parvalbumin promoter, which permits in these cells the activation of tdTomato marker, the target gene (Figure 2.1). Specifically, the 2 transgenic mice were Ai14Cre reporter mice (JAX008069) and PV-Cre knockin mice (JAX008069). Ai14 mice express a construct generated by Madisen et al. in 2010 and it consists of: Rosa-CAG-LSL-tdTomato-WPRE. Rosa26 and CAG have the role of ubiquitous strong promoter, while WPRE (woodchuck hepatitis virus posttranscriptional regulatory element) is inserted as promoting mRNA transcript stability.
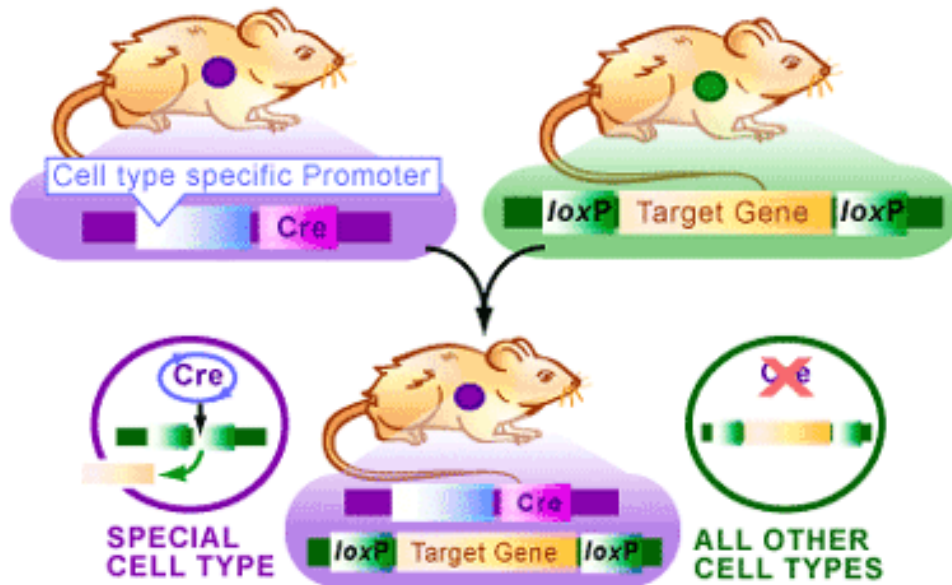
Figure 2.1 this scheme shows how from the crossing of two different transgenic mice, one has the gene of Recombinase Cre under control of a cell type specific promoter (parvalbumin promoter) and one has the transcription of target gene (tdTomato) inhibited from loxP sequences

2.2 MONOCULAR DEPRIVATION

Monocular deprivation was carried out in young mice during the critical period (P25-P28). The animals were put in anesthetic chamber with 4% of isoflurane and 0.5 l/min of oxygen until they were asleep. After that, they were placed in a styrofoam mould, lying on the side, and the isoflurane was reduced to 2% and the oxygen to 0.3 l/min. Using small scissors the inferior and superior lid margins were removed in order to facilitate cicatrization. A 6/0 silk suture with a curved needle (Johnson) was used to join the eye lids of the left eye with 2 mattress stitches. Antibiotic and anti-inflammatory eye ointment was applied on the stitches to avoid infection during the monocular deprivation period.

Animals were placed in a heated recovery chamber they were fully awake and then returned to their cage.

2.3 MOUSE REARING CONDITION

All the mice used for in vivo Optical Imaging experiments were subjected to a lid closure at P28 (during the CP) , so they were monocularly deprived for a period of 30 days and after that period the deprived eye was reopened and they were imaged 5 times in total. The mice were split in two groups:

1)   Dark group: subjected to dark rearing (DR) after the reopening of the eye, so they spent 7 day period in a complete dark room. This group was imaged at: 30 MD; 30 MD+ 7DR; 30MD+ 7DR +7LR; 30MD+ 7DR +14LR; 30MD+ 7DR +23 LR.

2)   No-Dark group: normally light recovery (LR) after the reopening of the closed eye, on standard light/dark cycle (12h + 12h). This group was imaged at: 30 MD; 30 MD+ 7LR; 30MD+ 14LR; 30MD+ 21LR; 30MD+ 30 LR.

For Immunohistochemistry experiments, seven different kind of brains under seven different conditions were analyzed:

1)   control group;

2)   dark born;

3)   monocular deprivation long term

4)   monocular deprivation + 7d dark rearing + short light rearing;

5)   monocular deprivation + 7d dark rearing + long light rearing;

6)   monocular deprivation + short light rearing;

7)   monocular deprivation + long light rearing.

Control mice were normally light reared, on standard light/dark cycle (12h + 12h), and sacrificed at age postnatal day 61 or 57 (P61,P57)

Dark born mice were born in a dark room and sacrificed between P55 and P65.

Mice long term monocularly deprived were deprived for 6 months from the age of 2 months and sacrificed at age P248 and P276.

Mice of the other 4 groups were subjected to MD during the critical period for 30 days and after that the eye was reopened. The animals were split and for recovery placed either in the dark room or in a normal light/dark cycle room. Dark rearing had a duration of 7 days and then animals were light reared (12h light + 12h dark). Short term LR means a recovery in a normal light/dark room for a period of 7 days or less, while a period longer

than 7 days was considered long term LR. The table 2.1 below summarizes the immunohistochemistry mice conditions used.

Condition	Age	Duration of MD	Sample size (n)
Ctrl	P61	-	3
DB	P50-65	-	4
MDlong term	P276,248	6 months	3
MD+ 7DR+ LR short term	P118	1 month	3
MD+ 7DR+ LR long term	P86-P125	1 month	2
MD+ LR short term	P60-P62	1 month	3
MD+ LR long term	P71-P90	1 month	4

Table 2.1 it shows the mice conditions used for immunohistochemistry experiments

2.4   IN VIVO OPTICAL IMAGING

2.4.1 Chamber implantation

For chronic optical imaging a stainless steel chamber was implanted, so that it was possible to analyze a mouse up to over several weeks to visualize the changes in its V1 map and the development state of visual cortex plasticity. The chamber was implanted about 10 days before starting the series of optical imaging because that permitted the stabilization of the cortex under the chamber and the monitoring of the animal's status. This procedure was done by a F. Sengpiel's lab member following the Goldey et al. protocol (2014).

Animals were anaesthetized in a chamber with 4% isoflurane in oxygen (0.5 L/min), subsequently an intramuscular injection of 0.03 ml chlorprothixine (Sigma, 1mg/kg) was given to induce sedation. A homeostatic heating blanket (Harvard apparatus, UK) was used to keep constant the body temperature during the surgery. The isoflurane was reduced to 2% and the oxygen to 0.3 L/min the wellness of the animals was monitored by ECG recording; heart rate should vary between 400 and 600 beats per minute (bpm).

A needle passing through the leg muscle was used as one of the two electrodes, and the ear bar as the second electrode both linked with the ECG amplifier. Isoflurane was reduced to 1.5% and a subcutaneous injection under the scalp of lidocaine was administrated to anaesthetize locally. The depilatory cream was used to remove the hairs on the head ethanol and iodine was applied to disinfect the skin area above V1 on the right side (contralateral to the deprived eye in case of MD animals); a rectangular flap was cut out by small scissors. Using fine tweezers, the skull was cleaned from the above periost. Saline was applied to avoid that the bone tissue alters its hydration status and to remove periost remains. After that, a small amount of dental cement was applied to fix the skin and, to glue the steel chamber ring all around the exposed visual cortex area. By a very fine drill a little square (1mm x1 mm) of the bone was drilled off gently, taking care not to touch the underlying brain and removing the bone powder using Sugi swabs (Kettenbach, Germany) and saline. Two glasses previously glued together, a circle (Ø 3mm) and a square (1mm x 1mm), were applied into the steel ring so that the square one was juxtaposed on the boneless area. The glasses were stuck using dental cement and waiting for some minutes till it was completely dried. The animals were put in the recovery chamber until they were awake and active. (insert chamber image)

2.4.2 Optical imaging session

Animals were anaesthetized in a chamber with 4% isoflurane in oxygen (0.5 L/min), subsequently an intramuscular injection of 0.03 ml chlorprothixine (sigma, 1mg/kg) was given to induce sedation. A homeostatic heating blanket (Harvard apparatus, UK) was used to keep the body temperature constant during the surgery. The isoflurane was reduced to 2% and the oxygen to 0.4 L/min and the metallic chamber was screwed to the holder to keep completely stable. This method prevents noise caused by the heart beat and breathing-related body movement. The wellness of the animals was monitored by ECG recording which should have a variation between 400 and 600 beats per minute (bpm). Two needles passing through the muscle were used as electrodes, one through left posterior leg muscle and the other one through the right anterior leg muscle, both linked with the ECG amplifier to receive the signal from the heart.

In case the animals were subjected to optical imaging for the first time, it was necessary to reopen the MD eye before starting the recording with fine scissors and forceps.

At that point the isoflurane was reduced to 0.9% in oxygen to avoid the brain activity being too depressed, taking care that the heart rate stayed in the correct range.

Using a green light of wavelength 546nm generated by a halogen light box and selected by band pass interference filter, the exposed part of the head, inside the chamber, was illuminated. Green light was chosen because hemoglobin absorbs maximally at wavelength 546nm, so that allows to visualize best contrast between the surface and deeper blood vessels in the Region Of Interest (ROI) which is visualized on the computer using VDAQ program (figure 2.2).
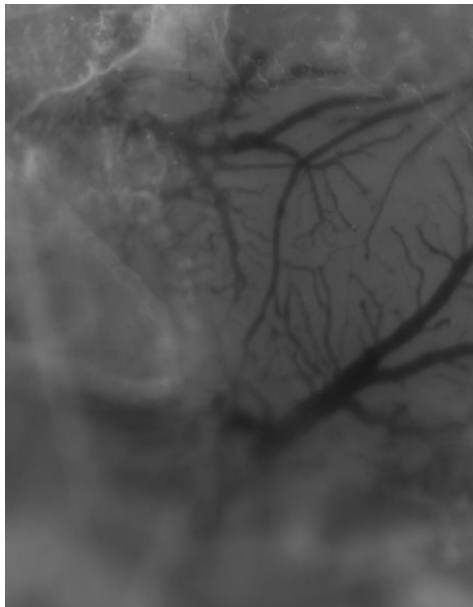
Figure 2.2. Typical Region of Interest (ROI). the big vessels could be noted which were a guide to find the correct V1 area into the occipital zone

The camera, linked to the VDAQ computer, was positioned and focused on the ROI (V1) in the right hemisphere. Intrinsic signal imaging was carried out using an Imager 3001 imaging system (Optical Imaging Inc, Mountainside, NJ) with the CCD camera focused 200μm below the cortical surface. A microscope tandem lens was used, constructed from two front to front photographic lenses (50mm, f1.2).

After selecting the correct anatomic area, the functional visual area and the intrinsic signal were detected by changing the illumination wavelength to 700 nm (red light), moreover the live display was changed to a pseudo color representation of the

intensity of the reflected light. Illumination was increased until saturation was near maximal (but without any over-saturation) since the cortical responses result in a decrease of reflection.

The 2 eye shutters were placed in front of the mouse eyes and a flat CRT monitor was positioned in the center 15 cm from the animal. Both shutters were connected, via a control box, to a computer on which periodic stimuli, either for OD or Retinotopy imaging, were pre- set up using VSG program (Visual Stimulus Generator, Cambridge Research System UK). (Figure 2.3)
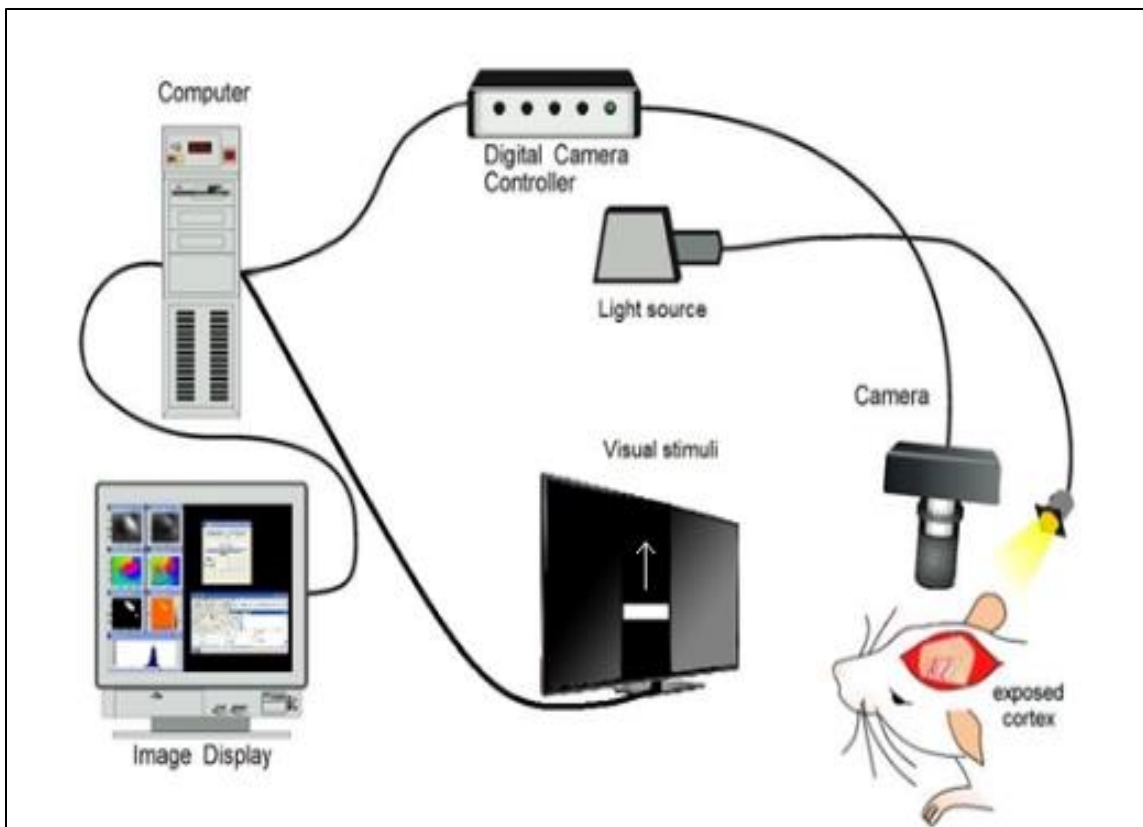

Figure 2.3. A typical optical imaging setup. The lightly anaesthetized mouse saw the stimulus on the opposite screen. The cortical surface was illuminated by a light source, while the camera acquired the images. The camera was linked with a computer in order to generate the stimuli and collect the images. ()

2.4.3 Periodic intrinsic signal

Periodic imaging was used to measure intrinsic signal in the visual cortex. Kalatsky and Stryker (2003) developed this paradigm that consists of a periodic, repetitive stimulus at a specific temporal frequency presentation able to evoke a periodic pattern of cortical activation. Our periodic stimuli consisted of a stimulus with a temporal repetition period of 8 seconds, thus stimulating at 0.125Hz.

The OD stimuli consisted of a white horizontal bar of diameter one degree and width 30 degrees moving upwards on a black background. The temporal pattern is 8 seconds, thus stimulating at 0.125 Hz and the acquired signal was at 8.33 Hz. The eye shutters work in alternation, so while the left eye is allowed to see the right eye is covered and vice versa. Each trial for each eye has a duration of 2 minutes and the entire OD recording comprises 12 trials, 6 for each eye. Using a custom-written Matlab program all the intrinsic signals obtained for each eye were averaged and a fast Fourier analysis (FFT) was carried out, calculating the amplitude and phase value of each pixel value in the ROI for the ipsi- and contralateral eye.

From the amplitude values for each pixel the ocular dominance index (ODI) was calculated using the formula:

ODI = (Contralateral - Ipsilateral)/(Contralateral + Ipsilateral)

The Retinotopy map was also obtained using the periodic paradigm, but in this case the eye shutters were not required and, moreover, different stimuli with the same temporal frequency were employed. Such stimuli consisted of a white bar of diameter 1 degree and width 60 degrees drifting alternatively 2 minutes horizontally and 2 minutes vertically on a black background. This stimuli has a temporal repetition period of 8 seconds, thus stimulating at 0.125 Hz and the signal was acquired at 8.33 Hz. Using again Matlab program all the functional images for horizontal and vertical stimuli were averaged. In this way it was possible to analyze the degree of organization of retinotopic cortical maps (Smith & Trachtenberg, 60 2007). The principal of this analysis is that in a well organized map one should observe a spatially smooth progression of phase values which are represented ion pseudo color. (Figure 2.4)
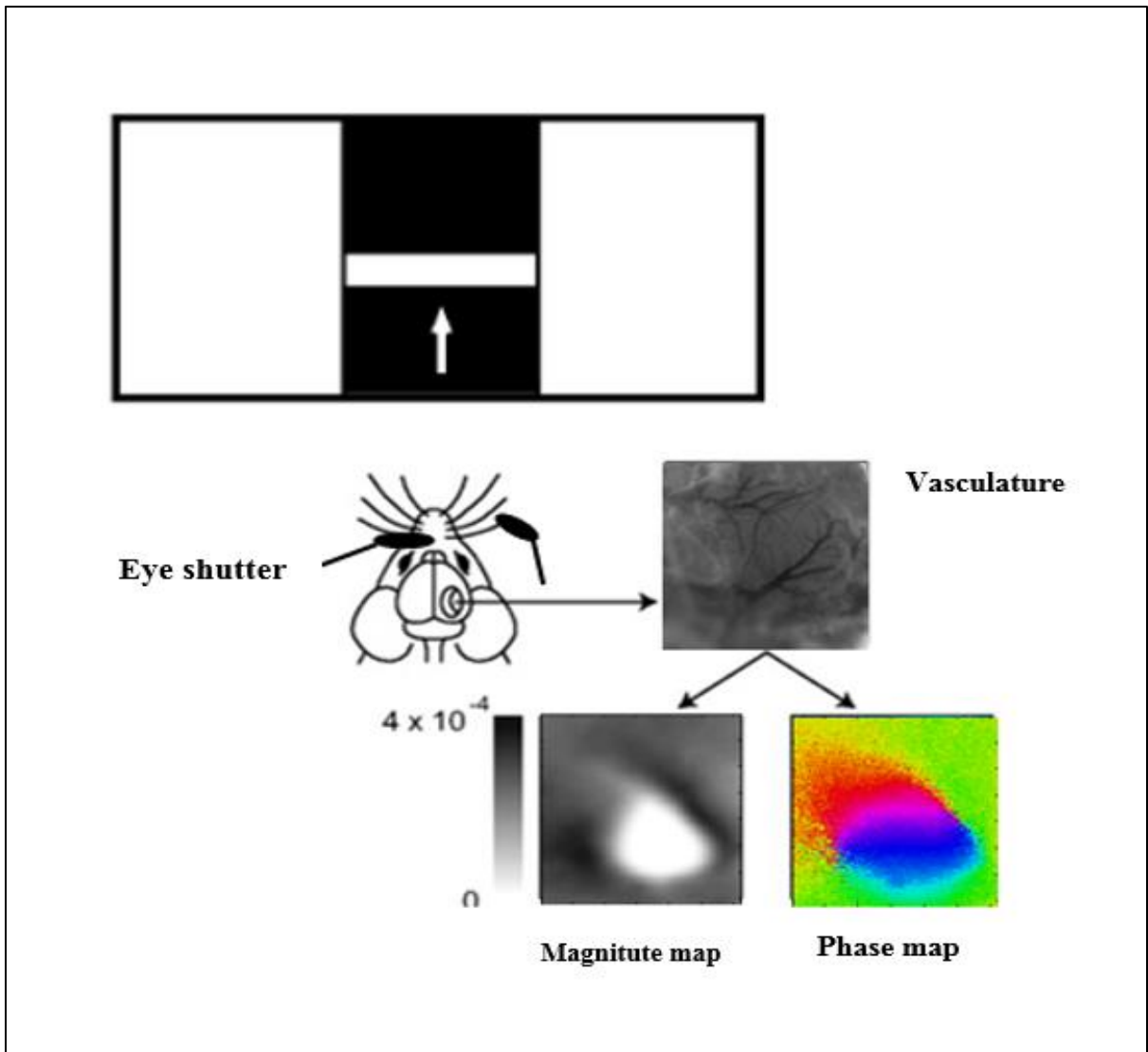
Figure 2.4. Typical Optical Imaging Output during ODI registration. The eyes of the mouse were alternatively covered by the eye shutters while visual stimuli were presented. The phase and the magnitude maps were generated from the acquired images.
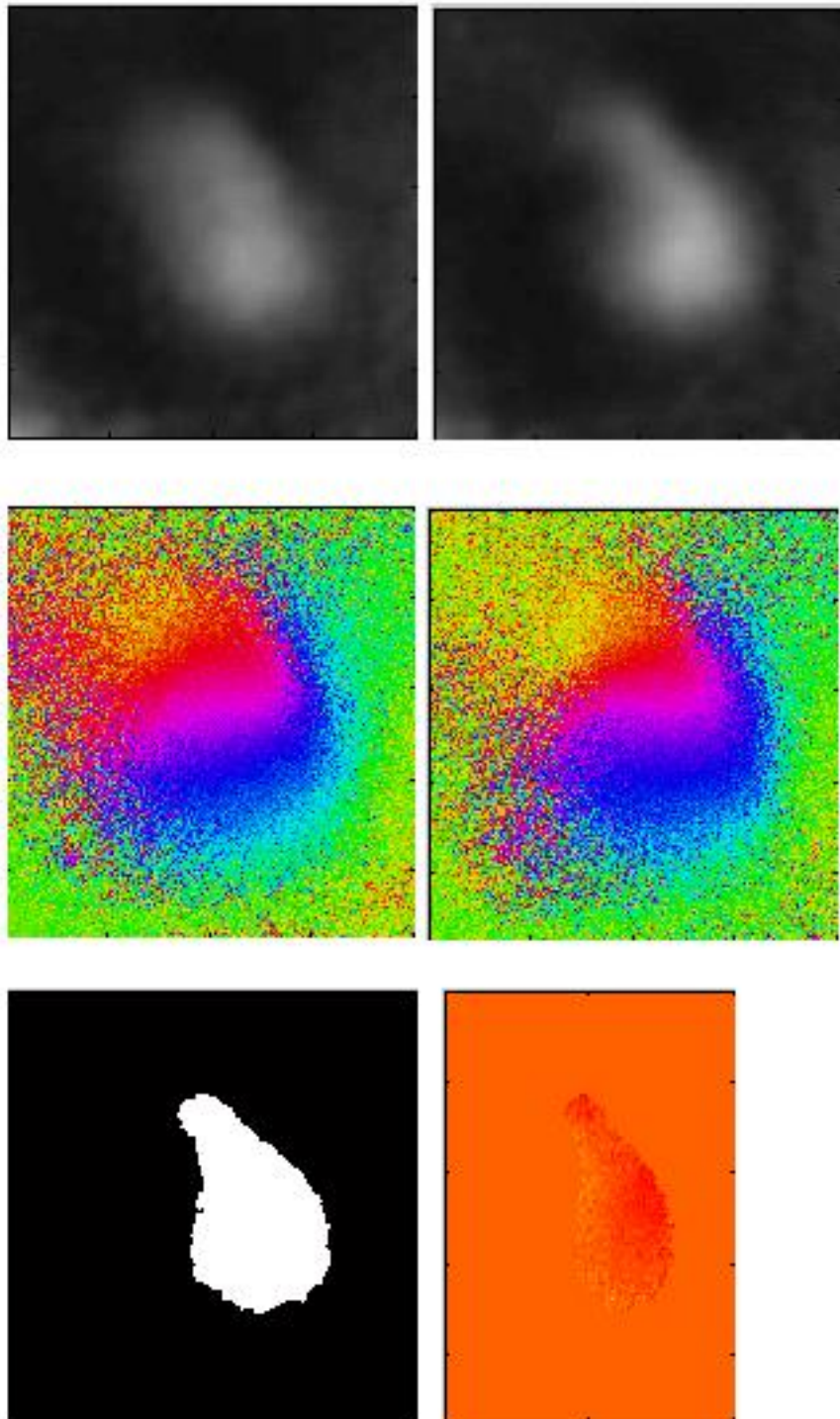
Figure 2.5. typical Optival Imaging analysis. It shows the ODI maps obtained from the average of 12 alternative recording of both eyes.

Figure 2.6. Typical Optival Imaging analysis. It shows the retinotopy map obtained with periodic horizontal and vertical stimuli

2.5 FREEZING BRAINS

After the final imaging session animals were killed by cervical dislocation. The brains collected were fixed in 4% paraformaldehyde (4% PFA) for 24 hours, subsequently they were placed into sucrose solution overnight for cryoprotection. At this point the brains had reached the bottom of the tubes, so they were extracted and carefully dried. Cube trays were half filled in with OCT (Optimum cutting temperature) and left to 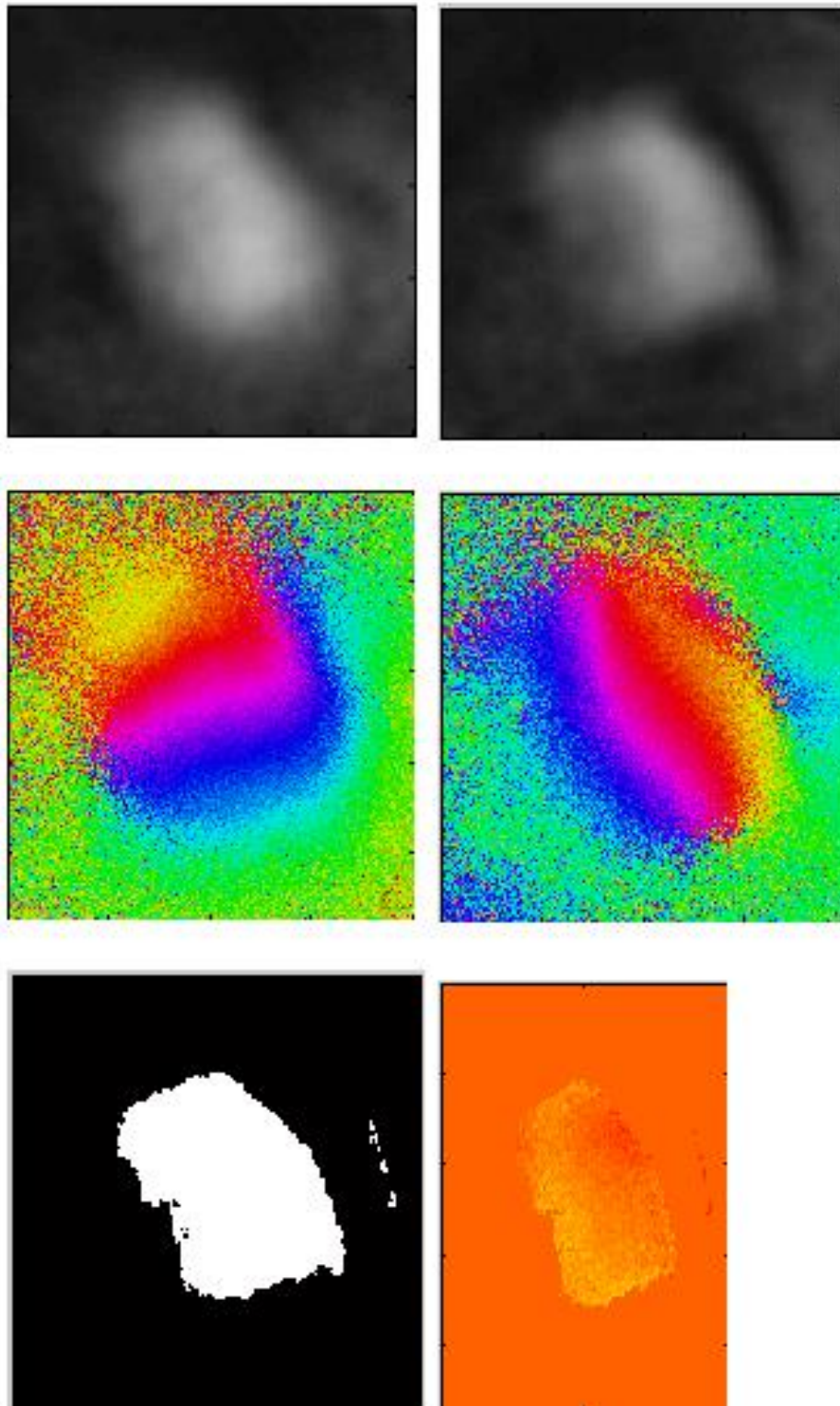solidify in a container of dry ice, then each brain was put in one of these cubes with the ventral surface down and covered completely with more OCT. Isopentane, previously cooled in liquid nitrogen, was used to freeze the cubes. It was important that the orientation were the same for all the brains in order to know their position even though it was completely impossible see them in the white cubes. The cube tray was covered with paraffin film and stored in a -30°C freezer. Using the cryostat coronal sections were cut of the whole visual cortex region. The thickness of the slices was 14μm and the ideal temperature to obtain the best cutting results was -18°C.

2.6 IMMUNOHISTOCHEMISTRY

2.6.1 Staining protocol

Two different histochemical staining protocol were carried out:

1)   WFA staining: it permits to visualize the perineuronal nets (PNNs) since WFA has an affinity for N-acetylgalactosamine;

2)   VGAT staining: it labelled the vesicular GABA transporter using antibodies specific for N- and C-terminal epitopes of VGAT to localize the protein. VGAT is highly concentrated in the axonal terminals of GABAergic neurons in the brain.

Six slides deriving from three different animals for each condition were randomly selected (6 slides x 7conditions =42 total slides). The sections were air dried for at least 30 minutes and the borders of each slide were lined by Pap pen, a special marking pen that provides a thin film-like hydrophobic barrier when a circle is drawn around a specimen on a slide. This water repellent barrier keeps staining reagents localized on the tissue sections and allows use of less reagents per section. Then the slides were rehydrated

by phosphate buffer solution (PBS) twice with an interval of 5 minutes. At this stage all the slides were split into 2 groups: one for WFA staining and one for VGAT staining.

In contrast to VGAT staining, WFA staining did not require the application of blocking serum because it has a vegetable origin, so it was possible to apply directly the primary antibody (WFA-biotinylated) with the correct concentration (1:100 in PBS) and stored in a humidifying chamber overnight at 4°C.

In the second group, before using VGAT antibodies to detect proteins by immunohistochemistry, all epitopes on the tissue sample should be blocked to prevent the nonspecific binding of the antibodies. Normal serum is a common blocking reagent, because the serum carries antibodies that bind to reactive sites and thus prevents the nonspecific binding of the secondary antibodies used in the assay. A critical factor, though, is to use serum from the species that the secondary antibody was generated in, as opposed to the species of the primary antibody. Serum from the primary antibody species would bind to reactive sites, but the secondary antibody would recognize those nonspecifically-bound antibodies along with the antibodies bound to the target antigen. Besides serum, concentrated protein buffers made with bovine serum albumin (BSA), gelatine or non-fat dry milk are often used to coat all proteins in a sample. This approach essentially forces primary antibodies to out-compete the blocking protein for binding to cognate ligands while reducing nonspecific binding because the antibodies have no greater binding affinity for nonspecific epitopes than do the buffer proteins.

Therefore, Normal Goat Serum (NGS) and Bovin Serum Albumin (BSA) in 0.5% triton PBS were used and left 1 hour. Just after that step it is possible to apply the primary antibody in these 21 slides. VGAT (1:250) was used and stored in the same way as the WFA slides.

The next day, the slides were rinsed three times with PBS and the secondary antibody was applied for 1 hour: Avidin-FITC (1:500 in PBS) was used for the WFA staining group and Rabbit Alexa488 (1:500in PBS) for the VGAT staining group. Sections were rinsed again and the Nuclear stain (Hoechst) was dropped on them and left for 5 minutes. At this point the coverslips were glued on top and the borders were fixed by nail vanish. The slides were then stored in a 4°C fridge until the imaging session which is necessary to take place within two week in order to not compromise the strength of the fluorescence.

2.6.2 Images analysis

The images of the slides, previously prepared, were scanned under a fluorescence microscope furnished with appropriate filters to excite tdTomato protein, PNNs and vesicular GABA transporter, VGAT. Images were taken from the contralateral and ipsilateral hemisphere of each slice.

These images were analysed by Fiji software and the number of red cells (PV+), the number of the green rings around the cells (PNNs+) and the colocalisation of both (PV+/PNNs+) were counted. On each image a square of 500x500μm was drawn, excluding layer 1 of V1, in order to count a region of the same size in all the images. All counts were then recorded on an Excel sheet. Densities recorded from different sections of the same brain were added up and converted from values per μm2 to values per mm2, resulting in one measurement of density for each hemisphere for each brain. Calculation and statistical comparison were carried out using Microsoft Excel. Percentage of PV cells surrounded by PNN (% colocalisation) were calculated by:

$$\% \text{ colocalisation} = \frac{\text{nr cells PV/PNN}}{\text{Nr PV cell}} \times 100$$

Two-tailed t-test were then calculated for this colocalisation, PNN density, PV density in order to compare the values obtained for each rearing condition. These test were carried out for each hemisphere separately. A P-value of < 0.05 was considered significant. Figure 2.7 shows is an example of images obtained from the analysis.
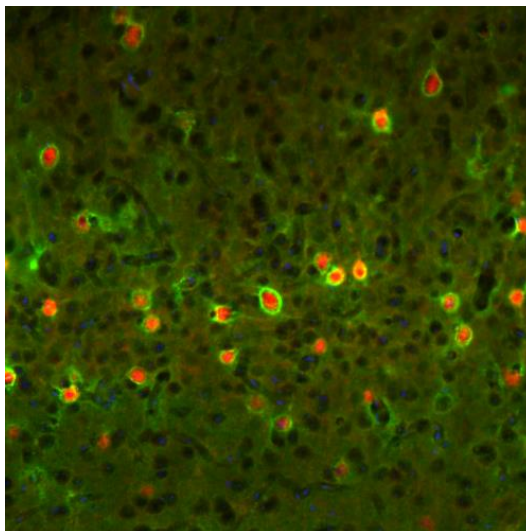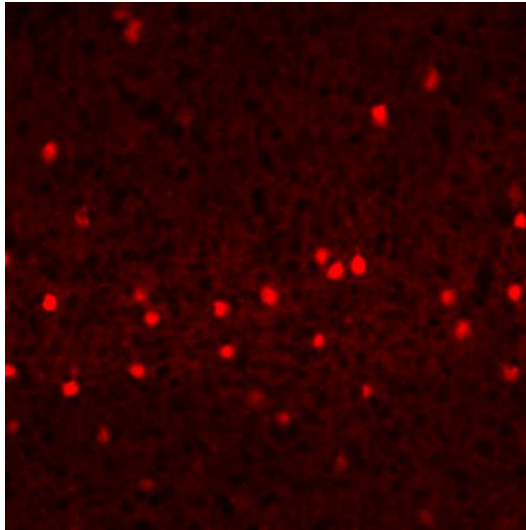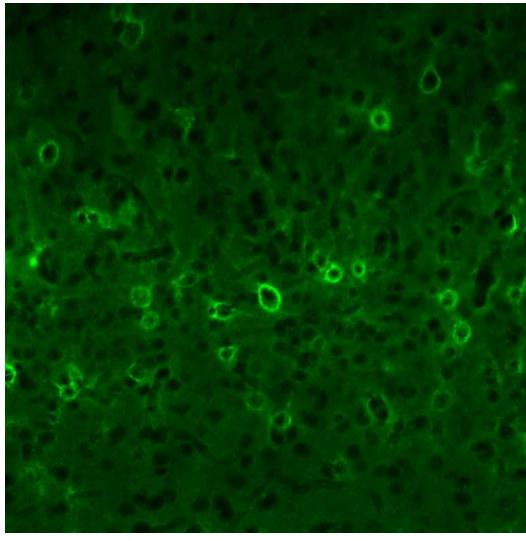
Figure 2.7. typical Images analysis of PNN immunostaining and PV. The belowest image shows the merge between PNNs and PV cells staining, so it is possible to observe that the perineuronal nets is typical for the PV cells. Moreover the colocalisation could be visualize.

Fiji software were used to analyze the VGAT images and the fluorescent mean intensity was measured in layers 2-5 of visual cortex.
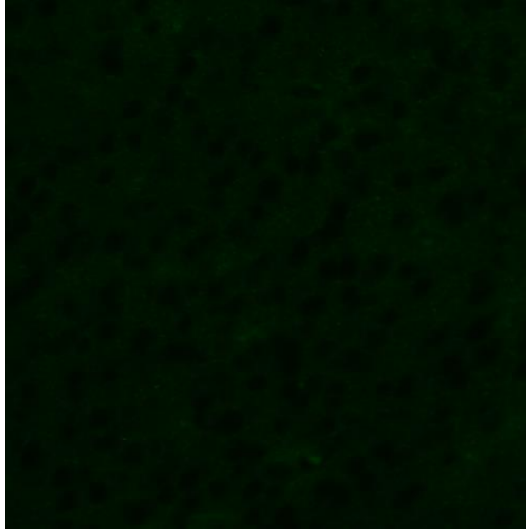
Figure 2.8. typical image analysis of VGAT immunostaining.

3. RESULTS

3.1 OPTICAL IMAGING

3.1.1 Ocular Dominance Index

The first part of our project was to investigate the brain activity in mice which had been subjected to different forms of recovery after MD. The chamber implantation gave us the possibility to do optical imaging not just once but up to five times in order to observe the evolution of the V1 activity after 30 day period of MD and after 1,2,3 and 4 weeks of recovery. Thus, in the best case we had 5 different values of ODI for each mouse.

The data of the animals were split in two groups:

1) Dark group: include the data of the mice subjected to 30 day period of monocular deprivation; after that they were put in completely darkness (dark-rearing, DR) and subsequently in a room where a normal cycle of light and dark was present (light-rearing, LR). Imaged at: 30 MD; 30 MD+ 7DR; 30MD+ 7DR +7LR; 30MD+ 7DR +14LR; 30MD+ 7DR +23 LR.

2) No Dark group: include the data of the mice subjected to 30 day period of monocular deprivation, but after the reopening of the closed eye recovery started immediately in a normal light/dark room. . Imaged at: 30 MD; 30 MD+ 7LR; 30MD+ 14LR; 30MD+ 21LR; 30MD+ 30 LR.

Mice were imaged using the procedure described before. The mice analysed were n=5 for the Dark group and n=4 for the No-dark group. Figures 3.1 and 3.2 illustrate the evolution of the magnitude maps in a mouse of the Dark group and in a mouse of the No-dark group. It shows the difference in the ODI shift before and after 7 days of light rearing in the two groups of mice. The P-value derived from two-tail test < 0.05 was considered significant.

The maximum ODI shift of the two groups (before vs. after recovery) was calculated and averaged. Another interesting comparison comes from the valuation of the ODI shift between before and after 7 day period of light rearing, that means the variation in ODI value revealed at 30MD + 7DR and 30MD+7 DR+ 7 LR in the Dark group and

between ODI values at 30 MD and 30 MD+ 7LR conditions . This will inform us about the difference in the cortex activity with and without the darkness. The comparison in the ODI value was done between 30MD and 30MD+ 7DR +7LR in the first group, instead of between 30MD and 30MD+ 7LR. The results are shown in the table below.

.

Dark Group

	Max shift	shift 30MD+7DR and 30MD+7DR+7LR	shift 30MD and 30MD+7DR+7LR
	0,53939	0,46903	0,25591
	0,37087	0,12981	0,37087
	0,190625	0,083242	0,018932
	0,39901	0,32405	0,39901
	0,43694	0,43694	0,18807
Mean	**0,387367**	**0,2886144**	**0,2465584**

No Dark Group

	Max shift	shift 30MD 30MD+7LR
	0,225424	0,04642
	0,161486	0,116246
	0,3819	0,03478
	0,037855	0,037855
	0,129365	-0,129365
Mean	**0,187206**	**0,0211872**

Table 3.1 A. it shows maximum ODI shift, the ODI shift between before and after 7 days of light exposure (both before and after Dark rearing) in Dark group. **B.** it shows maximum ODI shift and the ODI shift between before and after 7 days of light exposure in No-Dark group
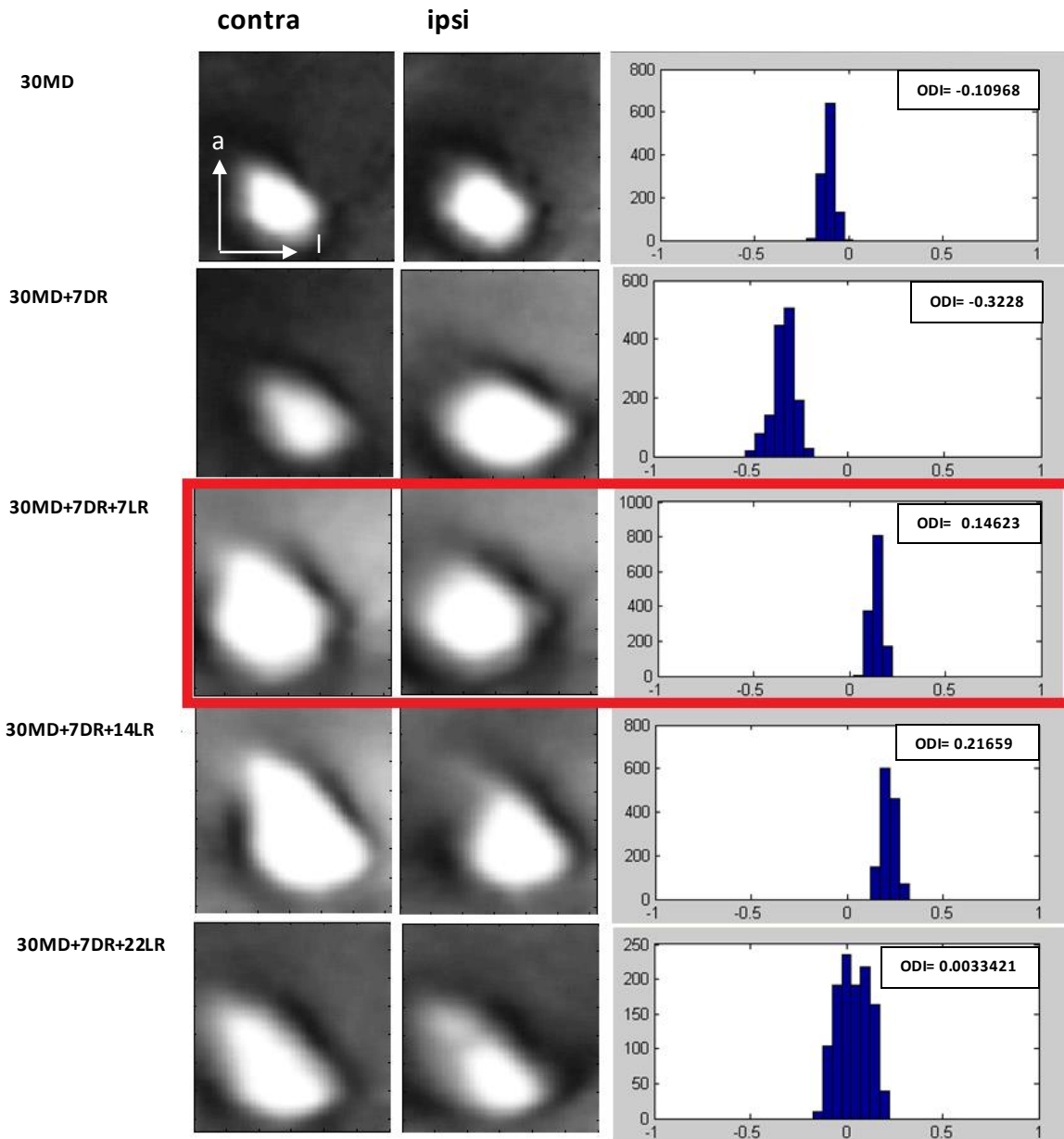
Figure 3.1 shows the evolution in the magnitude maps during the 5 optical imaging sessions in a sample mouse subjected to a 7 day period of darkness after the reopenig of the closed eye. The red square underlines the condition after 7 days of light. It is possible to observe to the right of the magnitude maps the histograms representing the shifts in ODI in favour of ipsilateral eye (negative) or contralateral eye (positive). The axes labelled a (Anterior) and l (Lateral) provide anatomical orientation.
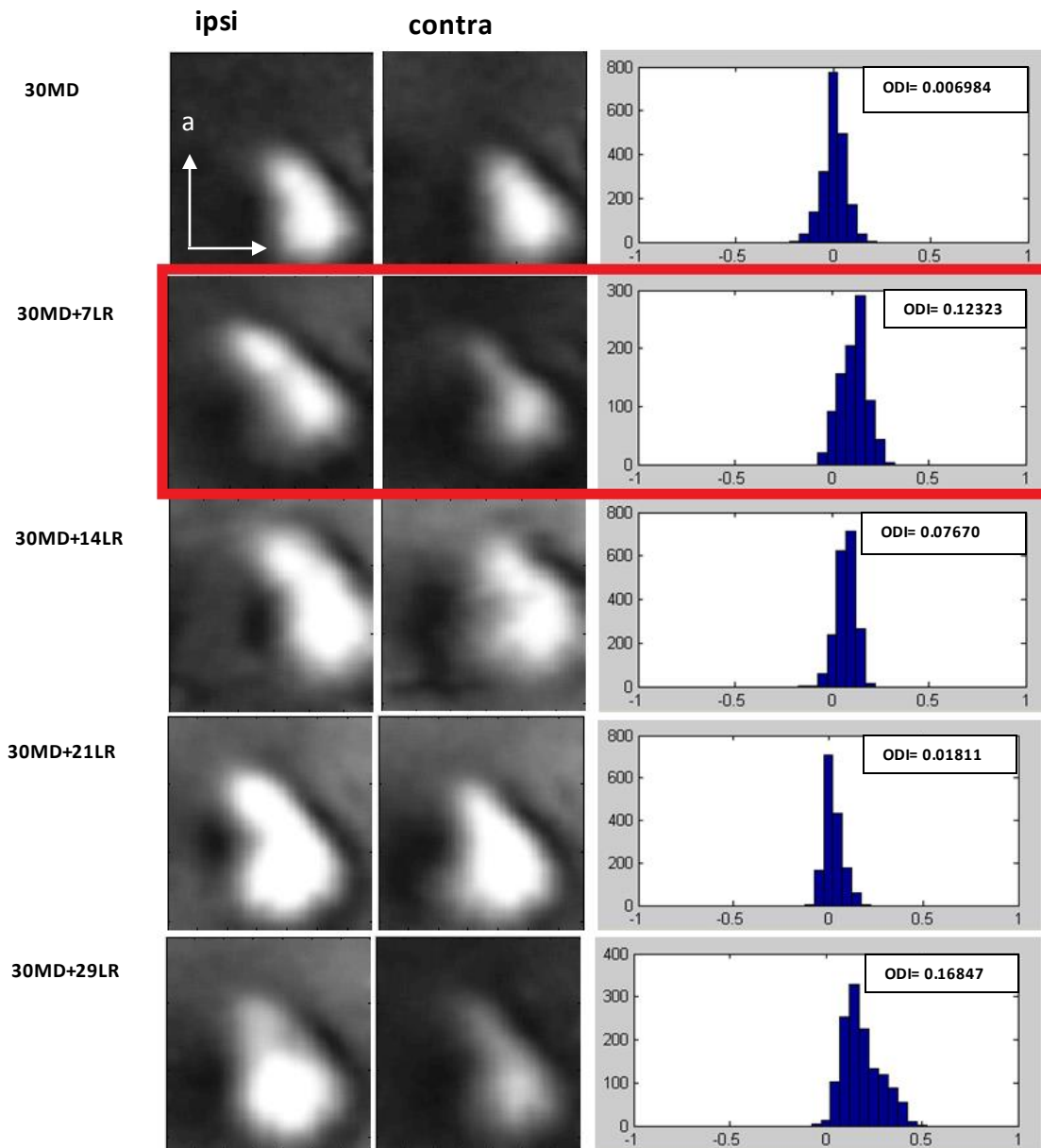
Figure 3.2 shows the evolution in the magnitude maps during the 5 optical imaging sessions in a sample mouse subjected to normal light/dark recovery after the reopenig of the closed eye. The red square underlines the condition after 7 days of light. It is possible to observe on the right of the magnitude maps the histograms representing the shifts in ODI in favour of ipsi eye (negative) or contra eye (positive). The axes labelled a (Anterior) and l (Lateral) provide anatomical orientation.
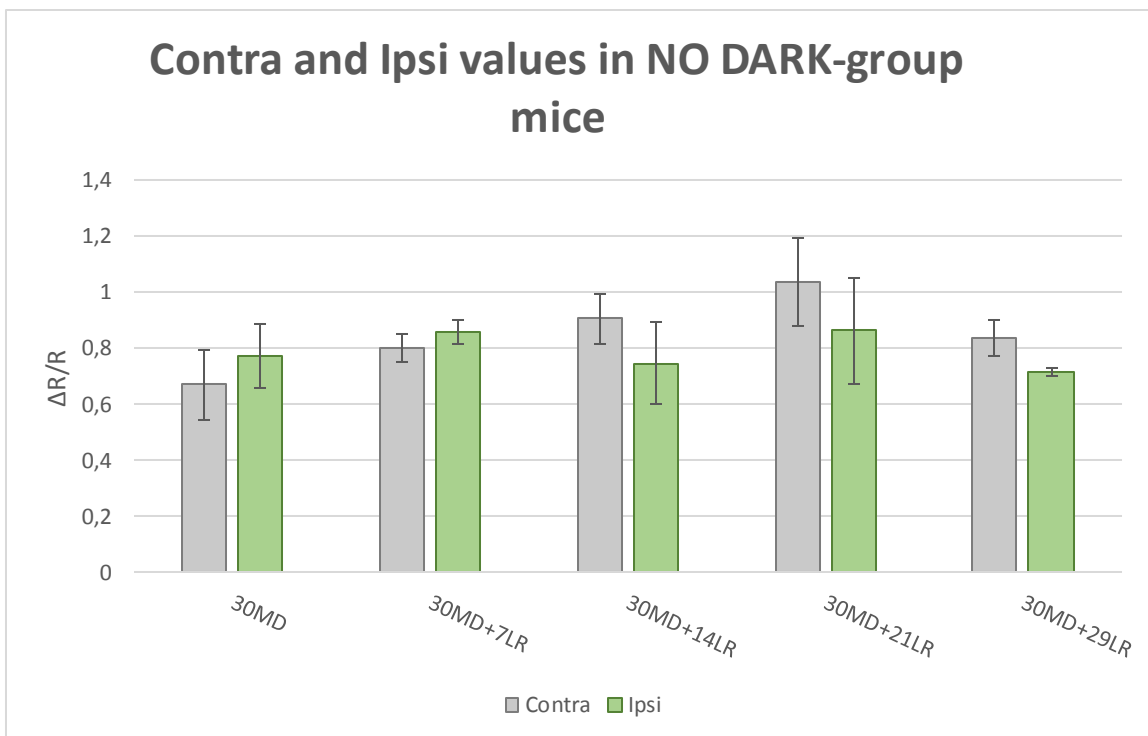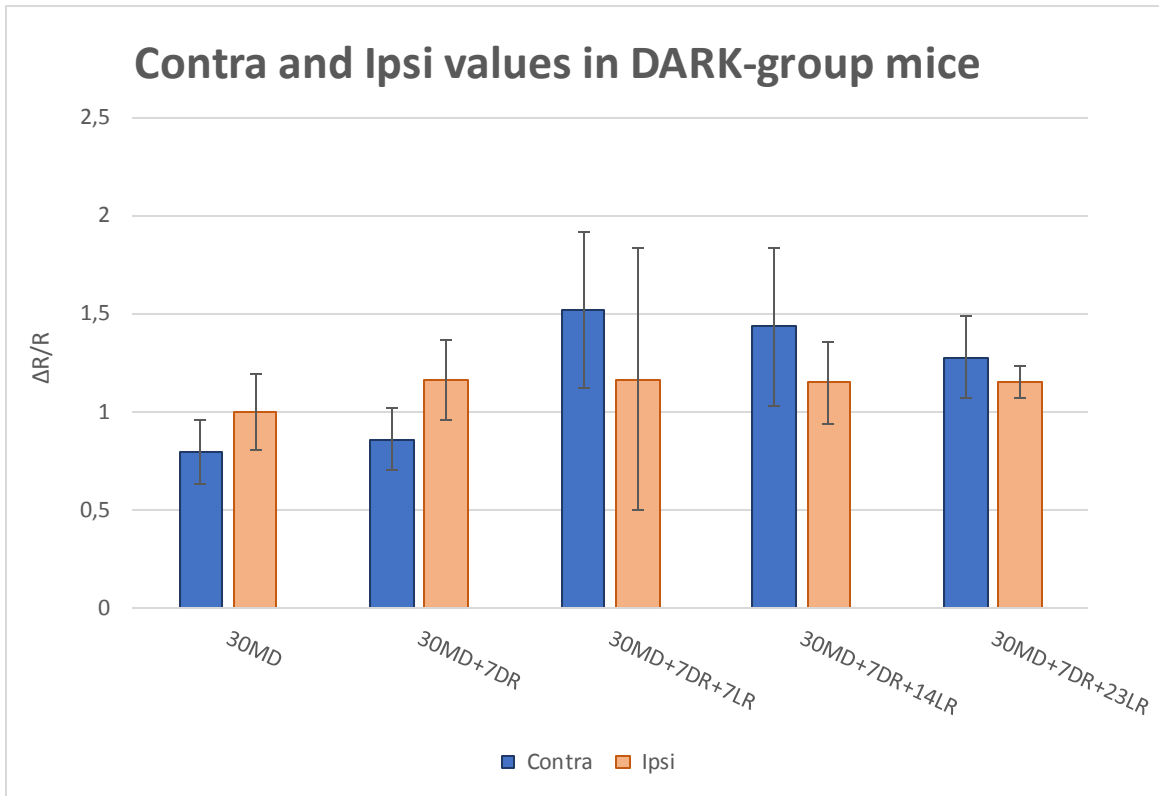
Figure 3 shows the variations of contra (previously deprived and ipsi (non-deprived) eye response magnitude value (ΔR/R) during 4 weeks of recovery. In the upper graph note the deprived-eye recovery in the Dark-group (third time point), compared with the lack of recovery in the No dark-group (second time point).
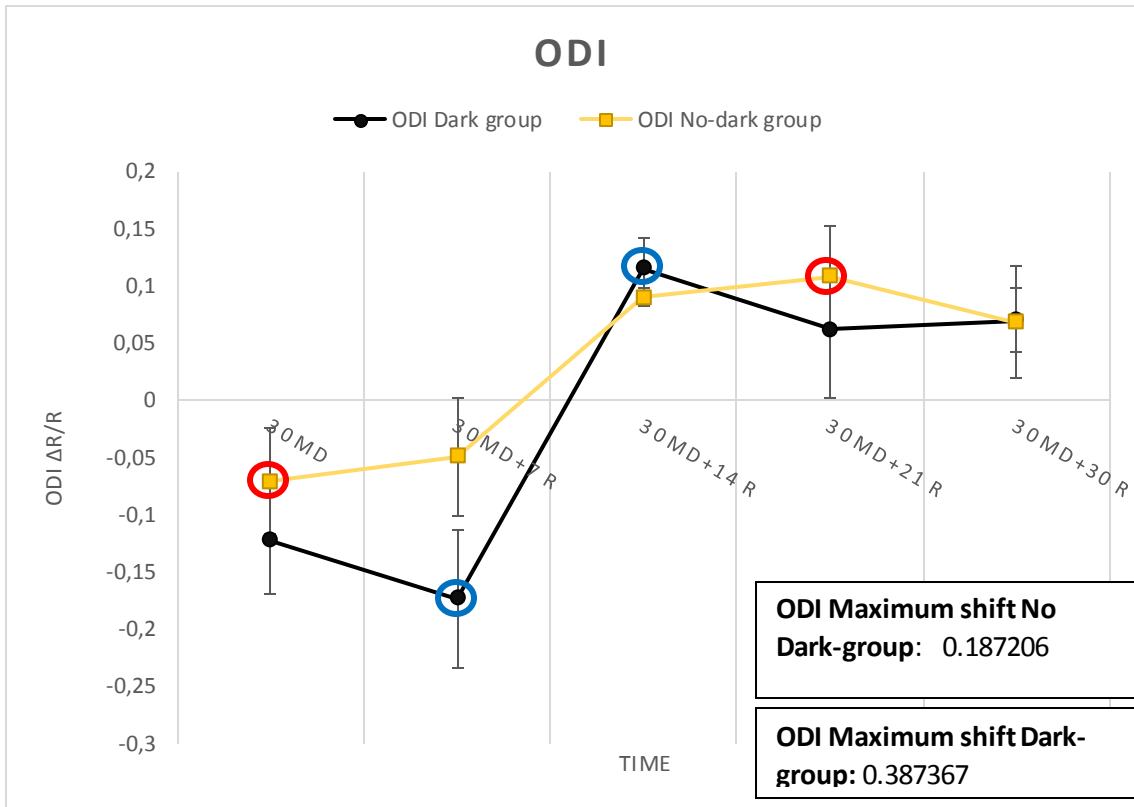
Figure 3.4 compares the ODI changes in Dark and No dark- groups. The black line follows the ODI variations in Dark group, the yellow one the No Dark-group. It is possible observe also the ODI maximum shift of the 2 groups expressed numerically in the bottom right side of the figure. Error bars show the standard deviation.

ODI shift after 7 days of light

ODI shift after 7 days of light
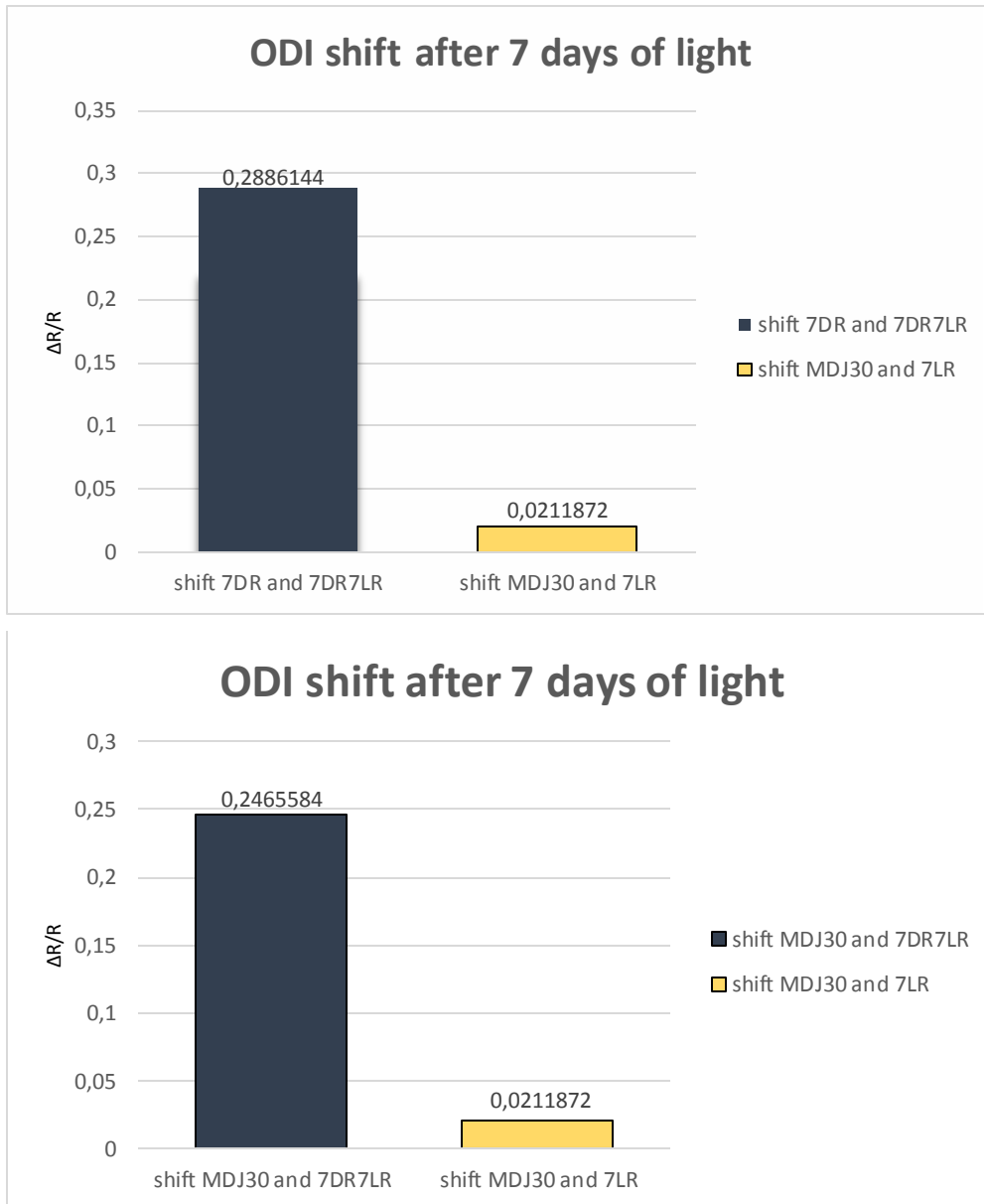
Figure 3.5. These graphs compare the degree of the ODI shift before and after 7 days of light in the 2 groups. The black bar represents the ODI shift in the Dark group between 30MD+7DR and 30MD+7DR+7LR conditions in the upper chart, in contrast in the second chart the shift shown is between 30MD and 30MD+7DR+7LR. In both graphs, the yellow bar represents the shift in ODI level in No dark-group between 30MD and 30MD+7LR conditions.

3.1.2 Retinotopic maps

Mice were imaged using the procedure described before and split in the same 2 groups to measure the retinotopic map. The mice analyzed were n=5 for Dark group, subjected to a 7 day period of dark rearing after the reopening of the eye lid closure, and n=4 for the No-dark group, put in a normal light/dark room after the reopening of the closed eye lid. The figures below show the retinotopic maps for a mouse of the Dark group (Figure 3.6) and a mouse of the No-dark group (Figure 3.7) for horizontal and vertical stimuli. The scatter value measures the difference in retinotopic position preference between neighbouring pixels and is the parameter which describes the smoothness of the retinotopic map.

Horizontal **Vertical**

30MD

Mean Scatter: 3.15

30MD+7DR

Mean Scatter: 3.0231

30MD+7DR+7LR

Mean Scatter: 2.9616

30MD+7DR+14LR

Mean Scatter: 3.2885

30MD+7DR+23LR
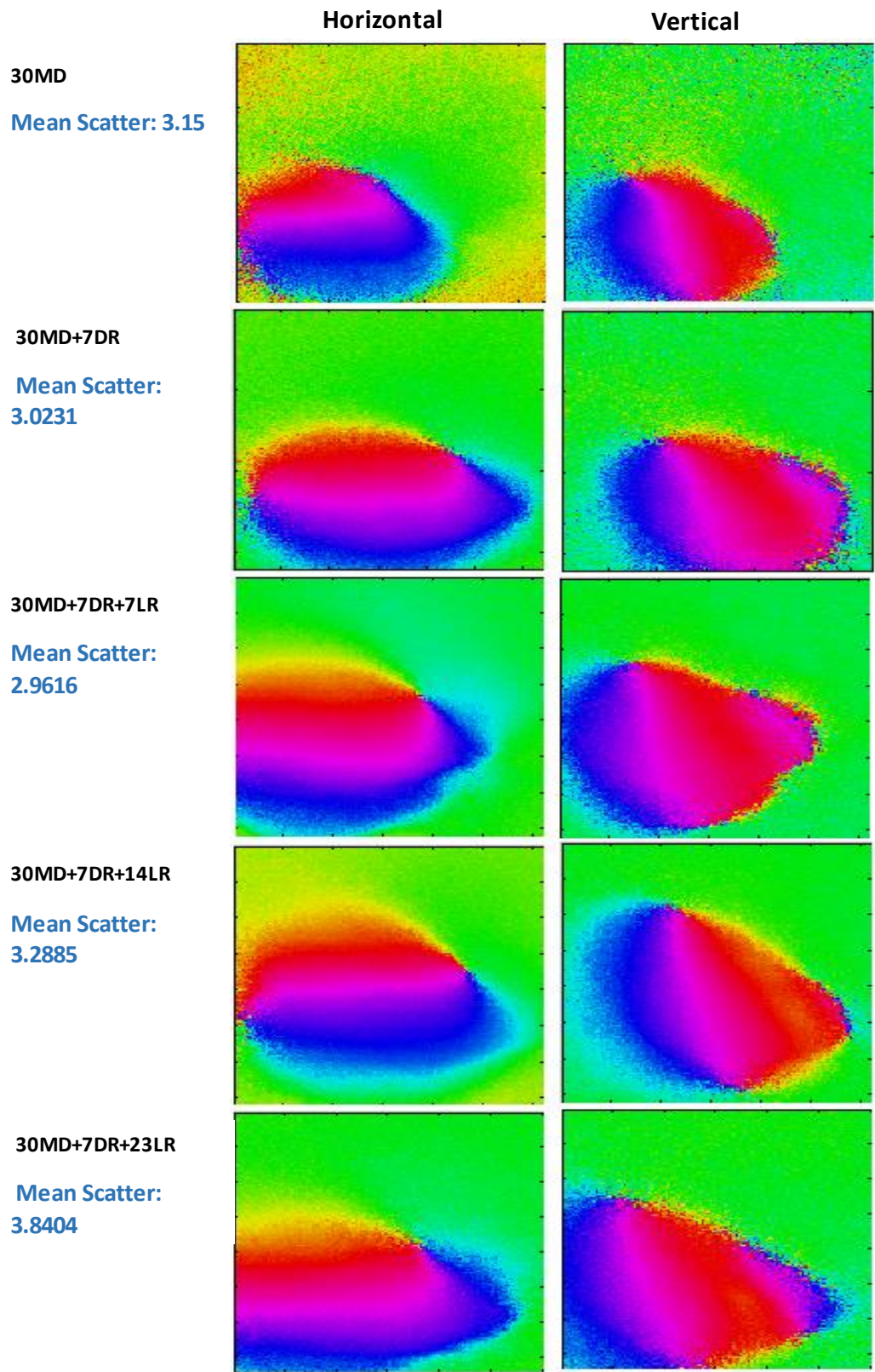
Mean Scatter: 3.8404

Figure 3.6. Retinotopic maps in a representative mouse of the Dark-group at the 5 time points. The left column shows the map obtained with horizontal bars, on the right side the map obtained with vertical bars is shown. Mean scatter values are also shown.

	Horizontal	**Vertical**

30MD

Mean Scatter: 3.9203

30MD+7LR

Mean Scatter: 5.1676

30MD+14LR

Mean Scatter: 7.8561

30MD+21LR

Mean Scatter: 3.3597

30MD+29LR
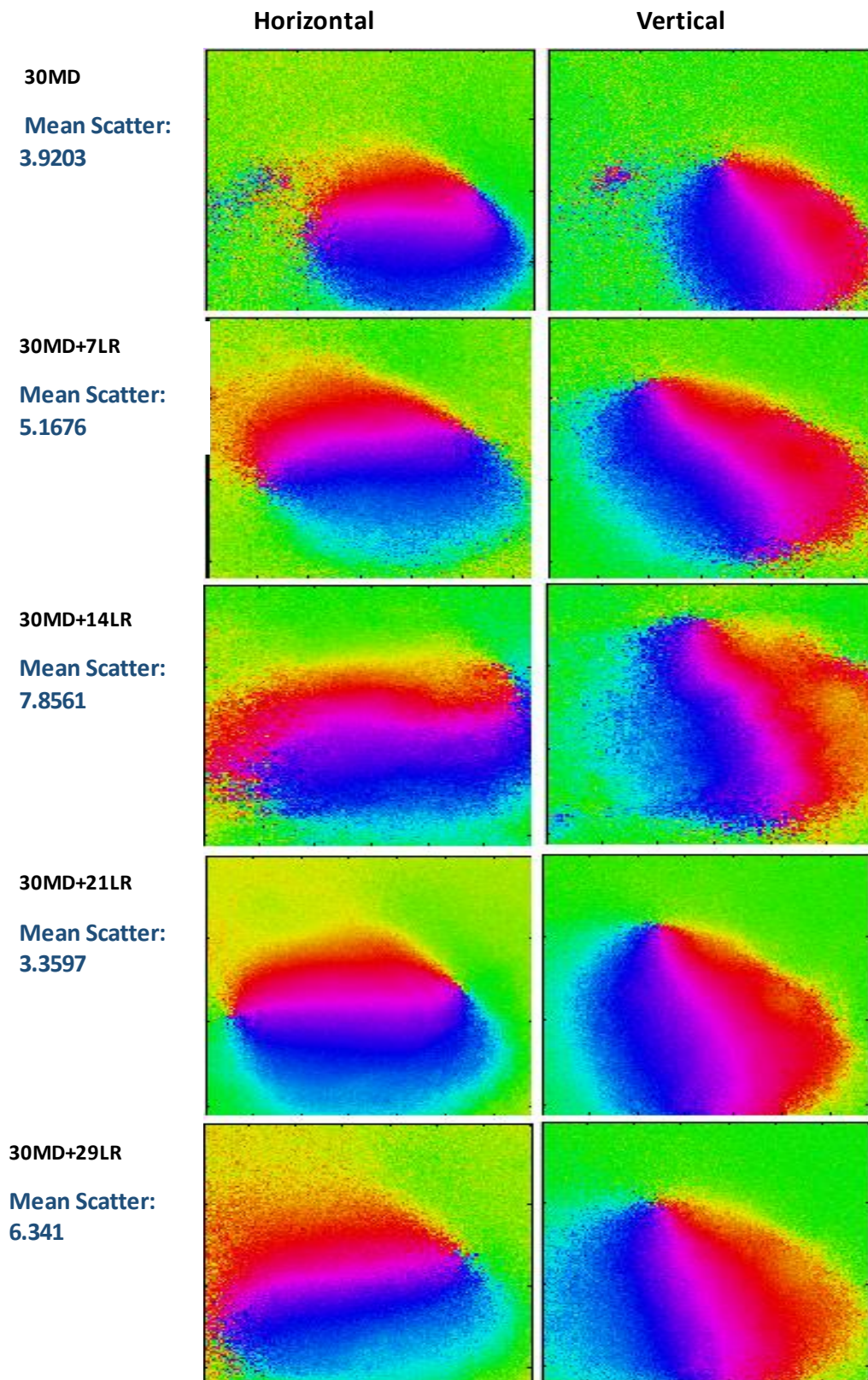
Mean Scatter: 6.341

Figure 3.7. Retinotopic maps in a mouse of the No-Dark-group at the 5 time points. The left column shows the maps obtained with horizontal bars, the right column the maps for vertical ones. Mean scatter values are also shown.
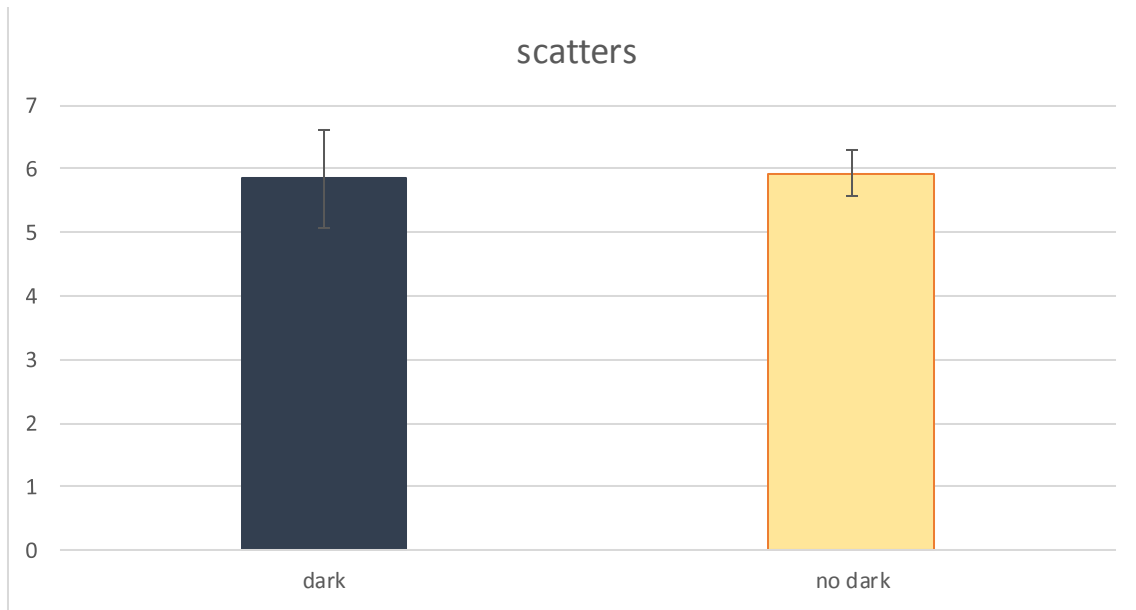
Figure 3.8 Graph shows the similarity between the scatters averaged in the two groups. Error bars show the standard deviation.

3.2 IMMUNOHISTOCHEMISRY

3.2.1 *PV-expressing cells density ,PNN density and percentage of Colocalization*

The counting of PV cells and PNNs and the colocalization percentage were performed using the methods described in detail in the previous chapter; n=3 mice were used for CTRL group, n=4 for DB group, n= 3 for MDlong term group, n=3 for MD+ 7DR+ LR short term, n=2 for MD+ 7DR+ LR long term, n=3 for MD+ LR short term, n=4 for MD+ LR long term. In the first part of the analysis of our project we investigated if differences occur between ipsi and contra hemisphere in terms of PV cell and PNN density and in the percentage of colocalization. In figure 3.9 PV, PNN and PV/PNN for each group considered are displayed and it discloses the features and the dissimilarity for each group which are analysed later in this section.
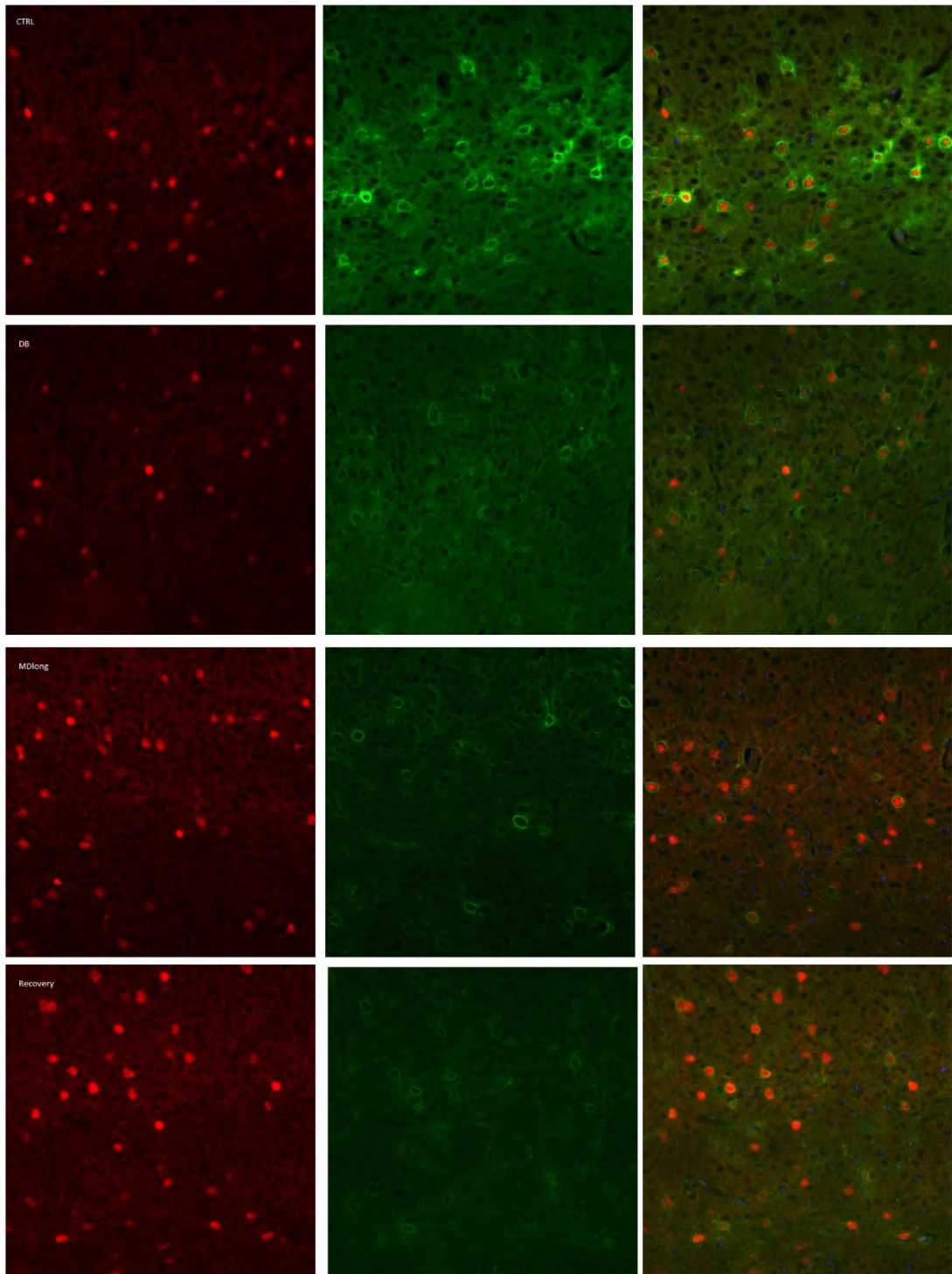
Figure 3.9. In this figure images of PV, PNN and PV/PNN for each group considered are shown. The images are taken in 500µm2 using fluorescence microscopy. All the PV cells images are lined in the first column, PNN in the second one, instead in the third column is possible to appreciate PV cells surrounded by PNN (colocalization).

The PV density was measured in a 0.25 mm2 area in V1 excluding layer 1 of the cortex. A comparison of left and right hemisphere were done and a P-value derived from two-tail test <0.05 was considered significant. The tables below show the averages with standard deviation in contra- and ipsilateral eye of PV, PNN density and the percentage of PV cells surrounded by PNN (% Colocalization), they show also the p-value obtained from the comparison in each group. As can be seen, there was no significant difference between the two hemispheres for any of the conditions, for neither PV cells, PNNs nor colocalisation (Tables 3.2).

A

Condition	PV density both hemisphere	PV density Contra hemisphere	PV density Ipsi hemisphere	P-Value
Ctrl	92.21±3.16	95.37±20.13	89.06±14.14	0.81
DB	70.05±1.44	71.48±10.38	68.61±15.90	0.88
MDlong term	90.74±3.92	86.83 ±16.40	94.67±13.57	0.73
MD+ 7DR+ LR short term	106.89±7.19	114.09±15.63	99.70±24.36	0.64
MD+ 7DR+ LR long term	101.99±5.15	107.14±25.45	96.83±25.07	0.79
MD+ LR short term	79±11.22	90.22±6.64	67.78±7.89	0.10
MD+ LR long term	110.95±1.75	112.71±30.75	109.20±20.81	0.90

B

Condition	PNN density both hemisphere	PNN density Contra hemisphere	PNN density Ipsi hemisphere	P-Value
Ctrl	46.84±1.22	45.62±6.29	48.06±8.96	0.83
DB	28.30±1.99	30.29±1.71	26.30±5.49	0.53
MDlong term	28.08±4.08	32.17 ±5.60	24±4.14	0.46
MD+ 7DR+ LR short term	34.36±3.77	38.13±8.44	30.58±4.90	0.47
MD+ 7DR+ LR long term	25.28±2.28	27.55±6.84	23±9	0.71
MD+ LR short term	28.5 ±0.4	28.11±6.56	29±11.59	0.95
MD+ LR long term	46.08±5.68	40.40±9.83	51.76±3.66	0.27

C

Condition	% Colocalization both hemisphere	% Colocalization Contra hemisphere	% Colocalization Ipsi hemisphere	P-Value
Ctrl	49.26±0.37	49.63±6.75	48.88±4.07	0.93
DB	47.87±4.14	43.73±13.57	52.01±18.30	0.73
MDlong term	30.93±6.86	37.79 ±6.45	24.07±6.26	0.20
MD+ 7DR+ LR short term	33.25±0.80	32.46±7.07	34.05±11.09	0.91
MD+ 7DR+ LR long term	28.51±0.86	27.64±9.91	29.37±15.80	0.93
MD+ LR short term	34.19±4.57	29.62±4.62	38.76±12.57	0.55
MD+ LR long term	37.17±10.25	26.92±12.42	47.43±8.40	0.46

Tables 3.2 A,B,C they show the PV,PNN density and Percentage of colocalization in both, ipsi and contra hemispheres in each condition. Values are represented with standard deviation. The P-value indicates the significance of the differences between contra and ipsi hemispheres (>0.05 non-significant)

Long and Short term light rearing both in mice subjected to darkness and not, were compared and no statistically relevant difference was found (t-test>0.05), so we analyzed the differences between Dark and No-Dark (Figure 3.10). The Dark group was obtained by pooling MD+7DR+LR long term and MD+7DR+LR short term, while for the No-Dark group MD+LR long term and MD+LR short term were pooled. The results from the comparisons are shown below. Again, there were no significant differences between the two hemispheres for any of the 3 parameters tested.

A

T-test Dark VS No-Dark		
PV	PNN	% Colocalization
0.678	0.259	0.589

B
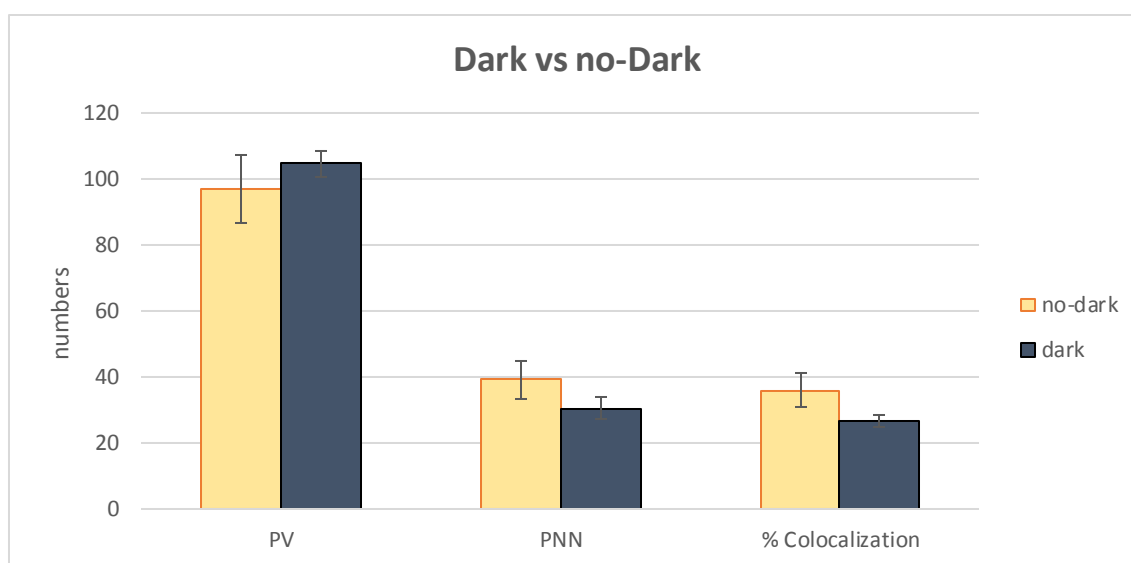
Figure 3.10 (A) Table with the two-tailed t-tests values for PV and PNN density and % of colocalozation. (B) Histogram illustrates differences in PV-density, PNN-density and percentage of Colocalization in V1 measured in Dark group (n= 5) and No-Dark group (n=7). Error bars show the standard deviation.

Since no statistically significant differences were observed, a Recovery group (n=13) was subsequently formed by pooling Dark (n=5) and No-dark(n=7) groups together.

CTRL was compared with all the other groups. CTRL (n=3) and DB (n=4) were compared in order to confirm a lower level of inhibition and/or a slower rate of maturation in mice subjected to darkness. In this case we assumed that differences should go in one direction, so a one-tail t-test was performed and the table below shows the results. The PNN density was significantly reduced in the DB group compared with the CTRL group (P < 0.05), moreover the results shows a trend where PV+ expressing cells are more in CTRL group than DB. The weak levels in DB of both PV+ expressing cells and PNN+ density causes an high percentage of colocalization, similar to the CTRL one (Figure 1.11).

A

| T-test CTRL VS DB		
PV	PNN	% Colocalization
0.171302311	**0.030564382**	0.413759644

B
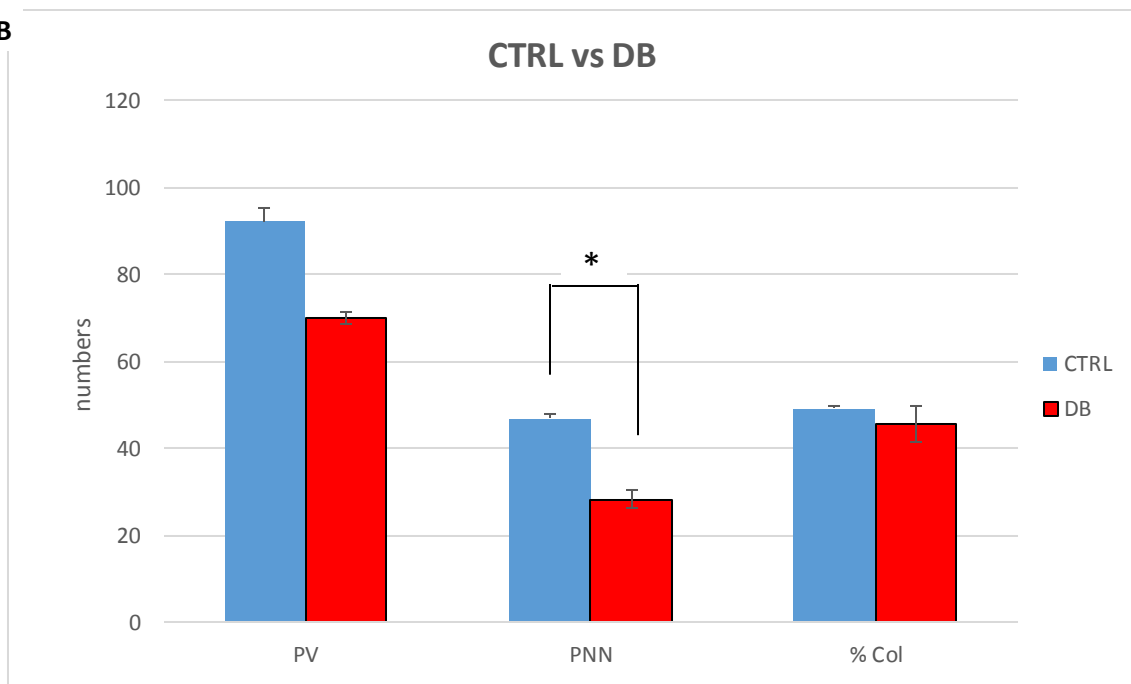
Figure 3.11 (A) Table with the one-tailed t-test values for PV and PNN density and % of colocalozation. (B) Histogram illustrates differences in PV-density, PNN-density and percentage of Colocalization in V1 measured in CTRL group (n= 3) versus DB group (n=4). P<0.05*, error bars show the standard deviation.

CTRL (n=3) and MDlong (n=3) were also compared in order to discover potential differences. We used a two-tailed t-test two tail and P-values < 0.05 were considered significant. The PV+ expressing cells values are very similar in both groups, but the PNN+ density shows a little difference. The percentage of PV cells with PNNs was found to be significantly lower in the MDlong group than in the CTRL group (Figure 3.12).

A

T-test CTRL VS MDlong		
PV	PNN	% Colocalization
0.952	0.125	**0.041**

B
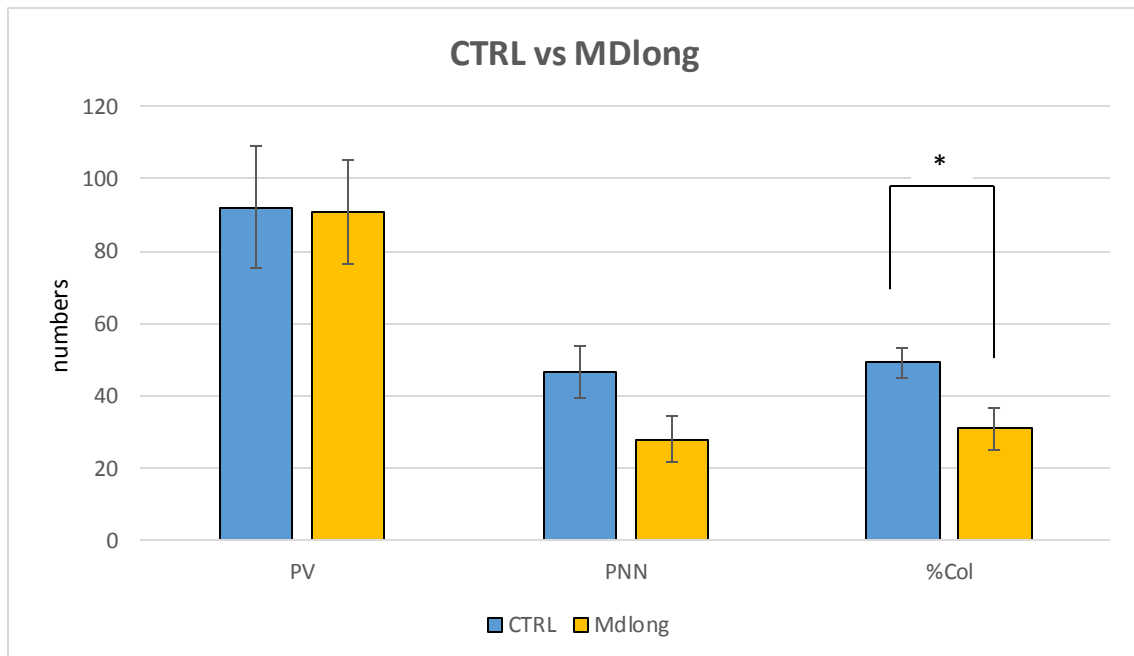
Figure 3.12 (A) Table shows the two-tailed t-tests values (B) Histogram illustrates differences in PV-density, PNN-density and percentage of Colocalization in V1 measured in in CTRL group (n= 3) versus MDlong group (n=3). P<0.05*, error bars show the standard deviation.

Recovery were compared both with DB and CTRL. The t-test used in the comparison between Recovery vs CTRL was two-tailed. PV+ expressing cells and PNN+ density show no difference between Recovery and CTRL, instead it is possible to observe a different balance, statistically significant, in the percentage of colocalization.

Instead a one tail t-test was used for comparison between Recovery and DB, since a trend toward a lower density of inhibition-related factors in the DB group was expected. Indeed the PV+ expressing level is statistically lower in DB group. PNN+ density and % of colocalization are similar in both groups. The Figure 3.13 summarizes the results obtained.

A

T-test Recovery VS CTRL		
PV	PNN	% Colocalization
0.665	0.200	**0.021**

T-test Recovery VS DB		
PV	PNN	% Colocalization
0.04	0.106	0.233

B
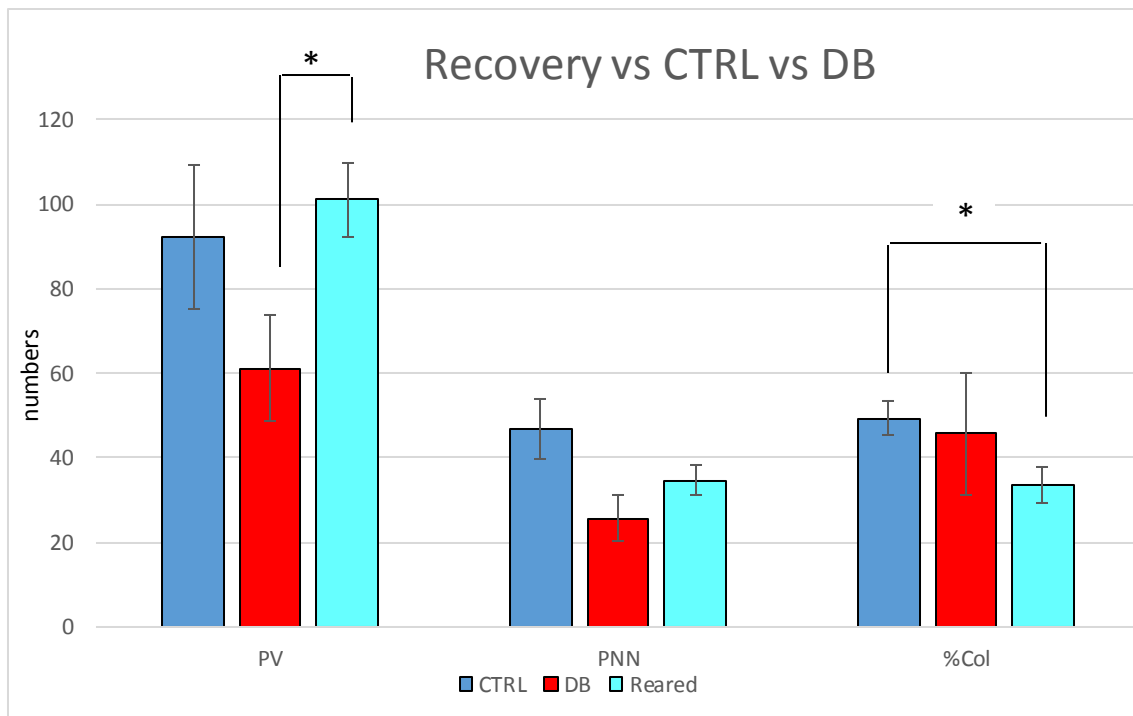
Figure 3.13. (A) Tables with the two-tailed t-tests values in Recovery versus CTRL and one tail t-test in Recovery versus DB groups. (B) Histogram illustrates differences in PV-density, PNN-density and percentage of Colocalization in V1 measured in in CTRL group (n= 3),DB group (n=4) and Recovery (n=13). P<0.05*, error bars show the standard deviation.

3.2.2 VGAT fluorescence index

VGAT immunohistological staining was performed using the methods previously described in detail. N=2 mice were used for CTRL group, n=3 for DB group, n= 2 for MDlong term group, n=2 for MD+ 7DR+ LR short term, n=4 for MD+ 7DR+ LR long term, n=3 for MD+ LR short term, n=2 for MD+ LR long term. (Figure 3.14)
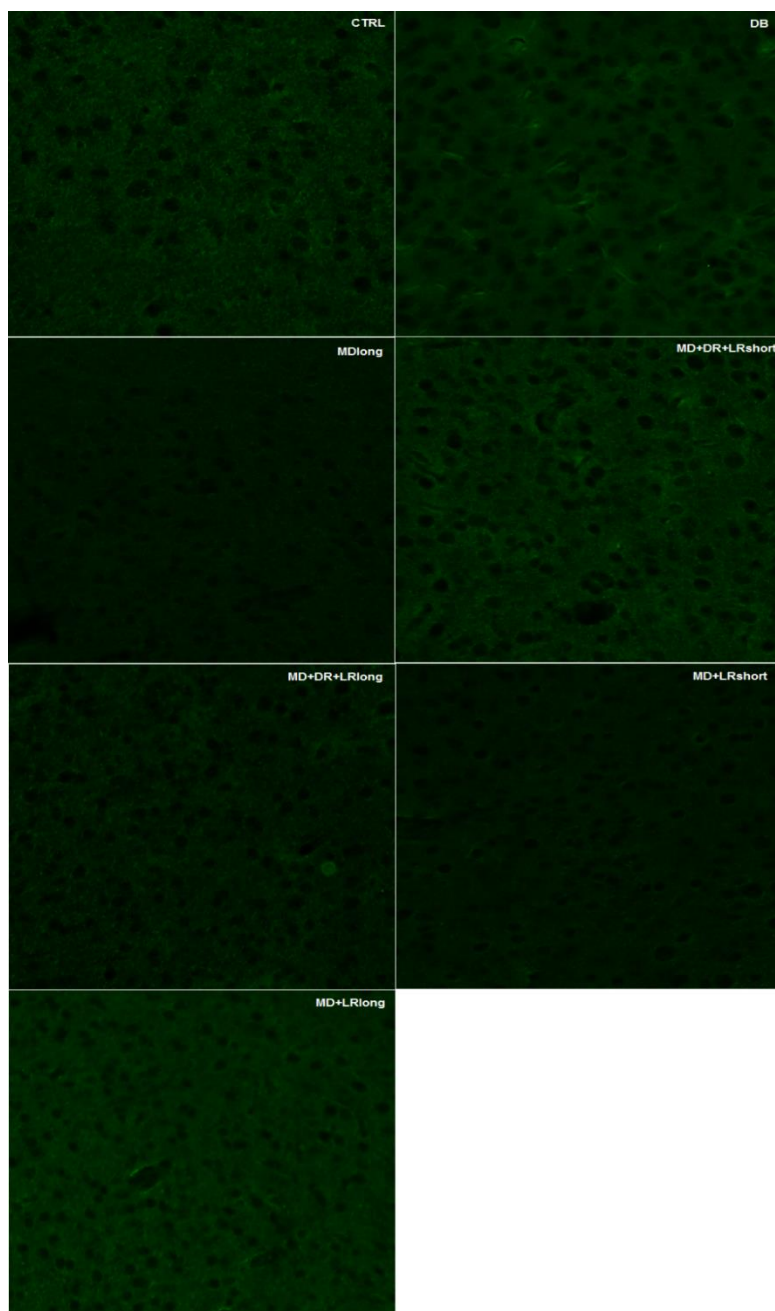
Figure 3.14. In this figure images of VGAT for each group considered are shown. The images are taken in 500μm2.

As for PV, PNN and Colocalization, in all groups no differences in the density of VAGT staining were found between contra and Ipsi hemisphere in terms of fluorescence intensity (P-value >0.05). Moreover, also in this case no significant differences occured between MD+DR+LRshort and MD+DR+LRlong and between MD+ LRshort and MD+ LRlong, so again, we formed 2 groups, the Dark group (n=6) pooling MD+DR+LRshort and MD+DR+LRlong and the No-Dark group (n=5) pooling MD+ LRshort and MD+ LRlong.

Using the same line of reasoning as for the previous analysis, we tested if there were variations in fluorescence intensity between Dark and No-dark group. The results are shown in the figure below (Figure 3.15). The density of VGAT staining was significantly higher in the Dark group than in the No-dark group.
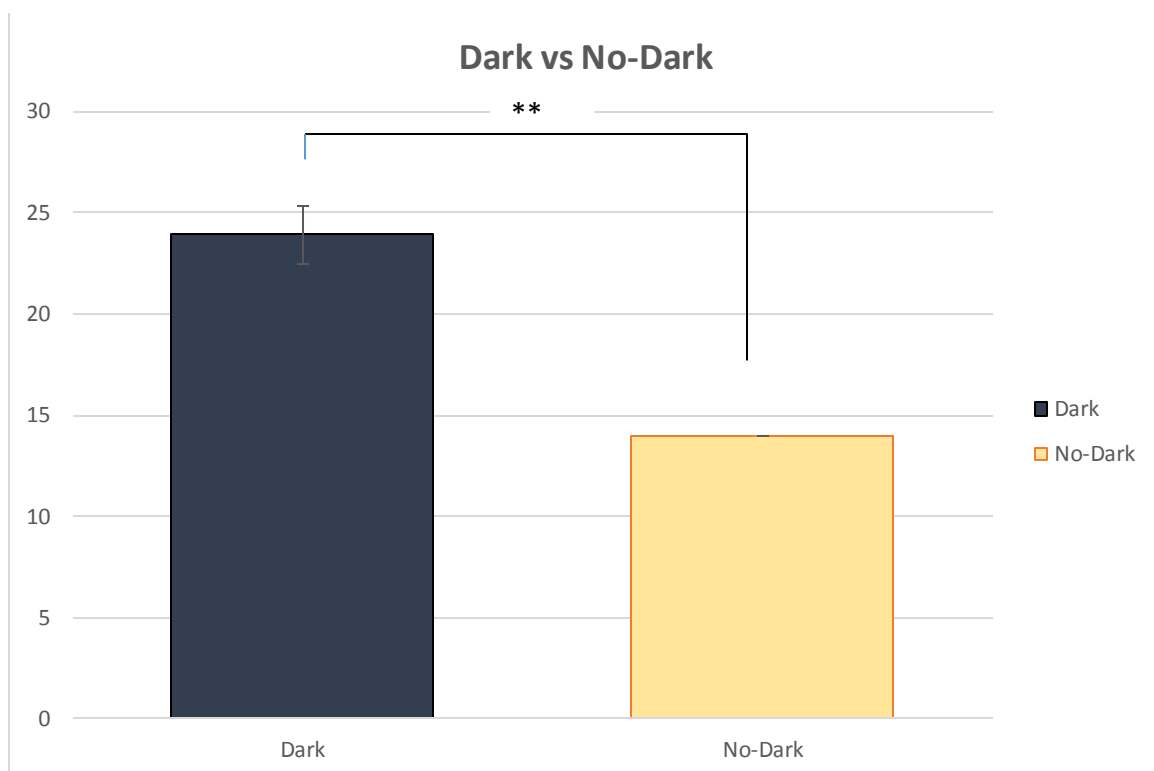
Figure 3.15 Histogram showing the difference between Dark and No-Dark groups in terms of VGAT Fluorescence intensity in V1. P-value <0.01**, error bars show the standard deviation.

Subsequently CTRL was compared with each group using a two-tailed t-test and considering P-values < 0.05 significant. No important differences were measured, moreover the negative control group fluorescence intensity seems to be very similar to No-Dark and MDlong groups (Figure 3.16).
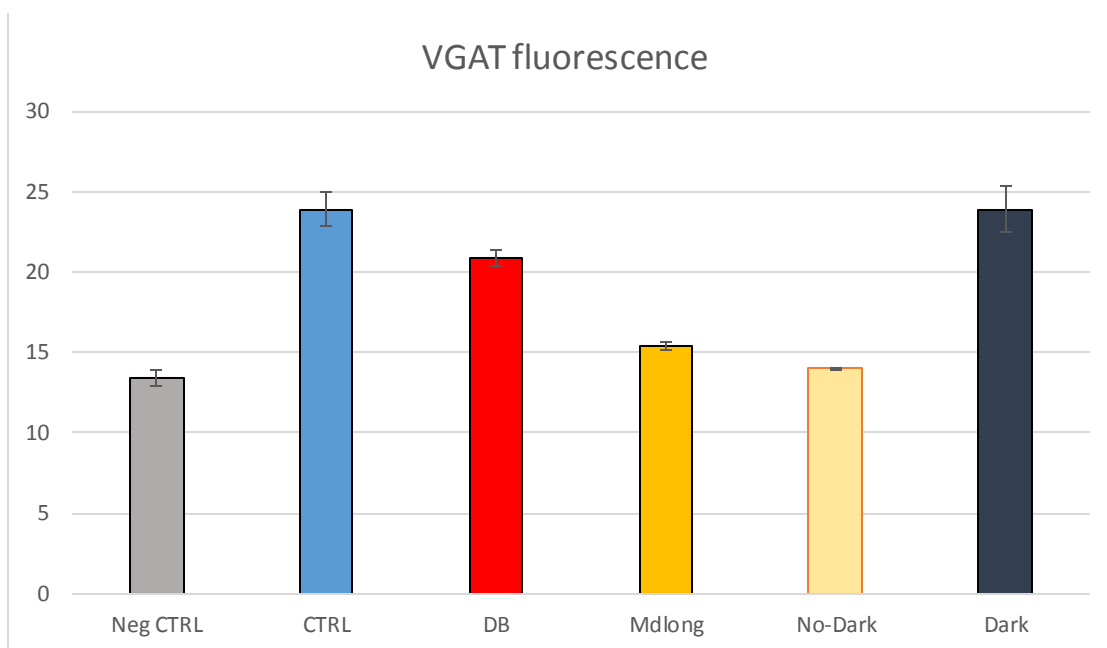
Figure 3.16. Histogram shows the differences among the groups analysed in the VGAT Fluorescence intensity. Error bars show the standard deviation.

4. DISCUSSION

The aim of the project is to improve the knowledge about the plasticity processes and to confirm the importance of dark rearing in enhancing plasticity, both at level of the brain activity, observed by optical imaging (ODI), and at cellular level, by immunohistochemistry.

4.1 SUMMARY OF MAIN FINDINGS

In vivo Optical Imaging

- Mice subjected to dark rearing after MD have a bigger ODI shift than the mice put just in a normal light/dark room

- The ODI shift measured between before and after 7 day period of light rearing is bigger in the Dark-group

- The endpoints for both groups appear to be similar.

- The retinotopy maps seem to be very similar.

Immunohistochemistry

- There were no differences in the number of PV cells, PNNs and in the colocalization of PV and PNN between dark and no dark groups.

- A significant difference occurs between DB and CTRL in PNN density

- The percentage of colocalization in CTRL group is significantly higher rather than in MDlong group;

- From the comparison between Recovery and CTRL groups we found that the percentage of PV/PNN colocalization in Recovery group is significantly lower than CTRL

- In Recovery group a greater number of PV- expressing cells was observed than DB;

- VGAT immune fluorescence in Dark group is more intense than in the No-Dark group.

4.2 GENERAL DISCUSSION

Chronical implantation gave us the possibility to follow the evolution of ODI in V1 of our mice during recovery from MD. The most important comparison was between mice subjected to Darkness and those not. In agreement with Gordon and Stryker (1996), after 30 day period of monocular deprivation in both groups we possible observed a low ODI indicating dominance of the non-deprived eye. Indeed, that transient deprivation of vision in one eye during a critical period in early life stimulates a reorganization of visual responses in primary visual cortex (V1) in favor of the ipsilateral (non-deprived) eye. A seven day period of darkness provokes an additional reduction of the ODI, which means a further enhancement of the cortical response to ipsilateral eye stimuli. Subsequently, according to Duffy and Mitchell (2013) discovery, when the mice were put in a normal light/dark recovery, they showed a great capability to restore the normal OD index, i.e. rapid cortical reorganization in favor of the contralateral (previously deprived) eye. In contrast, in the No-Dark group 7 day period of normal light/dark recovery induced only a small shift of ODI. Furthermore, the maximum shift, as can be observed in Figure 3.4 and in table 3.1 of the Optical Imaging results, is significantly bigger in the Dark group and, moreover, the maximum and minimum peaks in ODI values occurred after 1 week of recovery, while in No-Dark group the maximum shift is smaller and it occurred after a longer period, namely 3 weeks. Already Cynader & Mitchell in 1980 found a strong plasticity in dark reared adult cats explainable by a delay in the maturation of cortical circuits and more recently, it has also been demonstrated that brief complete visual deprivation in adult rats causes the visual cortex to be returned to a more immature state (He et al.,2006) Thus, the complete deprivation of stimuli leads the cortex to a more plastic state, becoming more receptive to stimuli from both eyes, resulting in rapid restoration of normal response properties. According to these results there are weaker and slower changes in No-Dark group compared with Dark group, so Darkness has been a valid instrument to make the neuronal circuitry more prone to reorganization and it could be considered a good a good paradigm to find a way to restore the plastic capability of the neurons in older subjects.

It is interesting to note that the end points of the two groups were very similar. Even though the shift in No-Dark group was smaller and slower than in the Dark group, the similar level of ODI at the end point proves that a certain degree of reorganization has occurred. This could find an explanation in the maintenance of plastic capability that has been reported for rodents even when the canonical Critical period is closed. As already mentioned, the neuronal plasticity reaches the maximum level during the Critical period, but plastic events have been recorded during the entire lifetime. Even if ODI shifts in adulthood are generally less pronounced (Hofer et al.,2006; Sawtell et al.,2003; Lickey et al.,2004), they can occur during different stages of life with different degree and using different mechanisms . So our recording of the ODI end points is a confirmation that plastic events can take place in adulthood, even without specific intervention. A goal for future research would be to find the best way to amplify this intrinsic feature of the cortex in order to treat neurodevelopmental conditions like amblyopia.

In the second part of our project, PV expressing cells and PNNs were counted in order find a molecular explanation underlying plasticity in terms of inhibitory factors. In Stodieck et al. work (2014) it was observed a reduction in PV-expressing cells and PNN density after a 10-14 day period of dark exposure as molecular changes which accompanied the restoration of Ocular Dominance Plasticity in adult and old mice. Thus, the percentage of colocalization of PV cells and PPNs was also calculated in our research, as a potential indicator of plasticity in each group. However, it is very important to be careful in drawing conclusions because we do not have enough knowledge to firmly link higher colocalization with lower plasticity. Indeed, we should note that the colocalization is not an absolute value, considering that PV and PNN are independent factors which could be influenced by different variables. Moreover we do know about the timing of their expression during development.

PV and PNN play an important role in the plastic ability of the cortex. Parvalbumin (PV), a calcium-binding protein, is expressed in a particular population of GABAergic neurons and PV expression in GABAergic neurons during the developmental period could be influential for neuronal maturation (Stichel et al., 1987; Del Rio & DeFelipe, 1994) and for the regulation of neural plasticity and critical period (Chevaleyre & Piskorowski, 2014). Moreover, PV-positive basket cells are known to play a key role in ocular dominance plasticity (Hensch, 2005; Saiepour et al., 2015)

The perineuronal net (PNN) is a reticular structure of the extracellular matrix enriched with chondroitin sulfate proteoglycans (CSPGs), and hyaluronan that enwraps the soma and dendrites of neurons (Bandtlow & Zimmermann, 2000; Giamanco & Matthews, 2012). PNNs play an important role in the regulation of neural plasticity (Wang & Fawcett, 2012) and in the closure of the critical period in the developing neocortex (Pizzorusso et al., 2002; Dityatev et al., 2007; Makara et al., 2007). Therefore, breakdown of PNNs may be one way to restore plasticity after the end of the critical period (Pizzorusso et al., 2002, 2006).

In this context, the first objective of this part of project was to test for differences between the hemispheres contralateral and ipsilateral to the deprived eye. In CTRL and DB, since no imbalance in visual input occurs we did not expect to see a difference in PV cell and PNN density. In the groups subjected to MD we might have found differences if MD influenced epigenetically the PV and PNN expression, but no differences were found in any of the groups. Even in the MDlong group, in which the brains were collected just after a period of unequal binocular visual stimulation (MD), no differences were found. We can conclude that MD has an effect on the percentage of eye-specific responses in the central part of the visual field, but not on the PV and PNN expression, even though this should be confirmed from a larger sample analysis.

Difference between Dark and No-dark group were detected in our analysis, but none were statistically significant, probably due to the light rearing period in both groups before the collection of the brains. Thus, it is possible that a period of light could reestablish the usual PV cell and PNN density even if mice had previously been subjected to a period of darkness.

As explained in the introduction of this project, the presence of PNNs is an important feature in the advanced stage of cortical maturation which reflects a reduction in the plastic capability of the neurons. As predicted, we found a higher PNN density in CTRL compared with DB animals (P-value = 0.030). Indeed, it is already known that darkness extend plasticity (Duffy and Mitchell, 2013) so it could delay the PNN formation surrounding the PV expressing cells. This result confirmed the use of PNNs in our project as a reliable indicator of the level of plasticity.

Although no statistically significant differences were found in PV density, but there was a trend where PV expressing cells have a higher density in the CTRL group

than the DB group. That point could explain why the percentage of PV/PNN colocalization between the 2 groups is so similar (P-value= 0.414). Such results could be explained from a lower neuronal stability in DB group. This hypothesis is supported from Gianfranceschi et al. (2003) discovery in which they found a BDNF down regulation in dark reared mice and a delay in the development of inhibitory innervation, so consequently a later critical-period onset. Since Parvalbumin is expressed in a particular type of GABAergic neurons (the basket cells), it is likely that dark rearing has an effect on plasticity, but we do not have enough knowledge about the timing of PV expression in the cells to be sure that dark rearing influences the maturation of PV expressing neurons themselves and not just the epigenetic expression of the Ca-binding protein.

For the same reasons, the density of PV expressing cells is statistically higher in the Recovery group than in the DB group (P-value = 0.021).

It is important to underline that in our project we did not analyze 30MD+7DR mice, i.e. we do not know the density of PV cells and PNNs immediately after dark-rearing in the Recovery groups. It would be very interesting to extend our analysis to that condition in order to investigate if dark exposure later in life can influence PV or PNN expression, and the mechanisms underlying the enhancement of the plasticity induced by DR previously mentioned.

In the comparison between MDlong and CTRL groups we can see a lower percentage of colocalization in the first group even though the PV expressing cell density is very similar. Indeed there is a trend of PNN reduction in the MDlong group, even though that difference is not statistically significant. This could mirror a form of cortex reorganization. Hofer and colleagues (2006) discovered that a second period of MD causes faster and longer lasting OD changes many weeks after a first period of MD in both juvenile and adult cortex, thus it is like MD leaves an intrinsic trace on the organization of the cortical network independent of the age at which the MD occurs (Hofer et al.,2006). It could be possible that the lower percentage of colocalization in the Recovery group is that mark mirroring the cortical reorganization.

The VGAT (vesicular GABA transporter) immune staining was done in order to investigate the degree of intracortical inhibition in the various rearing conditions. Indeed VGAT is a transporter specifically associated with synaptic vesicles highly concentrated in the nerve endings of GABAergic neurons, i.e. a presynaptic marker of inhibition. Like

PV cell and PNN density, VGAT fluorescence intensity showed no difference between the hemispheres, but statistically significant differences (P-value< 0.01) were found between dark and No-Dark group, with more VGAT seen in the Dark group than in the No-Dark group. This result is not in line with our expectations or with the markers of inhibition already analyzed (PV cells and PNNs), for which no difference was observed between these two groups. Bearing in mind that our staining was done in just one trial and using only a small number of samples, one possible explanation of this result could be that after a dark period in which a more immature state was reached, the light recovery could provoke an enhancement of the inhibitory system.

Furthermore, the fluorescence intensity degree between CTRL and DB is very similar, and that result is in contradiction to the hypothesis in which Darkness causes a delay in the maturation of inhibition circuitry. However it is not possible to speculate about reasons for this discrepancy because not enough data were collected and, moreover, we found that MDlong and No-Dark group show the same level of fluorescence intensity as the Negative control, casting doubt over the validity of our results. This point requires further investigation the Negative control were supposed to reflect the autofluorescence capability of the tissue since no primary antibody was used.

More analysis should be done in order to validate this system of measurement, but the GABAergic network continues to be one of the most important lines of investigation in order to improve our knowledge of Neuronal Plasticity.

It will also be important to analyze postsynaptic markers of inhibitory synaptic transmission, such as Gephyrin and study synapse turn-over under different conditions (Van Versendaal et al., 2012) since markers for GABA alone do not permit conclusions regarding the efficacy of transmission.

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