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NEUROINFLAMMATION AND DEFECTIVE MYELINATION IN POLYMICROGYRIA

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ABSTRACT

Polymicrogyria (PMG) is a condition characterized by abnormal prenatal brain development and excessive number of ectopic small gyri in the cerebral cortex. PMG patients present an excessive number of abnormally small gyri separated by shallow sulci, associated with fusion of the overlying molecular layer of the cerebral cortex. The topographic distribution of PMG may be focal, multifocal or diffuse; unilateral or bilateral; symmetric or asymmetric. Clinical manifestations have a large spectrum, ranging from isolated selective impairment of cognitive functions to severe encephalopathy and intractable epilepsy. The severity of neurological manifestations and the age at presentation are in part influenced by the extent and localization of the cortical malformations but may also depend on its specific aetiology. The pathogenesis is still poorly understood, several causative gene mutations have been recently found, but also other causes has been identified (prenatal infections, ipoxia).

Experimentally, the mouse model of polymicrogyria (PMG) displays the formation of ectopic microgyri in the mouse cortex, enhanced excitatory and inhibitory synaptic transmission accompanied by increased connectivity in the paramicrogyral cortex and higher susceptibility to epilepsy in vitro.

Besides the alteration in the cortical layering, the molecular, morphological and behavioural analysis of PMG mice reveal a significant astrogliosis and microglial activation, indicating the occurrence of an inflammatory process. In addition, a diffuse cortical hypomyelination is evident in brain slices stained for myelin basic protein (MBP). Furthermore, PMG mice displayed altered EEG profile and defective motor skills such as reduced brawn. All these features make PMG model suitable for the study of the pathology and to investigate possible therapeutic approaches.

Here we found that transplantation of human neural stem cells (hNSCs), which has been demonstrated to exert positive effects on inherited or acquired myelination disorders and to dampen brain inflammation, plays a beneficial effect on the pathological condition of PMG ameliorating the myelination defect by promoting oligodendrocyte precursors proliferation and remodelling of myelin fibres. Our data also show that hNSC transplantation restores normal EEG brain activity and improves motor performances. Moreover, we tried a pharmacological blockade of IL-1R

activation by the IL-1R antagonist: anakinra. We found that this treatment leads to a significant improvement of EEG and motor skills in adult PMG mice thus suggesting a possible role of inflammation at the root of the pathology and identifying a therapeutic time window for the treatment.

RIASSUNTO

La Polymicrogyria (PMG) è una patologia caratterizzata da uno sviluppo prenatale anomalo del cervello e dalla formazione di un eccessivo numero di giri ectopici a livello della corteccia. I pazienti polymicrogyriaci presentano un eccessivo numero di piccoli giri anomali separati da solchi superficiali associati alla fusione degli strati superiori della corteccia cerebrale. La distribuzione topografica della PMG può essere focale, multifocale o diffusa; unilaterale o bilaterale; simmetrica o asimmetrica. Le manifestazioni cliniche sono ad ampio spettro e vanno da eventi isolati di problemi cognitivi a severe encefalopatie ed epilessia farmaco-resistente. La severità dei sintomi neurologici e l'età a cui questi si manifestano sono in parte influenzati dall'estensione e dalla localizzazione delle malformazioni corticali ma dipendono anche dall'eziologia della patologia. La patogenesi della PMG è poco conosciuta, recentemente le sono stati associati molti geni ma sono state identificate anche altre cause (infezioni prenatali, ipossia).

Sperimentalmente gli aspetti patofisiologici della PMG possono essere riprodotti attraverso il modello di lesione focale a freddo (FFL) nei roditori in età neonatale, che provoca la formazione dei microgiri a livello della corteccia del topo. Studi precedenti nel modello FFL hanno mostrato un aumento della trasmissione sinaptica eccitatoria ed inibitoria accompagnata da un'incrementata connettività nella corteccia circostante il microgiro e un'elevata suscettibilità all'epilessia.

Analisi biochimiche, morfologiche e comportamentali del modello PMG hanno rilevato, oltre alle alterazioni nella struttura laminare della corteccia, una significativa astrogliosi e attivazione della microglia, il che indica la presenza di un processo infiammatorio. Inoltre, una diffusa ipomielinizzazione corticale è evidente a livello delle sezioni ottenute da cervelli di topi FFL sottoposte a colorazione immunoistochimica per il marcatore specifico per la mielina MBP. In aggiunta, gli animali PMG mostrano un profilo elettroencefalografico (EEG) alterato e difetti motori come debolezza muscolare e mancata coordinazione. Tutte queste caratteristiche rendono il modello FFL adatto allo studio della patologia e all'approccio con diverse vie terapeutiche in quanto mima perfettamente i sintomi ritrovati nei pazienti polymicrogyriaci. Nello specifico, abbiamo trovato che il trapianto di cellule staminali

neuronali umane, che sono state dimostrate essere in grado di esercitare effetti positivi su disordini di mielinizzazione acquisiti o ereditati e di avere effetti positivi in condizioni di infiammazione cerebrale, gioca un effetto benefico nelle condizioni patologiche associate a PMG migliorando i difetti di mielinizzazione, promuovendo la proliferazione dei precursori degli ologodendrociti e rimodellando le fibre mieliniche. I nostri dati dimostrano anche che il trapianto di queste cellule staminali porta ad una normalizzazione del tracciato EEG e ad un miglioramento delle performances motorie negli animali lesionati.

Inoltre, abbiamo usato anche un approccio farmacologico inibendo l'attività della citochina IL1 attraverso un antagonista del suo recettore: l'anakinra. Negli animali trattati con questo farmaco, abbiamo trovato che il trattamento porta ad un significativo miglioramento del tracciato EEG e delle performances motorie in età adulta, suggerendo un possibile ruolo dell'infiammazione alla base della patologia ed identificando una possibile finestra temporale terapeutica.

INTRODUCTION

Malformation of Cortical Development Disorders

The development of human cerebral cortex is a complex and tightly organised process, for this reason, disruption or deregulation of any of the steps that contribute to this process can give rise to a range of developmental disorders. Many of these disorders are classified as malformations and comprise a class of disorders named Malformations of Cortical Development (MCD). This term was introduced to define disorders characterized by defective cortical development such as microcephaly, megalencephaly and heterotopia. The classification for MCD is based on the developmental stages at which the process is first disrupted, the genes and biological pathways involved and imaging features [20, 21]. This system classified MCD into three principal groups that recapitulate the principal developmental points as malformations of cell proliferation, neuronal migration and postmigrational cortical organisation and connectivity. The complete knowledge of biological pathways at the root of these disorders is not yet available, indeed the boundary between disorders of neuronal proliferation, migration or cortical organisation is only suggested. Recent studies suggest that MCD-related genes are implicated in any developmental stages that are interdependent [4]. Most MCD can be differentiate using MRI scans as is shown in the Figure 1 in which arrows highline the presence of malformation (for instance in the upper layer are shown example of Polymicrogyric brains (A, B, C), in the second are shown lissencephalic brains (E). The principal features to look to identify distribution and severity of MCD are the cortical surface and the interface between white and grey matter (smooth or irregular) and cortical thickness. The better knowledge of the main morphological features of the normal pattern of cortical development is essential to have a prenatal diagnosis of some MCD, including regional Polymicrogyria at an early sulcation stage using neurosonography or foetal MRI. Diagnoses can be made before 24 weeks of gestation and can help to select the most appropriate genetic testing and counselling implementing the ultrasound analysis that can

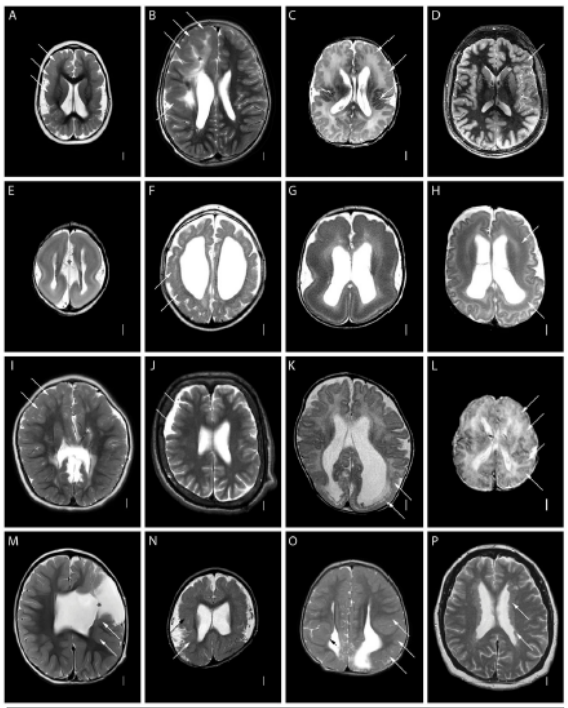


Figure 1. MRI of patients with different forms of MCD. Arrows highline the presence of malformation at level of the cortex. Figure adapted from [4].

unveil minor CNS anomalies or even non-CNS anomalies. But these imaging techniques have limitation. Many MCD can be detected because associated to early feeding problems, seizures or global developmental delay. Others can be identified by abnormally small or large head size, hydrocephalus or other congenital anomalies. Children with these clinical presentations present congenital microcephaly, dysplastic megalencephaly, lissencephaly, cobblestone malformation, polymicrogyria-like malformations or classic polymicrogyria. The most severe symptoms include deficits in language development and social interactions, stereotyped or other involuntary movements, autonomic (especially gastrointestinal) dysregulation, abnormalities in mood, sleep, and attention, and visual and hearing loss. Most have severe neurological disabilities and high risk for a reduced life span [4, 22].

THE PATHOLOGY

Polymicrogyria (PMG) is a disease of early brain development characterized by abnormal neuronal migration and cortical organization resulting in the formation of atypical and multiple small gyri [23]. Polymicrogyria presents a range of histologic features, all having in common a derangement of the normal six-layered lamination of the cortex and an associated derangement of sulci [24]. The aetiology of this pathology is poorly understood, but the main causes include congenital infection (particularly cytomegalovirus infection [25]), localized or diffuse in utero ischemia [26], or genetic mutations [27-31].

Patients may have a wide variety of symptoms, ranging from hemiparesis or partial epilepsy to developmental delay, motor disabilities, speech defects and intractable epilepsy. The severity and the age of the clinical presentation appear to depend both by the portion(s) of brain involved and the type of Polymicrogyria (Barkovich and Lindan 1994). Indeed, Polymicrogyria affects variable portions of the cortex: it may be focal, multifocal, or diffuse. Moreover, it may be unilateral, bilateral, and asymmetrical or bilateral and symmetrical. The most common location (in 60–70% of cases) is around the Sylvian fissure [12], however, any part of the cerebral cortex (frontal, occipital, and temporal lobes) can be affected [32, 33].

Polymicrogyria is a common endpoint of a variety of aberrations of cortical development, for this reason, to understand how and when this heterogeneous condition occurs, it is necessary to identify which specific developmental pathways are disrupted. The onset of PMG has not been established, it could be both early (depending on the impaired proliferation and migration of neuroblasts) and late (due to disorders of post-migrational maturation of the cortex) [24].

The clinical diagnosis and the majority of the studies on PMG patients have been based on radiological analysis of mature brains. The exam in the post-natal brain, when the development is complete, cannot reveal pathogenetic mechanisms [23]. Studies on foetal human brains were uniquely able to demonstrate human cortical development and its aberrations [34].

Pathological definition of polymicrogyria

The term Polymicrogyria (PMG) was used for the first time in 1916 by Bielschowsky to describe a cerebral cortex characterized by multiple excessive small convolutions [35]. PMG is a spectrum of cortical malformations which have as common features the excessive gyrification and abnormality of cortical structure and lamination [36]. PMG characteristic microscopic features have been described as abnormal arrangement of cells, intracortical fibre plexus, excessive folding of all or only the upper layers, and fusion of gyral surfaces with large sinusoidal intracortical vessels as is shown in the Figure 2. In a more detailed description five subtypes have been identified: 1- unlayered 'festooned', 2- four-layered cortex with a sinuous upper layer, 3- parallel four- layered cortex, 4- miniature gyri which are fused and 5- poorly laminated [37]. Cortical layering has been further classified as two, four and unlayered forms which are thought to reflect the onset of the pathology. Unlayered is considered "early", occurring at 16–24 weeks of gestation, and 4-layered is late [38].

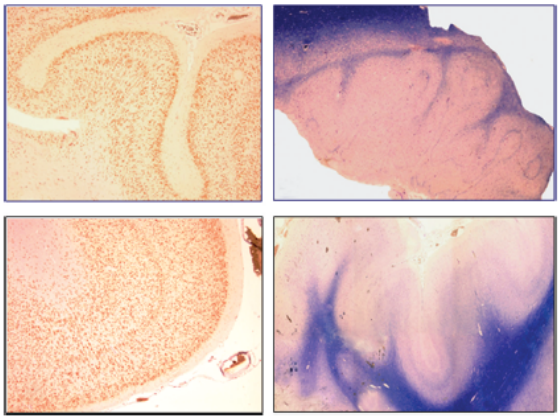


Figure 2. Histological imagines of human brains of patients with Polymicrogyria. Figure modified from [3].

It is evident that both fusion and cortical lamination are highly variable between cases and may not be reliable criteria to define the malformation. Moreover, PMG can be associated with other anomalies of the central nervous system (CNS) [21, 39].

Based on imaging, PMG has been classified into three different morphological subtypes: coarse, delicate and saw-toothed, even though the appearances are highly variable and depending on age and degree of myelination [12, 22, 23]. The Figure 3 shows a classification of most common type of polymicrogyria and the percentage of recurrence between the different forms.

Table 1 Topographic patterns and subtypes of polymicrogyria

Topographic pattern	Subtype	Number	Percentage
Perisylvian		200	61
	Bilateral symmetric	154	47
	Bilateral asymmetric	15	5
	Unilateral	31	9
Generalized		41	13
	Normal white matter	14	5
	Abnormal white matter	27	8
PNH/polymicrogyria		35	11
	Perisylvian	15	5
	Posterior	17	4
	Other	3	1
Frontal		18	5
	Frontal only	15	4
	Frontoparietal	3	1
Parasagittal parieto-occipital		11	3
	Unilateral	2	<1
	Bilateral	9	3
Other		23	7
Total		328	100

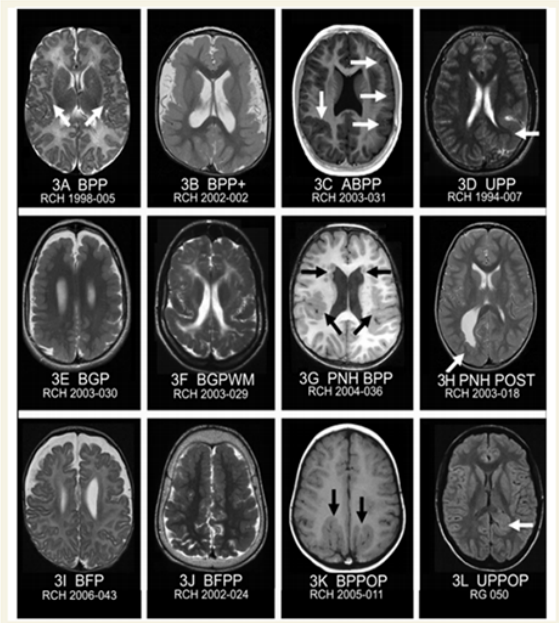


Figure 3. MRI features of common patterns and subtypes of polymicrogyria. Abbreviations: Bilateral perisylvian polymicrogyria with polymicrogyria either limited to the perisylvian cortex (**BPP**), or extending beyond it (**BPP+**); Asymmetric bilateral perisylvian polymicrogyria (**ABPP**) with polymicrogyria involving the posterior third of the right Sylvian fissure and the left Sylvian fissure along its entire length (arrows); Unilateral perisylvian polymicrogyria (**UPP)** with polymicrogyria lining the left Sylvian fissure that is abnormally extended postero-superiorly; Bilateral generalized polymicrogyria (**BGP**) showing no clear gradient or region of maximal severity; Bilateral generalized polymicrogyria with abnormal white matter (**BGPWM**); Bilateral periventricular grey matter heterotopia with bilateral perisylvian polymicrogyria (**PNH BPP**) (arrows); Right-sided posterior periventricular grey matter heterotopia (arrow) associated with overlying polymicrogyria (**PNH POST**); Bilateral frontal (only) polymicrogyria (**BFP**) with bilateral symmetric polymicrogyria involving the majority of the frontal lobes with abrupt cut-off in the mid-frontal regions; Bilateral frontoparietal polymicrogyria (**BFPP**) with bilateral symmetric polymicrogyria involving the frontal lobes and extension posteriorly into the parietal lobes; Bilateral parasagittal parieto-occipital polymicrogyria (**BPPOP**) with bilateral symmetric polymicrogyria lining abnormal gyri radiating antero-laterally from the parasagittal parieto-occipital region (arrows); A mild form of unilateral parasagittal parieto-occipital polymicrogyria (**UPPOP**) with polymicrogyria lining a deep and abnormally-oriented sulcus in the left parasagittal region (figure modified from [12]).

Radiological characterization of PMG is used in the clinical evaluation and it guides genetic studies, but in general this correlates poorly with aetiology. Indeed, radiology does not have the resolution to describe the detail of the malformed cortex. It is essential to have a pathological correlation for an accurate interpretation, but this is not often done. Frequently, the patients who survive until adult life do not receive a post-mortem autopsy and the material derived from epilepsy surgery is restricted to cases whose epilepsy was so severe as to necessitate surgery.

PMG is not easy to discriminate using computerized tomography (CT) since can appear as thickened cortex and so to be diagnosed as other malformations such as pachygyria (Congenital malformation of the cerebral hemisphere that results in unusually thick convolutions of

the cerebral cortex) or lissencephaly (set of rare brain disorders where the whole or parts of the surface of the brain appear smooth). MRI is useful to characterize PMG from other malformation of cortical development (MCD). In fact, with this technique is possible to have high quality images of microgyri and microsulci also in younger children in whose PMG cortex appears thinner than at later ages. This may be due to the presence of incomplete myelination in subcortical and intracortical fibres [40]. Therefore, using MRI it is possible to describe PMG in a number of recurrent topographic patterns, which most common are bilateral perisylvian and unilateral perisylvian (52% and 9% of all forms, respectively) [12]. The other commune patterns are all bilateral and includes generalized [27], bilateral frontal [41], bilateral frontoparietal [27] and bilateral parasagittal parieto-occipital [39].

Clinical Features

PMG is related to a variety of clinical associations and sequelae. The clinical features depend on a number of factors such as associated syndromic features, the presence of other brain anomalies, the extension and the localization of microgyri and other frequent complications such as epilepsy. Surely, the extension and the topography of PMG have a significant weight on the clinical manifestations [12, 42]. PMG was reported to be associated to multiple conditions including metabolic disorders, chromosome deletion syndromes and multiple congenital anomaly syndromes. The most common PMG associated symptoms are epilepsy (78%), global developmental delay (70%) and spasticity (51%) [12]. The combination of bilateral perisylvian PMG (BPP) and the seizure disorders has been defined “congenital bilateral perisylvian syndrome”. Patients affected by BPP have difficulties in tongue movements, expressive speech and facial diplegia [43]. They can present mild to moderate intellectual disability and motor dysfunctions [12].

CORTICAL DEVELOPMENT AND GYRIFICATION OF HUMAN CORTEX

The most common PMG condition is characterized by the disruption of the cortical surface and the interaction with the leptomeninges. Other types include fusion of the molecular layers of contiguous gyri, premature folding of the cortex and early lesions.

The cerebral cortex is one of most important region in the mammalian brain since it controls complex cognitive behaviours such as learning, memory, spatial orientation and the ability to experience complex emotions. Its growth is depending by the proliferation of neural stem cells (NSCs) and neural progenitors (NPs) that consequently generate the postmitotic neurons. Cortical size varies among different mammalian species and there is not a strict correlation between brain-to-body mass ratio and behavioural complexity and intelligence [44, 45]. However, cortical size is very important for the brain function. Indeed, patient affected by microcephaly or macrocephaly (small or enlarged brains, respectively) present a range of cognitive defects and deficits.

On the base of the cortical folding rate, mammals can be identified as lissencephalic species (e. g., mice) with smooth-surfaced cortices, and gyrencephalic species (ferrets and most primates) with convolution in the cortex, but the rate of gyrification can be considerably different between and within mammalian orders and can correlates with brain size [44].

The adult neocortex has a simple structure composed by six layers of cells, each characterized by specific circuit functions as is shown in the figures below. In the specific, the left panel of Figure 4 shows the denomination of six layers which take their names from the cell types by they are populated, while in the right part of the panel there are graphic representations of intra-layers' connections and between different brain's areas (Figures modified from Purves et al, Neuroscience 3rd edition; [6]).

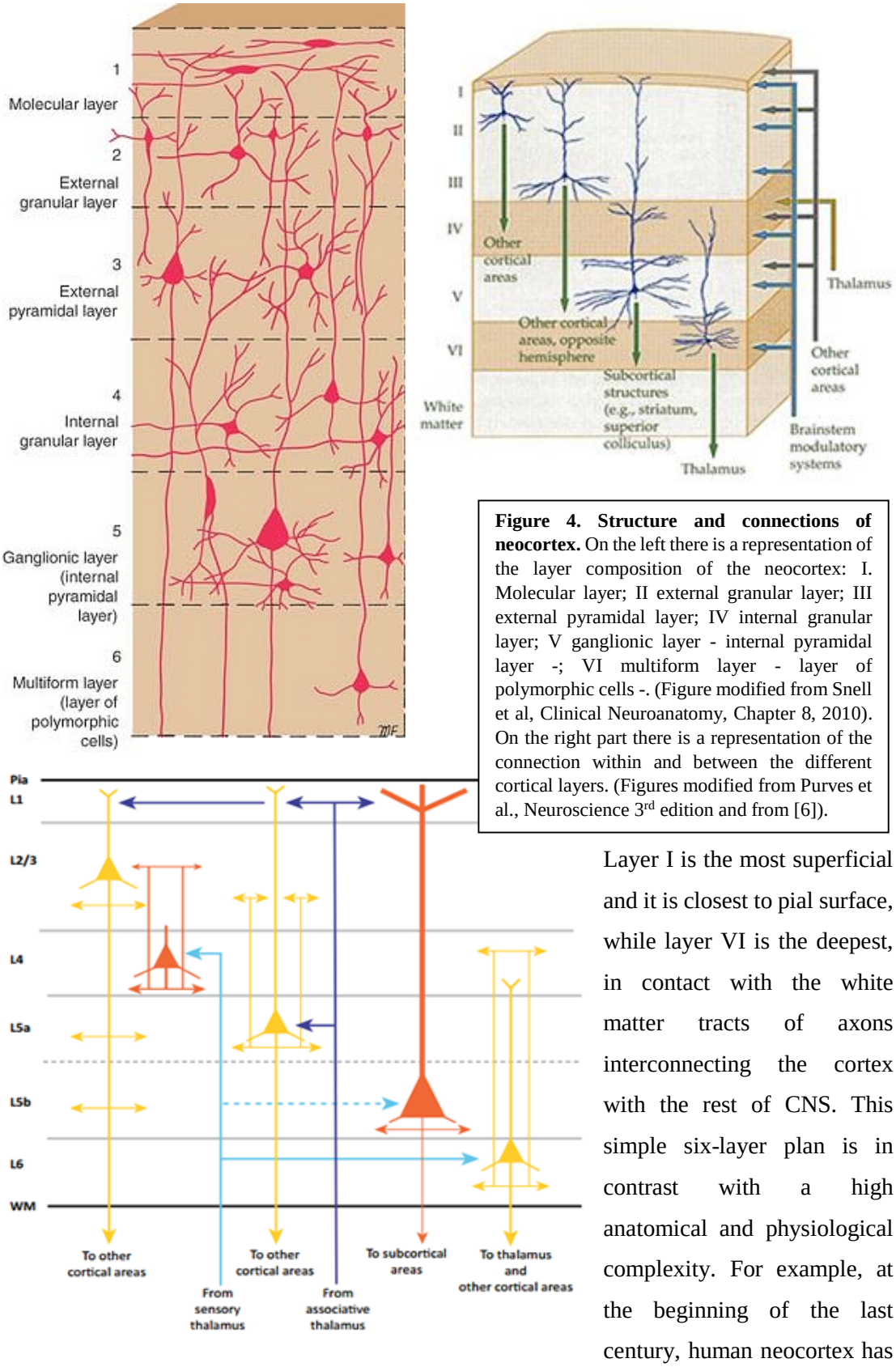


Figure 4. Structure and connections of neocortex. On the left there is a representation of the layer composition of the neocortex: I. Molecular layer; II external granular layer; III external pyramidal layer; IV internal granular layer; V ganglionic layer - internal pyramidal layer -; VI multiform layer - layer of polymorphic cells -. (Figure modified from Snell et al, Clinical Neuroanatomy, Chapter 8, 2010). On the right part there is a representation of the connection within and between the different cortical layers. (Figures modified from Purves et al., Neuroscience 3rd edition and from [6]).

Layer I is the most superficial and it is closest to pial surface, while layer VI is the deepest, in contact with the white matter tracts of axons interconnecting the cortex with the rest of CNS. This simple six-layer plan is in contrast with a high anatomical and physiological complexity. For example, at the beginning of the last century, human neocortex has been subdivided in many distinct regions in relation to density and thickness of cortical layers and it was correctly predicted that each area has different functional properties. Later, it was shown that the local circuitry of neocortex can be recapitulated as a series of vertically aligned

columns of interconnected neurons. In the columns the neurons accurately connect each other and with other neurons in nearby columns and distant cells of other brain regions and spinal cord. Cortical development is a complex process. The young cortical neurons migrate centrifugally to their adult location near the pial surface. At first stage of development, the cortex is a thin layer of subpial cells named preplate, then the neurons that will form the layer VI, the deepest, migrate to preplate in which they infiltrate and split in two layers: an upper marginal zone and a lower subplate. The next step is the migration of newly generated neurons through the subplate and the previous neuronal layers to successive layers, stopping themselves under the marginal zone. The final result is the stratification of cortical neurons: the oldest one, those of preplate, are positioned in layer I (derived from marginal zone) and deep portion of layer VI (cells derived from subplate) of the developing cortex; other cortical layers are in a "inside-out" conformation with respect to birth date, in fact layer II is composed by the youngest cells and layer VI contains the oldest [46]. The embryology and anatomy of neocortex have been studied for many years and in the last decades molecular and genetic pathway have been identified to partially explain the spatial and temporal organization of cerebral cortex. An important point has been the identification of gene defects responsible of neurological malformations. In particular, an important contribution in embryonal neocortical field, derives from the study of a naturally occurring mouse mutant, reeler in which migration patterns of neurons are abnormal. In fact, in this mouse, neurons are "born" at the correct development step and they assume the proper morphology and functional properties of layer VI at the correct date, but they do not migrate further the preplate. As a consequence, the preplate does not split and the subplate remains near to the marginal zone. Layer V cells stop beneath layer VI cells instead to migrate past. This give an abnormal cortex in which layer I cells are still superficial, but layers II and VI are inverted respect to wild-type cortex [47, 48].

Relatively recent studies, showed that the arrangement of cortical neurons into layers depends on the presence of a secreted glycoprotein, Reelin [49, 50]. This protein is produced by Cajal-Retzius (CR) cells and it is highly concentrated at level of the marginal zone (MZ), acting on neurons and radial glia. Reelin receptors and their cytoplasmic effector Disabled-1 (Dab1) are expressed by neuroblasts that are originated from the ventricular zone (VZ), that maybe respond to Reelin by migrating out in direction of the cortical plate (CP) situated below the MZ. Reelin is fundamental for cortical layering; in fact, mice deficient for genes of Reelin–signalling pathway have structural defects of neocortex [51]. Even though the large number of studies, it remains unclear how Reelin pathway is responsible of the correct assembly of neurons into sequential layers. At the moment, there are a series of theories and models that do not univocally respond to this question, given that Reelin function is complex and needs to consider both the location and the amount of Reelin expression [52].

A possible functional and evolutionary explanation of six layered structure of neocortex is that axon of neurons organized in this way may easier to reach the targets and more neuronal connection could be formed compared with neurons in a nuclear structure [53]. Moreover, this disposition is functional to maximize the ratio between brain volume and skull space.

Neural progenitors and cortical growth

Some of the major changes occur during the embryonic period and early foetal period. In humans the embryonic period begins at conception and extends through GW8. By the end of the embryonic period the rudimentary structures of the brain and central nervous system are established and the major compartments of the central and peripheral nervous systems are defined. The early foetal period, which extends to approximately midgestation, is a critical period in the development of the neocortex. Most cortical neurons are generated by that time and many have migrated to their positions in the neocortex and have begun to from essential brain networks for information processing [54]. The cerebral cortex origins in the most rostral region of the early embryonic neural tube, that is constituted by neuroepithelial (NE) cells [55] that can generate both neurons and glia. Radial glial cells (RGCs) are progenitors deriving from NE cells, which are located in the ventricular zone (VZ) and form bipolar radial fibres which connect ventricular and pial surfaces in the cortex. RGCs are similar to the glia cells, in fact they act like scaffold for migrating neurons, express glial markers (such as the glial fibrillary acidic protein, GFAP) and have the astrocytic-specific glutamate transporter (GLAST) [56]. Different studies have shown that RGCs can also produce neurons, astrocytes and oligodendrocytes [56, 57]. The so called radial unit hypothesis postulates that the cortex is assembled starting from radial progenitor units, that are the proliferative RGCs, and from more differentiated cells (neurons), which migrate radially along the RGC fibres forming the six-layered cortex, in an inside-out way [56] as is shown in the Figure 5 modified from [14]. In the specific the image represents the multiple progenitors in the mouse (a) and human (b) developing cerebral cortex during cortical development. Apical radial glial cells (aRGCs) and apical intermediate progenitors (aIPs) reside in the ventricular zone (VZ). Basal IPs (bIPs) are mostly positioned in the subventricular zone (SVZ) in the mouse cortex and in the inner SVZ (ISVZ) in the human cortex. Basal RGCs (bRGCs) are identified in the SVZ in the mouse cortex and the outer SVZ (OSVZ) in the human cortex. Migrating neurons mostly appear in

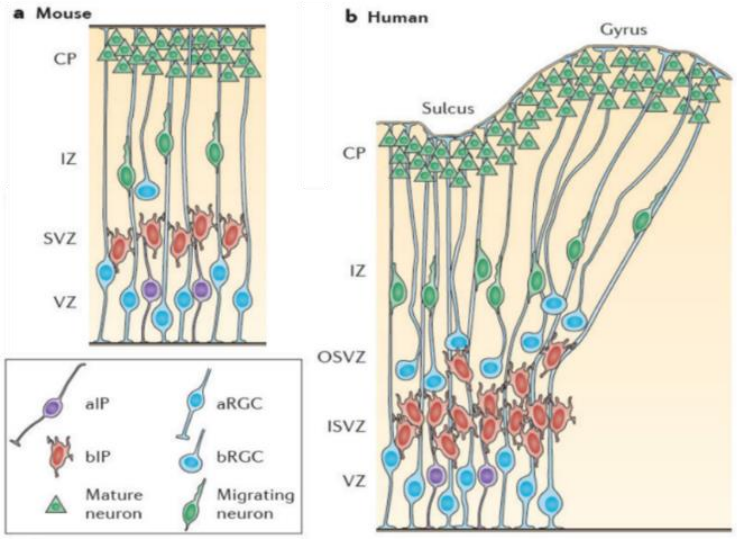


Figure 5. Progenitor's migration during cortical development. (a) mouse and (b) human developing cerebral cortex. Figure modified from [14].

the intermediate zone (IZ), whereas mature neurons form an inside-out six-layered structure in the cortical plate (CP). Cortical surface expansion in the human cortex results in folded structures forming sulci and gyri. This schematic representation also describe how RGCs do asymmetrical divisions, originating one RGC and one postmitotic neuron, or one RGC and one intermediate progenitor (IP) that stop in the subventricular zone (SVZ). Intermediate progenitors can be divided in two subclasses: the apical IPs (aIPs) and basal IPs (bIPs) with different molecular profile. aIPs are located in VZ and have short radial connexion to the ventricular (apical) surface; bIPs migrate from the VZ to the SVZ [58]. IPs divide symmetrically generating two postmitotic neurons, representing a major neurogenic cell pool [59].

The SZV can be histologically distinct into inner SVZ (ISVZ) and outer SVZ (OSVZ). Recently, a group of RGC, named basal (bRGCs), has been identified in the OSVZ of developing cortices. It has been supposed that these cells may be an additional source of progenitors contributing to cortical expansion and folding. Numerous studies show that few molecules and signalling pathway are crucial in the regulation of cortical growth and folding; so it is no strange that a dysregulation of these factors or pathways give rise to brain malformations especially in the human brain [60].

Gyrencephaly: a mammalian trait

The folding of the neocortex to form gyri and sulci, the gyrencephaly, evolved as a way to fold large cortical surface areas in a limited volume (skull) [10]. This package property is not exclusive of primates, but it is present in others mammals such as large rodents (as capybaras), large felines, aquatic mammals [61].

The complexity of this structure can be quantitate by the gyrification index (GI), which is defined as the ratio between the total neocortical surface area (including cortex inserted in the sulci) and the superficially exposed neocortical surface area; this parameter has therefore a strong positive relation with the brain mass [61, 62].

The gyrogenesis, that is the process of development of gyri, has been extensively studied. Recently this phenomenon raised interest thanks to progresses in the characterization of different types of cortical progenitor cells. In particular, the characterization of intermediate progenitors is the principal key of the “intermediate progenitor hypothesis”, which affirms that the growth of the gyri is mediated by differential proliferation of IPs [63]. This hypothesis is

dependent on the local proliferation and growth of the cortical tissue, but the mechanism that drives the process of "gyrus-building" is poorly known. Recent studies have investigated the role of bIPs and bRGCs in the process of gyrification, perturbing the genesis of these basal progenitors in the mouse cortex. In particular, a group focused on Trnp1, a gene that is express at high levels in the radial glia and in basal progenitor-producing radial glia, demonstrating that an over-expression of this gene in the embryonic neocortex (through in utero electroporation) induces a selective RGC self-amplification and decrease basal progenitor levels, leading to tangential expansion of the neuroepithelium. On the contrary, the knockdown of Trnp1 induces proliferation of basal progenitors with a consequent radial growth and folding of the cortex [64]. Another group found that gyri were induced in the normal lissencephalic cortex of mice by injecting FGF2 in the ventricles during early cortical development [65]. Interestingly, FGF2, that is a factor directly involved in the gyrification process, diffused in a bilateral way throughout the ventricles and had a very localized effect at level of the lateral neocortex, where increased the growth and the formation of new gyri, bordered by aberrant sulci. One of the aberrant sulci was positioned in correspondence of the Sylvian fissure (that is the lateral sulcus) in gyrencefalic species. The induced gyri and sulci showed a normal six-layered conformation at postnatal ages and were visible in adult mice [66].

Cortical surface area seems to be determined by the number of progenitors at the level of radial units in the embryonic VZ and by the shape, size and neuronal composition of the cortical columns that derive from each radial unit [56]. In this arrangement, the cortical surface could be expanded increasing the number of apical progenitors and the ventricular surface area. But, more recently, new studies have shown that ventricular surface expansion is an essential factor for the normal development of gyri and sulci (especially in the humans at the level of the occipital and temporal lobes) and it is involved in some neuropathological conditions. However, ventricular surface expansion contrasts with gyrus-building mechanism and seems to be present as mechanism of gyrogenesis only in the temporal and in the occipital lobes, where the skull represents a resistant surface that might contrast the cortical outward growth causing cerebral wall to fold. When excessive, ventricular surface expansion may lead to pathological gyrification [67].

Hypergirification, as pathological condition and as a result of ventricular surface overgrowth, in humans can derived from gain-of-function mutations in some genes associated, e. g., with dysplasia. This condition is characterized by a significant increase of the brain mass (megalencephaly), cortical hyperconvolution and hippocampal dysgenesis [68].

Different anomalies of gyrification have been described in the neuropathology and neuroimaging studies [69]. Some of these are associated to genetic pathways or to external

factors and cause neurological disorders such as intellectual disability and epilepsy. Lissencephaly is characterized by a smooth brain and is caused by genetic defects in the interaction between the pia meninges and glial cells, which cause a disruption of the brain surface; or by mutation in cytoskeletal-related genes that are involved in cell migration during the gyrus-building. Otherwise, Polymicrogyria is characterized by an excessive number of gyri and can be associated with viral infections and hypoxia-ischaemia, but also by genetic mutations [22]. A lot of other anomalies in the process of gyrification have been related to single-gene mutations and a more complex chromosomal disorders. The importance of gyral pattern in the brains has been at the centre of great interest and debate. There are evidences that suggest that gyral patterns are associated with the different borderlines of different cortex areas [70]. One clear example of this is the link between the language functions and the asymmetry of gyri in and around the Sylvian fissure where Broca's and Wernicke's areas are located. In the majority of the individuals, language functions are dominant in the left hemisphere that is characterized by morphological features such as a more pronounced development of the posterior temporal region. These cortical regions have an asymmetrical growth that can be detected in human foetal brains by MRI (20-23 gestational weeks). Asymmetry could represent the differential expression of genes [14, 71].

All these factors are the substantial demonstration that the brain surface is very complex and for all these reasons, the alteration in the structures of its components, such as leptomeninges (arachnoid membrane, pial cells), basement membrane, radial glial/astrocytic end-feet and Cajal-Retzius cells in the upper molecular layer, is implicated in cortical malformations [34].

In 1896 was already proposed the idea that the cortical gyri develop at level of proliferative spots. This idea was revalued in 2006 by Kriegstein and collaborators. They showed that the subventricular zone results to be ticker in correspondence of some developing gyri with respect to the proximity of emerging sulci [63]. This hypothesis however has some limitations; in fact, the increased proliferation can only create a gyrus by locally thickening the cortical plate. Instead,

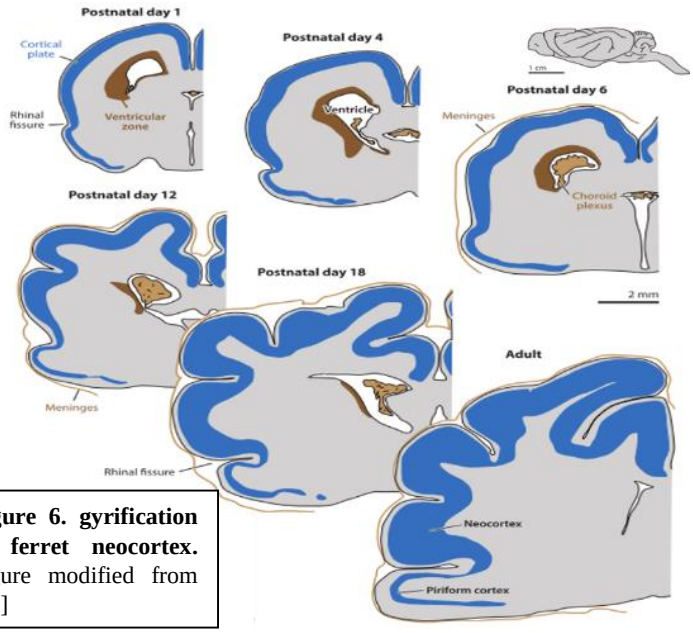


Figure 6. gyrification in ferret neocortex. Figure modified from [10]

as shown in the Figure 6 modified from [10], cortical folds develop because the cortical plate becomes undulate maintaining constant its thickness. In the specific it is here shown a graphic representation of coronal sections through the telencephalon of ferrets at the indicated ages (at comparable rostrocaudal levels). Cortical gray matter is shown in blue, and the proliferative ventricular zone is shown in brown. In the upper part of the panel is shown a lateral view of an adult ferret's brain.

This discordance between the two aspects can be explained considering the differential proliferation in combination with the radial migration hypothesis. In particular, the higher level of proliferation should lead to an increased local expansion of the cortical plate, which should cause a rise of the probability to develop one or more folds. To support this point of view, Nonaka-Kinoshita and collaborators performed an experimental increase of the proliferation of cortical progenitors that caused an increased cortical folding [72]. So, it is possible to affirm that there is a relationship between spatial variation in proliferation rates and the level of cortical folding. This event is affected by different molecules [14].

DIFFERENCES BETWEEN RODENT AD HUMAN BRAIN DEVELOPMENT

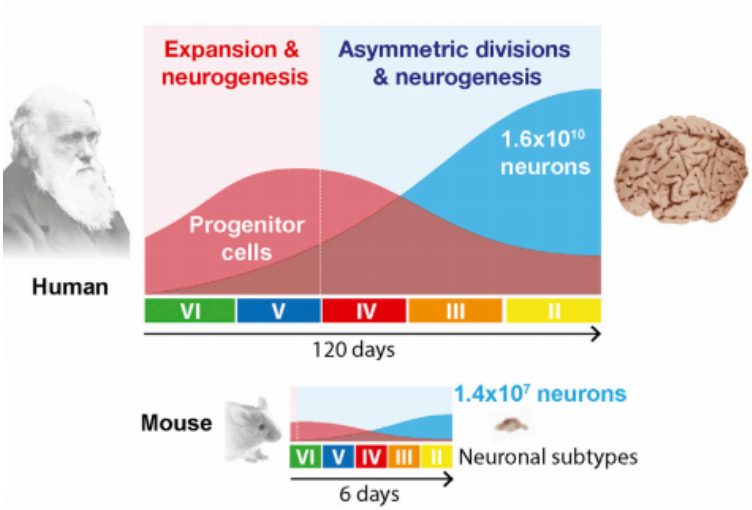


Figure 7. Modified from [1]

Our current understanding of adult brain structure and brain development is based on a handful of animal models. The common house mouse is usually used as a rodent model, while macaque and human brains serve as traditional primate models. The topology (size and shape) of the mammalian cerebral cortex presents variation across species, ranging from the small and smooth cortex of mice, to the large and profoundly folded cortex of humans. These features are determined during embryonic development and reflect the balance between radial and tangential growth of the neuronal layers [72]. The first major event of CNS development in all vertebrates is the formation of a specialized fold of ectodermal tissue called the neural tube, from which the spinal cord and brain subsequently differentiate. Neural tube formation occurs approximately mid-gestation in rodents, on gestational day (gd) 10.5–11 and 9–9.5 in rats and mice, respectively, with birth typically occurring on gd 20–21. In humans, this event occurs earlier during prenatal development, between gd 24 and 28 (3–4 weeks) out of a gestation period of 266–280 days (40 weeks) [73]. The key stages of cortical development during foetal brain formation are conserved between mammalian species and have been extensively described [74]. The most notable difference between the human and rodent developing brain is gyrification, which is essentially absent in the rodent brain. Sulci and gyri formation acquires complexity postnatally, maybe dependently to changes in cell density and maturation of subcortical fiber tracts [75]. The generative capacity of the immature brain depends on brain region features and cell types. In general, cell proliferative processes between rodents and humans are more or less parallel, although the time scales are substantially different. The neonatal mammalian brain contains a temporary layer of glutamatergic and gamma-aminobutyric acid (GABA)-ergic neurons between the immature cerebral cortex and white matter regions, which acts as a source of new neurons during development [76]. Although the human and rodent subplate have common gene expression patterns, there are species differences in structural organization and complexity; while in mice the majority of

cells form a single compact layer, in humans they are dispersed throughout a larger zone [77]. Rodent and primate species show much variation in cortical cytoarchitecture, in number of functional areas, in composition of cortical layers that have to be accurately considered in the study of cortical development and pathologies.

GENETIC OF PMG

The mechanisms of folding involve a lot of genes that regulate the process both in a positive and negative way when they are overexpressed or silenced. These genes act as mediators of cortical folding and are essential for the mechanisms described above. These include cytoskeletal proteins (such as those implicated in lissencephaly: LIS1 and DCX); others like L1cam, which affects terminal translocation in species with cortical gyri; and factors that have an effect on the proliferation rate and timing (e. g., some members of the fibroblast growth factor family which can be responsible of abnormal folding) [66, 78].

The spatial patterning of the cortex is controlled by specific genes and, for example, it was found a transcription factor responsible for the cortical area map, EOMES (eomesodermin), that has been shown to be defective in a syndrome which includes PMG, microcephaly and agenesis of the corpus callosum [79]. Originally described in association with congenital muscular dystrophy and dystroglycanopathy, pial basement membrane defects and over-migration are now recognised in association with mutations in numerous genes including GPR56, TUBB2B, COL4A1 and MARCKS [80-82]. In this view, other, well characterized, cortical malformations may be clarified studying the mechanisms and spatial distribution of PMG.

As anticipated above, a large number of genes have been associated with PMG [83]. PMG can be associated with mutations in genes which encode for proteins with very different functions, including centrosome-, cilia- and microtubule-related proteins, or proteins that maintain the integrity of the basement membrane [84, 85].

Many of these studies are based on the description of the human cortical malformation obtained by MRI. More precise characterization of the pathology in the human brain, together with animal models, are beginning to detect the specific pathways interested in individual syndromes. Two of the more important genes related to PMG are coding for tubulins and GPR56.

A central point of discussion about PMG is its timing of aetiology (i.e. before or after completed migration) and pathogenesis (i.e. developmental or due at cellular death). Recent studies support that most types of PMG are post-migrational disorders that occur after the migration of neurons to form the cerebral cortex from the periventricular region [20, 86].

In the last years has been produced a huge amount of reports on the association between PMG and genetic factors and numerous loci and more than 30 genes have been correlated to this pathology suggesting a causative role (Table2).

Cortical malformation	Pattern of inheritance	Gene	Locus	OMIM
PMG-like, microcephaly, ACC	AD	*DYNC1H1*	14q32.31	600112
PMG-like, microcephaly, ACC, CBLH	AD	*TUBA1A*	17q13.12	602529
PMG-like, microcephaly, ACC, CBLH	AR	*TUBA8*	22q11.21	605742
PMG-like, microcephaly, ACC, CBLH	AD	*TUBB3*	16q24.3	602661
PMG-like, microcephaly, ACC, CBLH	AD	*TUBB*	6p21.33	191130
PMG-like, microcephaly, ACC	AR	*EOMES*	3p24.1	604615
PMG and Goldberg-Shprintzen syndrome	AR	*KIAA1279*	10q21.3	609367
PMG and CK syndrome	X-linked	*NSDHL*	Xq28	300275
PMG and CEDNIK syndrome	AR	*SNAP29*	22q11.21	604202
PMG and Knobloch syndrome	AR	*COL18A1*	21q22.3	120328
PMG	AD	–	1p36.3pter	
PMG and microcephaly	AD	–	1q44qter	
PMG and facial dysmorphisms	AD	–	2p16.1p23	
PMG and microcephaly, hydrocephalus	AD	–	4q21q22	
PMG	AD	–	21q2	
PMG	AD	–	6q26q27	
PMG	AD	–	13q3	
PMG	AD	–	18p11	
PMG and Di George syndrome	AD	–	22q11.2	
Bilateral perysilvian PMG	AD	*PIK3R2*	19p13.11	603157
Bilateral temporooccipital PMG	AR	*FIG4*	6q21	609390
PMG and microcephaly	AR	*RTTN*	18q22.2	610436
Bilateral frontoparietal PMG	AR	*GPR56*	16q13	604110
Asymmetric PMG	AD	*TUBB2B*	6p25.2	612850
PMG and rolandic seizures, oromotor dyspraxia	X-linked	*SRPX2*	Xq21.33q23	300642
PMG and ACC, microcephaly	AD	*TBR2*	3p21.3p21.2	604615
PMG and aniridia	AD	*PAX6*	11p13	607108
PMG and microcephaly	AR	*NDE1*	16p13.11	609449
PMG and microcephaly	AR	*WDR62*	19q13.12	613583
PMG and fumaric aciduria	AR	*FH*	1q43	136850
PMG and 'band-like calcfication'	AR	*OCLN*	5q13.2	602876
Perysilvian PMG and CHARGE syndrome	AD	*CHD7*	8q12.1q12.2	608892
PMG and Warburg Micro syndrome	AR	*RAB3GAP1*	2q21.3	602536
PMG and Warburg Micro syndrome	AR	*RAB3GAP2*	1q41	609275
PMG and Warburg Micro syndrome	AR	*RAB18*	10p12.1	602207

AD = Autosomal dominant; AR = autosomal recessive; ACC = agenesis of the corpus callosum; CBLH = diffuse cerebellar hypoplasia; DMEG = dysplastic megalencephaly; ILS = isolated LIS; MCAP = megalencephaly-capillary malformation syndrome; MDS = Miller-Dieker syndrome; MPPH = megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome; PH = periventricular heterotopia; PMG = polymicrogyria; – = not known.

[a] Posterior to anterior or anterior to posterior.

Table 2. Modified from [11]

Autosomal Dominant Genes

Numerous chromosome anomalies have been associated with PMG even though the causal genes located in these regions have not been identified yet. The most common are the 1p36.3 and 22q11.2 microdeletions, both of which related to unilateral or bilateral perisylvian PMG [30, 87].

Tubulins

The role of cytoskeleton is essential for neuronal migration, for this reason genetic mutations which cause a disruption in the function of microtubules have been identified as crucial in the

onset of MCD including lissencephaly and PMG. In particular, PMG has been associated with the a-tubulin genes TUBA1A and TUBA8 [85, 88] and the b-tubulin genes TUBB2B and TUBB3 [80, 89]. Disruption of tubulin may affect several aspects of the protein, for example interference with the a-tubulin tertiary structure has been reported in PMG-related TUBA1A mutations suggesting that the disease may be directly related to the stability of microtubules and to their interactions with associated proteins [90]. The group of Jaglin identified five unrelated cases with de novo loss-of-function mutations in TUBB2B and bilateral, asymmetrical, anteriorly-predominant PMG [80]. In utero RNAi-based inactivation of TUBB2B gene in the rat showed that TUBB2B is dominantly expressed in post-mitotic neurons during their migration and differentiation and that these mutations affect the formation of tubulin heterodimer [80]. Heterozygous missense mutations in TUBB3, that encodes for the b-tubulin isotype III, can give a series of neurological disorders associated to different degree of intellectual impairment, facial paralysis and axonal sensorimotor polyneuropathy [91].

Given that TUBB3, TUBA1A and TUBB2B have similarities in expression, a cohort of patients presenting MCD has been studied to find mutation for TUBB3. From this analysis have been identified six novel missense mutations (one homozygous) in nine cases of PMG. In the specific was found that these mutations associated with PMG caused different amino acid substitutions. Functional studies showed that these mutations negatively influence microtubule resistance to depolymerization [89]. Moreover, the gene TUBA8 is thought to origin PMG in the 22q11 deletion syndrome because it is near to the low-copy repeat that is involved in this microdeletion syndrome and furthermore, it may have a positional effect of the deletion on TUBA8 expression [88].

Autosomal Recessive Genes

GPR56

GPCRs (G protein-coupled receptors) are a heterogeneous group of proteins characterized by seven-transmembrane α-helix, an extracellular N-terminus, an intracellular C-terminus and three inter-helical loops on each side of the plasma membrane. GPCRs are divided in five subgroups, one of that is Adhesion GPCRs that facilitate cell-cell and cell-matrix interaction and that is characterized by the presence of a longer extracellular N-terminal fragment and a GPCR proteolysis site (GPS). This is an auto-cleavage site that cleaves the receptor at level of N- and C-terminal fragments during the maturation process.

GPCR56 is the first and the only member of the adhesion G protein-coupled receptor factor to be linked to a human developmental disorder: bilateral frontoparietal Polymicrogyria (BFPP) [82, 92]. In fact, mutations in this gene cause a typical phenotype characterized by a bilateral, symmetrical malformation similar to PMG with a decrement in antero-posterior gradient, changes in white matter and brainstem and cerebellar hypoplasia [93]. In the GPR56 knockout mouse model this loss of function causes an abnormal neuronal localization with overmigration, damage in the pial basement membrane ad disorganization of the glia scaffold [20, 82].

GPR56 association with a severe human brain malformation suggests an its important role in brain development, for this reason, several studies have been performed to characterize its expression pattern in developing brain. In situ hybridization showed that mRNA is preferentially expressed in neuronal progenitor cells of cerebral cortical ventricular and subventricular zones during the neurogenesis [94]. Studies of immunohistochemistry revealed a diffuse expression of GPR56 in multiple cell types at level of the preplate, marginal zone, subventricular and ventricular zone [95].

Experiments performed to identify the putative ligand of GPR56 showed that it is the collagen III in the developing brain that localizes in the meninges and pial basal membrane [96, 97]. Fibrillary collagen type III is a major structural component of the extracellular matrix. Mutations in this gene causes excessive bleeding, bruising and vascular problems [98]. It was reported a case of a homozygous mutation in GPR56 gene which results in a diffuse cortical dysplasia. This suggests a putative role of collagen III in the developing brain. However, the cortical dysplasia does not occur in a uniform way along the cortex, but the malformation is most prominent in the frontal cortex [96]. This supports the hypothesis that the signalling of GPR56 in association with the collagen III, is important for the formation of the rostral cortex.

These findings support the hypothesis that GPR56 signalling, particularly in the context of GPR56 and collagen III, is important for the formation of the rostral cortex. In fact, the absence of these two elements gives abnormal neuronal migration through a discontinuous pial basal membrane into the arachnoid space [96, 97].

FIG4

Mutations of Factor-Induced-Gene 4 (FIG4) have been described in association with Charcot-Marie-Tooth disease type 4J (CMT4J) [99] and Yunis-Varon syndrome [100]. Recently a homozygous missense mutation of FIG4 was found to co-segregate with PMG. Brains of FIG4^{-}

/- mice showed abnormal neurodevelopment of cortex and cerebellum with malformed gyration and foliation [101].

FIG4 is a phosphatase which forms a complex with Vac14 at plasma membrane and regulates the production of phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) [102, 103] activating the 5′-kinase of PI(3)P known as Fab1 [104]. In the specific, FIG4 can decrease PI(3,5)P2 levels via its phosphatase action but also promote its synthesis by acting as a secondary scaffold for the complex. In particular, the second function seems to be dominant, in fact loss of FIG4 gives a reduction of PI(3,5)P2 [99]. PI(3,5)P2 is associated with intracellular early and late endosome vesicles [103]. Even though it was reported that mutation of FIG4 can be associated to these neurodegenerative pathologies, the mechanisms are not clarified yet. The capability to regulate PI(3,5)P2 levels in mammalian cells is crucial for cellular function. In fact, different external agents, such as hormones, growth factors, neurotransmitters, can give a dynamic regulation of PI(3,)P2 levels. In cultured cortical neurons, the absence of PIKfyve lead to the internalization of AMPA-type glutamate receptor (AMPAR) subunit, HA-tagged GluA2, whereas the knockout of Vac14 and/or Fig4 impairs the synapses' strength [105-107], suggesting that Vac14 and Fig4 are important in the regulation of synaptic strength mediating a positive regulation of PIKfyve. These results have been obtained with the combination of multiple approaches. In particular, it was shown that the chronic down-regulation of PIKfyve activity lead to an increase of postsynaptic strength, but a brief chemical inhibition of this enzyme gives the block of NMDA receptor (NMDA)-dependent long-term depression (LTD) and a reversion in homeostatic synaptic weakening. Specifically, PIKfyve levels are higher during the homeostatic downscaling and this increase mediated by a dominant-active PIKfyve mutant is sufficient to impair the postsynaptic strength. Moreover, these effects on synapse derive from PI(3,5)P2-dependent trafficking of AMPARs [105]. Furthermore, PIKfyve may affect intracellular calcium dynamics by interacting with some L-type calcium channels [106], whereas Vac14 interacts with PDZ domains at the synaptic level [108]. Mutations in FIG4 gene may impact on the complex formation and function thus ultimately affecting a broader range of physiological aspect. Indeed, the functional consequences of PIKfyve and Vac14 alterations –secondary to FIG4 disruption- need to be investigated

It is clear that PMG is characterized by an elevated heterogeneity and for this reason it is one of the most interesting brain malformation to characterize and understand. The gene mutations causing this pathology affect different cellular processes and for this reason it is not correct to define a single genetic mechanism at the root of PMG pathophysiology. In this view it is possible to consider the opinion of Guerrini et al., who affirm that these kind of pathologies can be strictly associated to somatic mosaicism and mutations that lead to partial loss of

function of the protein potentially involved in the onset of cortical diseases [109]. Also other authors support this view, affirming that high variability of MCD can be related to germline mosaicisms [110]. Understanding the molecular basis of PMG is important to have accurate prognostic and genetic counselling to patients. Furthermore, an accurate characterization of PMG can help to elucidate the mechanisms which rule the human brain development and its complex pattern of gyrification.

Considering these aspects of neuronal migration and cortical folding, it is possible to suppose that exist a temporospatial patterning of PMG. In fact, radiological studies have explained specific patterns of localization of the PMG [12, 23] that occurs most commonly in the perisylvian region, but the pathological literature has less considered the spatial distribution of PMG.

WHITE MATTER DEFECTS IN PMG PATIENTS

Patients affected by PMG also display many non-cortical abnormalities such as an irregular cortical–white matter appearance. Indeed, PMG patients show defect in myelination both as reduction of white matter volume and as defect of fibre structure and organisation [23]. In the CNS the conduction of action potentials is depending on the myelin sheath. This structure is multi-lamellar and lipid enriched structure that also has the function of trophic and metabolic support to axons. Myelin is produced by the plasma membrane of specialized glial cells: oligodendrocytes in the central nervous system and Schwann cells in the peripheral nervous system [111]. The study of glial development and myelination is very important to understand the aetiology of demyelinating disorders and to develop specific therapies. During the development, the migrating proliferative oligodendrocyte precursor cells (OPCs) prolong and withdraw many processes [112]. OPCs migrate along the blood vessels following the Wnt signalling which involves the receptor-ligand pathway of Cxcr4-Cxcl12 that is expressed by OPCs and endothelial cells, respectively. Furthermore, the OPCs differentiation is depending on specific factors such as SOX10, Yin yang 1 (Yy1), Olig1, Olig2, Nkx2.2 and Myelin regulatory factor (Myrf) [2, 113]. Recently, study on oligodendrocytes differentiation have identified the involvement of other signalling molecules: GPCRs, GPR17, GPR56 and GPR37 in the CNS [114-116]. OPCs differentiate from multipotent neuroepithelial progenitor cells that are located in the neurogenic niches of the developing and adult brain. OPCs are highly proliferative and mobile, they can divide themselves and migrate throughout the CNS. Once in their last position, they can have a final differentiation before to start to myelinate adjacent axons [112]. These are highly dynamic cellular process that occur during both development and adulthood and that are mediated by regulated changes of the expression and activity of many transcription factors and epigenetic programs.

The first transcriptional control for the specification of neural progenitor cell in Oligodendrocyte lineage (OL) is deeply depending on the transcriptional control of dorsoventral patterning of the neural tube that is established by gradients of expression of Sonic hedgehog (Shh) and bone morphogenic proteins (BMPs). During the first phases of spinal cord development, OPCs derive from the ventral pMN domain, that gives rise to motor neurons and then to OPCs. This domain is defined by the transcription factor Olig2 which is fundamental for the appearance of early ventrally derived OPCs [117]. Even if Olig2 is fundamental for this early ventral production of OPCs and is one of the principal marker of the OL lineage, during later moments of embryogenesis, OPCs arising from more dorsal region of neural tube do not initially express this marker. This probably suggest that Olig2 is not essential for the specification of OL lineage [118]. During development other transcriptional factors are

involved in OPCs differentiation such as Ascl1 that promotes the specification of OL lineage given that Ascl1 knockout mice display a relevant reduction in the number of OPCs [119]. Following OPCs specification to the OL lineage it is possible to distinguish a number of transcription factors that characterize their migration journey. These include Olig1 and Olig2 that induce the expression of Nkx2.2 and Sox10 [120].

A central point of regulation of OL lineage and CNS myelination is when an OPC exits from cell cycle and differentiates into an OL. Even though mature OLs can dedifferentiate in vitro in the presence of basic fibroblast growth factor (bFGF), in vivo the OL differentiation step seems to be a terminal event. For this reason, the differentiation event must be tightly controlled both to regulate the timing of myelination during development and to maintain a pool of OPCs capable to differentiate and divide during life.

As descript, OPCs express high levels of specific transcription factors such as Sox5, Sox6, Hes5, Id2, and Id4, that together mediate the inhibitory extracellular signals acting on the OPCs, to prevent their differentiation and myelination [121, 122]. Another key pathway of extracellular inhibition of OL differentiation during the normal development and in case of injury/remyelination is that of Wnt. Indeed, it has an important role during the differentiation step together with its main partners, b-catenin and another transcription factor, Tcf712. Tcf712 and b-catenin form a transcriptional complex that inhibits differentiation acting on Id2 [123]. Furthermore, Wnt/b-catenin signalling seems to be highly stage specific, since it inhibits specification and some aspect of differentiation of OPCs [124].

As shown in the Figure 8, in addition to this plethora of genetic factors that regulates timing and mechanisms of OPCs differentiation and myelination, there is another important factor: Epigenetic regulation. These processes that by definition act changing targeted DNA sequences, are crucial for the cell growth and differentiation. Epigenetic comprises a series of

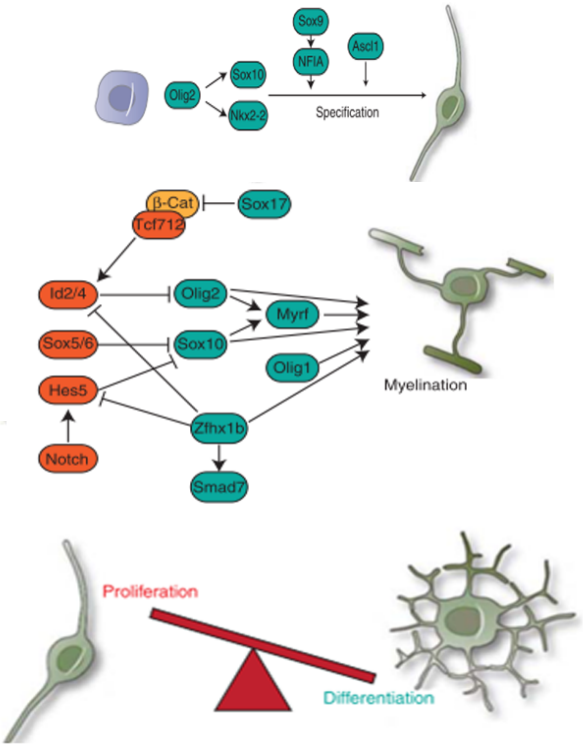


Figure 8. Genetic factors implied in Oligodendrocytes differentiation. Image modified from [2]

highly interconnected processes that can be summarized in four main classes of marks: histone modifications, ATP-dependent chromatin remodelling, a network of non-coding RNAs and DNA methylation [125]. It has been shown that the transition from OPCs to mature OL is associated to a rapid and significant chromatin remodelling. This suggests that chromatin reorganization is a crucial event for OL differentiation [126]. Activators and repressor of gene transcription that act during differentiation are modulate through the organization of the structure of the chromatin. This and other evidences suggest that epigenetic regulation has a pivotal role in OL development, myelination and re-myelination [2].

During the development glial cells intensely upregulate the production of their plasma membrane that they envelop around an axon segment. This intense activity produces a shape change of the cell that require extensive cytoskeletal rearrangements that drive myelin sheath formation. Experiments using zebrafish in vivo imaging and 3D electron microscopic reconstruction demonstrated that the plasma membrane inner tongue contacts the axon segment to wrap it and progressively extend itself to form the myelin internode. The initial movements of the inner tongue are promoted by the transport of mRNA and proteins through cytoplasmic channels and then are driven by actin. In vitro experiments showed that F-actin depolymerization caused by drug treatment increase cell spreading. Actin disassembly seems to be driven by competition of MBP protein for binding to PI(4,5)P2 which is responsible to release the actin disassembly factors gelsolin and cofilin [127]. The dynamic interchange between actin assembly during development and disassembly during myelination could suggest a form of temporal control. Since actin assembly is essential for OPCs development [128], the disassembly timing must be strictly regulated. One possible factor that could influence the timing is axonal activity as demonstrated with in vitro experiments by Wake and Lee in 2011 [129]. Oligodendrocytes present intrinsic myelinating capacity and for this reason it is necessary some factors to prevent the myelination of dendrites or other cells in the CNS. One of these molecules is JAM2, which was demonstrated to be a negative regulator of oligodendrocyte myelination as shown in the figure. In fact, it was demonstrated that loss of Jam2 causes an increase in myelinated neuronal cell bodies [130]. But also the variation in myelin distribution along single axons of the developing cortex needs to be regulated by an intrinsic program [131]. At this purpose, it was important to understand how activity can influence myelination. A series of works demonstrated that neurons form functional synapses on OPCs, suggesting that oligodendrocytes are more likely to myelinate electrically active axons in a way that is independent by synapse formation but that is sensible to vesicular release of glutamate and ATP [132, 133].

All these evidences support the idea that myelin is not only a static insulator deposited during development, but it is a player in nervous system plasticity. Myelination and oligodendrocyte maturation from precursors during development and in adulthood is regulated by animal's external experiences and myelin remodelling occur throughout life [111, 134, 135].

ASTROCYTES

Astrocytes take their name from their star-shape. These cells were described for the first time in 1891 by Lenhossek [136], and represent the most abundant cell type in most brain region [137]. Astrocytes were recognized to have an active function in brain since they can sense, integrate and respond to synaptic activity contributing to brain homeostasis and neuronal function [138, 139]. Their origin is common with that of neurons and oligodendrocytes and they are produced un the final stage of neurogenesis. Developing astrocytes differentiate into mature cells going through morphological, functional and electrophysiological properties changes [140]. Mature astrocytes form the so-called "tripartite" synapse together with pre- and postsynaptic terminals, helping to maintain brain homeostasis and modulating synaptic transmission via the release of neuroactive molecules such as glutamate, D-serine and ATP [141, 142] after the increase of cytosolic Ca^{2+} concentrations as a response to neuronal activity [143]. Furthermore, astrocytes are responsible of the neurotransmitter uptake with which they can terminate the synaptic transmission [144].

The Origin(s) and Development of Astrocytes

Astrocytes origin is from the neural lineage and starts at the end of neurogenic wave. In mice astrogenesis start at E18 and lasts at least until P7. The Figure 9 is a scheme of the Timeline of microglia invasion, gliogenesis and several developmental processes in the developing mouse brain. In the beginning, a homogenous pool of neural progenitors (NCs) (also called neuroepithelial precursor cells, NEPs) in the embryonic tube transform into radial glia. In the specific, these are pluripotent neural stem cells in the VZ that give rise to neurons and microglia [145-147]. At the end of neurogenesis, radial glia can both differentiate into astrocytes, or give rise to intermediate cells that later become astrocytes. Developing astrocytes differentiate into mature cells changing their morphology, connectivity and

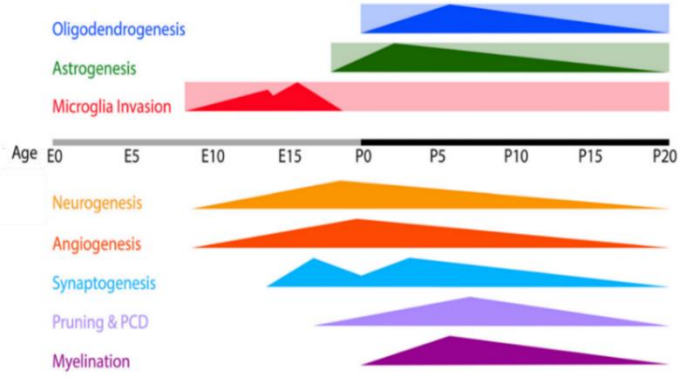


Figure 9. Representation of the estimated periods during/from which microglia, astrocytes and oligodendrocytes are present in the brain. Triangles indicate the onset and peaks of the developmental processes. (E, embryonic; P, postnatal. (Figure modified from [16].

electrophysiological properties [140]. Astrocytes are a heterogeneous population with differences both across brain regions and within the same brain region [148] and, since their discovery, they have been divided into two classes: protoplasmic and fibrous astrocytes that are located in the gray and white matter, respectively [149, 150]. Astrocytes with a spheroid shape present more complex branched extensions in comparison with fibrous astrocytes which have long extensions oriented in a longitudinal way along fiber bundles. Today it is known that there are many subclasses of astrocytes that differ in morphology, gene expression and physiological properties, characteristics that imply functional diversity. It is possible that different astrocytes subtypes arise from distinct progenitors, but the understanding of the precise process and timing of maturation from progenitors into distinct groups is incomplete [148]. In fact, there are some difficulties in this sense: 1) there is a lack of reliable and specific markers defining progenitor cells and immature astrocytes throughout their development steps; 2) it is difficult to precisely manipulate genes that only affect astrogenesis and not neurogenesis, since the identified astrocytes promoters are also active in neural stem cells [151]. Furthermore, these cells do not have a defined developmental endpoint since adult astrocytes are mitotic cells that can in principle continue to divide and differentiate [152].

Studies that describe the distribution and migration of astrocytes in neonatal brain are lacking due to lack of a general astrocyte marker. In 2005, Taft and al., analysed the distribution of astrocytes positive for one of the two main intermediate filaments expressed by these cells, the glial fibrillary acidic protein (GFAP) that is accepted as a general astrocyte marker [153, 154] and Vimentin, that is used as an embryonic astrocyte marker [155]. From these studies emerged that in the neonatal rat brain GFAP+ and Vimentin+ cells had a similar distribution and that these cells were located in high number along the whole brain, except the brainstem. This early postnatal distribution resembled the adult distribution, suggesting that astrocytes move right after their birth in specific domain and do not change their location under physiological conditions throughout the adulthood. GFAP is a well-documented marker of astrocytes, but it is not always expressed at high levels by all astrocytes subclasses especially in the gray matter [156, 157]. For this reason, it could be useful to use other specific markers such as calcium binding protein β (S100β), glutamine synthetase (GS), glutamate aspartate transporter (GLAST). These proteins underlie the existence of different subtype-specific function, different stages of astrogenesis and distinct astrocytes phenotypes [16].

NEUROINFLAMMATION: THE ROLE AND CONSEQUENCES

Inflammation is a response of the immune system that act in an innate way to protect and defend the body. Inflammatory response is regulated and driven by the mobilisation and interaction of different cell types and signalling molecules that produce a both local and systemic response. The principal cell types that operate to inflammatory response are white blood cells (leucocytes) and endothelial cells. Leucocytes include monocytes and derive from the monocuclear phagocyte system (bone marrow, lymph nodes and spleen). These cells can penetrate tissues and carry out their function: phagocytosis and antigen presentation. Signalling molecules are divided into locally acting small molecules (such as nitric oxide, NO), lipid compound (such as prostaglandins) and complex circulating proteins (cytokines). Innate immunity is generic, with non-specific responses of the immune system, and is generally triggered by pathogen-derived molecules known as pathogen-associated molecular patterns (PAMPs). This type of response is immediate and short-lived and differ from the adaptive immune response, which involves T and B lymphocytes. This response starts to produce effect after two or three weeks from the infection and produce highly specialized response to pathogens. At the beginning of inflammation, tissue resident leucocytes stimulate endothelial cells to exhibit cellular adhesion molecules that are important to recruit more leucocytes to the site of tissue damage. Adhesion molecules bind circulating leucocytes in a weak way, getting them slower and allowing them to bind the signalling endothelium, which undergoes a phenotypic change and becomes permeable to leucocytes that are attract by a high concentration of cytokine near the stimulus. At level of the tissue, monocytes differentiate to macrophage which act phagocytosis and secretion of additional signalling molecules that mobilise and recruit more effector cells from the periphery. In this way the response to inflammation is sustained and amplified, turning into a systemic response [158]. The principal aim of inflammation is to clear and control the initial stimulus through phagocytosis and activation of inflammasome which trigger apoptosis [159] to enable tissue regeneration. However, it is important to underlie that an excessive inflammatory response can be dangerous since can cause or contribute to tissue damage and pathological status. Once organised, activated cells target both the initial site of inflammation and remote ones that respond to inflammatory stimulus.

Peripheral inflammation gives rise to neuroinflammatory response which involves blood-brain barrier (BBB), glia and neurons. Neuroinflammation is the term that describes the wide range of immune responses of CNS. The BBB is a highly specialised form of endothelium that was

thought to separate the CNS from the peripheral immune system. This barrier is not permeable to pro-inflammatory mediators but can be stimulated to release and transmit these mediators allowing the migration of leucocytes into the brain [160]. This neuroinflammatory response gives synaptic impairment, neuronal death and promote several brain diseases [161].

CELLULAR COMPONENTS OF NEUROINFLAMMATION

Microglia

Microglia represent approximately 5-15% of all cells in the human brain [162]. Initially, microglia were considered "resting" immune cells that only activated in response to pathological triggers starting phagocytose and secreting cytokines and chemokines. In this view, the only function of microglia was to help to protect the brain against damage and infection [163]. Only recently, evidence shows that these cells are high dynamic, since they check their environment and regulate tissue homeostasis under both pathological and physiological conditions [164]. Furthermore, microglia seem to can influence synaptic transmission and synaptogenesis [165] and participate to the maturation of neural networks [166]. Maybe the most interesting discovery about microglia's functions is that these cells are able to sense and respond to local neural activity, since they express neuro-transmitter receptors [167] and also they can secret neuroactive molecules [168]. In this view the term "activated microglia" must be reconsidered, in fact a change in activation state should be depending on the kind of the stimulus [169].

The Origin of Microglia and invasion into the brain

Microglia are the first glial cells that were observed in the CNS. It is known that these cells develop at the same time of neurons during the critical period of brain development [170], but their origin has been matter of debate. Their first description was by Nissl in the 1899, Robertson in 1899 and Ramon and Cajal in 1909 as "reactive glial elements", "mesoglia" and "the third neural element" respectively [171-173]. "Mesoglia" described by Robertson are oligodendrocytes [174]. The name "Microglia" was coined by Del Rio-Hortega, who described cells that were different from neurons and macroglia [175, 176]. In 1999, was proposed the yolk-sac theory [177] and recently it was experimentally confirmed. According to this theory,

Yolk-sac derived macrophages invade the brain at early embryonic stages and represent the vast majority of microglia in the adult [178, 179].

Migration into and colonization of embryonic brain by yolk-sac derived macrophage precursors starts between E8 and E10 [178]. In particular, microglia invasion into CNS seems to occur in two waves. In mice, the first wave occurs between 8.5 and E.14.5 during which microglia progenitors initiate the colonization of the brain and the number of microglia cells increase in a gradual way [177]. This first gradual increase is caused by a rapid proliferation of microglia already present together with the invasion of new microglia precursor [180]. Around E9.5 microglia invade the brain via extravascular routes [181]. At this purpose, seem that there are two routes by which embryonic microglia reach the brain, but it is not established yet. Microglial cells enter either from the meninges by crossing pial surface or from the ventricles, where they are free-floating cells or attached to the ventricle wall that then they cross to reach the brain parenchyma. Between E14 and E16 there is a second increase in microglia number that cannot be explained only by proliferation because the number of proliferating microglia decrease from E14.5. At E17.5 microglia increase end and the cells scatter through the brain [180]. Physiologically, microglia proliferate during the period of embryogenesis and self-renew constantly throughout life; in this way the cell number is constant [182]. Also the patterns of colonization and distribution of microglia in the embryonic mouse brain have received a great attention. Between E10 and E12 the microglia progenitors are localized at the PS in the meninges and within the lateral ventricles. At these early embryonic stages, it is possible to detect only few proliferative and highly motile microglia at level of neuroepithelium. Then amoeboid microglia transform into ramified microglia. It is possible that the dynamic and mobile features of microglia reflect their ability to explore in an efficient way their environment. It has been suggested that the increase in length of microglia processes reflects not only their maturation or activation state but may also indicate functional changes such as in response to inflammatory insults [180, 183]. It is important to classify microglia according to their phenotype. Three stage in microglia development have been established: very early (E10.5-E14), pre- (E14-P9) and adult (4 weeks and onwards) microglia. In these stages cells express different sets of genes reflecting their activities in the brain. Many studies hypothesize that genetic and environmental perturbation cause changes in stage-related expression profile and function of microglia, altering the precise timing of the microglial developmental programs, influencing in a negative way the brain development and causing neuropathology. In addition to temporal variations in gene expression profiles, region-specific differences have also been found. Furthermore, different types of microglia have been characterized in different brain regions, sometimes relate to neuropathological disease [184]. Neurons and microglia communicate via ligand-receptor way, that play an important role in

microglial development and activation state [185]. During the embryonic brain development, microglia are re-distributed in a non-uniform manner throughout the brain parenchyma [186]. Maybe an important role in this process is operated by neural progenitor cells that are involved in migration and positioning of microglia in the prenatally developing cortex via secretion of specific chemokines [187]. An important point is that until E16.5 microglia do not enter in the cortical plate (CP) and before this stage these cells are detected in the ventricular and intermediate zones (IZ) that are regions rich in neural progenitors. Around E17, microglia gradually invade the CP starting from deeper layers. During this stage those cells acquire ramified shape [180]. In adult brain microglia have an equal distribution over the brain, while in embryonic age they are a localization in four specific "hotspots" [170, 180]. One hotspot is near the radial glia in the VZ and SVZ, where cells play a role in the regulation of the size of the precursor cell pool [188]. The second is near newly forming blood vessels, where microglia contribute to angiogenesis [189]. In the third phagocytizing microglia are near dying cells at level of choroid plexus and in the developing hippocampus [180]. The fourth is near developing axons, where maybe microglia are involved in developmental axonal pruning and/or axonal growth and guidance mechanisms [190].

Another important microglia characteristic is that there is a microglial cell number, morphology and distribution differences between male and female brains [191, 192]. Maybe it should be a sex-dependent mechanism that influence microglial function during brain development. This could contribute to the sex-dependent susceptibilities to certain developmental psychiatric disorders [16, 193]. However, more studies are needed to clarify the signals the mediate the direction, speed and distribution of microglia over the embryonic brain, because the embryonic microglia distribution can give a suggestion of their function in developing brain.

A role in neuroinflammation is played by Brain Blood Barrier (BBB). Inflammatory cytokines and other proteins were thought to be capable to easily reach brain from the blood, but numerous transport mechanisms have come to light over the last two decades. Indeed, active transport systems that facilitate the delivery of TNF and IL cytokines into the brain have been found [194]. It is well known that cytokines levels modulate the BBB permeability by altering the resistance of tight junction in endothelial cells at level of brain vasculature, and damage to tight junction proteins cause an increase of permeability [195]. The movement of leucocytes across the BBB is regulated by chemokines such as CCL19 and CCL21 which enable T cell adhesion to BBB. Many of these mediators are secreted by astrocytes [196].

Macrophages are activated in different ways divided into M1 and M2 activation. M1, or classically activated macrophages are effector macrophages that are stimulated by interferon

(IFN)-γ and tumour necrosis factor and produce a primary aggressive immune response. M2, or alternatively activated macrophages are all other types of macrophages. These are usually stimulated by IL-4 and act in wound healing and in the regulation of the macrophage response. Macrophages can switch from M2 state to pro-inflammatory M1 state with an effect on the intensity and development of peripheral inflammation. Astrocytes are the other family of glial cells that release pro-inflammatory mediators such as TNF-α at level of cortex and midbrain [197].

Although microglia release the great amount of cytokines, the combination of glial response can influence in an important way the development of neuroinflammation, since a dynamic crosstalk between BBB endothelial cells, glia and neurons exists and it seems that the response of a cell type can directly impacts on another [198].

The inflammatory response in CNS is very complex and it take advantage of a multitude of receptors on cells that play a pivotal role in this process. Toll-like receptors (TLRs) are important proteins that transduce the signal in the innate immune system and inflammatory response. These receptors are activated in presence of a foreign element and initiate the downstream signalling cascades. Astrocytes and microglia express TLRs that cause and activation of these cells to initiate the neuroinflammatory response. Microglia also express the two classes of major histocompatibility complex, MHC class 1 and class 2, that are thought to play a role in the development of neuroinflammation [199].

Also microglial cells play a crucial role in the neuroinflammation since in response to cytokines and other signalling molecules deriving from acute inflammation, these cells change themselves from a ramified, inactivate state to an activated phagocytic one and release pro-inflammatory mediators. In case of chronic inflammation microglial cells can remain activated for long periods and release cytokines and neurotoxic factors that contribute to long-term neurodegeneration [200].

Microglia are the principal actors in neuroinflammation. This is because these cells perform the first primary immune surveillance producing cytokines and chemokines operating the innate immune response in the CNS. Other microglial activities related to immune response include the propagation of inflammatory signals from the periphery. These responses are essential to coordinate the communication between the immune system and the brain. For example, after an infection, microglia become activated and operate as inflammatory cellular mediators rapidly altering their transcriptional profile and producing cytokines and chemokines to facilitate the recruitment of leukocytes in the CNS [201]. Activated microglia is also responsible of cytoskeletal rearrangements that cause the alteration of the pattern of

receptor expression on the cell surface. Furthermore, thanks to these alterations microglia can migrate where sites of injury or infection are and increase their phagocytic efficiency [202]. But microglia also have not CNS protective functions. In fact, a massive or chronic microglial activation can cause pathological changes and neurobehavioral complications [203].

Molecular components of neuroinflammation

Neuroinflammation is mediated by several pro-inflammatory cytokines [interleukin (IL)-1β, IL-6 and tumour necrosis factor (TNF)α], chemokines (CCL2, CCL5, CXCL1), secondary messengers (NO and prostaglandins), and reactive oxygen species. Many of these molecules are secreted by activated cells that are located in CNS including microglia and astrocytes [204]. But also endothelial cells and macrophages are important for the production and propagation of these inflammatory signals within the CNS.

Cytokines are cell-signalling proteins which function is to mediate neuroinflammation exacerbating or reducing it. Pro-inflammatory cytokines such as interleukin-1 (IL-1) and tumour necrosis factor (TNF) have a role in pathological inflammation and acceleration of disease, but also exist several cytokines, such as IL-4, that are anti-inflammatory [205]. Pro- and anti-inflammatory cytokines are not rigidly separated since in all cases of physiological or pathological condition the effects of a signalling molecule may be different, depending on its localization and on the context of the disease. Cytokine release can cause the production of others signalling molecules. An example is IL-6 that active T cells and stimulate the production of other inflammatory markers such as C-reactive protein (CRP) and fibrinogen [206].

When TNF receptor-1 bind TNF-α, gives rise to a number of signalling cascade that influence gene transcription. An example is the cascade that leads to inflammation and degeneration in which TNF receptor-1-associated Death domain protein (TRADD) and TNF receptor-associated factor 2 protein (TRAF2) recruiting enzymes that activate transcription factor NF-κB. These proteins induce c-jun N-terminal kinase (JNK) pathways that is responsible of the activation of another transcription factor which modulate apoptosis and inflammation [207]. TNF signalling can directly induce apoptosis through the Fas-Associated protein with Death Domain (FADD) mediated production of enzyme Caspase-8 which is strongly linked with apoptosis and neurodegeneration [208].

Chemokines are small chemotactic cytokines important in the neuroinflammation. They have a very low physiological concentration in CNS, but levels of some chemokines, such as

monocyte chemoattractant protein-1 (MCP-1) are strongly upregulated in chronic neuroinflammation [209]. These mediators are involved in the upregulation and chemotaxis of astrocytes and microglia after an inflammatory insult and they can impair neuronal function and neurogenesis.

The complement cascade is an important feature of immunity and inflammation that is activated by the alternative, classical and lectin-binding pathways. It is important in CNS in different processes such as mast cell degranulation, chemotaxis and cell lysis since complement proteins act in relationship with glial cells during the neuroinflammation [210]. In this view the complement cascade can be considered a double-edged sword in the CNS because exhibits a protective effect at physiological and acute levels but causes damage in chronic condition.

The enzyme cyclooxygenase (COX) converts arachidonic acid to eicosanoid groups [211] such as prostaglandins and thromboxanes and has many function during the inflammatory process. COX presents two isoforms, COX-1 and COX-2, which pathways are shown to be associated with neuroinflammation and neurodegeneration. The roles of both isoforms are different in normal physiology and pathology. COX-1 expression in microglia leads to prostaglandin synthesis and are involved in pro-inflammatory pathways [212]. COX-2 is mainly expressed in neurons and it is associated with synaptic function [213]. It has anti-inflammatory properties [214] and its activation is promoted by cytokine signalling since IL-1β induces MAPK activation which promotes COX-2 gene expression [215]. Then, the activated cyclooxygenase pathway stimulates the secretion of IL-6 that has positive feedback loops in the systemic inflammatory response [216]. COX-2 production also influence leukocyte migration across the damaged BBB; this suggest that the effect of increased peripheral inflammation can propagated sustaining neuroinflammation in a positive feedback loop [217].

IL-1β

In the last years, huge advantages have been made in the clarification of the interactions between CNS and the immune system. Foremost among these is the realization that cytokines are able to exert a number of neuromodulatory effects within the CNS under both physiological and pathological conditions. In line with this, it has been demonstrated that many cytokines and their receptors are present in the brain [218]. Much attention has focused on the pro-inflammatory cytokine interleukin-1, especially the variant β (IL-1β), since it has demonstrated to be produced in CNS in response to different stimuli such as the peripheral administration of lipopolysaccharide (LPS), traumatic brain injury and acute stress [219]. The distribution of IL-1R mRNA in rat brain showed its presence in the hippocampus where it seems to have implication in the event of Long Term Potentiation (LTP) [220]. IL-1R family contains three immunoglobulin-like domains which are required for the binding od IL-1, which downstream signalling result in a change of gene expression (up to 90 genes have been shown to be affected by IL-1 signal. These changes result in the upregulation of mRNA transcription for cytokines, growth factor, adhesion molecules and acute-phase proteins [220]. IL-1β, when bind the IL-1 Receptor complex, is a critical initiator of a number of signal transduction cascades involving the mitogen-activated protein kinase pathways (MAPK) [221]. p38 MAPK is a stress-activated protein kinase and its action is pro-inflammatory producing IL-8 and IL-6 [206].

It was demonstrated that intracerebral injection of very low doses of IL-1β in rat can cause fever, sickness behaviour, slow wave sleep, effects on immune function and effect on the hypothalamus-pituitary-adrenal axis [222, 223]. Furthermore, IL-1β is involved in neuronal cell death and damage and can activate the c-jun N-terminal kinase (JNK) which may result in programmed cell death. Long-term action of IL-1β in the brain is associated with activation of glial cells and with the occurrence of chronic disorders such as AD and epilepsy, but it is not clear if interleukinergic neurons are involved in brain responses to acute stress stimuli [224]. The possibility that IL-1β gives rise to age-related changes in brain was extensively reviewed [225, 226]. Furthermore, since in the last part of LTP are implicated MAPKs that is directly regulated by IL-1β, a dysfunction of this cytokine can result in a malfunction of learning processes [220].

Neuroinflammation and epilepsy

Epilepsy is the most common serious neurological condition, since at least 40% of all epilepsies have structural or metabolic causes as a consequence of diverse brain injuries. Infections are among the most common risk factor for seizures and acquired epilepsy [227]. Of the estimated 70 million people with epilepsy, around 30 million are children [228]. The relationship between immune-mediated inflammation and excitatory activity in the brain was largely investigated from at the beginning of the 20th century [229]. Neuroinflammation seems to affect seizure severity and recurrence, how is evident by extensive clinical and experimental data [230]. In fact, is well described how patients with autoimmune diseases and encephalitis characterized by severe and long-lasting neuroinflammation are subjected to seizures [231], suggesting that the immune and inflammatory processes are involved in some forms of epilepsy. Thanks to recent studies, it now becomes clear that pro-inflammatory mediators such as COX-2, PGE2, IL-1β, IL-6, HMGB1, TLR4, TNF-α, TGF-β and NOX2 play important roles in seizure generation and exacerbation [232]. For instance, systemic inflammatory processes induced by fever in children can cause seizures and the pro-inflammatory cytokines such as IL-1β are believed to play crucial roles [233]. Furthermore, experimental systemic administration of LPS in rodents can change the seizure threshold enhancing epileptogenesis [234, 235], also involving cytokines IL-1β and TNF-α, and COX-2 activation in the brain. Thus, as a consequence of epilepsy, neuroinflammation in turn can cause or facilitate epilepsy. The mechanisms related to the pro-convulsant effects of IL-1β and HMGB1 include rapid post-translational changes in N-Methyl-D-aspartate (NMDA) receptor phosphorylation leading to increased receptor function. In the specific there is the induction of Src kinase-dependent phosphorylation of the NR2B subunit of the NMDA receptors [236] that is responsible of the potentiation of NMDA-dependent Ca2+ influx [237]. The implication of this inflammatory pathways is demonstrated by the preclusion of the pro-convulsant activity of these pro-inflammatory molecules by the administration of specific pharmacological compound [236]. Inflammatory molecules can also contribute to hyper-excitability by inhibiting the astrocytic glutamate reuptake and by inducing changes in glutamate receptor subunit expression, thus leading to increased glutamatergic neurotransmission [231]. All these evidences are a clear proof of the strictly interconnection between the two events, neuroinflammation and epilepsy and suggest a possible therapeutic approach to their more severe and invalidating forms.

AIM OF THESIS AND EXPERIMENTAL APPROACHES

Aim of my PhD project was to characterize a mouse model of PMG and to investigate the cellular and molecular mechanisms occurring during cortex formation inn this model in order to envisage possible therapeutic approaches.

To this purpose we chose the focal freeze-lesion (FFL) [238], which reproduces the morphological aspects of the pathology such as the formation of microgyri (Figure 10). Indeed, P0-P1 freeze lesioned mice present a focal cortical malformation with a small sulcus, and a 3- or 4-layered microgyric cortex that resemble the human 4-layered microgyria [238, 239]. Moreover, an enhanced excitatory and inhibitory synaptic transmission accompanied by increased connectivity and increased neuronal activity has been described in vitro [77].

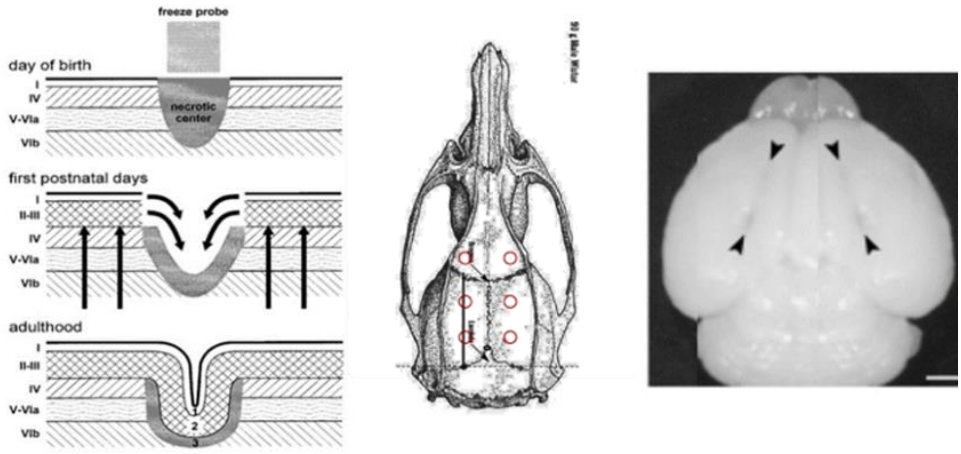


Figure 10. FFL mouse model. Focal freeze lesions (FFL) induce a morphological and structural reorganization in the mice brain detectable as the formation of the microgyrus in the cortex and alterations in the cortical laminar structure (Fig. modified from Dvorak et Feit, 1977, 1978).

In this model we have performed a through morphological and biochemical analysis and we have found the appearance of the microgyri at the level of motor cortex, and astrogliosis [240]. Furthermore, we also found a diffuse cortical demyelination (as shown by a reduction of the specific marker for mature oligodendrocytes, such as myelin basic protein (MBP)) and microglia activation (as shown by increased number of CD11b-positive IBA1 cells). To define whether microglial activation was accompanied by increased inflammatory response in PMG mice we assessed the IL-1β levels by ELISA and RT-PCR and a panel of different cytokines.

For the functional characterization of the mice, we employed a battery of specific behavioural tests for motor skills and coordination, and electroencelography (EEG) recordings for assessing cortical activity in collaboration with Dr. Mariaelvina Sala (University of Milan, CNR – Institute of Neuroscience of Milan).

After the molecular and functional characterization of PMG model, we have tested possible therapeutic approaches to treat the pathological symptoms of PMG. Based on our results regarding the defective myelination and the increased inflammation, we have employed two different strategies.

At first, in collaboration with Prof Angelo Vescovi (University of Milano-Bicocca) we have investigated the potential effect of transplantation of human CNS neural stem cells (hNSCs), which have been demonstrated to exert positive actions on inherited or acquired myelination disorders [241]and improve neurological functions by dampening the inflammatory process [242] in the cortex of PMG mice. As second strategy we used a specific pharmacological approach to counteract the increased activation of IL-1 receptor by using the recombinant version of the endogenous interleukin 1 receptor antagonist (IL1-RA, anakinra), which competitively inhibits the binding of IL-1 to the Interleukin-1 type receptor.

RESULTS

CHARACTERIZATION OF ANIMAL MODEL

Morphological analysis: gyri formation, defects of lamination, myelin defects

Nissl staining of coronal brain slices of P7 and P30 PMG mice reveals the appearance of cortical microgyric alterations that resemble those observed in patients affected by polymicrogyria. In the specific, at level of microgyri, as it was better detectable in P30 mice brain slices, it was visibly evident the invagination of cerebral surface and a zone without neuronal bodies that appear like a white scar (Figure 11).

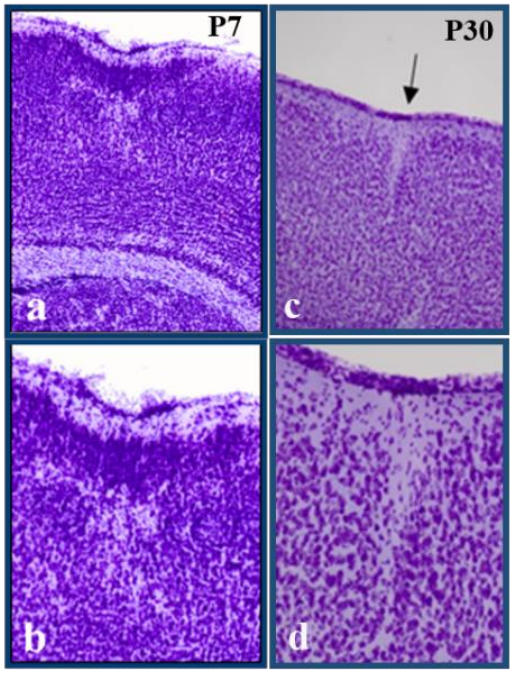


Figure 11. Microgyrus formation. Representative images of Nissl staining of coronal mice brain slices at P7 (a, b) and P30 (c, d) after focal freeze-lesion. Representative images show cortical microgyric alterations similar to those observed in patients affected by polymicrogyria. Scale bar: 200µm.

So this result clearly suggests that PMG brains display a structural reorganization at level of the ectopic microgyri detectable as alterations in the laminar structure of the cortex. Immunofluorescence staining for NeuN, a specific marker for neuronal cell bodies, allowed to evaluate neuronal distribution in cortical layers of P30 PMG mice coronal brain slices (Figure

12 A-B). Staining for the total neurofilaments (NF) highlighted a clear disorganization of the cortical structure. NF in PMG mice, respect to the age matched controls, appeared to have loss the canonical disposition in favour of a more imprecise one (Figure 12 C-D).

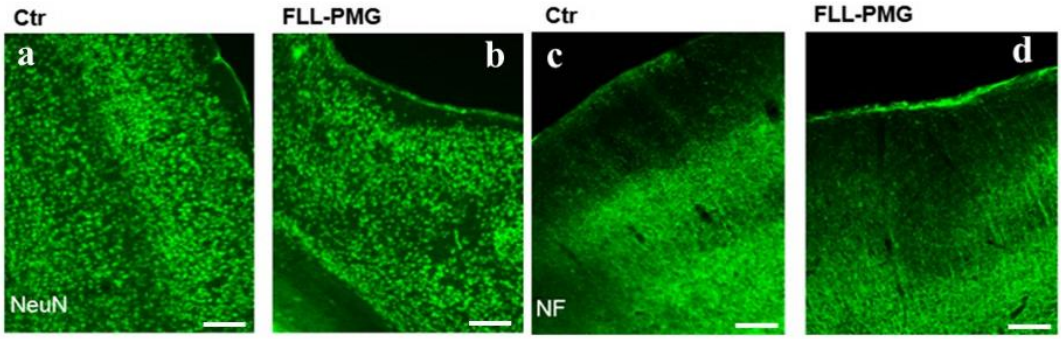


Figure 12. Reorganization of laminar structure in PMG mice. Representative immunofluorescence images of structural reorganization in PMG mice brain detectable as alterations in laminar structure. A-B) Representative images of immunostaining experiments against NeuN (green) allow to identify neuronal cell body in cortices of control and polymicrogyric (PMG) mice and their abnormal distribution in the cortical layers of PMG mice. C-D) Representative images of control and PMG cortices stained for the total neurofilaments (NF) (staining against neurofilaments' proteins Smi31/32). A clear disorganization of the cortical laminar structure consequent to the focal freeze-induced lesion is evident. Scale bar: 200µm.

Another important aspect of PMG brain that we detected, was a diffuse cortical demyelination as is well shown by the reduction of the specific marker of mature oligodendrocytes, such as myelin basic protein (MBP, Figure 13 A). Reduced myelination in the cortex of PMG mice, was analysed by quantifying MBP expression as area fold increase. In this way, we found a reduction about the 50% of MBP total area in PMG mice respect the controls (Figure 13 B). This analysis was performed in coronal brain slices taken at level of freeze-induced microgyri in P30 mice.

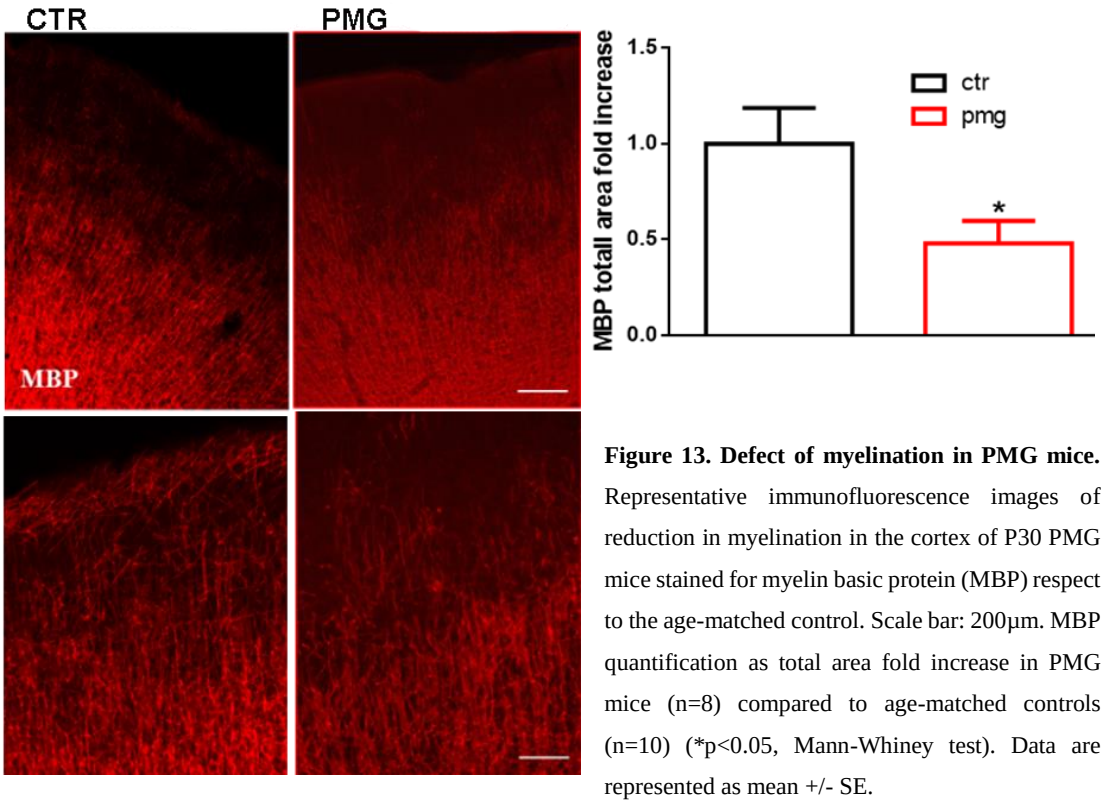


Figure 13. Defect of myelination in PMG mice. Representative immunofluorescence images of reduction in myelination in the cortex of P30 PMG mice stained for myelin basic protein (MBP) respect to the age-matched control. Scale bar: 200µm. MBP quantification as total area fold increase in PMG mice (n=8) compared to age-matched controls (n=10) (*p<0.05, Mann-Whiney test). Data are represented as mean +/- SE.

Taken together, these data show a morphological reorganization of PMG brains that reproduce in a reliable way the morphological aspect of PMG patient brains that are reported in literature [26, 41].

Astrogliosis, microglia activation

Several non-neuronal damages have been reported in the brain of patients affected by polymicrogyria, in particular affecting white matter integrity [4, 12, 21, 243]. To investigate this issue in the PMG mice we carried out immunofluorescence and confocal analysis of astrocytes and microglial markers. Using a staining for glial acid fibrillary protein (GFAP) that is a specific marker of astrocytes which increased signal is found during astrogliosis phenomenon, we obtained the detection of an increase of GFAP expression as area fold increase, approximatively of 20 times in PMG mice respect the controls (Figure 14 F). These data suggest the migration and the activation of astrocytes at level of the focal lesion that also persist after the induction of the damage (P30).

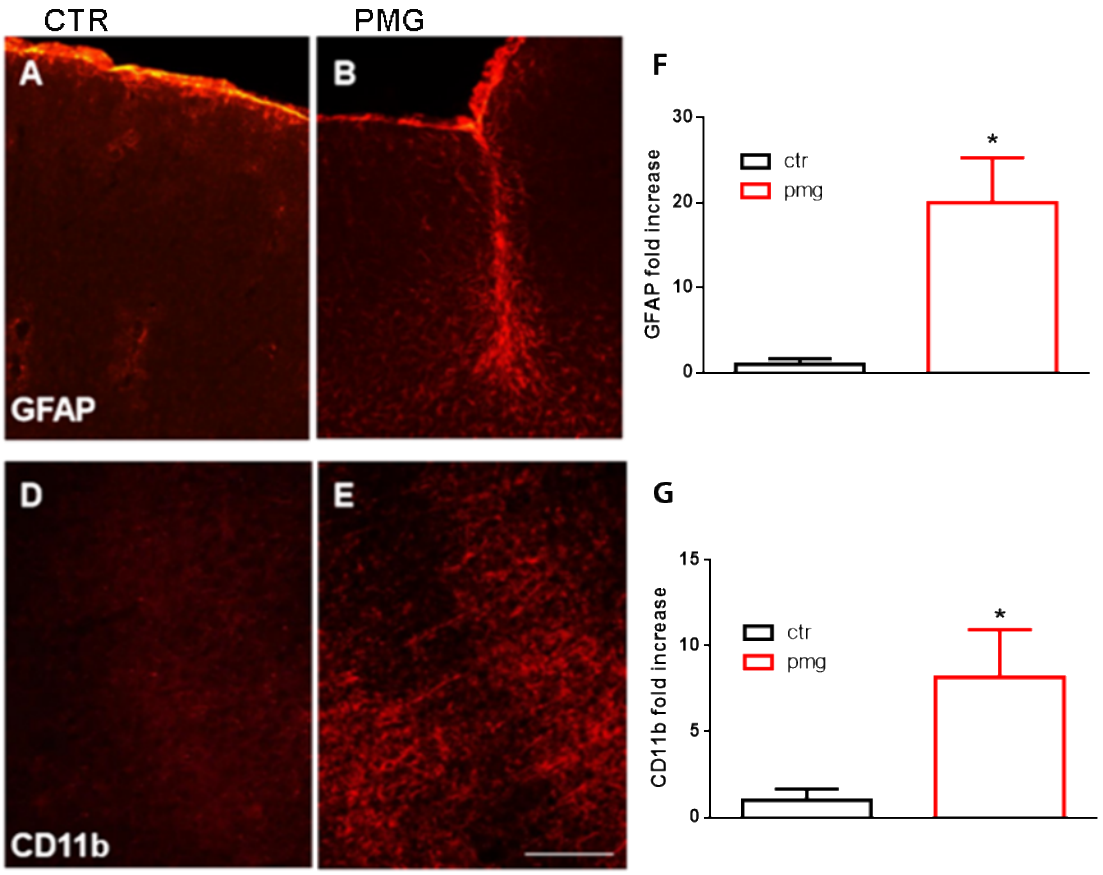


Figure 14. Astrogliosis and microglia activation in PMG mice. Representative immunofluorescence images of increased astrogliosis detected by glial acid fibrillary protein (GFAP) immunofluorescence (A, B) and microglial activation stained for the microglia-activated marker CD11b (D, E) in the cortex of P30 PMG mice compared to age-matched controls (Scale bar: 200µm). C, F) Quantization of GFAP (n=3) and CD11b (n=4 CTR vs n=8 PMG) expression as area fold increase in FLL-PMG cortices versus age-matched controls (*p<0.05; Mann-Whitney test). Data are represented as mean +/- SE.

Another sign of a protracted inflammation was the increased microglia activation as shown by confocal analysis of the microglial markers Iba1 and CD11b (Figure 15). Here we used Iba1 (Ionized calcium Binding Adapter molecule 1) is a microglia/macrophage-specific calcium-binding protein uniformly distributes in the cytoplasm and process of ramified microglia [244]. CD11b protein is a component of the type 3 complement receptor (CR3) often reported as CD11b/CD18, MO1 or Mac-1, even if it is a single complex. In the specific CR3 is an integrin while CD11b is the α subunit of this integrin that binds the β subunit-CD18. The complement receptor, of which CD11b is part, is located at level of the plasma membranes of neutrophil granulocytes and most mononuclear phagocytes and NK cells. Even though CD11b is not a highly specific microglial marker, immunocytochemical reactions for this protein are used as the principal method to detect activated microglia [245]. For this reason, at first, we quantified

the CD11b expression as area fold increase in PMG cortical slices versus age-matched controls finding a seven time increased expression of this inflammation's marker at level of freeze-induced lesions (Figure 14. G), furthermore to quantify the number of activated microglial cells we counted the number of IBA1 and CD11b positive cells (Figure 15. B). Results show an increased number of CD11b-positive Iba1 cells in PMG brains (Figure 15 B-C), that indicate the presence of a high level of activated microglia reminiscent for an ongoing inflammatory process.

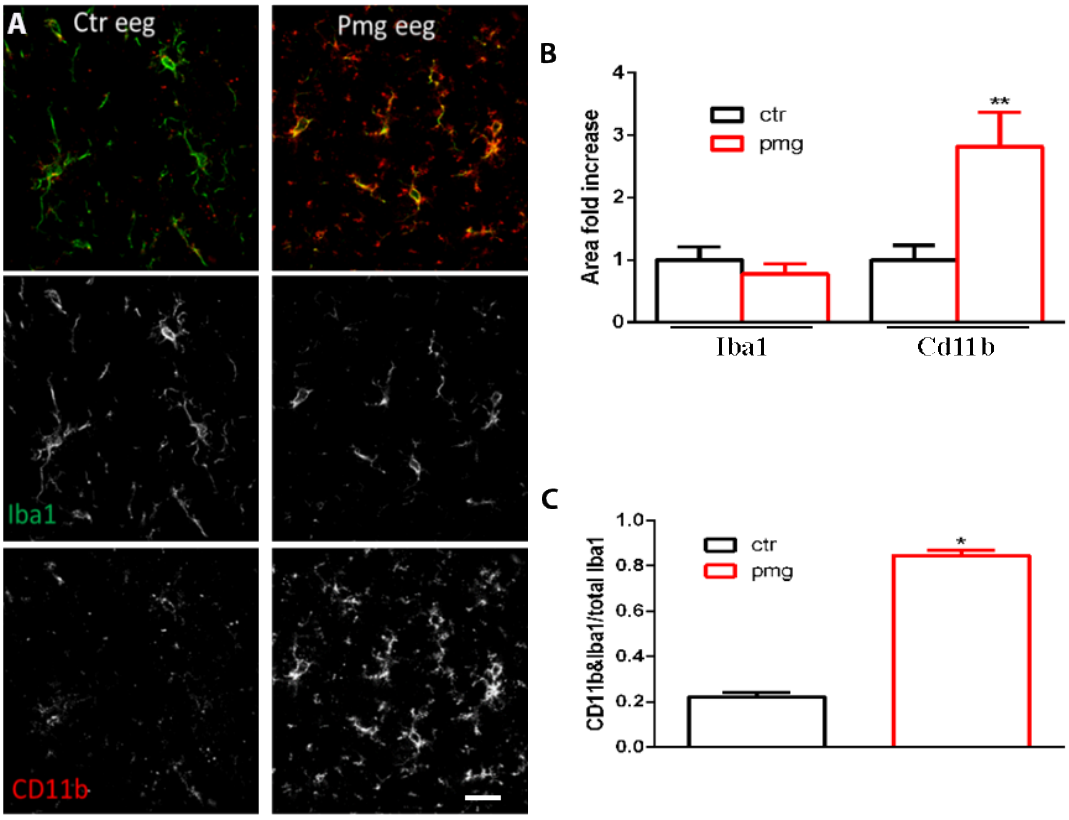


Figure 15. Microglia activation in PMG mice cortex. A) Representative images of immunocytofluorescence staining for the microglia marker Iba1 (green) and microglia-activated marker CD11b (red) after EEG recording in the cortex of P90 PMG and age-matched control mice (Scale bar: 200µm). B) Quantization of Iba1 (n=8) and CD11b expression as area fold increase in FLL-PMG cortices versus age-matched controls (Iba1 n=8, Cd11b CTR=8 vs PMG=7; *p<0.05 Mann-Whitney test Cd11b CTR vs PMG). C) Analysis of colocalization of Cd11b and Iba1 respect the total amount of Iba1 as area fold increase (n=4; **p<0.01 Mann-Whitney test). Data are represented as mean +/- SE.

Analysis of cytokines content

To define whether microglial activation was accompanied by increased inflammatory response in PMG mice we assessed IL-1beta levels either by ELISA or RT-PCR in brain tissue. We performed this analysis in P7 and P90 mice comparing the microgyric tissue and the

perilesioned areas to follow the temporal and spatial course of IL1beta levels. Results show that in P7 PMG brains the levels of IL-1β were increased only at level of the lesion (increase of five times compared to the controls), while in adult mice we found higher levels of this cytokines also in perilesion regions (increase of three times in lesioned cortex and of two times in perilesioned cortex of PMG mice brains respect to aged-matched controls) (Figure 16).

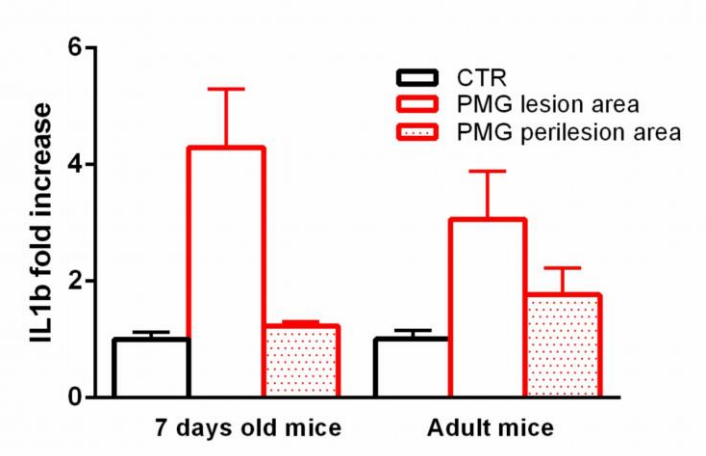


Figure 16. Quantification of IL-1beta content. ELISA analysis was performed at the level of the lesion and perilesion areas in P7 days old and adult mice cortex (7 days old mice: CTR=4; PMG lesion ad perilesion area=4; adult mice: CTR=6; PMG lesion ad perilesion area=4). Data are represented as mean +/- SE.

These data suggest that the inflammatory process in PMG mice starts from lesion and then becomes generalized. That evidence allows to suppose that this process can spread from the microgyri to the near zones that are in that way more easily subjected to seizure not only for the structural disorganization but also for a sustained inflammatory process.

We then carried out a specific analysis of a panel of inflammatory molecules by using a Multicytokines array [Box 1]. We chose this approach because simultaneous detection of multiple cytokines undoubtedly provides a powerful tool to study cell signalling pathways. Regulation of cellular processes by cytokines is a complex, dynamic process, often involving multiple proteins. As such, unravelling the role of individual cytokines in physiologic or pathologic processes generally requires consideration and detection of multiple cytokines rather than of a single cytokine. We performed this analysis on brain tissues of PMG mice and relative controls at age P7 and 3 months. Results showed an increased level of several cytokines

such as GM-CSF, GCSF, IL-2, IL-5, IL-6, IL-10, INF-gamma and MCP-1/5 in lesioned mice with respect to controls (Figure 17).

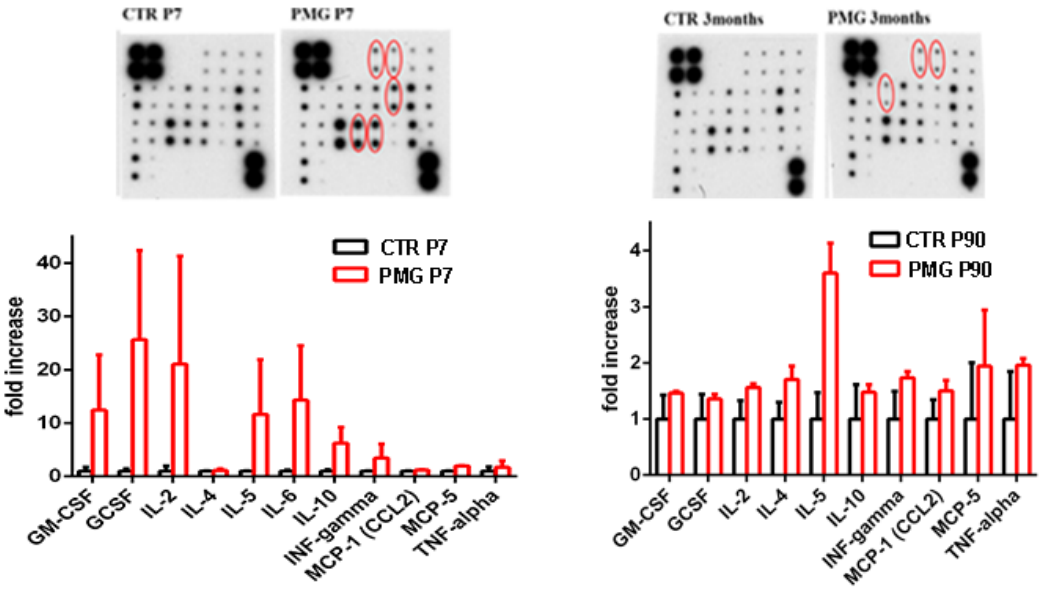


Figure 17. Multicytokines assay. Representative images of Multicytokines' membranes after signal detection. Quantification of inflammatory markers in PMG and age-matched control mice using a Multicytokines array. In the panel in the upper part of the image are shown the membranes on which are underlined with red circles some of cytokines which showed a higher expression in PMG mice with respect to age-matched controls. The cytokines with higher expression (P7 PMG mice: GM-CSF, GCSF, IL-2, IL-5, IL-6, IL-10, INF-gamma, MCP-1/5, TNF-alpha; P90 aged PMG mice: GM-CSF, GCSF, IL-2, IL-5, IL-10, INF-gamma, MCP-1/5, TNF-alpha) have been normalized on the controls' mean and represented as fold increase compared to relative controls (CTR=2, PMG=2). Data are represented as mean +/- SE.

The result of this analysis allows to speculate about a direct involvement of inflammation in the PMG progression since it is not only an event related to the initial phase of the pathology but it is also sustained in the late stages of animal model life. Moreover, a relevant aspect of our analysis is that inflammation is not related only to the microgyri, but spread on the surrounding area. We have supposed that this inflammatory process, which involves a series of different inflammatory molecules, is a fundamental event for the re-assembly of cortex and network structure, formation and development in the pathology. In this view, we can also speculate on the role of sustained inflammation during early life stages as a pivotal event for the onset of severe form of epilepsy. Indeed, how I described above, one of the most invalidating symptoms of PMG is the drug resistant epilepsy. For this reason, correlate a clear event to this phenotype might permit to investigate specific possible therapeutic strategies.

BOX 1 - Cytokines detected by Multicytokines Array

G-CSF is a polypeptide growth factor that regulates the production of neutrophilic granulocytes, a physiologic process which is on the basis of host defence systems and occurs on a large scale in vivo. G-CSF plays a role in the basal regulation of neutrophil production and has the function of primary regulatory factor since it controls the neutrophil response to inflammatory stimuli. The ability to produce G-CSF is characteristic of a variety of cell types following appropriate stimulation, but the most prominent sources of this chemokine are the cells of the monocyte/macrophage lineage. G-CSF is used in clinic to treat neuroinflammation and neurodegenerative pathologies such as Parkinson's disease, stroke, AD and ALS since it mediates its protective effects via the antiapoptotic P13/Akt pathway [8].

Granulocyte-macrophage colony-stimulating factor **(GM-CSF)** is an example of a cytokine growth factor which promotes the production of white blood cells by stem cells. GM-CSF plays an important role in normal brain and neuronal function, normal CNS development and neuronal repair after ischemia or trauma by diminishing expression of apoptosis-related genes. Normal resting human astrocytes can synthesize both G-CSF and GM-CSF and for many functions these two factors can crossover [9].

IL-2 is involved in checkpoints or brakes on the immune system. IL-2 is required to regulate the expression of T cells, and plays a crucial role in activation-induced cell death (AICD), a process that leads to the elimination of self-reactive T cells. It is a protein that regulates the activities of white blood cells (leukocytes, often lymphocytes) and it is part of the body's natural response to microbial infection, and in discriminating between foreign ("non-self") and "self" [17].

IL-5 is produced by Th2 lymphocytes and plays a role in eosinophil maturation, migration and activation and also induces immunoglobulin A switch in B lymphocytes. Astrocytes and microglia produce IL-5 in vitro and astrocytes respond to exogenous IL-5 by an increased production of nerve growth factor. However, it is still unknown which is its receptor in adult brain [19].

Interleukin-6 **(IL-6)** is a pro-inflammatory cytokine that regulates the immune response haematopoiesis, the acute phase response, and inflammation [5].

IL-10 is generally known as an anti-inflammatory cytokine that exerts a plethora of immunomodulatory functions during an inflammatory response with a determinant role during the resolution phase. Expression of IL-10 in the brain increases during pathology, promoting neuronal and glial cell survival, and dampening of inflammatory responses via a number of signalling pathways. Interleukin-10 is produced by Th2 lymphocytes, B cells, and macrophages [7].

IFN-γ, or type II interferon, is a cytokine that is critical for innate and adaptive immunity against viral, some bacterial and protozoal infections. IFNγ is an important activator of macrophages and inducer of Class II major histocompatibility complex (MHC) molecule expression. Aberrant IFNγ expression is associated with a number of autoinflammatory and autoimmune diseases. The importance of IFNγ in the immune system stems from its immunostimulatory and immunomodulatory effects. IFNγ is produced predominantly by natural killer (NK) and natural killer T (NKT) cells [18].

Functional characterization

In collaboration with Dr. Mariaelvina Sala (University of Milan and CNR Institute of Neuroscience) we carried out a functional analysis of muscular strength, motor skills by a battery of and electrophysiological activity of PMG mice to test if the lesioned mice also presented the phenotypic features of PMG patients such as muscular weakness, problems in motor coordination and seizures. This analysis was done in adult PMG and control mice at 3 months of age.

Brief description of motor tests

Body Tone assessment

With this analysis it is possible to evaluate the muscular tone of the animals. This type of test has a subjective evaluation because is the operator to assign the score to the analysed subjects, handily verifying the consistence of the musculature [246].

Spontaneous horizontal and vertical movements assessment

This evaluation was done as in Corradini et al., 2012 as a count of spontaneous movements of animals either in horizontal and in vertical while the animals are left in the cage. This test permits to estimate if there are any motor deficits and difficulties in movements in the experimental group to respect with the age-matched controls [247].

Wire Hanging

The wire hanging test can be used to assess global muscle function and coordination over time. This test is based on the latency of a mouse to fall off from an elevated cage top. The animal is placed on the cage top, which is then inverted and suspended above the home cage; the latency to when the animal falls is recorded. This test is performed three days per week with three trials per session. The average performance for each session is presented as the average of the three trials. Testing typically lasts one week, but can continue up to three weeks. The wire hanging test is performed in order to demonstrate a motor neuromuscular impairment and motor coordination (Figure 18) [13].

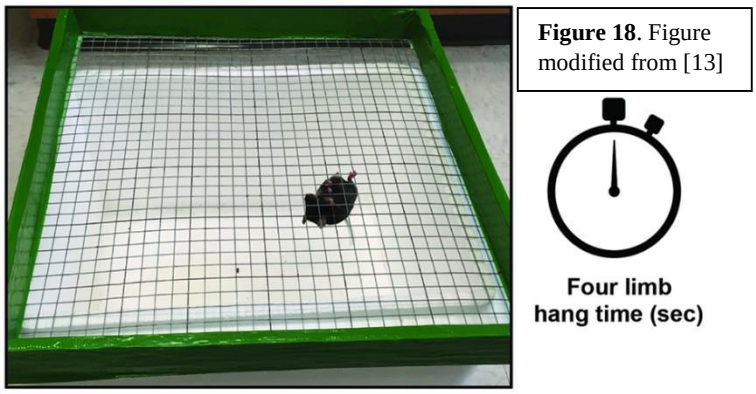


Figure 18. Figure modified from [13]

Rotarod and Rotarod Resistance tests

Rotarod test is a test of motor coordination and motor learning in which is measured the latency to fall from a rotating rod. Motor coordination can be tested by comparing the latency to fall on the very first trial between treatment groups. Motor learning can also be assessed by comparing the first trial with subsequent trials and is evident as an increased latency to fall over time. The acceleration step and time should

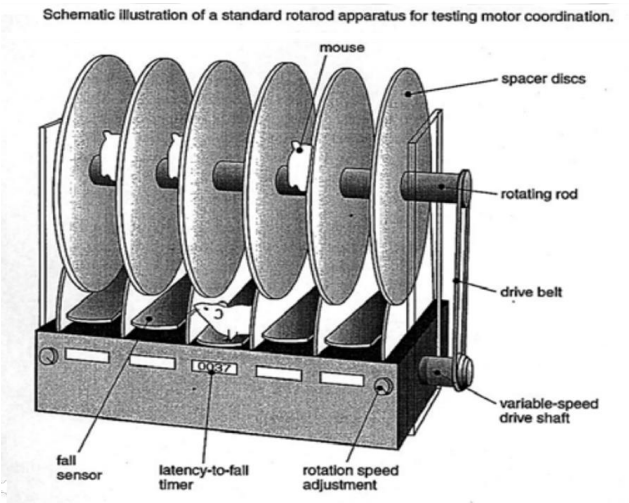


Figure 19. Figure modified from Current Protocols in Neuroscience, 2001.

be determined empirically. In general, the mice require 4-6 trials per day for 3-6 days to see significant improvement in controls. The variant of the test that we decide to use is based on an accelerate speed of the session trials thanks to which it is possible to better and faster evaluate the muscular resistance and the motor coordination of mice. At the moment we are not interested in the evaluation of the learning component (Figure 19) (Current Protocols in Neuroscience, 2001).

Pole Test

The Pole Test is used to assess movement disorders in mice. Animals are placed head-up on top of a vertical wooden pole. The base of the pole is placed in a cage filled with bedding material. When placed on the pole, animals orient themselves downward and descend the length of the pole back into the cage. After two days of training, animals receive five test trials. The time to orient downward (t-turn) and total time to descend (t-total) are measured and the scores are inversely proportionated at the goodness of the performances (lower is the time that mice need to carry out the task, higher is their motor coordination) [15] (Figure 19).

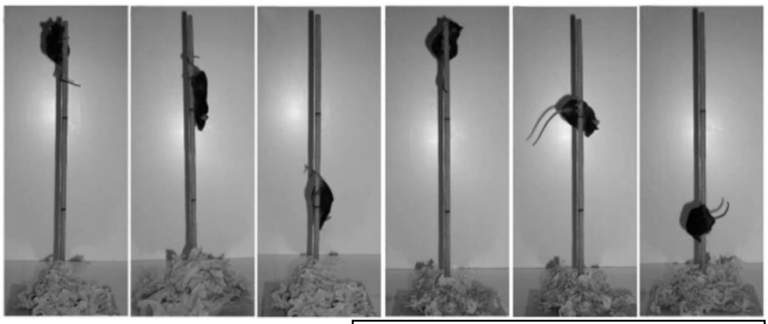
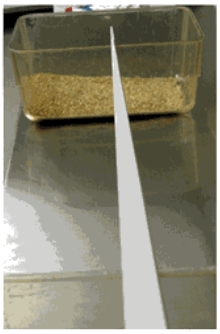


Figure 20. Figure modified from [15].

Balance Beam

The balance beam is a test of motor coordination and it can be more sensitive than rotarod for some types of motor coordination deficits. In this test the animals are positioned on a beam, that can have different widths during the different trial sessions, and it is scored the number of slips and latency to cross. As it is for the Pole Test, a lower score is sign of a better performance (Figure 20) (http://med.stanford.edu.).



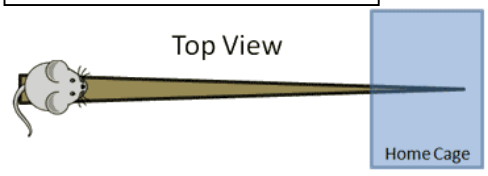
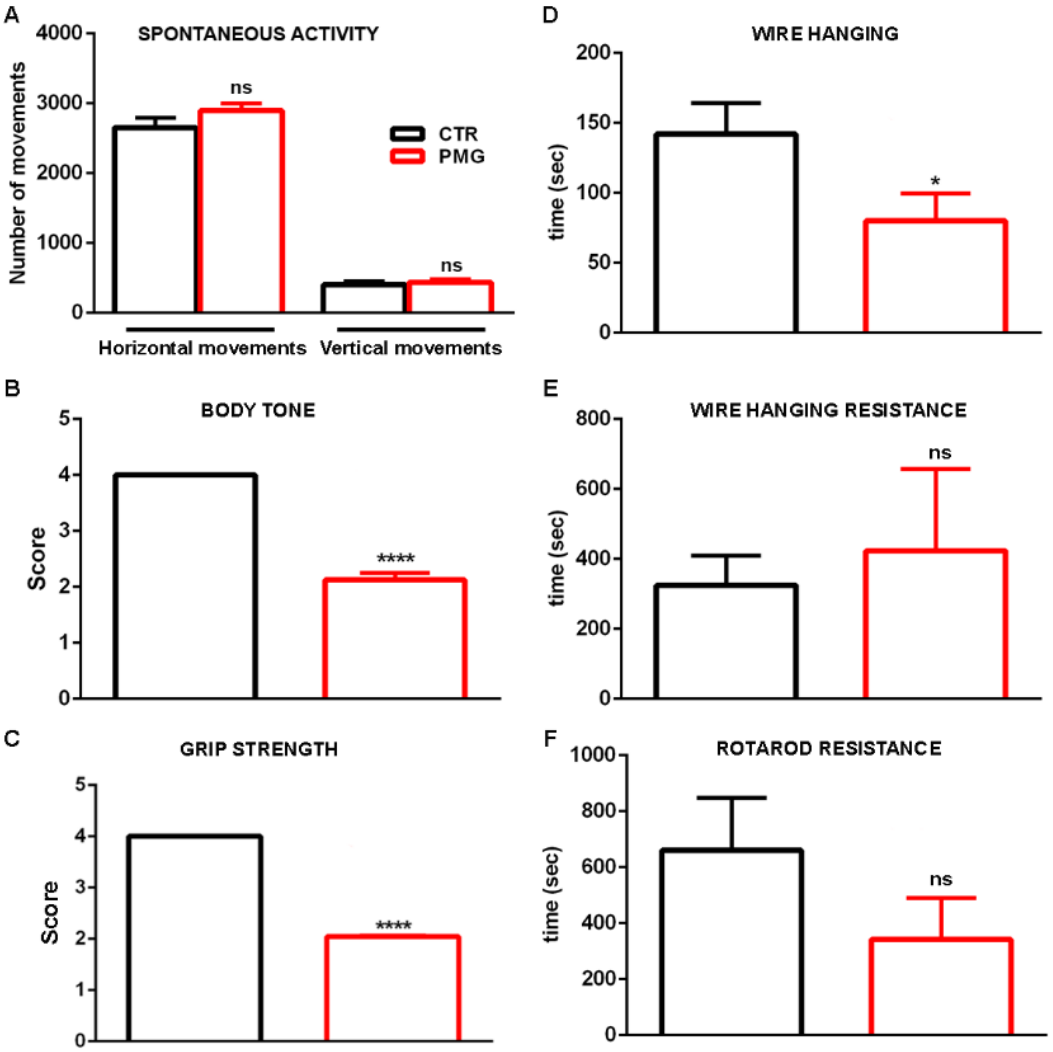


Figure 20. Figure modified from http://med.stanford.edu.

The mice tested are left in their cages until they do not reach the three months of age that was the moment we established for the functional tests. The motor tests were then performed in PMG mice and age-matched controls during different trial sets to guarantee to mice a successful performance. With these behavioural tests we have been able to assess the functional defects of PMG mice. Indeed, we found that PMG mice displayed a reduction of muscle strength and motor coordination and balance according to observations in patients with PMG (Figure 21). In particular, the Wire Hanging test (Figure 21 D) shows that PMG mice have a worse muscular resistance that is related to impaired coordination performances clearly unmasked by Pole test (Figure 21 H) and Balance beam test (Figure 21 I). Indeed, we found that in Wire Hanging test (Figure 21D), the time that PMG mice can be stay hanged to cage top was halved respect to controls and the time employed to carry out the task in Pole test and Balance Beam test (Figure 21 H-I) was doubled. In the Balance Beam test (Figure 21 I) in particular, the goodness of the score is less related to the width of the beam, since the differences of the scores obtained during the two tasks are not very different.



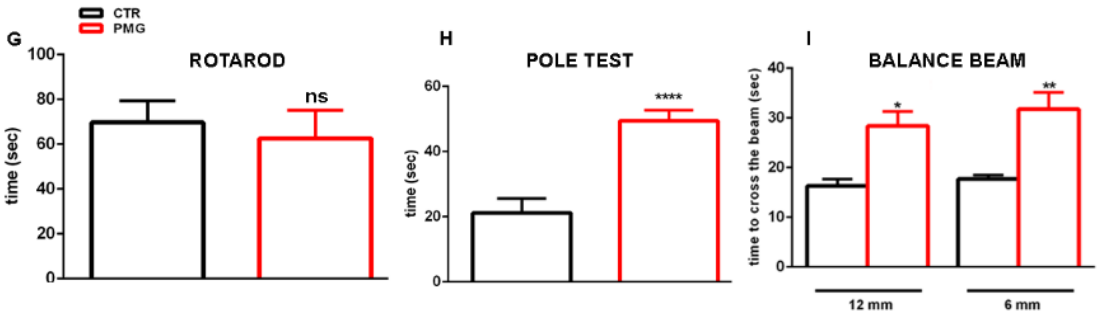


Figure 21. Motor impairment in PMG mice. These graphs represent the impairment in muscular strength and motor coordination typical of P90-P150 PMG mice to respect with age-matched controls. A) quantization of Spontaneous activity as number of spontaneous horizontal and vertical movements recorded in 30 minutes (CTR=21 vs PMG=18); B) Body tone (****p<0.0001 Mann-Whitney test, CTR=20 vs PMG=16); C) Grip Strength (****p<0.0001 Mann-Whitney test, CTR=20 vs PMG=16); D) Wire hanging (*p<0.05 Mann-Whitney test, CTR=23 vs FLL-PMG=15); E) Wire hanging resistance (CTR=7 vs FLL-PMG=8); F) Rotarod Resistance (CTR=17 vs PMG=13); G) Rotarod (CTR=25 vs PMG=16); H) Pole test (***p<0.001 Mann-Whitney test, CTR=7 vs PMG=8); I) Balance Beam test (*p<0.05, p<0.001 Tukey's test, ONE Way ANOVA, CTR=7 v PMG=8). Data are shown as mean +/- SE.

With this tests we have unmasked the motor and muscular defects of PMG mice correlating them with the structural disorganization. More important, these results gave us a clear proof that the motor deficits are directly related to pathology and comparable to the human PMG disease.

EEG recordings of cortical activity

Adult PMG and healthy control mice were subjected to EEG recordings in order to assess basal cortical activity. Electroencephalography recording is a technique that reads scalp electrical activity generated by brain structures. The electroencephalogram (EEG) is defined as electrical activity of an alternating type recorded from the scalp surface after being picked up by metal electrodes and conductive media. In our experiments we performed recordings from the cortical surface of mice using electrodes positioned in correspondence of lesioned area. During the EEG, mice were free to move and explore the cage. Recordings last two hours and immediately after, mice were sacrificed for morphological and molecular analysis as described in Menna et al., 2013 [248]. EEG analysis is a good tool to investigate network activity because it allows to unmask that phenomenon that are not physiological but that are not so clear to interpret with canonical behavioural tests. In particular, PMG mice do not display a spontaneous epileptic phenotype characterized by seizures, but EEG recordings unmasked the

occurrence of poly-spikes and frequent spikes of high amplitude (Figure 22, EEG traces). The mean number and the mean amplitude of spikes were significantly higher in PMG mice than in control mice as is shown in the quantization of the number of spike/hour in PMG mice and relative controls (Figure 22).

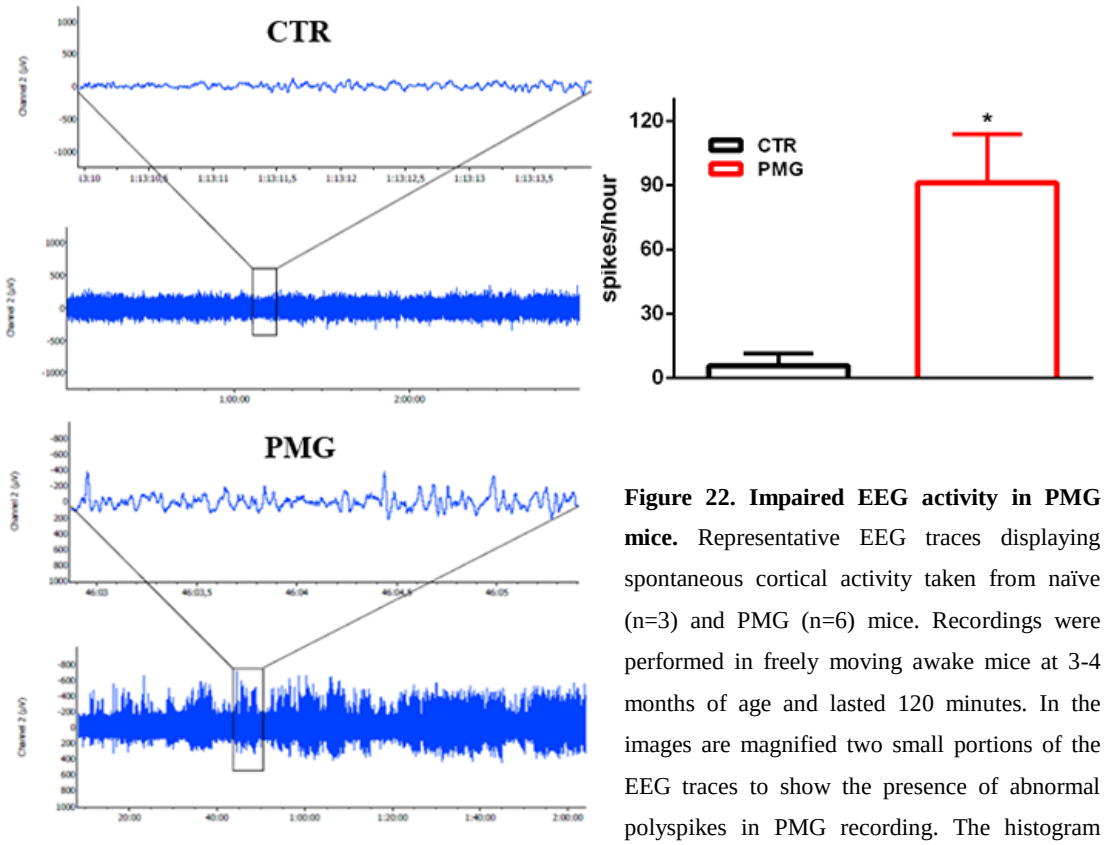


Figure 22. Impaired EEG activity in PMG mice. Representative EEG traces displaying spontaneous cortical activity taken from naïve (n=3) and PMG (n=6) mice. Recordings were performed in freely moving awake mice at 3-4 months of age and lasted 120 minutes. In the images are magnified two small portions of the EEG traces to show the presence of abnormal polyspikes in PMG recording. The histogram represents the quantitation of the number of spikes/hour (*p<0.05 t Student's test). Data are shown as mean +/- SE.

In all the behavioural test and EEG recordings we initially considered sex as two independent groups separating female and male mice. (Figure 23). This is an important point especially considering the inflammatory processes and the susceptibility to neurodevelopment diseases between two sexes. In fact, there are different papers affirming that there is a difference in cytokines level expression principally during the sexual development period that is around P30-P40 that approximately correspond to the time window that we consider for our experiments [191-193]. At the end given we could not find any substantial difference in performances between male and female (Figure 23), we decided to pull the two groups together.

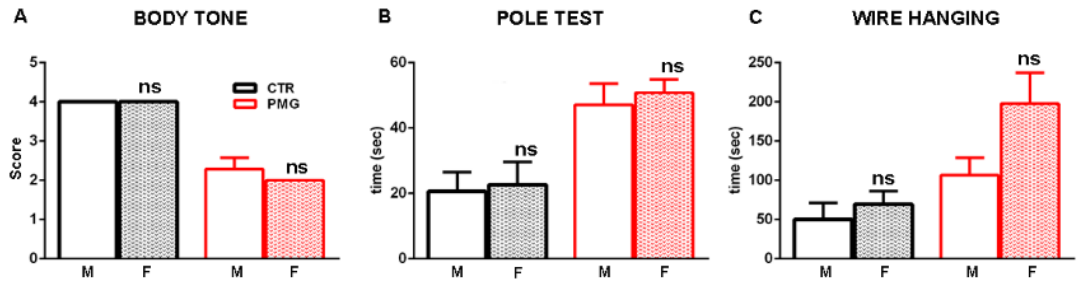


Figure 23. Functional differences between male and female mice. The graphs show the performances of male (M) and female (F) mice aged P90-P150 of different groups (CTR and PMG) relative to A) Body tone (CTR=14m, 6f; PMG=7m, 9f), B) Wire hanging (CTR=16m, 9f; PMG=8m, 11f) and C) Pole test (CTR=5m, 2f; PMG=3m, 5f). As is shown, there is not a difference in the score between members of the same group (CTR and PMG mice) for different functional tests. Data are shown as mean +/- SE.

HUMAN NEURAL STEM CELLS (hNSCS) TRANSPLANTATION IN PMG

Description of experimental design

Human Neural Stem Cells have been demonstrated to exert positive actions on inherited or acquired myelination disorders [241] and to dampen the inflammatory process and to improve neurological functions [242]. In this part of my project we collaborated with Professor Angelo Vescovi (University of Milano Bicocca). hNSCs have been isolated and cultured in Vescovi laboratory, as described in [249], and then they were injected in PMG brain mice at the age of P30 (Figure 24). The injections were performed bilaterally in the cortex with the help of a stereotaxic apparatus. A group of PMG mice were injected with the saline buffer used to resuspend hNSCs for control (HBSS or vehicle). Also naïve not PMG wild type mice (CTR) were injected with the vehicle or the hNSC. In this way, we obtained six experimental groups to compare: naïve wt control mice, PMG mice, wild type mice injected with cells, wild type mice injected with vehicle, PMG mice injected with cells and PMG mice injected with vehicle. At the age of three months, mice were subjected to behavioural tests and EEG to evaluate the effect of hNSC treatment on the pathology. Finally, they were sacrificed for the morphological and molecular analysis.

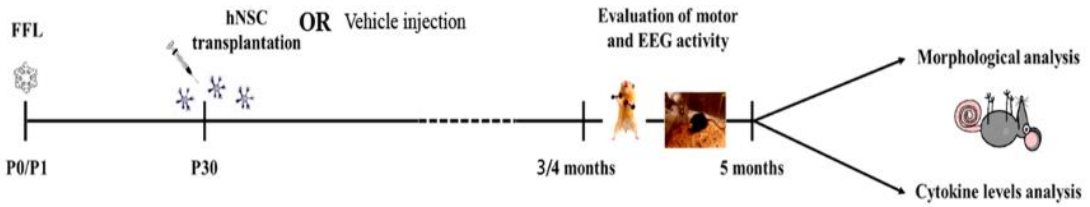


Figure 24. hNSCs treatment experimental design. The scheme shows the temporal line of hNSCs treatment or vehicle injection in FLL-PMG mice and relative age-matched controls.

Morphological analysis

At first we evaluate whether injected hNSCs survive by performing a temporal course analysis (1 week and 2 weeks after transplantation). For this aim hNSC injected PMG mice were perfused 1 week and 2 weeks after injection. The brain was removed and processed for immunofluorescence on coronal slices by using specific antibodies recognising human nuclei antigen (huN, green) the astrocytic marker GFAP (red) and DAPI. hNSCs were found to be localized at the level of the subventricular zone (SVZ) (Figure 25).

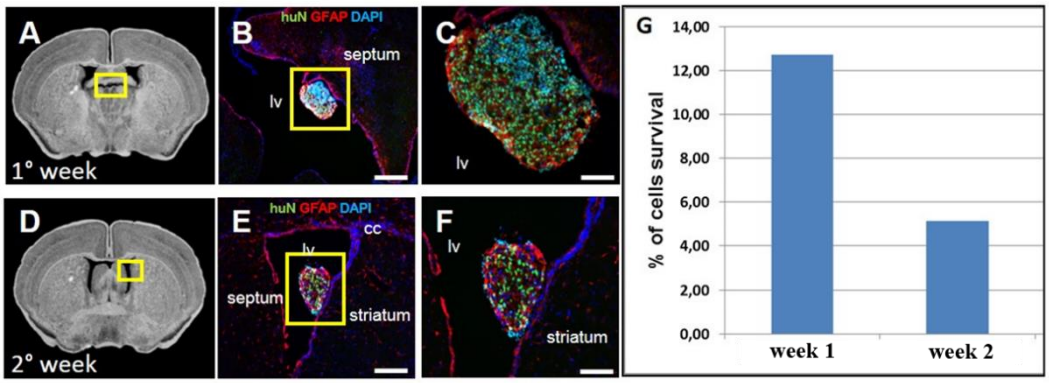


Figure 25. Intrathecally-implanted hNSCs into PMG mice survive during time after the transplantation and localize at the level of the third ventricle. A-D) Representative images of coronal section maps showing at different time points the regions of subventricular zone (SVZ) engrafted by cells. B-E and C-F) Immunofluorescence staining showing the presence and the localization of the injected cells one week and two weeks after transplantation. hNSCs were identified with an anti-huN antibody (human nuclei, green) and by their colocalization with GFAP positive cells (red) in the SVZ at low (B-E) or high (C-F) magnifications. Scale bars: 200µm, 100µm respectively. G) Cell survival quantification show a physiological decrease in the number of vital cells that does not affect their functions (n=4). cc: corpus callosum, lv: lateral ventricle.

Cortical layers and Myelination

After to have verified the survival and the grasping of the injected hNSCs in the brain of mice, we performed a series of immunofluorescence staining to verify if the presence of the stem cells might have some positive effects on the pathological defects which we characterized in our model.

At first we performed the staining against the marker of oligodendrocyte progenitor cells (Ng2) to verify if hNSCs were differentiated in oligodendrocytes as is demonstrated in literature [250]. In PMG mice injected with hNSCs we observed a significant increase in the number of Ng2 positive cells with respect to vehicle-injected PMG mice at every time points analysed (Fig. 26 and 27). However, the Ng2-positive oligodendrocyte progenitors were not positive for the human nuclei markers thus indicating that these cells are endogenous murine oligodendrocyte progenitors (OPCs) and not derived from differentiation of hNSCs (Fig 26 and 27).

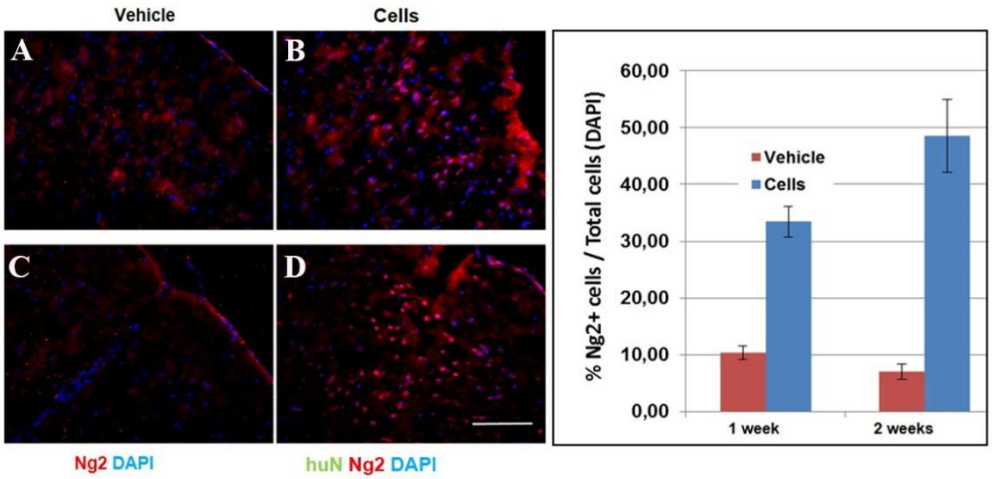


Figure 26. hNSC transplantation promotes the recruitment and the proliferation of endogenous oligodendrocytes progenitors into the cortex of PMG mice. A-D) Representative images of cell-treated or vehicle- treated PMG mice brain slices stained for Ng2 (early oligodendrocyte marker, red) and huN (human nuclei, green). Total nuclei are shown by DAPI staining (blue). Note that Ng2 expression is enhanced in the cortex of cell-treated PMG mice (B, D) with respect to the cortex of vehicle-treated PMG mice (A, C). Interestingly Ng2 positive cells were not positive for huN staining (green) thus indicating an increase proliferation of endogenous oligodendrocytes. Scale bars: 100µm. The histograms show the quantification of the percentage of Ng2 over total DAPI positive cells (n=4) in the first two time courses. Data are shown as mean +/- SE.

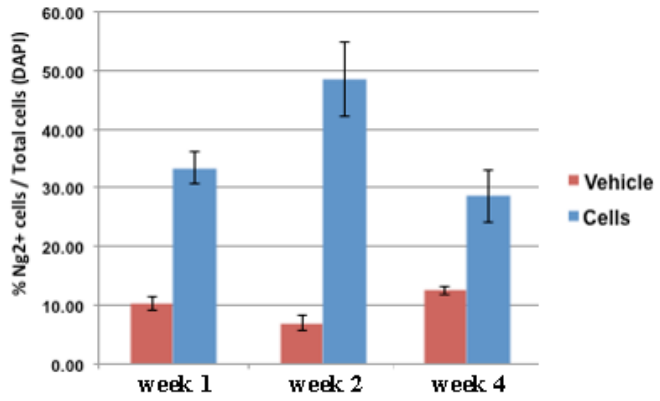


Figure 27. Quantization of recruitment and proliferation of endogenous oligodendrocytes progenitors in the cortex of PMG mice at different time points. The graphs show the percentage of Ng2 over total DAPI positive cells (n=4) since one week (1° time course) to 1 month from stem cells transplantation in PMG mice and relative age-matched controls treated with vehicle. Data are shown as mean +/- SE.

We performed the same analysis after 1 months from the hNSCs transplantation also in CTR mice founding that the hNSCs transplantation promotes a normalization of Ng2-positive cell number in PMG cell-treated mice that is comparable with physiologic condition (Figure 28).

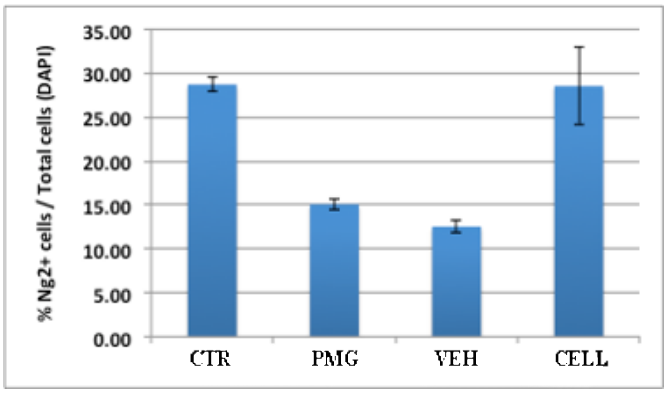


Figure 28. Quantization of Ng2+ cells in PMG cells-treated mice and relative age-matched controls. The graphs show the percentage of Ng2-positive cells over total DAPI positive cells (wt=3; PMG-CTR=3; PMG-vehicle=6; PMG-cells=4). Data are shown as mean +/- SE.

To further assess the effect of hNSC on oligodendrocyte proliferation and maturation we stained brain slices of P30 vehicle-treated and hNSC-treated PMG mice for CNPase, which is a myelin-associated enzyme [251]. In hNSC-injected PMG mice we found that the number of

CNPase-positive OPCs positive for was significantly increased with respect to vehicle treated PMG mice at thirty days after hNSCs transplantation, thus confirming that hNSC may promote endogenous oligodendrocytes proliferation which may in turn have beneficial effect on the myelination defects present in PMG mice.

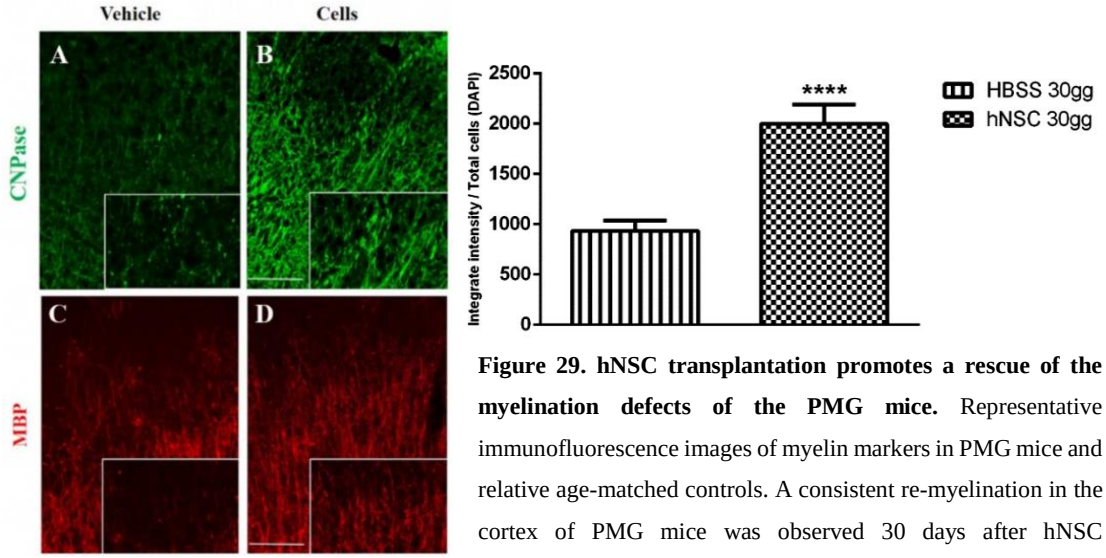


Figure 29. hNSC transplantation promotes a rescue of the myelination defects of the PMG mice. Representative immunofluorescence images of myelin markers in PMG mice and relative age-matched controls. A consistent re-myelination in the cortex of PMG mice was observed 30 days after hNSC transplantation (C, D) with respect to vehicle-treated PMG mice (A, B) by the increased expression of CNPase (early oligodendroglial marker) and MBP (late oligodendroglial marker) thus suggesting that both early and late re-myelination are enhanced by hNSCs. These results suggest that hNSC treatment may stimulate oligodendroglial progenitors to migrate into the lesion area of the cortex and to differentiate into mature oligodendroglial cells. Scale bars: 100µm. The histogram shows the quantification of CNPase positive oligodendrocyte precursors in the cortex of PMG mice 30 days after hNSCs transplantation. (****$p<0.0001$ t Student's test). Data are shown as mean +/- SE.

Reduced microglial activation upon hNSC treatment

To verify if hNSCs had an effect on the inflammatory process that we have characterized in PMG mice, we decided to perform a series of immunostaining against the inflammatory markers that we found more expressed in lesioned mice, such as CD11b (Figure 30). How is shown in the Figure 30 C, after the treatment with hNSCs, PMG mice present a reduction of CD11b-positive cells.

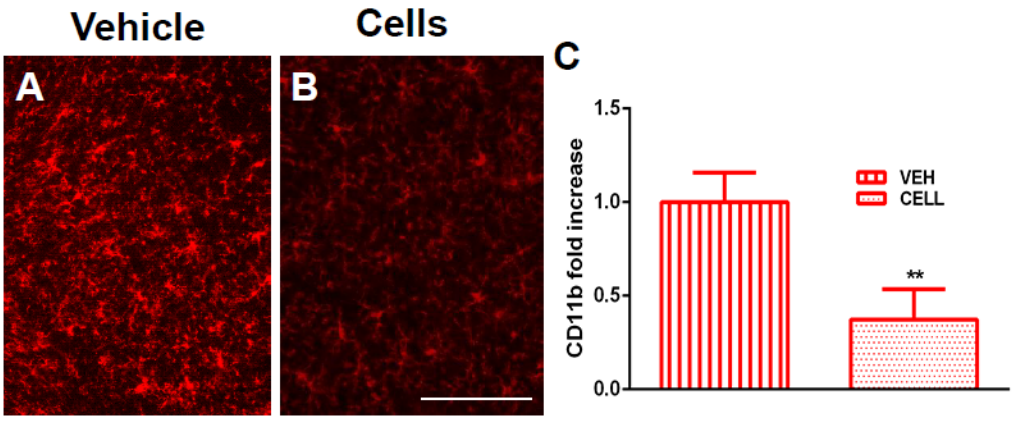


Figure 30. Reduction of microglial activation in cortex and a rescue of the motor defects have been observed after treatment with hNSC. Representative images of Cd11b in brains of PMG cells-treated and vehicle-treated mice. FLL-PMG mice, injected with cells or vehicle as control at P30, were evaluated for microglial activation 3 weeks after hNSC transplantation. (A, B) Representative images of cortices of cells-treated or vehicle-treated FLL-PMG mice stained for the specific marker of microglial activation, CD11b. Note the decrease of CD11b signal in the cortex of cell-treated with respect to the vehicle treated PMG mice, thus indicating a decrease level of microglial activation upon hNSC treatment. Scale bar: 80μm. (C) Quantitation of CD11b signal in the cortex of cell-treated and the vehicle-treated PMG mice (** p<0,01, Mann-Whitney test; n=4). Data are shown as mean +/- SE.

Improved motor function and EEG profile in PMG mice upon hNSC treatment.

After the recovery following the hNSCs transplantation at three months of age, PMG mice and relative controls performed the behavioural tests to assess the motor skills. As is shown by the Figure 31, hNSC-treated PMG mice show an improvement of motor functions with respect to the PMG and the PMG vehicle treated mice. In the specific we found a recovery in the muscular strength as is possible to see in graphs relative to Body tone and Grip strength (Figure 31 B-C), and in motor coordination as is shown by the graphs relative to Wire hanging (Figure 31 D), Rotarod (Figure 31 E) and Pole test (Figure 31 H). Even if the functional rescue is not generalised, it can be well observed an improvement in the motor and neuromuscular skills.

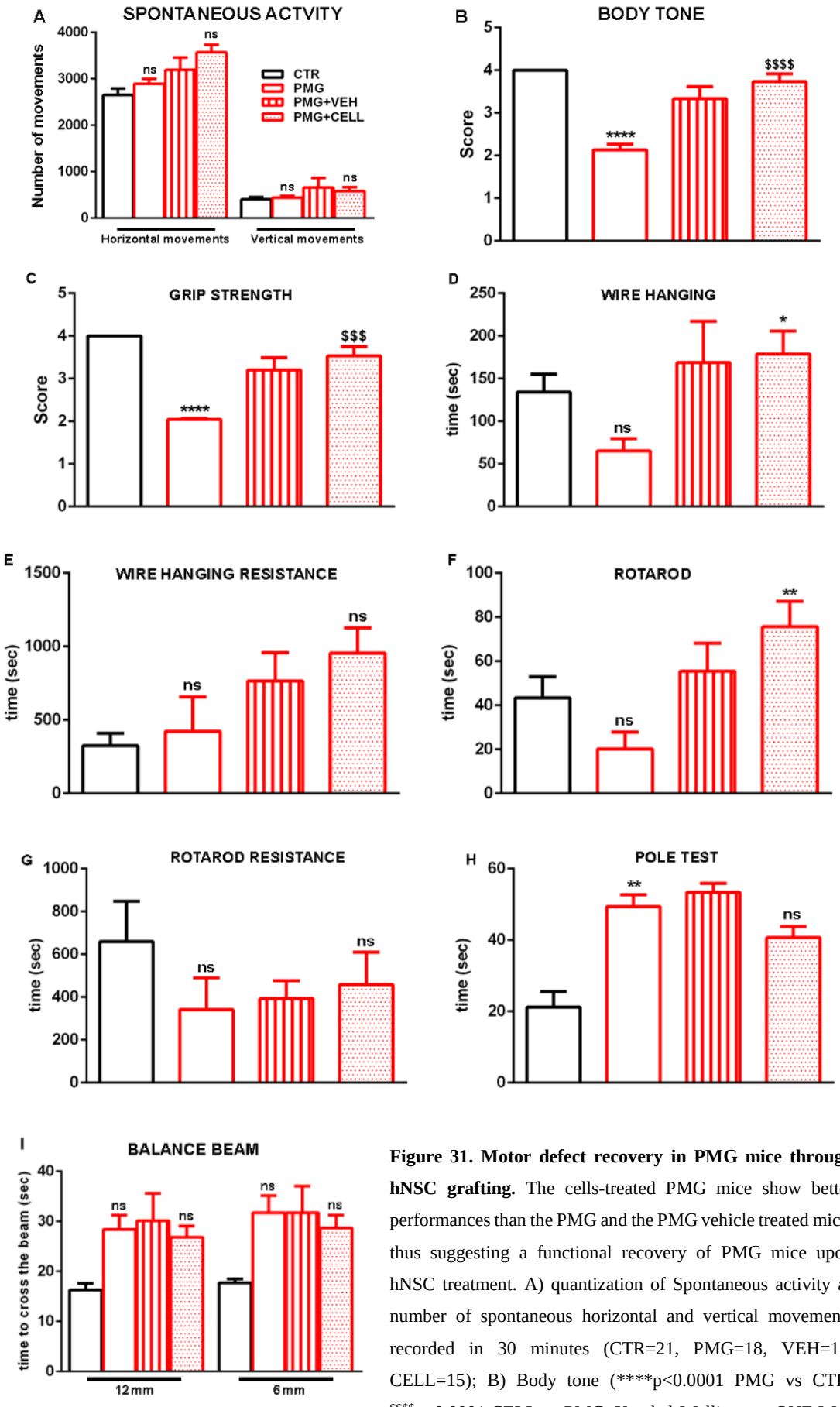


Figure 31. Motor defect recovery in PMG mice through hNSC grafting. The cells-treated PMG mice show better performances than the PMG and the PMG vehicle treated mice, thus suggesting a functional recovery of PMG mice upon hNSC treatment. A) quantization of Spontaneous activity as number of spontaneous horizontal and vertical movements recorded in 30 minutes (CTR=21, PMG=18, VEH=11, CELL=15); B) Body tone (****p<0.0001 PMG vs CTR, $^{\$\$\$\$}$p<0.0001 CELL vs PMG, Kruskal-Wallis test, ONE Way

ANOVA, CTR=20, PMG=15, VEH=12, CELL=15); C) Grip Strength (****p<0.0001 PMG vs CTR, $^{\$\$\$}$p<0.001 CELL vs PMG, Kruskal-Wallis test, ONE Way ANOVA, CTR=20, PMG=16, VEH=10, CELL=15); D) Wire hanging (*p<0.05 CTR=23, PMG=15, VEH=12, CELL=15); E) Wire hanging resistance (CTR=7, PMG=8, VEH=12, CELL=15); F) Rotarod (**p<0.01 CELL vs PMG, Kruskal-Wallis test, ONE Way ANOVA, CELL vs PMG , Kruskal-Wallis test, ONE Way ANOVA, CTR=21, PMG=16, VEH=12, CELL=15) G) Rotarod Resistance (CTR=17, PMG=13, VEH=12, CELL=15);; H) Pole test (**p<0.01 PMG vs CTR, Kruskal-Wallis test, ONE Way ANOVA, CTR=7, PMG=8, VEH=7, CELL=10); I) Balance Beam test (CTR=7, PMG=8, VEH=7, CELL=11). Data are shown as mean +/- SE.

Even if the test which are more specific in the evaluation of muscular strength and motor coordination did not shown a significant improvement of this faculties but only a tendency to ameliorate them, analysing the percentage of animals which display defective motor skills in the different animal groups it is possible to define the percentage of impaired animals. This was possible valuating the scores of single animals and confronting them within and between the different groups. In this way we obtained a clear indication for every test. In the specific we obtained that the percentage of impaired animals in the Body Tone test were 89% PMG, 33% PMG vehicle-treated, 14% PMG cell-treated; in the Wire Hanging test 87.5% PMG, 67% PMG vehicle-treated, 34% PMG cell-treated; in the Rotarod Test 93% PMG, 58% PMG vehicle-treated, 40% PMG cell-treated.

Notably mice treated with vehicle display a partial rescue of some functional skills (Figures 31-32). Given all animals injected with hNSC and controls received cyclosporine treatment for 2 weeks to prevent rejecting cells, we tested whether immunosuppression in PMG mice might have some beneficial anti-inflammatory effect on the pathology thus producing some recovery in motor skills. A group of PMG mice were treated with cyclosporine for two weeks starting at P30 and their behavioural performances were compare with those of other experimental groups. In the Figure 32 the scores obtained by cyclosporine-treated mice are shown together with the scores obtained from the other PMG groups.

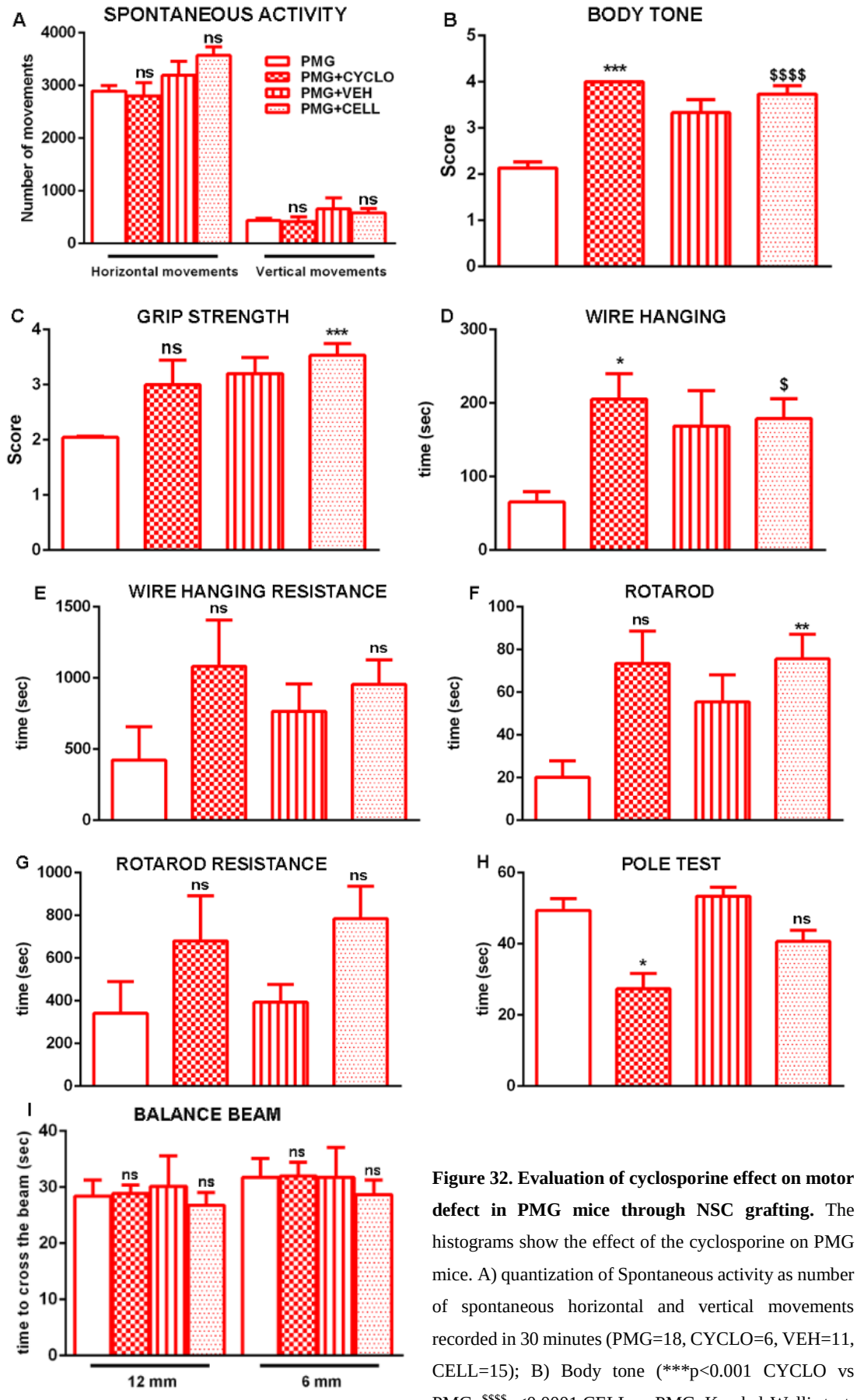


Figure 32. Evaluation of cyclosporine effect on motor defect in PMG mice through NSC grafting. The histograms show the effect of the cyclosporine on PMG mice. A) quantization of Spontaneous activity as number of spontaneous horizontal and vertical movements recorded in 30 minutes (PMG=18, CYCLO=6, VEH=11, CELL=15); B) Body tone (***p<0.001 CYCLO vs PMG, $^{\$\$\$\$}$p<0.0001 CELL vs PMG, Kruskal-Wallis test, ONE Way ANOVA, PMG=15, CYCLO=6, VEH=12, CELL=15); C) Grip Strength (***p<0.001 CELL vs PMG,

Kruskal-Wallis test, ONE Way ANOVA, PMG=16, CYCLO=6, VEH=10, CELL=15); D) Wire hanging (*p<0.05 CYCLO vs PMG, $p<0.05 CELL vs PMG, Kruskal-Wallis test, ONE Way ANOVA, PMG=15, CYCLO=6, VEH=12, CELL=15); E) Wire hanging resistance (PMG=8, CYCLO=6, VEH=12, CELL=15); F) Rotarod (**p<0.01 CELL vs PMG, Kruskal-Wallis test, ONE Way ANOVA, CELL vs PMG , Kruskal-Wallis test, ONE Way ANOVA, PMG=16, CYCLO=6, VEH=12, CELL=15) G) Rotarod Resistance (PMG=13, CYCLO=6, VEH=12, CELL=15);; H) Pole test (*p<0.05 PMG vs CTR, Kruskal-Wallis test, ONE Way ANOVA, PMG=8, CYCLO=6, VEH=7, CELL=10); I) Balance Beam test (PMG=8, CYCLO=6, VEH=7, CELL=11). Data are shown as mean +/- SE.

From this experiment, we obtained that there was not a significant difference in the percentage of impaired animals in the PMG treated with cyclosporine respect to PMG vehicle-treated mice indicating immunosuppression di per se might have contributed to the recovery however the effect of hNSC treatment was greater. Indeed, the percentage of impaired mice are: PMG 75%, Vehicle-treated PMG 57%, hNSC-treated PMG 54%, Cyclosporine-treated PMG 100% for the Balance beam test 12mm; PMG 75%, Vehicle-treated PMG 57%, hNSC-treated PMG 73%, Cyclosporine-treated PMG 100% for the Balance beam 6mm; and PMG 100%, Vehicle-treated PMG 25%, hNSC-treated PMG 66%, Cyclosporine-treated PMG 100% for the Rotarod.

Furthermore, hNSC treatment was effective in rescuing EEG defects reducing the number of spikes and polyspikes in EEG traces (Figure 33).

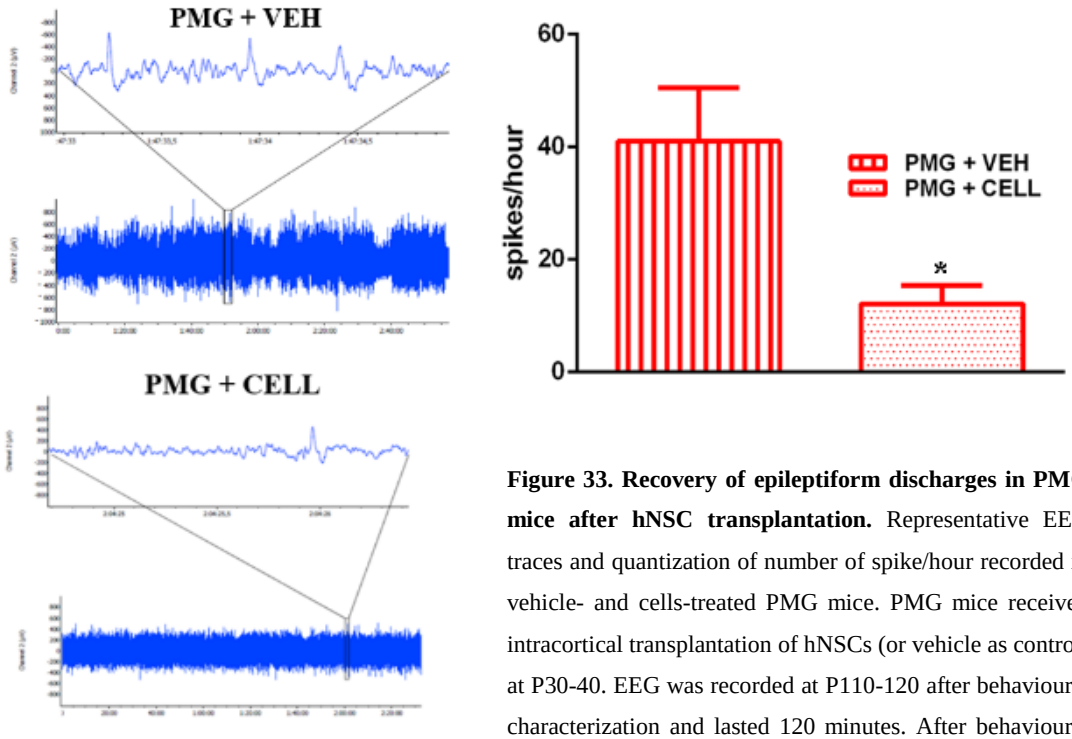


Figure 33. Recovery of epileptiform discharges in PMG mice after hNSC transplantation. Representative EEG traces and quantization of number of spike/hour recorded in vehicle- and cells-treated PMG mice. PMG mice received intracortical transplantation of hNSCs (or vehicle as control) at P30-40. EEG was recorded at P110-120 after behavioural characterization and lasted 120 minutes. After behavioural

tests and EEG recordings the mice are sacrificed for the morphological analysis. (*p<0.05, t Student's test, PMG vehicle-treated mice=12; PMG cells-treated mice=5). Data are shown as mean +/- SE.

In a general view of hNSCs treatment, we can affirm that these cells exert a generalised positive effect on PMG mice that can principally be related to the ability of endogenous mechanisms to respond to positive stem cells feedback. In fact, it seems that the cells-treated mice are more responsive to the damage and that the myelin loss is the triggering factor from which depend the majority of pathological symptoms present in the PMG mice. Of note no side effects were reported in control mice. So this therapeutic approach could be a promising via to treat PMG patients.

PHARMACOLOGICAL BLOCKADE OF IL1-R ACTIVATION BY ANAKINRA IN PMG

Description of experimental design

An important point on which we decided to focus our attention during the developing of this project was inflammation. As I well described above, inflammatory processes are sustained and largely present in the brain of PMG mice, not only at level of the microgyri, but they also spread in surrounding areas. Moreover, these processes are present along all lifespan of PMG mice. Results obtained by ELISA and multicytokines array analysis indicated that an increase content of IL1beta was present in PMG brain already at P7 and was still present at P30. To stop this process, we decided to treat the PMG mice with the specific compound that acts on IL-1R pathway. We chose a recombinant version of the endogenous interleukin 1 receptor antagonist (IL1-RA, anakinra), which competitively inhibits the binding of IL-1 to its receptor. This drug specifically counteracts the increased activation of IL-1R that it was shown to be sustained during neuroinflammation and during that correlated phenomena such as epilepsy [252].

We chose Anakinra because it is a compound already available in commerce that has been well tested and largely used in childhood pathology such as juvenile idiopathic arthritis and NOMID syndrome [219, 224]. So it can be considered safe in children, who are the principal subjects of PMG disease, since it has a brief half-life (4-5 hours), it showed to be well tolerated in the majority of patients and severe side effects are rarely observed [219].

PMG mice were treated with anakinra 24mg/day I.P. for 1 week starting at P30 (Figure 34) [253] to mimic the hNSC treatment which displayed a certain grade of efficacy. At three months of age, PMG mice treated or not with anakinra (ANAK) were subjected to behavioural test and EEG recordings and then they were sacrificed for morphological and molecular analysis.

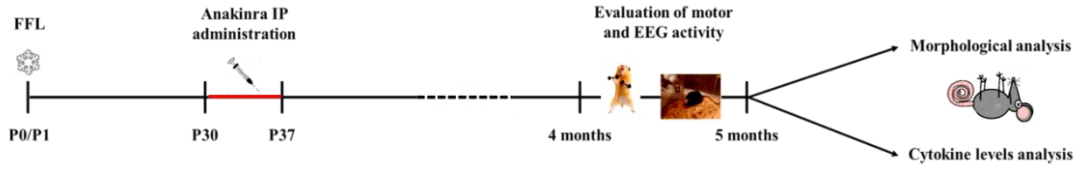


Figure 34. IL1Ra treatment experimental design. The scheme shows the temporal line of Anakinra treatment in FLL-PMG mice and relative age-matched controls.

Morphological analysis: Myelin defect

PMG mice treated with anakinra showed a partial rescue of NF disposition in the upper cortical layer respect to PMG untreated mice. Immunofluorescence at level of microgyri, seemed to show a slightly better organised lamination and connection even if the complete and definitive morphological rescue cannot be reached (Figure 35). The formation of microgyri remained unchanged after the administration of anti-inflammatory compound as the four layered conformation of the cortex, but the relevant thing is that at level of the lesion and in the perilesion area, the nervous fibres seems to reorganize themselves to create a network the more possible similar to that of physiological condition.

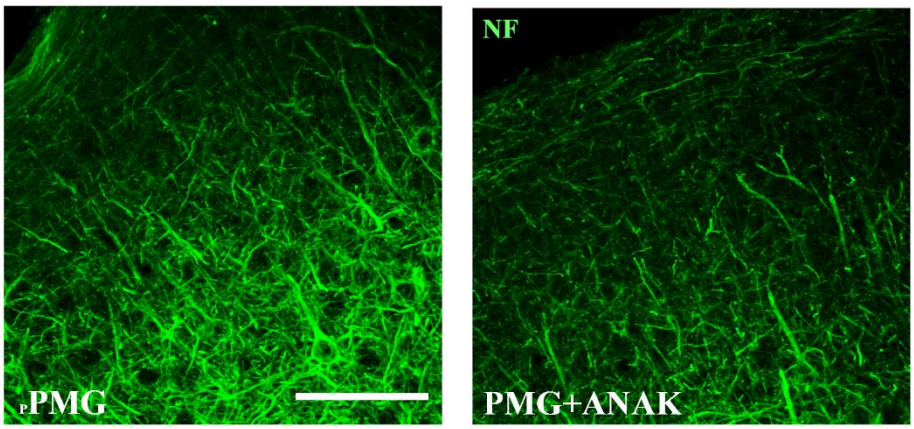


Figure 35. Partial rescue of the defects of neurofibres organisation after Anakinra treatment in PMG mice. Representative immunofluorescence images of a qualitative representation of reorganization of neurofilaments (NF: Smi31/32, green) after Anakinra injection in PMG mice with respect to the age matched controls. It is visible a more organised disposition of fibres in the upper part of the cortex (Scale bar: 60μm).

Staining for MBP, the marker for mature oligodendrocytes, however does not reveal any substantial difference in anakinra treated PMG mice with respect to untreated PMG mice (Fig. 36)

Indeed, how is shown in the Figure 36, it was possible to detect a slightly better disposition of myelin fibres (stained with an antibody against MBP, red) and OPC (stained with an antibody against Olig2, green) with respect to the PMG untreated mice. As is possible to see from the immunofluorescence images, the number of myelinated fibres is not higher after anakinra injection, but their organization ca be resembling more similar to that found in control mice. Moreover, the disposition of oligodendrocytes might indicate an action of this endogenous

cells. Probably, the action of this cells is to better myelinate the nervous fibres present in the region of the lesion to permit to have a better function of those.

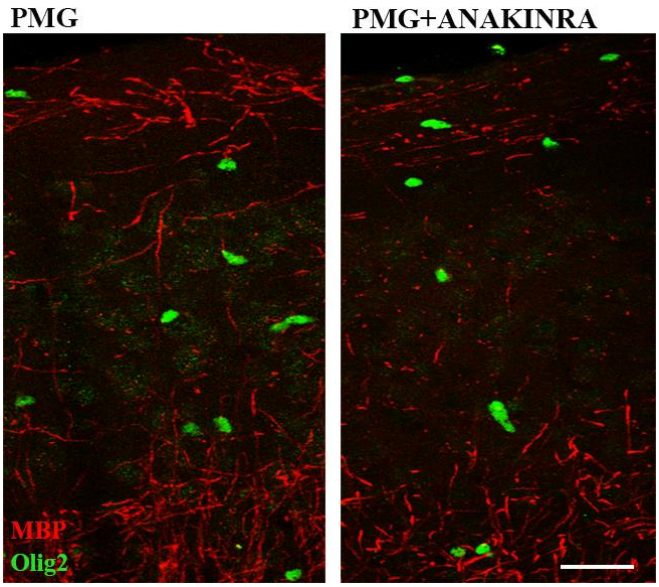


Figure 36. Effect on myelination defects of Anakinra treatment in PMG mice. The image is a qualitative representation of reorganization of myelin fibres (MBP, red) after Anakinra injection in PMG mice with respect to the age matched controls. It is also visible a migration and a myelin orientated disposition of OPCs after the treatment (Olig2, green). Scale bar: 80μm.

Inflammation

In order to assess whether anakinra dampened the inflammatory responses we carried out a qualitative confocal analysis of microglia activation by CD11b and Iba1 immunofluorescence. Qualitative confocal analyses highlighted a decrease signal of CD11b suggesting a reduced microglial activation upon anakinra treatment (Fig. 37)

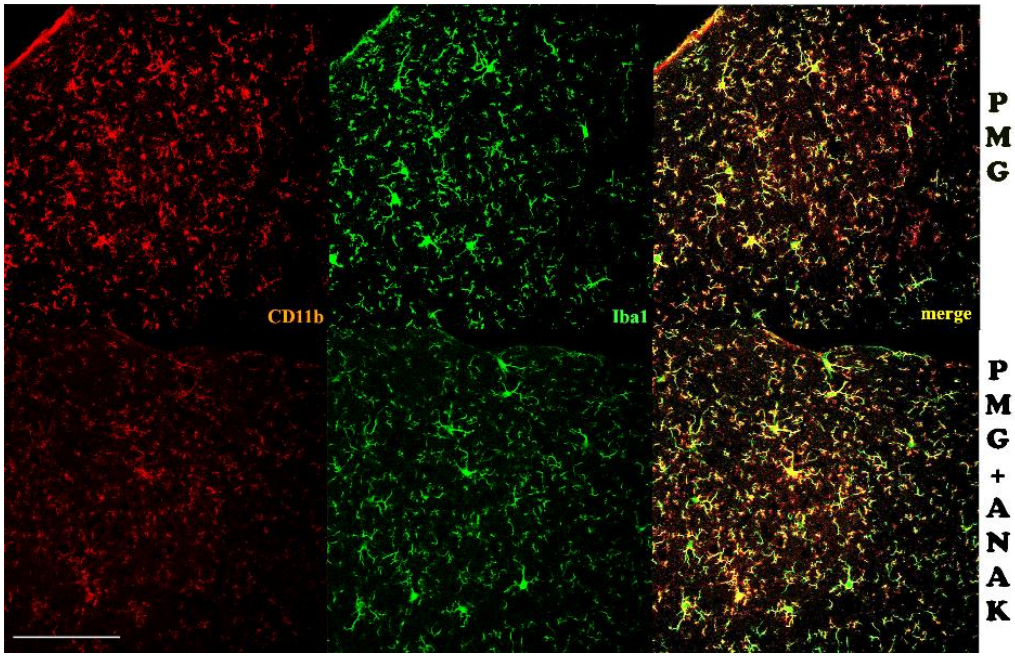


Figure 37. Microglia activation in PMG anakinra-treated mice cortex. A) Representative images of immunofluorescence staining for the microglia marker Iba1 (green) and microglia-activated marker CD11b (red) after anakinra administration in the cortex of P90 PMG and age-matched control mice (Scale bar: 80µm). Representative images showing a decrease of Cd11b marker after anakinra treatment.

We therefore carried out a multicytokines array analysis on brain tissues of CTR, PMG and anakinra-treated PMG (PMG+ANAK) at P90 (2months after the end of the treatment started at P30 and lasted 1 week) in order to get a picture of the levels of several different cytokines and inflammatory molecules in the brain parenchyma of PMG and anakinra treated PMG mice. This analysis allows to identify those inflammatory molecules (GCSF, GM-CSF, IL-2 and INF-gamma) which display greater changes in PMG condition and were partially rescued by anakinra (Figure 38).

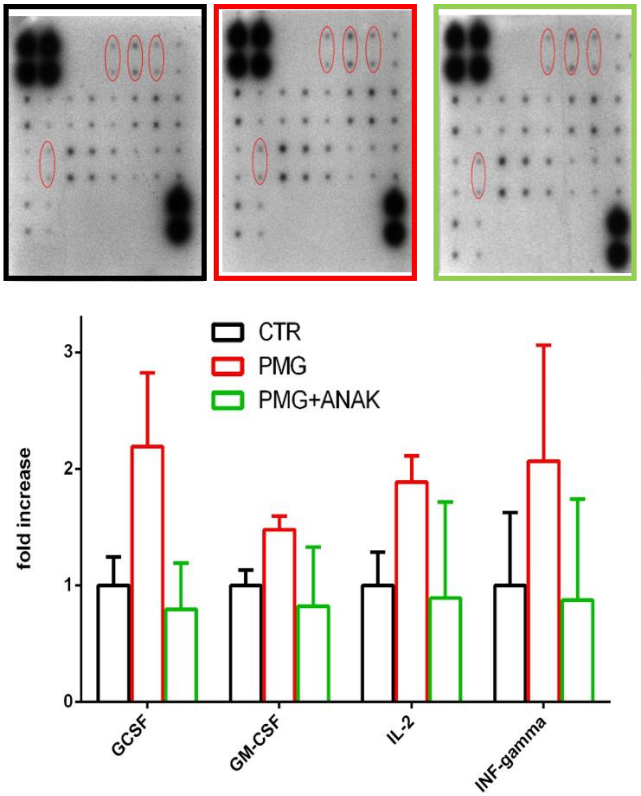


Figure 38. Multicytokines assay. Representative images of Multicytokines' membrane after signal detection. Quantification of inflammatory markers in PMG anakinra treated mice and age-matched control mice using a Multicytokines array. In the panel in the upper part of the image are shown the membranes on which are underlined with red circles some of cytokines which showed a higher expression in PMG mice with respect to age-matched controls. The quantification was performed in motor cortex samples of P90 mice (CTR=3, PMG=3, PMG+ANAK=3). Data are shown as mean +/- SE.

Functional recovery of PMG mice treated with anakinra

At the age of three months, anakinra-treated PMG mice and relative controls were subjected to the same set of behavioural tests to assess neuro- muscular function and motor coordination abilities. The more important effects of treatment that we recorded were in Body tone (Figure 39 B), Rotarod resistance (Figure 39 G) and Pole tests (Figure 39 H). In these tests, PMG mice treated with anakinra showed better performances with respect to untreated PMG mice that were comparable with aged matched CTR mice and CTR anakinra-treated mice (Figure 39). In particular, the rotarod resistance, pole test and also the assessment of body tone showed a statistically significant rescue of PMG deficits whereas wire hanging and wire hanging resistance did not (Figure 39).

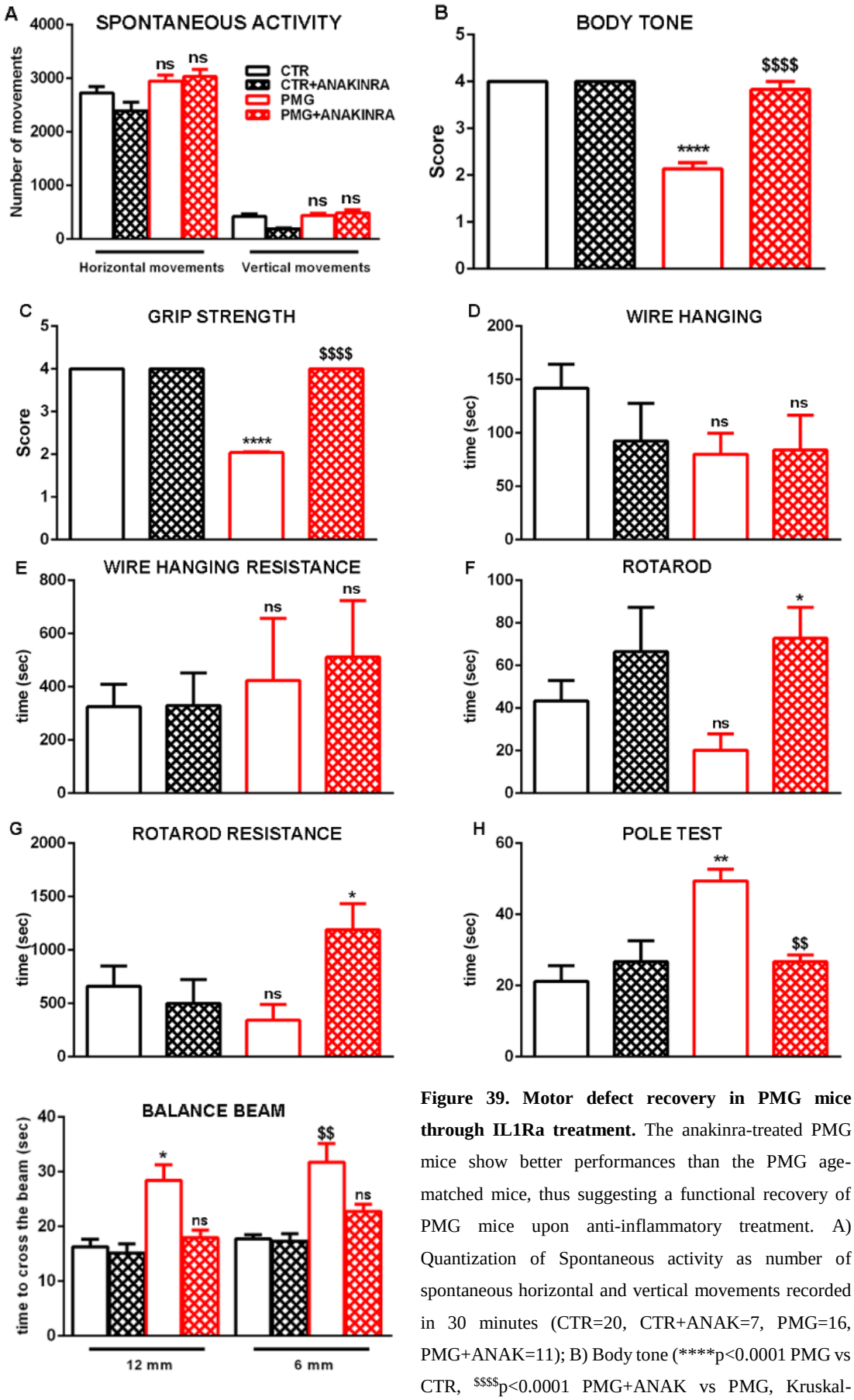


Figure 39. Motor defect recovery in PMG mice through IL1Ra treatment. The anakinra-treated PMG mice show better performances than the PMG age-matched mice, thus suggesting a functional recovery of PMG mice upon anti-inflammatory treatment. A) Quantization of Spontaneous activity as number of spontaneous horizontal and vertical movements recorded in 30 minutes (CTR=20, CTR+ANAK=7, PMG=16, PMG+ANAK=11); B) Body tone (****p<0.0001 PMG vs CTR, $^{\$\$\$\$}$p<0.0001 PMG+ANAK vs PMG, Kruskal-Wallis test, ONE Way ANOVA, CTR=20, CTR+ANAK=7, PMG=15, PMG+ANAK=12); C) Grip Strength

(****p<0.0001 PMG vs CTR, $^{\$\$\$}$p<0.001 PMG+ANAK vs PMG, Kruskal-Wallis test, ONE Way ANOVA, CTR=20, CTR+ANAK=7, PMG=17, PMG+ANAK=12); D) Wire hanging (CTR=21, CTR+ANAK=7, PMG=17, PMG+ANAK=11); E) Wire hanging resistance (CTR=7, CTR+ANAK=7, PMG=8, PMG+ANAK=11); F) Rotarod (*p<0.05 PMG+ANAK vs PMG, Kruskal-Wallis test, ONE Way ANOVA, CTR=17, CTR+ANAK=7, PMG=13, PMG+ANAK=12) G) Rotarod Resistance ((*p<0.05 PMG+ANAK vs PMG, Kruskal-Wallis test, ONE Way ANOVA, CTR=17, CTR+ANAK=7, PMG=13, PMG+ANAK=12); H) Pole test (**p<0.01 PMG vs CTR, $^{\$\$}$p<0.01 PMG+ANAK vs PMG Kruskal-Wallis test, ONE Way ANOVA, CTR=7, CTR+ANAK=7, PMG=8, PMG+ANAK=12); I) Balance Beam test ((*p<0.01 PMG vs CTR, 6mm, $^{\$\$}$p<0.01 PMG vs CTR, 12mm, Kruskal-Wallis test, ONE Way ANOVA, CTR=7, CTR+ANAK=7, PMG=8, PMG+ANAK=12).

Mice were then subjected to EEG recordings. As it is shown in the figure after anakinra administration, PMG mice showed a normalization of EEG activity with a statistically significant reduction of the number of spikes in EEG traces (Figure 40). This recovery could be possibly due to the blockade of IL1R pathway activation as it was partially suggested also by the immunomodulatory effect of hNSC treatment.

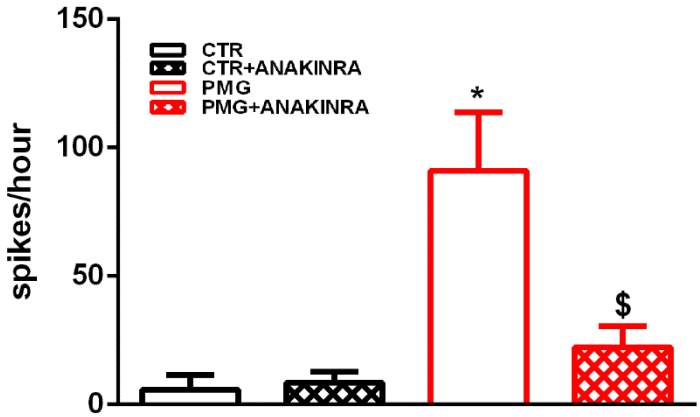


Figure 40. Recovery of epileptiform discharges in PMG mice after IL1R treatment. PMG mice received IP injection of anakinra at P30-P37. EEG was recorded at P110-120 after behavioural characterization and lasted 120 minutes. (*p<0.05 PMG vs CTR, $^{\$}$p<0.05 PMG+ANAK vs PMG, Kruskal-Wallis test, ONE Way ANOVA, CTR=3, CTR+ANAK=3, PMG=6, PMG+ANAK=6).

Altogether these experiments indicate that interfering with the inflammatory cascade in PMG may exerts positive effect on the pathology.

DISCUSSION

In this study we have performed a morphological, biochemical and functional characterization of a mouse model of polymicrogyria. This analysis allows to identify a diffuse hypomyelination and microglia activation as hallmarks of the pathology. Notably these features are also found in the brain of patients [4, 12, 23, 39]. Furthermore, by using ELISA, RT-PCR and multicytokines array analysis we have demonstrated that microglia activation is accompanied by an increased content of IL1beta in brain tissue and altered pattern of inflammatory mediators. Starting from these evidence we employed two different therapeutic approaches one based of hNSC injection into the cortex of PMG mice and the other based on the systemic treatment with the anti-inflammatory drug anakinra. Both of them display beneficial effects on the pathology. In particular, hNSC treatment ameliorates the hypomyelination by stimulating the endogenous OPC proliferation and partially but significantly improves behavioural and EEG defects; anakinra normalizes some of the cytokine alterations and results in an almost complete rescue of behavioural and EEG alteration in our preclinical model.

Polymicrogyria is characterized by its complexity and heterogeneity. There are numerous forms of PMG and sometimes it is no easy to associate this pathology with well-defined symptoms. The outcome of polymicrogyria might be severe ranging from language deficits, impaired motor abilities and drug resistant epilepsy. There is not a specific therapy to counteract this disease since the onset is too precocious and the diagnosis is very difficult to obtain. There are some palliative treatment comprising canonical antiepileptic drugs and in some case surgical intervention [12, 23, 27, 32]. Furthermore, this disease presents a plethora of etiologic causes that can be genetic, environmental, determined by infections or by trauma [23, 41]. The aim of this thesis was to better characterize the polymicrogyria pathology investigating the possible molecular and cellular mechanisms of the pathology to envisage more appropriate therapeutic approaches.

Morphological and Functional Characterization of FFL Mouse Model

Our first goal was to characterize the model proposed by Dvorak and Feit [238, 239] in a more extensive manner. As described in literature, the PMG mouse model resembles both the histopathology and the focal cell loss [254]. Nissl staining analysis allowed to visualised the formation of the microgyri at level of the upper layers of the PMG cortex already at P7 (Figure

11). This microgyrus, caused by the neuronal death seems to be the more accurate reproduction of the onset of the pathology, that in humans is reported to be not due to defect of neuronal migration but to neuronal death [26]. An important aspect of this model is that it guarantees the stabilisation of the microgyri that are easily defined and detectable also in adult PMG mice. According to literature the reorganization of the cortical lamination is a consequence of neuronal loss and migration defects and the formation of the pathological microgyrus follows the stages of the physiological formation of the correct cortical gyri unless for the layer formation [23, 24, 26, 38, 82, 83, 92, 114, 255]. Indeed, an important issue is that in coincidence with the formation of the ectopic gyrus there is the loss of deepest layers that are important for the propagation of the signal within and between the brain areas. In line with these evidences we found a clear disorganization of cortical layering (as shown in neurofilaments staining. Figure 12) the loss of cell bodies (stained with an antibody against NeuN) and the neurofilaments (stained with antibodies against NF. Figure 12). In addition to this structural defects we found a diffuse hypomyelination and some clear histological signs of inflammation such as astrogliosis and microglia activation. Myelin is an important component not only for the maintenance of the fibres structure, but principally it acts as an insulating sheath that is fundamental for the propagation of the action potentials [111]. The loss of myelin is clearly detectable in PMG mice and patients. The loss of myelination and the misplacement of neuronal cells and neurofilaments might be also related to the inflammatory condition. Inflammation is easily detectable by immunofluorescence staining against specific markers such as CD11b, Iba1 and GFAP. These markers are reported to be reliable in which are expressed by activated microglia (double labelled by CD11b and Iba1. Figure 15) and activated astrocytes (GFAP. Figure 14) that actively take part to the inflammatory response giving rise to a series of inflammatory pathways through the release of specific cytokines and chemokines [139, 153, 163, 245]. Starting from these evidences, the first cytokine that we investigated was IL-1β in which it is known to be involved in neuroinflammation and in drug resistant epilepsy by a mechanism that involves the expression and function of NMDARs [231, 253, 256, 257]. Moreover, the IL-1 downstream signalling results in a change of gene expression (up to 90 genes have been shown to be affected by IL-1 signal [220]). These changes result in the upregulation of mRNA transcription for cytokines, growth factor, adhesion molecules and acute-phase proteins [220]. IL-1β, when bind the IL-1 Receptor complex, is a critical initiator of a number of signal transduction cascades involving the mitogen-activated protein kinase pathways (MAPK) [221]. p38 MAPK is a stress-activated protein kinase and its action is pro-inflammatory producing IL-8 and IL-6 [206]. Our results showed a sustained inflammatory process in PMG brain tissue either at P7 and P30 that seems initially restricted to the lesion area and then spread to the surrounding areas. This spreading can be associated either to the migration and activation of microglia at level of damaged area or the diffusion of immune

mediators along the cortex. the functionality of the cortical tissue exposed to increased content of IL-1β and other immune molecules can be severely impacted. Indeed, several lines of evidence demonstrated a synaptic damage but also a neurodegenerative process in these conditions [258, 259]. Moreover, cytokines elevation can be directly involved in seizure and myelin defects, not only of IL-1β but also of other chemokines such as MCP-5 and GM-CSF [260-262]. These molecular alterations directly correlate with functional problem such as muscular weakness, problem in motor coordination and presence of atypical EEG activity. All of the features that we could characterize in PMG mice can be reported also in patients as is well described in literature [23, 39, 41, 243]. These evidences allow us to affirm that the FLL model is a good model to study this pathology and it is suitable to test possible therapeutic approaches that can be focused on the myelination problems and the inflammation.

Consideration about the sex differences

Considering the relevant number of papers that in the last years have been focused on the differences between male and female features in mice in terms of predisposition to neurological disease of early and late life and to neuroinflammation [191-193], suggesting that a gender issue might be relevant also in preclinical-rodent based- studies. We therefore decided to carry out our experimental procedures considering the results deriving from male and female mice in a separate way. For this reason, we initially performed immunofluorescence analysis and behavioural tests trying to obtain a considerable number male and female subjects for each experimental groups. Our analysis, both in terms of inflammatory marker levels and motor skills scores does not reveal any significant differences between the two sexes.

hNSCs based treatment ameliorates morphological and functional defects

hNSCs are well characterised to be suitable to treat myelination defects and neuroinflammation [249, 250, 263, 264]. Our data support this evidence highlighting the efficacy of stem cells in a pathology which principal symptoms are caused by demyelination and inflammatory response. A first strong evidence of the positive action of stem cells treatment in PMG mice arise from the morphological data obtained from immunofluorescence staining. Indeed, hNSCs significantly improved the altered neurofilaments disposition and myelin loss. Also the neuronal cell bodies appear to have a quite better arrangement at level of the upper layer even if the structural malformation is never rescued. A very exciting data was the increased endogenous OPCs proliferation (that were positive for the specific markers NG2 and CNPase but not for the human antigen) that were recalled at the level of the lesion after the hNSCs transplantation. This is an important evidence because it suggests a possible molecular

mechanism involving the endogenous capability of brain repair operating in condition where myelin loss is present. In hNSCs-treated PMG mice, since the first month of the cells injection, it is possible to evaluate a rescue of principal pathological aspect that characterise the mouse model, including the microglia activation which results slightly diminished. More importantly at the functional level, mice treated with stem cells showed an improvement of several motor skills abilities and a significant recovery of EEG abnormalities. The recovery of neuromuscular strength and motor coordination can be attributable to the partial rescue of myelin defects and reorganization of neurofilaments and cell bodies redisposition. Moreover, the well-known immunomodulatory effect of these cells could play a crucial role in the normalization of cortical activity.

These results indicate that stem cell therapy could be beneficial I this complex pathology. Further, investigations will be necessary to define the best temporal window for the treatment and to set the best experimental procedures in terms of cell dosage and way of administration, taking advantage also from the extensive research involving stem cell therapies in neurodegenerative conditions.

Pharmacological blockade of IL1Ra activation in PMG

Inflammation is a strong event that we have found in PMG brains either in early stages after the lesion or, more significantly, in adult age. This process is highly invalidating since it can be related to cellular death, defect in cognition and to status epilepticus [257]. Starting to these forewords, as a focused and more specific treatment we choose to use Anakinra that is a recombinant version of endogenous IL1Ra. It has been demonstrated that this compound is able to counteract the pro-inflammatory action of IL-1β directly competing to bind its receptor. For its short half-life (4-6 hours), tolerability and approximatively lack of side effects, anakinra is considerate safe and largely used in the treatment of childhood diseases such as rheumatoid arthritis and NOMID syndrome [224, 252, 253, 265, 266]. Treatment with anakinra has been recently demonstrated to prevent also the detrimental effect induced by levels of IL-1β on synapse formation and plasticity [267]. For this reason, we considered it a good candidate to treat the neuroinflammatory processes that have been also observed in PMG patients. Anakinra administration results in a partial rescue of morphological and molecular defects but a large recovery of the functional impairment (behavioural and EEG). The positive effect of anakinra on myelin integrity and MBP density could be possibility due to the anti-inflammatory action of the drug as was already suggested by the results obtained with hNSCs treatment. In this study we carried out a detailed analysis of immune molecules which are deregulated in PMG conditions, some of which (i.e. GCSF, GM-CSF, IL-2 and INF-γ) might also have specific effects on myelin which could be further investigate. Furthermore, after the anakinra

administration, PMG mice showed better performances both in neuromuscular strength and motor coordination. This can be referred to a positive action of anakinra on structural defects and on the promotion of myelin production or reorganization. Moreover, treated display an almost complete rescue of cortical activity, in fact, EEG traces of treated mice lack the atypical multi-spikes events that characterised PMG. This can be related to direct action of anakinra on IL-1β action that is upstream to the activation of the majority of cytokines and chemokines involved in neuroinflammation. We think that this approach can be reliable and promising not only for the safety of the compound, but also for the well documented implication of IL-1β in epilepsy [231, 232, 257, 268]. This is a pivotal aspect since the drug resistant epilepsy is the most sever and debilitating symptoms present in PMG patients.

EXPERIMENTAL PROCEDURES

Animals

All the experimental procedures that required the use of animals followed the guidelines established by the Italian Council on Animal Care and were approved by the Italian Government Decree No. 27/2010. All efforts were made to minimize the number of subjects used and their suffering.

Focal Freeze Lesions and recovering

The protocol of Focal Freeze Lesions used in this work was the same used by Dvorak and Feit [238, 239]. P0-P1 mice were anesthetised using ice. In the specific the animals were wrapped in a wet paper sheet and put on ice for 5 minutes. There is briefly recapitulate the method and development of the micro-sulcus: contact freezing of the conus through the exposed calvarium, lasting 2-3 s; freezing apparatus - a copper rod 10cm long, diameter 4 ram, length of point 5 ram, diameter of point at place of contact 2 ram. The rod was immersed in liquid N2. Freezing of the calvarium caused total necrosis of the pia mater, with thrombosis of the blood vessels, necrosis of the molecular layer and layer IV beneath (layers II and III were not yet developed) and partial necrosis of layers V and Via, with neuron extinction and persistence of capillaries. Layer VIb, supplied with blood from the connections with the subcortical vascular network, was preserved, and formed layer 4 of the atypical four layered microgyric cortex (AMC). Neuroblasts destined for layers II and III of the normal cortex migrated over the region of partial necrosis, came to a halt at the border of total necrosis, and formed layer 2 of the AMC, their processes also participating in the formation of layer 1 of the AMC. Layer 3 the AMC was formed in the region of partial necrosis, was colonised by glia, and also contained individual neurons from the original layer. After the freeze lesions, animals were put in the origin cages with mother and a nurse. 30 days after lesions, FFL mice and controls were put in new cage separating male from female.

hNSCs transplantation and recovery

hNSCs transplantation was performed in collaboration with Professor Angelo Vescovi (University of Milano Bicocca). Mice at P30 of age were treated with hNSCs or vehicle as described in Neri et al. 2010 [250]. Starting from a day before the surgery for two weeks later, mice were treated with the antirejection drug cyclosporine (300uL/Kg/day). The injections were performed intrathecally with the help of a stereotaxic apparatus in correspondence of

motor and premotor areas. After the surgery, mice were put singularly in cages and monitored for the following five hours.

IL1Ra treatment

P30 mice were treated with Anakinra intraperitoneally (24mg/kg, Kineret; Swedish Orphan Biovitrum, Sweden) as described in Noe 2013 for a week once a day [253]. We treated indistinctly male and female. After the treatments mice were monitored and left in their cages until behavioural tests.

Motor tests

Motor tests for the assessment of motor skills and muscular tone were performed I collaboration with Professor Mariaelvina Sala (university of Milan). The motor test performed were Body Tone, Wire Hanging, Rotarod and Rotarod Resistance, Balance Beam and Pole tests.

a. Spontaneous motor activity evaluation

With this test is evaluated the spontaneous motor activity of mice considering the number of spontaneous horizontal and vertical movements that the animals perform in 30 minutes.

b. Body tone evaluation

This evaluation was operated by the experimenter and for this reason it was a subjective analysis of the tone of the musculature of the animals. The experimenter valued handily the tone of the musculature at level of anterior and posterior limbs of the animal of each experimental groups. This test was performed in animals at P30 of age as first evaluation of muscular skills.

c. Grip strength

This test is an easy way to objectively quantify the muscular strength of mice. Grip Strength Test allows the study of neuromuscular functions by determining the maximal peak force developed by a rodent (rat or mouse) when the operator tries to pull it out of a specially designed grid or bar, which are available for both fore and hind limbs.

d. Wire Hanging

In this test each mouse was placed on a wire lid of a conventional housing cage and the lid was turned upside down. The latency from the beginning of the test until the mouse stood with at

least two limbs on the lid was timed. The animals had three attempts to stand for a maximum of 180 s per trial, and the longest latency was recorded. Mice are trained to suspend their body by holding on to a single wire stretched between two posts 50 to 60 cm above the ground.

e. Rotarod and Rotarod Resistance

The Rotarod was first described for rats and later adapted to mice [269] for evaluation of motor function and ataxia. The set-up consists of a rotating cylinder (3.0 to 3.2 cm in diameter) and an attachment with several compartments that are separated by thin walls to test multiple mice simultaneously. Mice placed on the rotating rod will walk and try to remain on the rod to avoid falling onto a platform below. Hence, the 'latency to fall' is typically used as a quantitative end point to evaluate motor function. The Rotarod is either used at a constant speed or with gradual acceleration to evaluate the resistance as a fast reliable marker of motor coordination and muscular strength. The acceleration chosen were 12 and 32 RPM.

f. Balance Beam

The beam apparatus consists of 1 meter beams with a flat surface of 12 mm or 6 mm width resting 50 cm above the table top on two poles. A black box is placed at the end of the beam as the finish point. (Plywood and other types of wood for the beams, poles, and box can be found at most hardware stores). Nesting material from home cages is placed in the black box to attract the mouse to the finish point. On training days, each mouse crosses the 12 mm beam 3 times and then the 6 mm beam 3 times. Mice are placed at one end of a beam and the time required to cross to the escape box at the other end (80 cm away) is measured. C57BL/6 mice typically cross the beam with a minimal amount of stopping or stalling. However, when they do stall, sniff or look around without proceeding forward, the investigator should encourage the mouse to continue moving forward by prodding, poking, or pushing it from behind with gloved fingers. Once the mice are in the safe box, they are allowed some time (~15 secs) to rest there before the next trial. After each training session, the mice are returned directly to their home cages and taken back into their housing room. Of note, the mice may stall more if they are over-trained and become too familiar with the task and apparatus. On the other hand, training can be continued for additional days if the mice still do not traverse the entire beam successfully after 2 days of training.

g. Pole test

The pole test was performed as previously described [270] with minor modifications. The mouse was placed head-upward on the top of a vertical rough-surfaced pole (diameter 8 mm; height 55 cm) and the time until it descended to the floor (locomotor activity time: TLA) was

recorded with a maximum duration of 120 s. Even if the mouse descended part way and fell the rest of the way, the behaviour was scored until it reached to the floor. When the mouse was not able to turn downward and instead dropped from the pole, TLA was taken as 120 s (default value) because of the maximal severity. TLA was measured in each experimental group each day for 7 days.

EEG recordings

EEG recording were performed in collaboration with Dr Marielvina Sala (University of Milan and IN-CNR). Mice were anesthetized with intraperitoneal injection of 5% chloral hydrate dissolved in saline and given a volume of 10 ml/kg. Four screw electrodes (Bilaney Consultants GMBH, Dusseldorf, Germany) were inserted bilaterally through the skull over cortex (anteroposterior, +2.0– 3.0 mm; left–right 2.0 mm from bregma) as previously described [271] according to brain atlas coordinates (Paxinos and Franklin, 2004); a further electrode was placed into the nasal bone as ground. The five electrodes were connected to a pedestal (Bilaney, Dusseldorf, Germany) and fixed with acrylic cement (Palavit, New Galetti and Rossi, Milan, Italy). The animals were allowed a week for recovery from surgery before the experiment. EEG traces were analyzed as [271] for spike activity. Basal cerebral activity was recorded continuously for 2 hours in freely moving mice. For each 2-h EEG recording, the mean number of spikes was evaluated in both experimental conditions. After the recordings, the EEG was analysed for the incidence of spontaneous cortical spike activity and the percentage of animals displaying spike activity, as previously described [271].

Immunofluorescence staining on free-floating sections

Immunofluorescence staining was carried out on free-floating sections as described in Frassoni et al., 2005 [272]. Brain section were obtained from P37, P44, P51, P58 and P90 PMG and aged-matched control mice. Brains were coronally cut with a Vibratome in 50µm thick serial sections. Antibodies used were: against activated microglia marker CD11b and neurofilaments SMI31 and SMI32 from Biolegend (London, United Kingdom; Bi94776); against microglia marker Iba1 from Wako (Neuss, Germany; 019-19741); against astrocytes' marker GFAP from Synaptic System (Goettingen, Germany; 173002); against MBP from Merck Millipore (Vimodrone, Italy; AB980); against Human Nuclei from Chemicon Millipore (Vimondrone, Italy; MAB128); against NG2 chondroitin sulphate proteoglycan Chemicon Millipore (Vimondrone, Italy; AB5320); against Olig2 Merck Millipore (Vimondrone, Italy; AB9610); DAPI Life Technologies (Monza, Italy; 62248). Secondary antibodies were conjugated with Alexa-488, Alexa-555 or Alexa-633 fluorophores (Invitrogen, San Diego, CA, USA). Free-floating sections were mounted in Fluorsave (Calbiochem, (Calbiochem, San Diego, CA,

USA). Sections were examined by means of a Zeiss LSM 510 META confocal microscope (Leica Microsystems, Wetzlar, Germany). To resolve microgyri formation, defects in lamination and microglia activation, the images (2048x2048) were acquired using the x40 and x20 oil immersion lens (numerical aperture 1.0). The gain and the offset were lower to prevent saturation in the brightest signals. The pinhole size was kept at the minimum setting (1.0- 1.8). Analysis was carried out on each mouse of different experimental conditions and three different sample from cortex area were taken at two coronal levels. Fluorescence images processing and analyses were performed with ImageJ Software (National Institutes of Health).

Evaluation of inflammatory cytokines

a. ELISA analysis of IL-1β

To measure the levels of IL-1β in brain tissue we used a mouse ELISA kit (R&D System; #DY401-05). Enzyme-linked immunosorbent assay (ELISA), also known as an enzyme immunoassay (EIA), is a biochemical technique used in immunology to detect the presence and the amount of an antibody or an antigen in a sample. In the specific, ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilised on a solid support (usually a polystyrene plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen in a "sandwich ELISA" conformation). After the antigen is immobilised, the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody that is linked to an enzyme through bio-conjugation. The part of antibody incubation of ELISA is similar with that of western blot. Between each step, the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step, the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample. Homogenate were obtained from lesion and perilesion areas of PMG mice and from the equivalent brain areas of control mice. The actual IL-1 β concentration was estimated on the basis of a standard curve at known concentration of IL-1 β. At the average the duplicate readings for each standard, control, and sample was subtracted the average zero standard optical density (O.D.). The data were linearized by plotting the log of the mouse IL-1β concentrations versus the log of the O.D. and the best fit line was determined by regression analysis.

b. Multicytokines assay

Evaluation of cytokines' concentration was performed by a Multicytokines array (RayBio® Mouse Cytokine Antibody Array 1; # RAY-AAM-CYT-1). This technique is based on a membrane support coated with specific antibody for different cytokines (in our case we chose the array for the detection of different twenty-two cytokines) which interact with the sample during the incubation. The signal was acquired after ulterior incubations with biotinylated antibodies and HRP-streptavidin via chemo-luminescence detection. The kit that we used can assay 22 cytokines. Samples were collected from motor cortex of PMG and control mice both anakinra-treated and untreated at P7, P30 and P90. Detection was performed by chemiluminescence-imaging device and relative expression levels of cytokines was made by comparing the signal intensities normalized on the Positive controls from different membranes. Measurements regarding the chemoluminescence have been carried out using ImageJ public domain software from the National Institutes of Health (http://rsb.info.nih.gov/ij/).

Statistical analysis

Morphological analysis of cortices of PMG mice was performed using ImageJ software. Statistical analysis was performed using SigmaStat 3.5 (Jandel Scientific) or PRISM 5 software (GraphPad, Software Inc., San Diego, CA,). After testing whether data were normally distributed or not, the appropriate statistical test has been used, see figure legends. Data are presented as mean±s.e.m. from the indicated number of elements analysed. For behaviour, the continuous data were analysed using the one or two- way ANOVA test. The differences were considered to be significant if $P<0.05$ and are indicated by an asterisk; those at $P<0.01$ are indicated by double asterisks; those at $P<0.001$ are indicated by triple asterisks; those at $P<0.0001$ are indicate by four asterisks.

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