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Tesi di laurea:

Alterations of hippocampal neurogenesis in a mouse model of X-linked intellectual disability

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ABSTRACT

Intellectual disability (ID) is a complex disease of the central nervous system (CNS). The genetic contribution to the etiology of ID is well established and, among the genetic conditions, the most frequent are the X-linked intellectual disability (XLID) forms. Among the XLID genes, Oligophrenin-1 (OPHN-1) encodes a synaptic Rho GTPase- activating protein that regulates neuronal morphology, proliferation and maturation. The involvement of OPHN-1 in XLID was well established by the identification of mutations within the gene, in patients with XLID. OPHN-1 gene is expressed in brain areas that are characterized by high synaptic plasticity: in particular, the olfactory bulb and the hippocampus. However, it is not clear how mutations in OPHN1 result in impaired neuronal development and consequent cognitive deficits.

To address these issues, I have used a mouse model of XLID based on germline deletion of the OPHN1 gene (OPHN1 KO; Khelfaoui et al., J Neurosci 2007). In particular, as adult hippocampal neurogenesis recapitulates the processes of neuronal differentiation, I have studied the development of newborn cells in the hippocampus of wt and OPHN1 KO mice. Using labelling of newborn cells with the thymidine analogue bromo-deoxy-uridine (BrdU), I found that cell proliferation in the subgranular zone of the hippocampus was not impacted by OPHN1 deficiency. Importantly, reduced numbers of BrdU-positive neurons were found 50 days after BrdU pulse labelling in OPHN1 KO mice, indicating impaired neuronal differentiation. In keeping with these data, the number of migrating neuroblasts (stained with a doublecortin – Dcx -antibody) was also decreased in the dentate gyrus of KO animals. We also found reduced numbers of cells double positive for Dcx and the neuronal marker NeuN, confirming the impaired integration of newborn neurons in the hippocampus of OPHN1 KO mice.

Prompted by these results, we tested a novel therapeutic strategy based on inhibition of the RhoA pathway, whose activity is potently stimulated by loss of OPHN-1. In particular, we administered via the drinking water fasudil, an inhibitor of the ROCK kinase. Preliminary data indicate that fasudil treatment restores normal numbers of Dcx-NeuN double positive cells in the hippocampus of OPHN1 KO animals.

Altogether, these data demonstrate robust alterations in hippocampal neurogenesis in OPHN1 mice, and suggest a possible strategy for counteracting defects in neuronal differentiation triggered by loss of OPHN1.

1- INTRODUCTION

1.1 INTELLECTUAL DISABILITY

1.1.1 DEFINITION

According to the tenth revision of the WHO, the Intellectual Disability (ID) "is a disorder defined by the presence of incomplete or arrested mental development, principally characterized by the deterioration of concrete functions at each stage of development and that contribute to the overall level of intelligence, such as cognitive, language, motor and socialization of functions; in this anomaly, adaptation to the environment is always affected. For ID, scores of intellectual development levels must be determined based on all of the available information, including clinical signs, adaptive behavior in the cultural medium of the individual and psychological findings" .

On the other hand, the American Association on Intellectual and Developmental Disabilities (AAIDD) indicates that in addition to a significantly sub-average intellectual functioning, concomitant limitations are observed in two or more areas of adaptive skills, such as communication, home life and personal care; also, it's been observed that the disorder presents itself before the age of 18.

Altogether, ID is a pathological condition defined by cognitive and adaptive impairment, the latter expressed as social and practical skills. According to the AAIDD, there are five dimensions which define the ID:

- *Intellectual aptitudes*
- *Adaptation level*

• *Participation, interaction and social roles*

• *Health*

• *Social context*

1.1.2 CAUSES

The factors which have been related to induce ID are classified as: genetic, acquired (congenital and developmental) environmental and sociocultural.

• Genetic factors

Patients with Down Syndrome are the greatest group suffering from ID. Other less frequent genetic abnormalities are: Rett syndrome, fragile X syndrome and Will syndrome, among others.

Moreover, there are some hereditary factors that can produce ID, like: galactosemy, Tay-Sachs disease, glycogen deposit disease and other related conditions.

• Congenital factors

These can be classified as: metabolic (neonatal hypothyroidism), toxic (such as lead poisoning, fetal alcohol syndrome, etc…) and infectious (like syphillis, toxoplasmosis, etc…)

• Developmental factors

These factors can come into play during the prenatal, perinatal and postnatal period: complications related with the pregnancy, the birth and the first moments of life could cause the onset of the ID.

• Environmental and sociocultural factors667

Different studies analyzed the correlation between socioeconomic poverty and ID. In particular, the insurgence of ID may be related to: scarcity of prenatal, perinatal and postnatal health care, adolescence care and family instability, inadequate health treatment and low level of stimulation, in addition to infant mistreatment (Katz G. et alia, *Salud publica de Mexico* 2008)

1.1.3 CLASSIFICATION

Cognitive disabilities has a common denominator: a subnormal intellectual functioning level; however, the extent to which an individual is unable to face the requirements established by society are been divided into four degrees of severity: mild (IQ between 50 and 69), moderate (IQ between 35 and 49), severe (IQ between 20 and 34) and profound(IQ lower than 20). The characteristics of individuals must be found to define intervention guidelines and to promote a valid therapy (*fig.1*) (Katz G. et alia, *Salud publica de Mexico* 2008).

CLASSIFICATION OF INTELLECTUAL DISABILITY, STRATIFIED BY THREE AGE GROUPS

	Age	
0 to 5 years *Maturation and development*	*6 to 20 years* *Training and education*	*21 years and older* *Social and vocational adequacy*
Degree: Mild General develop communicative and social skills. May not be distinguishable until beginning school.	Can learn up to 4th/5th primary school grade skills when reaching the ages of 18 or 19 years. Can be integrated into society.	Is capable of acquiring social and work skills for integration into the work force at minimum wage.
Degree: Moderate Can speak or learn to communicate. Some difficulties with motor skills.	Difficulty meeting 2nd primary school grade academic objectives.	May be able to partially maintain oneself economically in manual work under protected conditions
Degree: Severe Marked limitations in motor skills. Minimal language ability.	Can speak or learn to communicate. Can learn elemental self-care and health habits.	Can partially contribute to maintaining oneself economically under total supervision.
Degree: Profound Significant delay, minimal functional ability in sensorimotor areas. Needs basic care.		Some motor and language development. Can learn very limited personal care skills.

Fig.1: Criteria to classify ID, based on the age of patients (Reproduced from: Gregorio Katz, Salud publica de Mexico, vol.50, supplement 2)

1.1.4 DIAGNOSIS

To treat efficaciously the Intellectual Disability, it's necessary to diagnosis it as soon as possible; however, the lack of legislation in developing countries, the stigmata

and discrimination imposed by society and structure capable of diagnose the pathology, delays significantly the possibilities for interventions.

A complete anamnesis, of course, is the first step to follow, especially during prenatal, perinatal and postanatal period, including a study of the genealogical tree for at least three generations and a deepen search for family antecedents. In second place, a physical exam is required, focusing on genetic abnormalities and malformations, followed by behavioral and neurological evaluations: if a clinical case is discovered, it's necessary to perform specific analysis. However, if the clinical diagnosis is not established, cytogenetic evaluations, besides metabolic analyses, are needed: one of the most useful is the Fluorescence *in situ* hybridization (FISH), a technique which permits to analyze the entire subtelomeric region. If any result is not find yet, submicroscopic chromosomal disorders are evaluated.

The use of Neuroimaging techniques (such as magnetic resonance imaging) is also critical to detect abnormalities in the anatomical structure of the brain.

Metabolic analyses include: urinary tests for amino acids, organic acids, oligosaccharides, mucopolisaccharides and uric acid; in addition, sometimes it may be useful to study disorders of glycosilation or problems in the distal cholesterol pathways (Katz G. et alia, *Salud publica de Mexico* 2008).

1.1.5 X-LINKED INTELLECTUAL DISABILITY

Ten percent of cases of ID in boys are caused by genes located on the X chromosome: these are defined as the X-linked Intellectual Disabilities (XLID). Usually, in this type of syndromes, hemizygous boys are affected, while heterozygous girls are asymptomatic or have milder symptoms (due to the presence of a normal allele on one of the two X chromosomes). However, girls can be affected in two ways: the first one, involving X-linked dominant diseases, like Rett syndrome; the second one, in

which the girls are symptomatic, because the X chromosome that carries the mutation is not well inactivated.

Thanks to the increase and development of more sophisticated techniques to diagnose and analyze the major characteristics of these pathologies, it's been found out that dozens of genes are involved; in particular, microdeletion or X/autosome traslocation are been used for the characterization. Traditionally, the patients presenting the symptoms have been divided into two major groups (Vincente des Portes, *Handbook of clinical neurology,* 3rd series):

- S*yndromic XLID*, which are recognizable thanks to some clinical signs, such as brain malformation or extraneurological problems;
- *"Nonspecific" XLID*, the most frequent group, which cannot be determined on well-defined clinical grounds.

1.1.6 FRAGILE X SYNDROME

Fragile X syndrome is the second major cause of ID after Down syndrome. Lubs, in 1969, discovered that, in this kind of syndrome, there is a chromosomal break in the Xq27.3 region in chromosome X: thus, the origin of the name. From 1991, thanks to J.L. Mandel's team, it's been discovered the molecular mechanism of fragile X. It consists of a dynamic mutation with a CGG triplet amplification which takes place in two steps along generations: upstream of the promotor region of the FMRI gene, repeat sequences (CGG triplets) comprise less than 55 repeats in the general population. Generally, a grandfather or a grandmother carries a premutation, that is, between 55 and 200 CGG repeats, which is responsible for instability of this structure. Usually, if carried by female, the premutation tends to amplify; instead, if it's carried by males, it is transmitted to all daughters, who could transmit it to 50% of their children, as pre- or complete mutation (>200 CGG). The above mentioned complete mutation causes a methylation of Fragile Mental Retardation 1 (FMR1) gene, which

blocks its expression: thus, the lack of FMR protein is the main cause of the symptoms of Fragile X syndrome. The gold standard to individuate the methylation of FMR1 remains the southern blot technique (Vincente des Portes, *Handbook of clinical neurology* 3rd series).

1.2 Role of Oligophrenin-1 gene in Intellectual Disability

1.2.1 The Rho GTPases signaling

Because learning deficiency is a constant feature of ID patients, it's been conjectured that this trait is due to a synaptic alteration: in first place, there are been evidences that ID related protein are enriched at pre- and/or post-synaptic compartments (Humeau Y. et alia, *J. Neurochem.* 2009); second, in ID patients, there are alterations concerning dendritic spines, actin-rich structures on which most excitatory synapses are located. Third, functional evidence has been provided for alterations in synaptic strength in models of ID (A. Pavlowsky et alia, *Mol. Psychiatry*, 2012).

However, not much is known about the gene alterations implicated in intellectual impairment. Because of the involvement of the dendritic spines, recently, the family of Rho GTPases family has received much attention, due to their role in regulating actin dynamics and organization (N. Nadif Kasri et alia, *Eur. J. Physiol.* 2012).

Rho family GTPases belongs to the Ras superfamily of small protein (about 21 KDa) GTPases. They have been divided into 8 different subgroups: Rho, Rac, Cdc42, RhoD, Rnd, RhoH/TTF, RhoBTB, Miro (P. Aspenström et alia, *Biochem. J.* 2004). Rho proteins are guanine nucleotide binding proteins, which act as molecular switches that cycle between an active GTP-bound form and an inactive GDP-bound form; moreover, their activity is spatiotemporally regulated by positive regulator guanine nucleotide exchange factors (GEFs) and negative regulators GTPases activating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (*fig.2*).

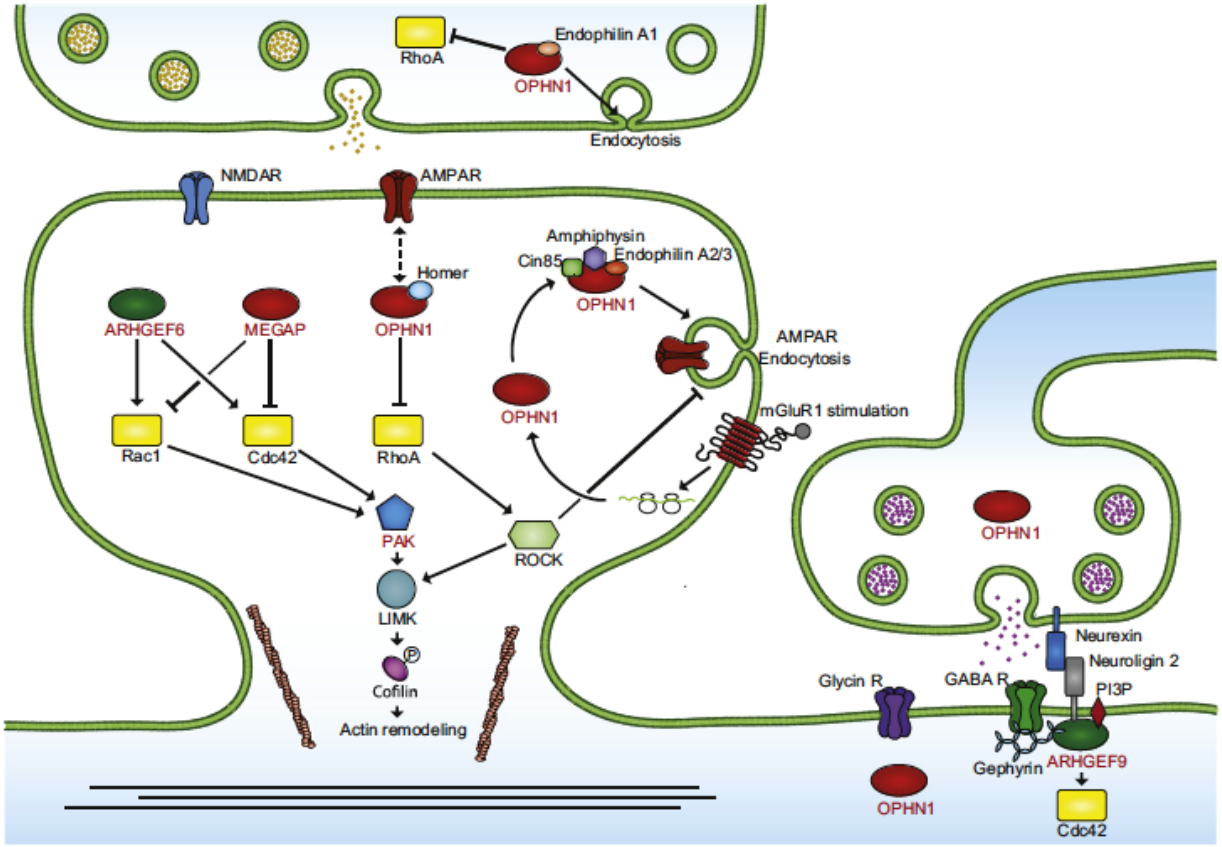


Fig. 2- Overview of ID genes and their role in Rho-GTPase signaling at the synapse. On the left an excitatory synapse and, on the right, an inhibitory one. The yellow squares represent the Rho-GTPases, the red ovals are GAP proteins, while green ovals are GEF proteins; finally, ID genes are written in red. When activated, Rac1, Cdc42 and RhoA interact with downstream effector proteins, which, eventually, results in actin remodeling. (Reproduce from Wei Ba et alia, *Exper. Cell Resea.* 2014)

In particular, it's been demonstrated that Rac1, Cdc42 and RhoA have a major role in controlling the changes in actin cytoskeleton in neurons (E.-E. Govek et alia, *Dev. Neurobiol.* 2011): indeed, at the synapses, they are key regulators of formation and morphogenesis of spines, and are also implicated in synaptic plasticity (S.E. Newey et alia, *J. Neurobiol.* 2005). Specifically, Rac1 and Cdc42 have been shown to promote the formation, growth and maintenance of spines, whereas RhoA induces spine retraction and loss. The changes in morphology of dendritic spines are associated with Long-term potentiation (LTP) and long-term depression (LTD), two processes needed to remodel the synapse strength depending on their activity and which are believed to be the cellular basis of memory and learning. Indeed, to produce stable LTP and LTD, enduring actin-dependent structural changes in spine heads are

required. These changes are majorly linked with incorporation (LTP) or removal (LTD) of synaptic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPARs) (L.a Cingolani et alia, *Nat. Rev. Neuro.* 2008): indeed, inhibition of actin polymerization attenuates LTP maintenance, whereas LTD is associated with filament disassembly (N. Honkura, *Neuron*, 2008).

1.2.2 THE OLIGOPHRENIN-1 GENE

Oligophrenin-1 (Ophn-1) gene is a gene located in X chromosome, in the q12 band. It is the first identified Rho-linked ID gene, characterized by 21 exons: it is ubiquitously expressed in the developing and adult brain.

The Ophn-1 protein, is a RhoGAP, specifically stimulates GTP hydrolysis of members of the Rho subfamily, thereby negatively regulating Rho-GTPase activity; moreover, its central GAP domain inhibits RhoA, Rac and Cdc42 without any specificity (Govek et alia, *Nature Neuro.* 2004). Its N-terminal end contains a BAR (Bin, amphyphysin, RSV) domain, which binds endophilin, amphyphysin and Cin85, thereby controlling clathrin-mediated endocytosis (CME), through the RhoA/Rho-associated protein kinase (ROCK) pathway. Beyond that, a pleckstrin homology (PH) domain follows, which should confer to OPHN-1 some specificity to the membrane binding, through the interaction with phosphoinositides. Finally, the C-terminal domain contains three proline rich regions at amino-acid positions 575, 629 and 740 with multiple Src homology 2 (SH3) binding domains (Khelfaoui K., *Hum. Molec. Genet.* 2009).

The protein is abundantly expressed in all brain regions, including cortex and hippocampus. It is present in axons, dendrites and spines (E.-E. Goveck, *Nat. Neurosci.* 2004); at the synapse, it must be noted that Ophn-1 is present both in presynaptic and in postsynaptic regions of excitatory and inhibitory synapses. Finally, Ophn-1 is present in glia cells, where is colocalized with F-actin, because of its role in active membrane movements.

1.2.3 Loss of function of OPHN-1

At present there is only a partial understanding of the cellular mechanisms in which Ophn-1 is implicated. However, several evidences indicate that loss of Ophn-1 function leads to synaptic impairments, that could contribute to ID.

From a molecular point of view, constitutive loss of Ophn-1 leads to abnormally high level of PKA activity and to the uncoupling of the cAMP/PKA signaling pathway from synaptic function and plasticity. Specifically, it has been observed that presynaptic PKA-dependent long term plasticity in both amygdala and hippocampus was severely compromised by absence of Ophn-1 (Khelfaoui M. et alia, *Phil. Trans. R. Soc.* 2014). Because of cAMP/PKA signaling role in learning and memory processes, dysregulation of this pathway following loss of Ophn-1 may result in learning deficits (Selcher JC et alia, *Neuroscientist*, 2002). Moreover, it has been demonstrated that the loss of function of Ophn-1 leads to an hyperactivation of the RhoA/Rock pathway, responsible for receptors endocytosis through endophilin phosphorylation and interaction with CIN85.

In hippocampal slice preparations, acute downregulation of Ophn-1 expression (using siRNA approaches) leads to alterations in the density and morphology of postsynaptic spine densities, along with changes in AMPARs and NMDARs mobility and postsynaptic LTP (Kasri N.N. et alia, *Genes Dev.* 2009). These effects are dependent on interaction of Ophn-1 with Homer 1b/c, a scaffolding protein involved in the organization of postsynaptic glutamate freceptors. Conversely, overexpressing Ophn-1 selectively enhanced AMPAR-mediated synaptic transmission and increased spine density (Kasri N. N. et alia, *Genes Dev.* 2009). Finally, Ophn-1 is also implicated in inhibitory synaptic transmission: indeed, impairments in presynaptic vesicle recycling and a reduction in the readily releasable pool were recently observed in inhibitory synapses in Dentate Gyrus (DG) of Ophn-1 KO mice (A.D. Powell et alia, *J. Physiology*, 2012).

1.2.4 OPHN-1 ID- RELATED PHENOTYPE

The human phenotype of ID caused by Ophn-1 mutations was linked, initially, with a non-syndromic form of XLID. However, it was later demonstrated that the loss of Ophn-1 induced a precise clinical case of ID. Generally, the boys affected by this syndrome presented, as a consistent feature, epileptic seizures, manifested as myoclonic events associated with absences and tonic-clonic fits. Furthermore, these patients showed micropenis and small testes without hormonal imbalance: this character has been linked with some neuron migration defects (Pradham M. et alia, *Am. J. Med. Genet*. 1999). Cartsen Bergmann and colleagues (*Brain*, 2003) analyzed also the neuro-morphological consequences of loss of Ophn-1 in male patients with ID. The neuroradiological examination showed a frontally and temporally pronunced reduction in cerebral volume with a widened interhemispheric fissure. Beside that, also the lateral and third ventricle presented an increase of volume, while the fourth ventricle appeared normal. There was lower vermian agenesis and bilateral cerebellar hypoplasia with asymmetric involvement.

Female carriers seem to be hardly detectable at the clinical levelsince strabismus represented the only consistent character. Other possible features are: impairment in verbal skills and moderate kinetic deficits, probably due to a skewed X-inactivation (Bergmann C. et alia, *Brain*, 2003).

1.3 THE MOUSE MODEL OF X-LINKED ID

1.3.1 GENERATION OF OPHN-1 KNOCK-OUT MOUSE

To observe and study the pathophysiology of Ophn-1-related ID, Malik Khelfaoui and his coworkers have created a mouse model in which they inactivated the Ophn-1 gene.

In order to do this, they disrupted the open reading frame of the Ophn-1 gene by the insertion of 100 bp in the coding sequence of exon 9, which leads to premature stop codon after the BAR domain (amino acids 1- 242) and before the GAP domain.

The exon 9 was targeted by homologous recombination leading to the insertion of a phosphoglycerolkinase-hygromycin resistant gene cassette (Hygro) flanked with LoxP sites. The Cre recombinase catalyzed the deletion of sequences between LoxP sites and led to the removal of the selective cassette in the final Ophn-1 mutated allele. Therefore, they created chimeric mice, by aggregation into a C57BL/6 blastocyst. The male chimeras, then, were crossed to C57BL/6 females from a transgenic line expressing the Cre recombinase early in development to generate N1 heterozygous females with a pure mutant allele deleted of the pgk-hygromycin-resistan gene cassette. The residual 100bpsequence consists of one remaining LoxP site after excision by the Cre recombinase plus adjacent polylinker sequence from the targeting vector (*fig.3*)(Khelfaoui M. et alia, *J. Neurosci.* 2007).

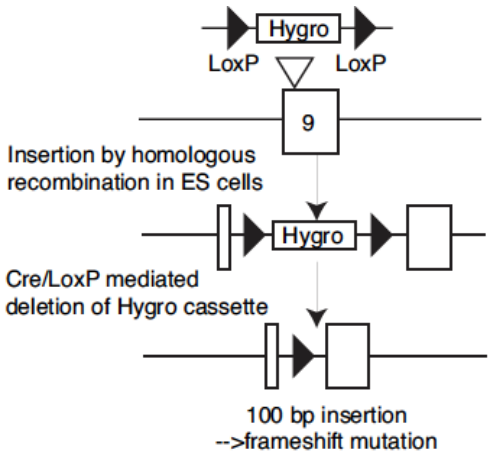


Fig. 3: Scheme that represent the inactivation of Ophn-1 gene (Reproduce from Khelfaoui et alia, *J. Neurosci.* 2007)

1.3.2 Behavioral, social and cognitive impairments

The generated strain was analyzed from a behavioral, social and cognitive point of view. No great differences were observed between mutant males and their wild-type (WT) in any anxiety-related variables as tested in the elevated O-maze and the light-dark box. However, the mutant males presented higher exploration scores in the center of the open field compared with control animals, suggesting that they were less anxious.

Moreover, the KO mice showed a less aggressive behavior than WT mice, as seen by introducing an intruder in their cage.

To study their capacity of learning and memorization, the Morris water maze test was used. This is a classical hippocampus-dependent task of spatial learning and memory, in which mice rely on spatial cues to navigate from start locations around the perimeter of an circular swimming pool to locate a submerged escape platform. The authors showed that *Ophn1*$^{-/y}$ spent less time floating compared with *Ophn1*$^{+/y}$; moreover, the KO mice showed no preferences for the target quadrant during the probe trial, indicating a spatial memory impairment (Khelfaoui M., *J. Neurosci.* 2007).

1.3.3 Neuranatomy of Ophn1$^{-/Y}$ mice

For what concerns the anatomy of the Ophn-1 KO mouse brain, the main character of these animals was the enlargement of lateral and third ventricles, shown in 70% of *Ophn1*$^{-/y}$, whereas this phenotype was detected only in 10% of *Ophn1*$^{+/y}$ mice. Because it's been observed that this enlargement was rather mild during the weaning period, this phenotype likely worsens with age. However, KO mice showed no other significant neuroanatomical deficit at the macroscopic level. However, studying the fine structure of neurons in hippocampus and cortex, it's been found that the main changes were involving the dendrite spines in both regions, indicating that, in KO

mice, the loss of function of Ophn-1 leads to immaturity of dendritic spine in adult hippocampus, compared with WT animals (*fig.4*) (Khelfaoui M., *J. Neurosci.* 2007).

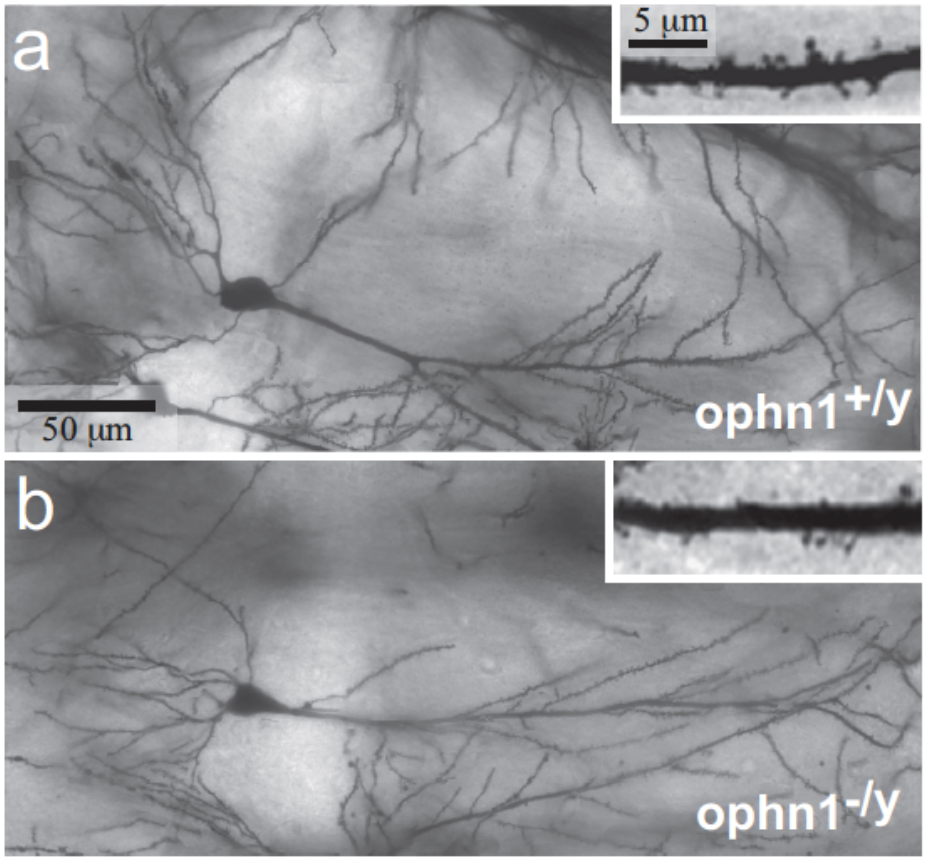


Fig.4: Reduced apical spine density and basal spine lenght in KO and WT mice CA1 hippocampal neurons. It's possible to see the decreased spine density in KO mice. (Reproduced from Khelfaoui et alia, J. Neurosci. 2007)

1.3.4 RESCUE OF THE PHENOTYPE

These data described above make clear that the ID caused by loss of function of Ophn-1 gene is a complex syndrome, in which many neuronal processes are impaired, and not all the molecular, cellular and synaptic mechanisms are presently known. However, it's been demonstrated that is possible to act on different pathways in order to restore the physiological function of CNS in ID patients. One of this strategy imply the use of inhibitors of the Rho Kinase (ROCK). Rock is one of the best known downstream target of RhoA and is a member of the ACG kinase family, which

is a group of kinases with a highly conserved amino acid sequence in the kinase domain. ROCKs have three distinct major domains, an RBD located in the coiled coil region, a catalytic kinase domain and a cystein-rich domain (Schmandke A. et alia, *Neuroscientist* 2007).

In the ROCK inactive state, the C-terminus acts as an autoinhibitory loop by folding back onto the kinase domain. This inactive state is reversed when the active Rho-GTP binds to the RBD breaking off the inhibitory loop. Once ROCK is biochemically active, it can phosphorylate many target proteins/substrates such as myosin light chain, LIM kinase, intermediate filaments, microtubule-associated proteins and collapsin response mediator protein: these substrates are highly involved in cytoskeletal re-arrangements, contractility, neuronal development, etc. (Mueller, *Nat. Rev. Drug Discov.* 2005) (*fig.5*).

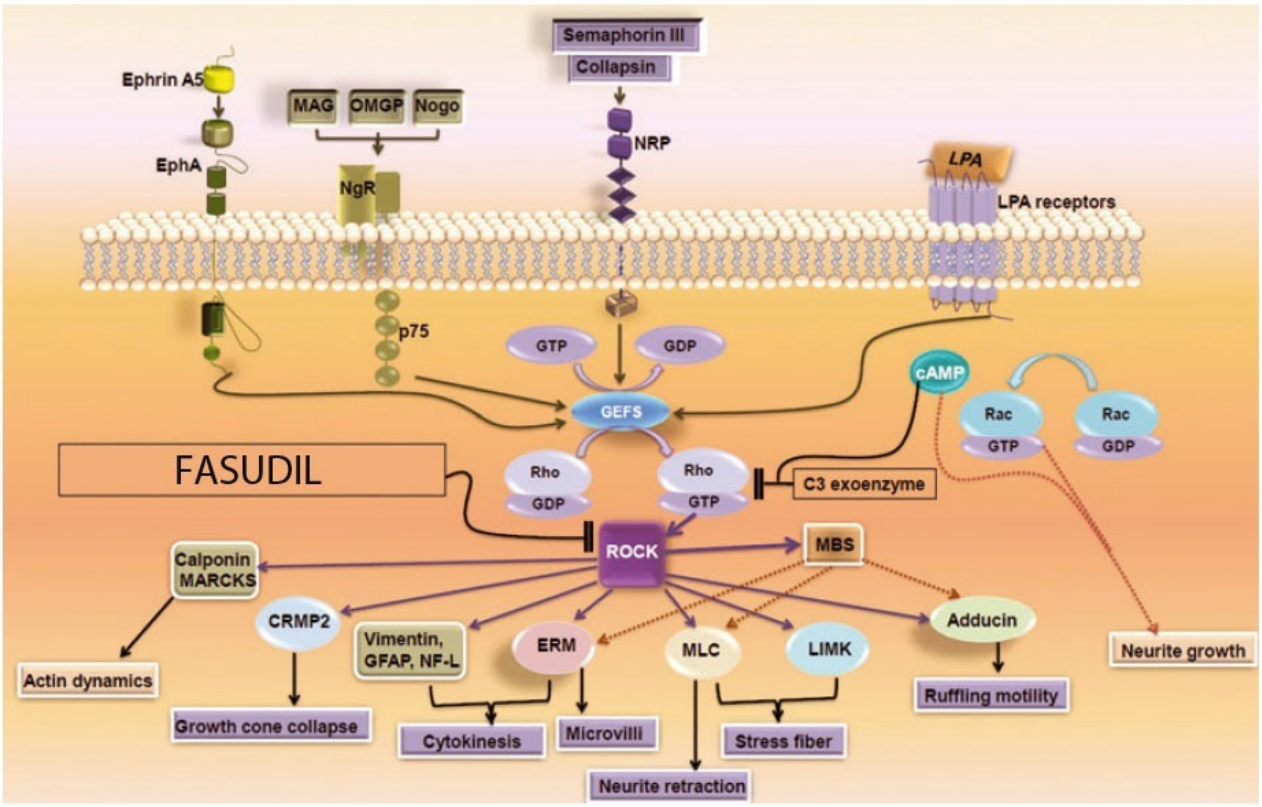
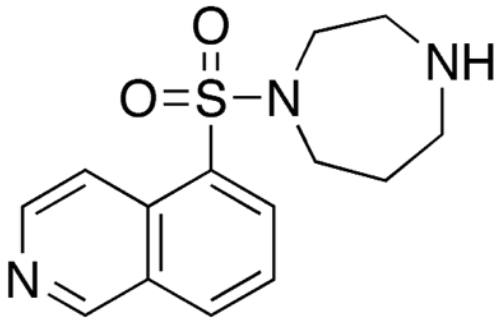


Fig. 5: The ROCK signaling pathways and their downstream targets and their affected biological processes. On the left, it's represented one of its inhibitors, Fasudil.(Reproduced from Mohamad R. et alia, Electrophor. 2012)

As shown by Khelfaoui and colleagues, one of the most prominent inhibitors of ROCK is Fasudil, an isoquinoline derivative drug approved for clinical trials in United States (Bowerman, *BMC Med.* 2012). Its structure can be seen below:



Fasudil has a structure containing isoquinolines and a homopiperazine ring, connected by a sulfonyl group. Fasudil acts on ROCKs by binding to a hydrofobic cleft between the N- and C- terminal lobes of the kinase domain of ROCK through the insertion of the planar isoquinoline ring of fasudil into the adenine binding pocket of protein kinases. Fasudil's homopiperazine ring is situated at the door of the cleft where the active residues are clustered (Yamaguchi, *Structure*, 2006). Khelfaoui and his coworkers (*Hum. Molec. Genetic*, 2009) used administration of Fasudil in the drinking water in their mouse model of Ophn-1-related ID and showed that Ophn1$^{-/y}$ mice restored internalization rate of AMPARs, specifically the GLUR1 subunit, rescuing the N-metyl-D-aspartic acid (NMDA)-dependent LTD, reactivating synaptic plasticity and transmission in KO mice hippocampal neurons.

Prompted by these observations, we decided to investigate whether the process of neurogenesis in adult mammalian brain was affected by the loss of function of Ophn-1 in mice, and whether treatment with fasudil could rescue the pathological phenotypes.

1.4 NEUROGENESIS IN ADULT MAMMALIAN BRAIN

1.4.1 DEFINITION

Neurogenesis is the process of generating functional neurons from precursor cells. This process was traditionally viewed to occur only during embryonic and perinatal stages in mammals. However, Altman's studies provided the first anatomical evidence for the presence of newly generated dentate granule cells in the postnatal rat hippocampus (Altman J. and Das G.D., *J. Comp. Neurol.* 1965). The field of adult neurogenesis took off after the introduction of bromo-deoxyuridine (BrdU), a nucleotide analog, as a lineage tracer (Khun H.G. et alia, *J. Neurosci.* 1996) and demonstrations of life-long continuous neurogenesis in almost all mammals examined, even humans (Eriksson P.S. et alia, *Nat. Med.* 1998). Active adult neurogenesis is spatially restricted, under normal conditions, to two specific brain regions: the subgranular zone (SGZ) in the dentate gyrus (DG) of hippocampus, where new dentate granule cells are generated, and the subventricular zone (SVZ) of the lateral ventricles, where new neurons are generated and, then, migrate through the rostral migratory stream (RMS) to the olfactory bulb, to become interneurons (Gage F.H. *Science* 2000). In this thesis, I focused on the SGZ of the DG of hippocampus, in which the population of stem cells is called Radial Glia cells (RGCs), expressing glial fibrillary acidic protein (GFAP).

1.4.2 ORIGIN OF THE SGZ IN THE DG

The DG of hippocampus originates from the dorsal neuroepithelium of the cortical hem, from a ventricular indentation called the dentate notch, where the first proliferative zone, called the primary matrix of the DG, locates (Altman J. et alia, *J. Comp. Neurol.* 1990). Next, the progenitor cell pool expands, forming the secondary

matrix and the first granule cells are generated (Altman J. et alia, *J. Comp. Neurol.* 1990). Thus, the first wave of granule cells originates from the progenitors located at the lateral margin of the hippocampal primordium, around embryonic day 14 (E14) in mice. With the formation of the dentate primordium, the progenitors are found along the ventricular germinal zone, in the dentate notch and the fimbrodentate junction (E15 in mice). These progenitors form the primordial radial glia scaffold and extend their cytoplasm as a single process from the germinal zone to the pial surface and are thus denominated radial glia cells. Next, the newborn granule cells migrate along this radial scaffold into the incipient DG (Rickmann et alia, *J. Comp. Neurol.* 1987). Later, during the first days after birth, a secondary radial glia scaffold develops after significant anatomical changes. The orientation of the radial processes shifts and the main proliferative area locates into the dentate gyrus, in the hilar zone beneath the forming blades of the granule cells layer, forming the tertiary matrix (Rickmann et alia, *J. Comp. Neurol.* 1987). Later on, around postnatal day 15 (P15), proliferation is mostly absent from the hilar region and remains only in the SGZ, where the somata of the RGCs, forming the secondary radial glia scaffold are located. This secondary radial glia scaffold is fully developed by the second postnatal week in mice, and than regresses: its size decreases and the radial process retracts and arborizes in the molecular layer (Brunne B. et alia, *Glia* 2010). By P21, the development of the DG is considered complete; however, the population of radial glia responsible for adult neurogenesis remains in the SGZ, maintaining the same morphological and marker-expression characteristics as during development. A population of non-radial/horizontal population of neural stem cells (nr/hNSC) has been suggested to exist in adult DG: these cells would be capable to generate radial neural stem cells, astrocytes and neurons, however, their characteristics, properties and origin are still poorly defined (Encinas J.M. et alia, *Int. J. Devl. Neurosci.* 2013).

1.4.3 Development of Neural Stem Cells in adult DG

Adult neurogenesis recapitulates the complete process of neuronal develoment in embryonic stages. In the adult SGZ, proliferating radial and non radial precursors give rise to intermediate progenitors, which in turn generate neuroblasts which migrate into the DG (Ge S. et alia, *Nature*, 2006). After a prolonged maturation phase, adult-born neurons exhibit similar basic electrophysiological properties as mature neurons, such as firing behavior and the amplitude and kinetics of GABAergic and glutamatergic inputs (Mongiat L.A. et alia, *Eur. J. Neurosci.* 2011). There are some principles that emerged from the study adult neurogenesis. First, the most important milestones of neuronal development are conserved among embryonic, postnatal and adult neurogenesis: during the embryonal period, immature neurons receive GABAergic synaptic inputs before the formation of glutamatergic inputs and are depolarized by GABA, due to high expression levels of the chloride importer NKCC1 (Ge S. et alia, *Nature*, 2006). One notable difference is a significantly slower timing of neuronal maturation in adult, compared to embryonic development (Duan X. et alia, Cell 2007). Second, neural progeny survival exhibits two critical periods: one at the intermediate progenitor and neuroblast stage (Platel J.C et alia, *Neuron* 2010) and one at the immature neuron integration stage (Mouret A. et alia, J. Neurosci. 2008). Newborn neurons also exhibit enhanced synaptic plasticity of their glutamatergic inputs within a critical period. Therefore, it's been suggested that such enhanced plasticity may give adult-born neurons an advantage in the competition with mature neurons for selective formation and stabilization of afferent and efferent synaptic connections (Tashiro A. et alia, *Nature* 2006) (*fig.6*).

Such properties may also allow integrated adult-born neuron to make a unique contribution to information processing during this period.

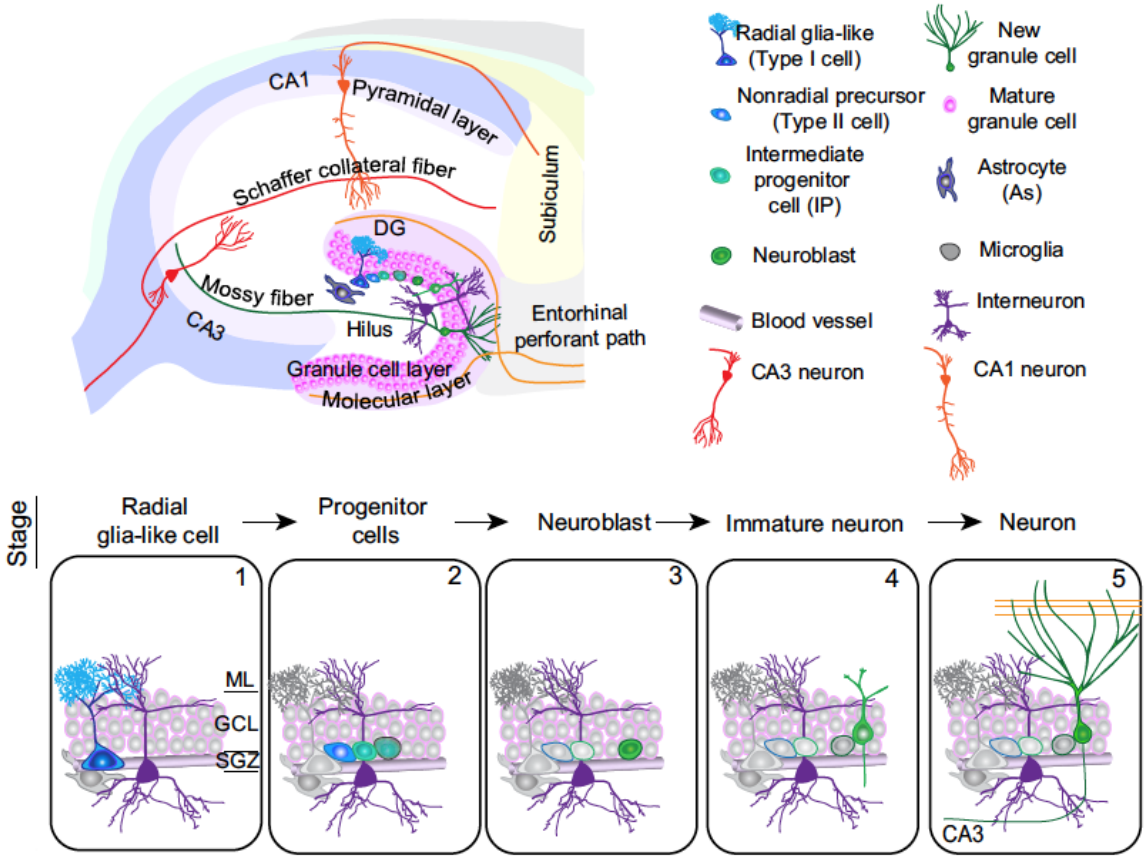


Fig. 6: Summary of five developmental stages during adult hippocampal neurogenesis: (1) activation of quiescent radial glia-like cell in the SGZ; (2) proliferation of non radial precursor and intermediate progenitors; (3) generation of neuroblasts; (4) integration of immature neurons and (5) maturation of adult-born dentate granule cells. ML: Molecular Layer; GCL: Granule Cell Layer; SGZ: Subgranular Zone (Reproduced from Ming G.-Li et alia, *Neuron* 2011).

1.4.4 Regulation of adult neurogenesis

1.4.4.1 Neurogenic niche in the adult brain

Both intrinsic and extrinsic mechanisms regulate different aspects of adult neurogenesis. For a better understanding of these processes, it's necessary to define the concept of "niche". Niche, as suggested by Schonfield (Schonfield R., *Blood Cells*, 1978), is the microenvironment that anatomically houses stem cells and functionally

controls their development in vivo. In this specific case, the niches in which we can identify neurogenetic processes are the SGZ and SVZ. Endothelial cells, astrocytes, ependimal cells, microglia, mature neurons and progeny of adult neural precursors are among major cellular components of the adult neurogenic niche. Vascular cells play an important role in regulating proliferation of adult neural precursors: it's been discovered, indeed, that in SGZ, dense clusters of dividing cells were found to be anatomically close to the vasculature, especially capillaries.

Moreover, astrocytes form gap junctions and are closely associated with the vasculature and its basal lamina in the adult SGZ, likely modulating the influence of endothelial and circulation derived factors as well as the availability of cytokine and growth factors in the basal lamina. Microglia has, also, shown a prominent role in regulating adult neurogenesis. Under basal conditions, apoptotic corpses of newly generated neurons are rapidly phagocytosed from the niche by unactivated microglia in adult SGZ. Under inflammatory conditions, reactivated microglia can have both beneficial and damaging effects on different aspects of adult neurogenesis, depending on the balance between secreted molecules with pro- and anti-inflammatory action (Ming G.-L. et alia, *Neuron* 2011).

Moreover, it's been suggested that newborn cells progeny can regulate the behavior of neural precursors. In the SGZ, quiescent radial glia-like cells are rapidly activated to support continuous neurogenesis after eliminating rapidly proliferating progeny with AraC® (Cytarabine) treatment. In the SGZ, local interneurons release GABA, which in turn regulates cell proliferation as well as maturation, dendritic development and synaptic integration of new born neurons. On the other hand, glutamate regulates survival of newborn neurons, through an NMDAR-dependent mechanism (Ming G.-L. et alia, *Neuron* 2011).

1.4.4.2 MOLECULAR REGULATION OF NEUROGENESIS

A number of morphogenes, i.e., signaling molecules that acts directly on cells to produce specific cellular responses depending on their local concentration, serve as niche signals to regulate maintenance, activation and fate choice of adult neural precursors. Growth factors, neurotrophins, cytokines and hormones are major regulators of adult neurogenesis. Different phases of adult neurogenesis are subject to regulation by pharmacological manipulations, mostly through various neurotransmitters systems. The DG is enriched with inputs from many brain regions that release different neurotransmitters and neuropeptides. Among classic neurotransmitters, GABA, glutamate and acetylcholine directly regulates migration, maturation, integration and survival of newborn neurons, although it is not perfectly clear if pharmacological manipulations act by directly affecting neural precursor and newborn neurons, or through indirect modulation of the niche. In the DG, an important role is played by reelin. Reelin is a large secreted extracellular matrix glycoprotein that helps regulating processes of neuronal migration and positioning in the developing brain by controlling cell–cell interactions. Besides this important role in early development, reelin continues to work in the adult brain. It modulates synaptic plasticity by enhancing the induction and maintenance of long-term potentiation. It also stimulates dendrite and dendritic spine development and regulates the continuing migration of neuroblasts generated in adult neurogenesis. Specifically, reelin prevents new neurons from migrating into the hilus region (Ming G.-L. et alia, Neuron 2011).

At the intracellular level, cell-cycle regulators , transcription factors and epigenetic factors are major intracellular regulators of adult neurogenesis. Cell-cycle inhibitors like p16, p21 and p53 play major roles in maintaining the quiescence of adult neural precursors; deletion of these factors, indeed, leads to transient activation and subsequent depletion of the precursor pool. Sequential development of different transcription factors ensures proper development of adult neural precursors. One of

this transcription factor is Sox2, which plays a role as major mediator of Notch signaling in maintaining the precursor pool in the adult SGZ. Moreover, various epigenetic mechanisms play important roles in fine tuning and coordinating gene expression during adult neurogenesis, including DNA methylation, histone modifications and non-coding RNAs (Sun B. et alia, *Cell Stem Cell* 2009).

1.4.4.3 ENVIRONMENTAL REGULATION OF NEUROGENESIS

One hallmark of adult neurogenesis is its sensitivity to physiological and pathological stimuli at almost every stage, from proliferation of neural precursors to development, maturation, integration and survival of newborn neurons. Among the physiological stimuli that enhanced neurogenesis, it's been demonstrated that physical exercise promotes proliferation in SGZ, while an enriched environment promotes neuronal survival (Rossi D.J. et alia, *Cell* 2008). Also, it's been seen that learning modulates adult neurogenesis (Zhao C. et alia, *Cell*, 2008); even seizures increase cell proliferation in SGZ, but also lead to mis-migration of newborn neurons to the hilus, aberrant dendritic growth, mossy fibers recurrent connections and altered electrophysiological properties of GABAergic and glutamatergic synaptic inputs for newborn cells. (Kron M.M. et alia, J. Neurosci. 2010). Another potent inducer of adult neurogenesis is focal or global ischemia: stroke induces cell proliferation and migration of newborn neurons to infarct sites, the vast majority of which fail to survive over the long term, presumably due to the lack of functional connections and trophic support (Arvidsson A. et alia, *Nat. Med.* 2002). Conversely, physiological stimuli that decrease neurogenesis are: chronic stress, which lead to decreased cell proliferation in the adult SGZ, whereas the effect of acute stress on cell proliferation and new neuron survival depends on paradigms of stress and species (Mirescu C. et alia, *Hippocampus*, 2006). Also diabetes impairs cell proliferation in the adult SGZ through a glucocorticoid-mediated mechanism (Sun B. et alia, *Cell Stem Cell* 2009). Another major negative regulator of adult neurogenesis is inflammation,

induced by injuries, degenerative neurological diseases and irradiation (Carpentier P.A. et alia, *Neuron* 2009). It's clear, then, that the processes involved in neurogenesis are of very different types, but the better understanding of these mechanisms could lead to find new therapeutic strategies to enhance functional neurogenesis for regenerative medicine.

1.4.5 STUDYING NEUROGENESIS IN ADULT DG OF HIPPOCAMPUS

The current studies about neurogenesis have allowed to clarify in detail the series of steps by which neural stem cells and progenitors give rise to new neurons in the DG. However, the first true step ahead was done by Khun and his coworkers, who demonstrated that the thymidine analogue Bromo-deoxyuridine (BrdU) is a useful lineage tracer to investigate the state of proliferation in the DG of hippocampus (Khun H.G. et alia, *J. Neurosci.* 1996). Nowadays, the identification of the various subtype cells involved in neurogenesis can be performed employing different cellular markers, which permits to analyze the different stages of maturation of cells in DG.

1.4.5.1 BRDU

Two strategies can be used for examining cell proliferation: the first involves the treatment of the animals with a compound that labels mitotically active cells and later detecting the label; the second, instead, involves the detection of an endogenous protein that is only expressed in mitotically active cells. However, the downside of the first method is that it requires treatment who could lead to a potential alteration of proliferation. The downside of the second type of method, instead, is that it's possible to see only proliferation at a one time point.

Currently, the gold standard for investigating the state of proliferation in DG is the use of BrdU, a thymidine analogue that is stably and specifically incorporated into the DNA in place of thymidine during the DNA synthesis S-phase of the cell cycle. DNA that has incorporated BrdU can be detected using standard immunohistochemical methods after denaturing the DNA. BrdU immunostaining in the adult brain is specific for dividing cells, even when the level of DNA repair is dramatically increased; moreover, it remains specific even when BrdU id given at high doses (Christie B.R. et alia, *Hippocampus* 2006). The only flew of BrdU, although it marks the mitotic active cells, is that it doesn't tell anything about the cells which are stained. Fortunately, there are various markers that can give information about the phenotype of the cells that are labelled. In this thesis, I focused especially on two of them, in addition to BrdU: Doublecortin (DCX), to study migrating neuroblasts, and Neuronal Nuclear antigen marker (NeuN), to identify the mature neurons. I also examined the status of inhibitory circuitry in the hilus using staining for Neuropeptide Y (NPY), which marks a subpopulation of GABAergic inhibitory interneurons.

1.4.5.2 DOUBLECORTIN

Doblecortin (Dcx) is a microtubule-associated protein: it is expressed by migrating neuroblasts (from about 4 days to about 28 days after cell birth). Though a microtubule-associated protein, it regulates filamentous actin structure in developing neurons, also regulating the microtubule of axonal growth cones. In the dentate gyrus of the developing hippocampus, Dcx is expressed in cells with begin their dendritic growth, but diminishes when the neurons mature. Dcx also promotes the migration of human progenitor cell-derived neurons in the DG. Dcx is expressed early in neuroepithelial cells from their first expression of neuronal lineage and during migration (Christie B.R. et alia, *Hippocampus* 2006).

1.4.5.3 NEURONAL NUCLEAR ANTIGEN

NeuN is a low molecular weight (46-48 kDa) nucleoprotein. It's recognized by a specific antibody and may be demonstrated immunohistochemically in most, but not all, neurons of the central and peripheral nervous system in humans. NeuN is now identified as *Fox3,* a new member of the Fox1 gene family and regulatory splicing factors. Glial and ependimal cells are non-reactive for NeuN. NeuN is a particularly valuable marker because of its late expression in neuronal maturation. In the hippocampus, both granule cells of the DG and pyramidal cells of Ammon's horn are labeled by NeuN as they mature (Christie B.R. et alia, *Hippocampus* 2006).

1.4.5.4 NEUROPEPTIDE Y

Subgroups of hippocampal interneurons are characterized by the expression of NPY. NPY is a 36-amino acid neuropeptide that acts as a neurotransmitter in the brain and in the autonomic nervous system of humans: it is contained in at least four types of GABAergic interneurons in the dentate gyrus, many of which also contain somatostatin, and give rise to the dense NPY innervation of the dentate outer molecular layer. Y1 and Y2 receptors are the most abundant NPY receptors expressed in the dentate gyrus. Y1 receptors are postsynaptic receptors, primarily located on granule cell dendrites in the molecular layer and some interneurons, while Y2 receptors are presynaptic receptors mediating inhibition of glutamate release, and potentially that of NPY and GABA depending on their presynaptic localization, and may also be expressed on some hilar interneurons (Sarnat H.B., *Clin. Neuropath.* 2013).

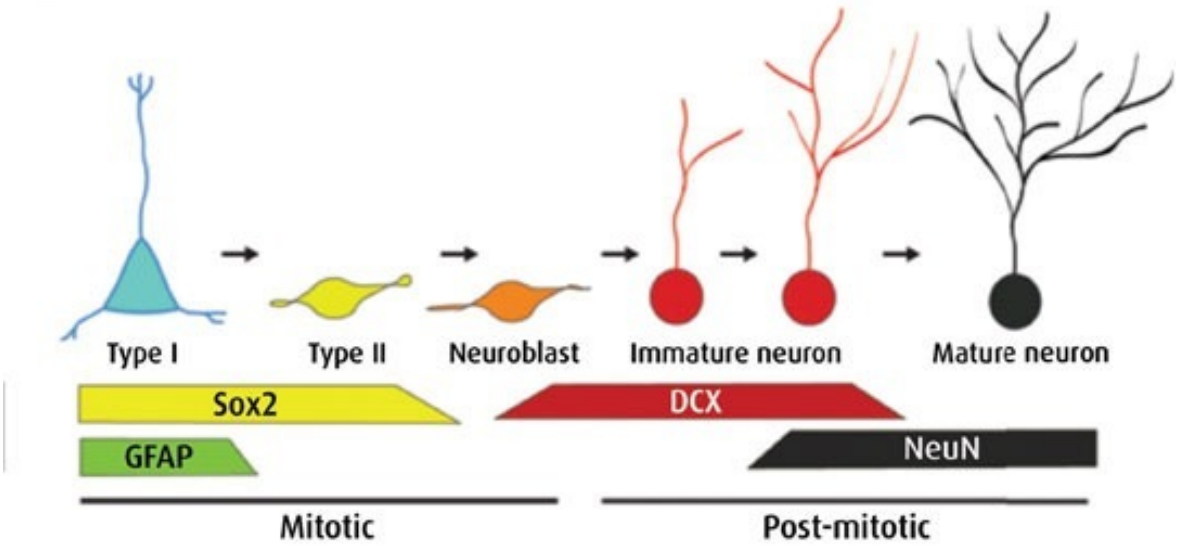


Fig. 7: Scheme of adult neurogenesis in the hippocampal DG. (A) During the mitotic phase, two types of progenitors proliferate in the subgranular zone (SGZ) of the DG: radial glia-like progenitors (type I cells, expressing Nestin, GFAP and Sox2) and amplifying progenitors (type II cells, expressing Sox2 only). Before becoming post-mitotic, the progenitors undergo a short intermediate stage (neuroblasts) during which they become committed to the neuronal fate and begin expressing doublecortin (DCX). In the post-mitotic phase, DCX-positive newborn neurons derived from neuroblasts undergo morphological and physiological maturation with the final expression of the mature neuron marker, NeuN (Reproduce from: Gasparini L. et alia, *ACNR* 2013).

1.5 AIM OF THE THESIS

The goal of this thesis is to evaluate alterations in adult neurogenesis in a mouse model of ID characterized by loss of function of Ophn-1. XLID is a complex syndrome that implies genetic, metabolic and environmental factors, and there is currently no real cure for it. From a basic science point of view, the role of Oligophrenin-1 in the brain is not clear and it remains to be seen how mutations in Ophn-1 affect neuronal development and function and contribute to ID. In this context, adult hippocampal neurogenesis allows to study in detail different aspects of neuronal development, including proliferation, migration, elaboration of dendrites and axons, and integration into the neuronal network. All of these steps are essential for normal function of the newborn neurons. Indeed distortions in neural circuit formation are associated to altered information processing, which is likely to be responsible of the cognitive impairment present in ID.

Understanding the molecular mechanisms underpinning XLID is crucial to develop any future therapeutic strategies. In particular, in this thesis I have tested a possible therapeutic strategy for rescuing specific deficits in adult neurogenesis, based on fasudil-mediated inhibition of ROCK signalling, a major pathway set in motion by loss of Ophn-1.

2 - MATERIALS AND METHODS

2.1 ANIMALS AND EXPERIMENTAL PROTOCOL

Every effort was made to minimize the number of animals and their suffering. The experimental design and all procedures were in accordance with the guidelines of Italian Ministry of Health for the care and use of laboratory animals. *Ophn-1*$^{-/y}$ mice and wild-type littermates were generated by breeding heterozygote females (*Ophn-1*$^{+/-}$) with wild-type males (*Ophn-1*$^{+/y}$), resulting in ~50:50 *Ophn-1*$^{+/y}$:*Ophn-1*$^{-/y}$. Mice were housed in an animal room with a 12-hour/12 hour light/dark cycle, with food and water available *ad libitum*. A total of 20 male animals were used in the experiment. At post-natal day (p) 60, animals were divided into three experimental group:

- 9 animals *Ophn-1* $^{+/y}$ wild type animals (*Control Group*)used as controls
- 7 animals *Ophn-1* $^{-/y}$ without drug treatment (*Sham Group*)
- 4 animals *Ophn-1*$^{-/y}$ treated with fasudil (*Rescue Group*)

The three groups of animals were housed in standard conditions as above; the *Sham Group* was treated with Fasudil (0,64 mg/ml, in H_2O) for 3 weeks in drinking water. The solution was renewed every week. Mice of *Control Group* and *Sham group* were given the same amount of water per animal and the average of water consumed was constantly monitored to ensure that the presence of the drug had no impact on water intake. In order to label newborn neurons in the Dentate Gyrus (DG) of the Hippocampus, all mice received, between p60 and p70, in one day, 4 intraperitoneal injections of Bromo-deoxyudridine (BrdU,50 mg/kg) spaced by two hours. Mice were sacrificed by perfusion at 1 day (to assess proliferation), and 15 and 50 days after BrdU (to evaluate incorporation of new neurons in the adult DG).

2.2 GENOTYPING

The genotype of mice was determined by PCR analysis on small samples of tail tissue taken from pups at P7. Samples were incubated overnight in 0.5 mL of Lysis Buffer and 4 μL of 20 mg/mL Proteinase K at 55 °C.

Then, samples were centrifuged at 13000 rpm for 10 min to pellet undigested hair and obtain the supernatant. After that, 0.5 ml of isopropanol was added, inverting the tubes a few times to reveal the denatured DNA: so, samples were centrifuged at 13000 rpm for 10 min at 4 °C. After eliminating the supernatant, pellets were washed with 200 μL of 70% ethanol; then, samples were centrifuged again at 12000 rpm for 5 min at room temperature (RT). The DNA pellet was finally resuspended in 300 μL of TRIS-EDTA buffer (TE).

The genotype of all 11 mice were revealed through PCR procedure: all samples of DNA (4 μL) were added with 1 μL of primers Forward (F; 5'-GCCCATGTTGTGAGCAGAGAAATC) and Reverse (R; 5'-GGAAGCTAGAGGATGACCCTG) (Sigma Aldrich), 6.5 μL of distilled water (Sigma) and 12,5 μL of Ready Mix (Sigma) for a total volume of 25 μL, with the following protocol:

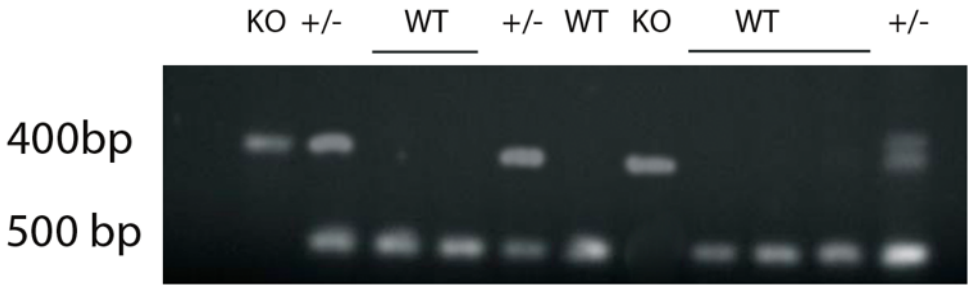
- A first denaturation phase at 95 °C for 3 minutes.
- 35 cycles of amplification, composed by 3 steps at 94 °C, 55 °C and 72 °C, 30 seconds each.
- A final step of incubation at 72 °C for 10 minutes

After amplification, all samples were made run in agarose gel (1.5%), with 1:10.000 Ethidium bromide(Sigma), in TRIS-Borate-EDTA buffer (TBE, Sigma) for 1h at 110 V in Biorad apparatus.

Analysis of the results was performed using Chemidoc XRS System (Biorad), that allows to visualize the bands of our interest by UV illumination:

- *Ophn-1*$^{+/y}$ animals have a 400 bp band
- *Ophn-1*$^{-/y}$ animals have a 500 bp band

• *Ophn-1*$^{+/-}$ animals have both strips



2.3 *Fixative preparation*

Paraformaldehyde solution (4% in distilled water) was used to fix brain tissues. Stock solution at 8% was prepared by dissolving the opportune weight of paraformaldehyde powder in distilled water under a chemical hood. Solubilization was favored by heating the solution between 50 - 55 °C, while stirring. To end the procedure, solution were clarified by adding NaOH 10N (2.5 drops every 100 mL). The clarified solution was cooled at RT and preserved at 4 °C.
The stock solution was, then, diluted at 4% with TBS at pH 7.4

2.4 *Brain preparation*

Two weeks after BrdU injections, animals were sacrificed to extract brain tissues after intracardiac perfusion with Paraformaldehyde 4% solution. This process allows to fix brain tissues, preserving cells integrity and morphology by a prompt arrest of autolysis processes and the conferral of structural stability to the chemical components of the cytoplasm. Briefly, mice were deeply anesthetized with overdose

of chloral hydrate (10.5%, in saline); the deep state of anesthesia was assessed by verifying the absence of reflexes after pinching the tail and the digit of the animal. Then, mice were positioned on a surgery table under chemical hood and a rapid thoracotomy was performed to expose the heart.

After that, the decendent aorta was clamped with hemostatic pincers, to restrict passage of the fixative to the superior circulation. A sharpened needle was connected to a peristaltic pump, regulated on a flux speed of 40 mL/min, and inserted into the left ventricle to allow passage of the fixative. The right atrium was pinched to allow flowing out of the fixative solution.

Before the fixative administration , blood vessels were washed with phosphate Buffer Saline (PBS, 0.1 M) until the all blood was completely removed. Finally, at least 50 mL of paraformaldehyde at 4% was administrated, permitting the fixation of tissues. At the end of the process, the skull was carefully opened to expose the whole dorsal face of the brain. After assessing the good result of the perfusion by evaluating the white aspect of tissues and the absence of clots, the entire brain was gently removed with a surgical spoon. Finally, isolated brains were post-fixed for 2 hr. Brains were frozen and coronal sections (40 microns) were cut using a freezing microtome. Sections were maintained in a serial order, and kept in PBS solution at 4°C.

2.5 IMMUNOHISTOCHEMISTRY

One of every six sections were selected to perform immunohistochemical analysis.

For double immunostaining with Doublecortin (Dcx) and NeuN, free-floating sections were blocked for 1h at RT with 10% rabbit serum (RS), 0.3% Triton X-100 in PBS. Slices were, then, incubated overnight at 4 °C with anti-Dcx and anti-Neun primary antibodies (respectively goat polyclonal, 1:1000, Sigma and guinea pig, 1:1000, Millipore) 2% RS and 0.2% Triton in PBS. The primary antibodies were

revealed by incubation for 2h at RT with secondary antibodies (respectively, donkey anti-goat Rodamine Red X (RRX), 1:200, Jackson and Donkey anti- guinea pig Alexafluor 488, 1:300, Jackson) diluted in 1% RS and 0.1% Triton in PBS.

For BrdU staining, free-floating sections were initially treated for 30' at 37 °C in HCl 2 M to denature DNA. Then, sections were blocked for 30' at RT with 10% normal goat serum (NGS), 0.3% Triton in PBS. Slices were incubated overnight at 4 °C with anti-BrdU primary antibody (rat monoclonal, 1:200, Abcam), NGS 5%, Triton 0.1% in PBS. The primary antibody was revealed by incubation for 2h at RT with secondary antibody (Donkey anti rat Cy3, 1:500, Jackson), diluted in Triton 0.1% in PBS.

For Neuropeptide Y (NPY) labeling, we blocked the specific sites with 10% NGS, 0.3% Triton in PBS. Free-floating were, then, incubated overnight at 4 °C with anti-NPY primary antibody (rabbit polyclonal, 1:5000, Peninsula), 2% NGS and 0.1% Triton in PBS. To reveal the primary antibody, we incubated the slices with Donkey anti-rabbit RRX (1:400, Jackson) with 1% NGS and 0.1% Triton in PBS. For Hoechst staining, slices were submerged in an aqueous solution with 3 mL of Hoechst stain for three minutes.

2.6 STEREOLOGICAL QUANTIFICATION

All cells were counted in blind conditions with respect to the treatment using a fluorescence microscope and Stereoinvestigator software (MicroBrightField): a 40x objective (air, 0.75 NA) was used for Dcx and Dcx/NeuN positive cells, while a 20x objective (air, 0.50 NA) was chosen for BrdU and NPY positive cells.

The counting of stained cells was performed using every six sections spaced 300 μm apart, counting 10 to 11 sections per mouse. To investigate the number of progenitor cell-derived neurons, or Dcx labelled cells, every label cells within DG was counted: during this phase, the contour of all somas was traced.

Then, to clarify the numbers of immature neurons which were on the verge to become mature, or Dcx/NeuN positive cells, all the cells whose soma matched the contour previously described were counted. Eventually, the percentage between the total number of Dcx positive cells and Dcx/NeuN positive cells for every section was calculated. In order to find out the effective number of proliferating cells, or BrdU positive cells, all the labelled cells in the DG were counted. To analyze the number of inhibitory interneurons or NPY positive cells, all the stained cells in the hilus of DG were quantified. Finally, to investigate the morphology of the DG and hilus, a stereological volume measure was performed. The measure was performed by contouring the DG and the hilus of every slice of each group.

The stereological count for each label cells was performed summing all positive cells per animal and multiplying it for 6 (spacing factor). Finally, all representative images hippocampal sections were acquired with a Leica confocal microscopy using a 25x objective (oil, 0.75 NA) and a 40x objective (oil, 1.25 NA).

2.7 STATISTICAL ANALYSIS

Statistical analysis was performed using Sigmaplot 11.0 software (Systat Software Inc. Chicago, IL), considering the value of significance at $p = 0.05$. Data normally distributed are summarized by mean * SEM. T-test and one way test ANOVA were used for statistic analyses. We used Student's t-test pairwise comparison of quantitative phenotypes between mice of different groups. Moreover, we performed One way test ANOVA to analyze the variance among control, sham and rescue group.

3- RESULTS

3.1 VOLUME OF THE DENTATE GYRUS AND HILUS OF THE HIPPOCAMPUS

To evaluate the possible macroscopic changes in the DG morphology, a stereological measure of the volume of the blades and hilus of DG in *Ophn-1*$^{-/y}$ animals was performed. The data indicate no significant changes in *Ophn-1*$^{-/y}$ animals with respect to control (*Ophn-1*$^{+/y}$ DG volume= 0.31 mm^3 , hilus volume= 0.30 mm^3, n= 5; *Ophn-1*$^{-/y}$ DG volume= 0.24 mm^3, hilus volume= 0.21 mm^3, n= 5. Student's t-test, Two tailed p value > 0.05 *fig.1a-1b*).

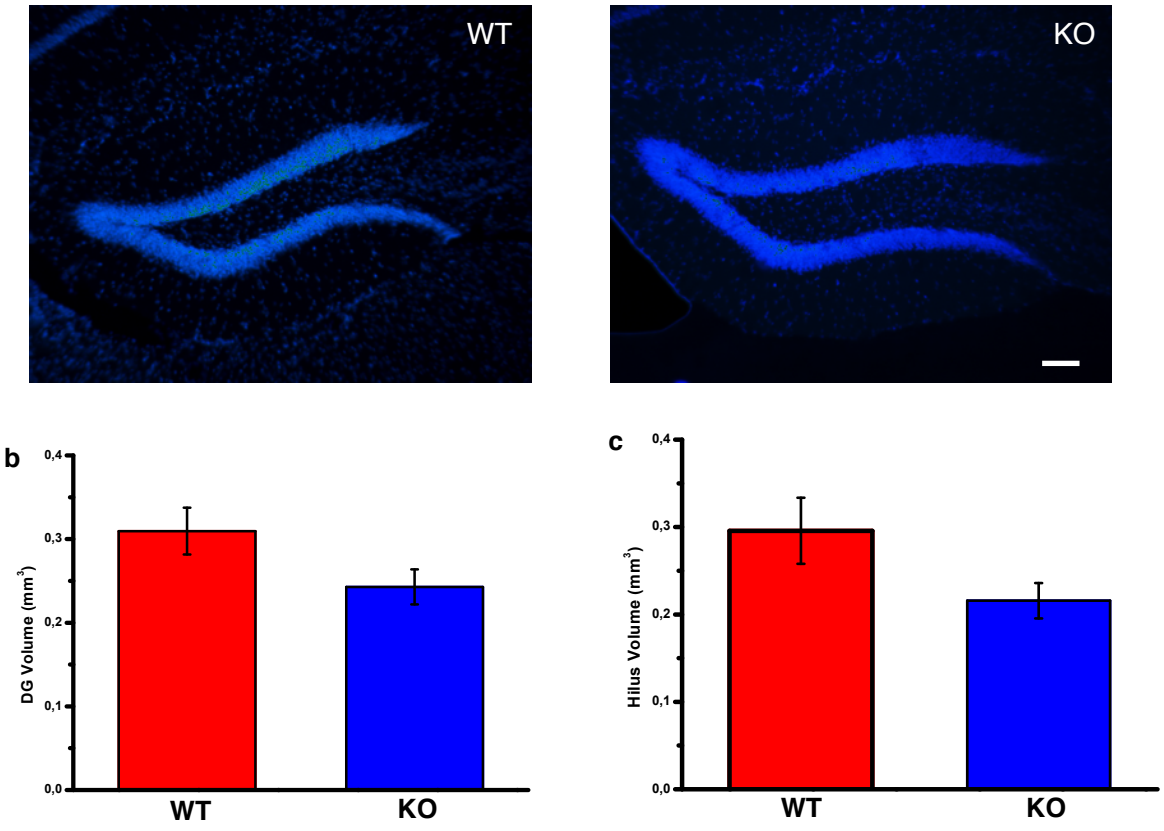


Fig. 1: Morphological analysis of the DG: a) Representative images of WT and KO DG . b) Volume of the DG. The results show that there is no significant difference between KO and WT animals. c) Volume of the hilus of the DG. The data show no significant difference between KO and WT mice. Histograms represent mean ± SEM.

3.2 PROLIFERATION IN THE DENTATE GYRUS OF HIPPOCAMPUS

In order to evaluate the state of proliferative cells in the DG of hippocampus in Ophn-1 KO mice, we used BrdU to label the mitotic cells, and the animals were sacrificed at 1d. To assess the number of newly generated neurons, additional animals were examined 15d and 50d post BrdU injection. Slices of one hemisphere were analyzed for each animal. Then, a stereological count was performed in order to identify the total number of BrdU positive cells in the DG of hippocampus. The results showed that no significant differences between *Ophn-1*$^{-/y}$ and *Ophn-1*$^{+/y}$ were found at day 1 (*Ophn-1*$^{+/y}$ total number of cells= 1517, n= 4; *Ophn-1*$^{-/y}$ total number of cells= 1111, n= 4. Two tailed and one tailed p value > 0.05 *fig.2a*) and 15d post-injections (*Ophn-1*$^{+/y}$ total number of cells= 1281, n=4; *Ophn-1*$^{-/y}$ total number of cells= 968, n=3. One tailed p value <0.05; Two tailed p value > 0.05 *fig.2b*). Instead, analyzing the slices 50d post-injections, we found out that there was a strong decrease of BrdU positive cells in KO mice (*Ophn-1*$^{+/y}$ total number of cells= 1315, n= 5; *Ophn-1*$^{-/y}$ total number of cells= 648, n= 5. Mann-Whitney sum test p value < 0.05 *fig.2c*). These data allow to conclude that loss of Ophn-1 has no impact on precursor proliferation. However, reduced numbers of BrdU-positive cells are evident in the long-term, likely as a result of impaired migration/maturation/network integration of the newborn neurons.

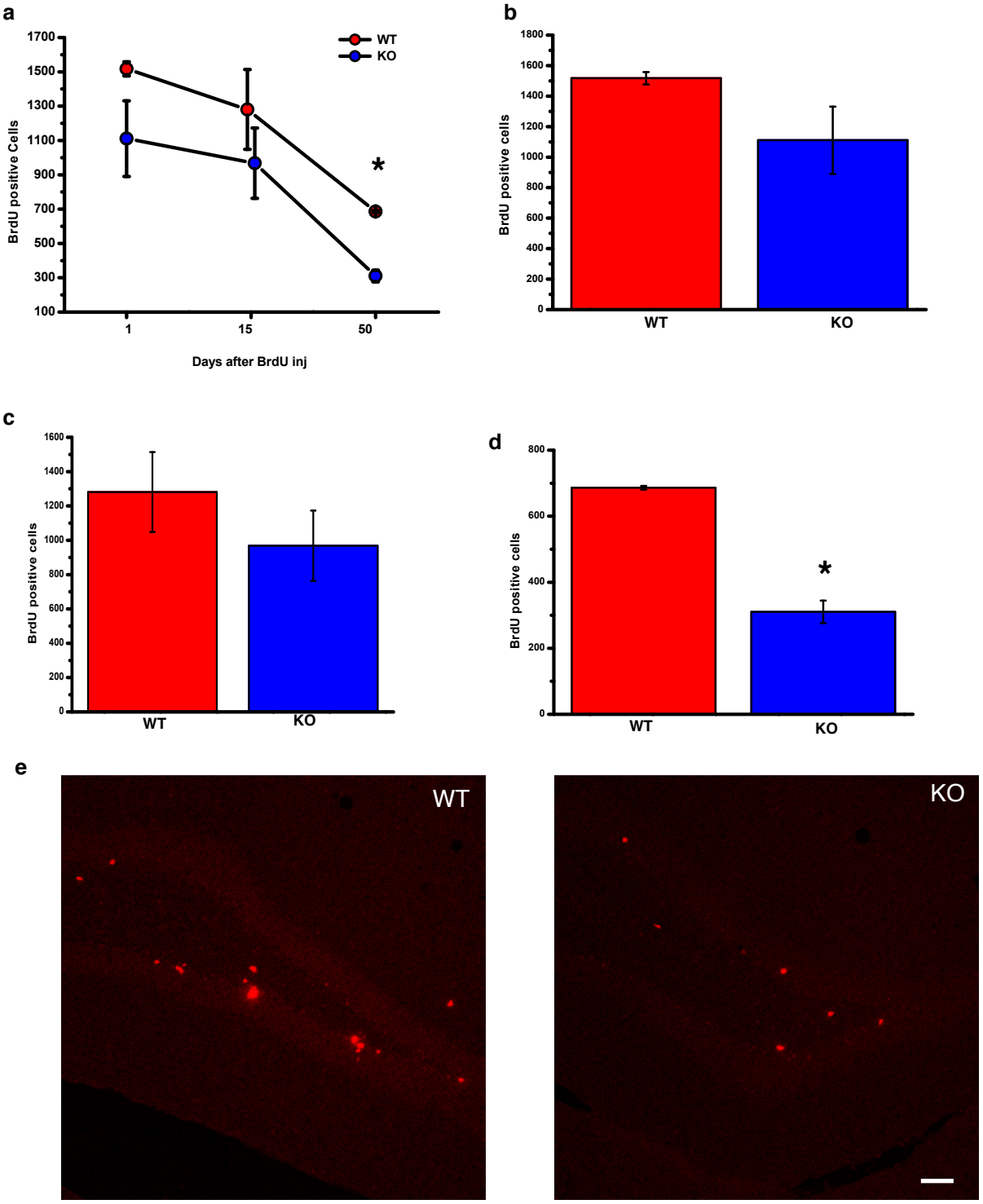


Fig.2: State of proliferative cells in the DG of hippocampus. *a)* Time course of the state of proliferation in the DG at 1d, 15d and 50d post injections: it's clear that the decrease of BrdU positive cells is grater in *Ophn-1*$^{-/y}$ animals. *b) c) and d)* The data of BrdU positive cells in the DG, respectively at day 1, 15d and 50d post injections, showing that *Ophn-1*$^{-/y}$ mice, at 1d and 15d, present no differences respect to controls, indicating that, at this time, no impairments can be detected. Instead, at 50d post injection,there is a drastic decrease in *Ophn-1*$^{-/y}$ mice, evidencing that, at this point, there is a greater impairment between the two groups. Histograms represent mean ± SEM. *e)* Confocal images, showing the BrdU positive cells at 50d post injection. Scale bar: 100 μm. Statistical significance, *P< 0.05.

3.3 State of the neurogenesis in the Dentate Gyrus of hippocampus

In order to investigate the migration and integration of newborn neurons in the DG of hippocampus in Ophn-1 KO mice different markers were used, to identify possible deficits at distinct steps of the maturation process. Therefore, Dcx positive cells and cells double stained cells with Dcx and NeuN were analyzed (*fig.3*). Moreover, thanks to Marco Canossa and his team, axon elongation by the newborn neurons was investigated.

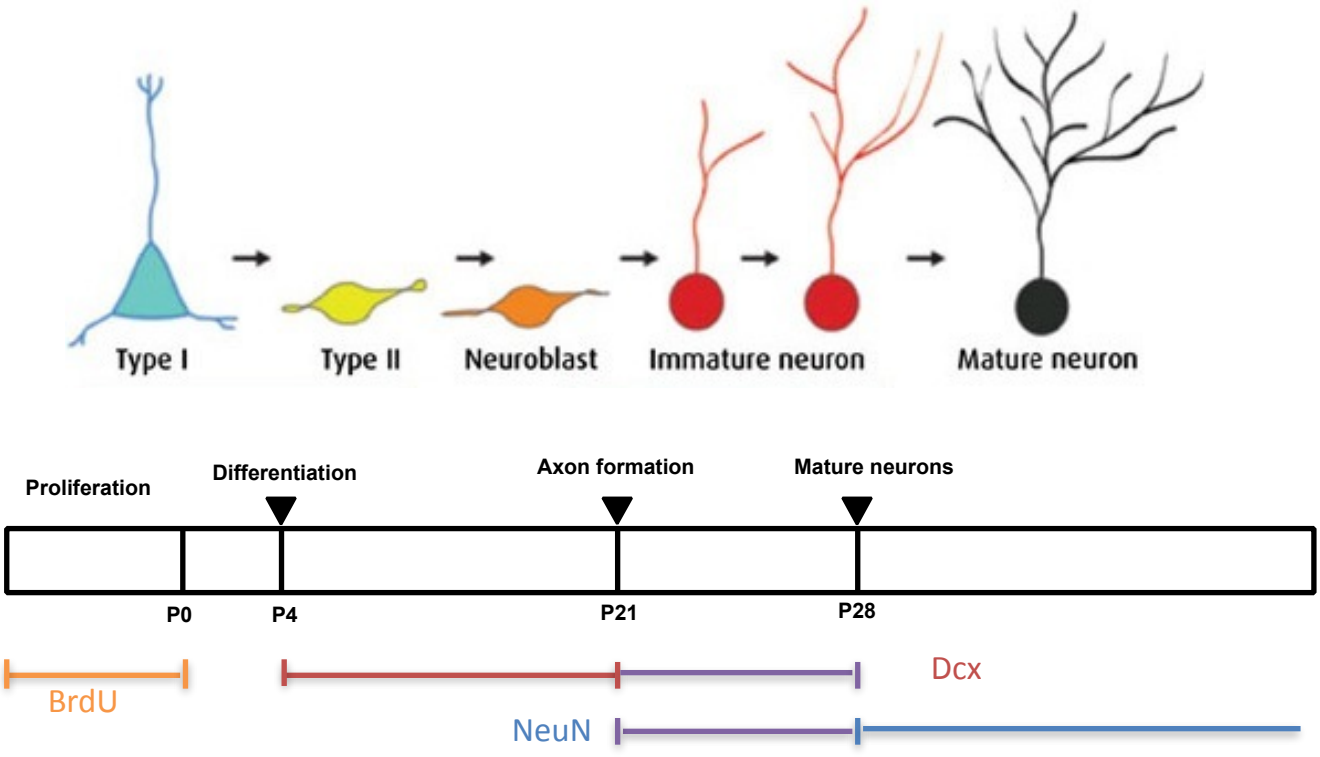


Fig. 3: Scheme of the timing of maturation. After the proliferative phase, at p4, the cells start their process of differentiation. At this point, they begin to express the Doublecortin (Dcx) protein. At p21, the maturating cells start forming the axon, and they begin to express the Neuron nuclear protein (NeuN). At p28, we have the complete formation of new neurons.

3.3.1 State of the migratory cells in Dentate Gyrus of hippocampus

To verify the state of migratory cells in DG of Ophn-1 KO mice, we used Dcx as a marker. The animals were sacrificed at p75, and the brain slices of one hemisphere were stained with Dcx antigen. Then, a stereological analysis was performed to count the total number of Dcx positive cells in *Ophn-1*$^{+/y}$ and *Ophn-1*$^{-/y}$ animals. The data clearly indicated a substantial reduction of Dcx-positive neuroblasts in KO animals (*Ophn-1*$^{+/y}$ total number of cells= 7531, n=9; *Ophn-1*$^{-/y}$ total number of cells= 5144, n=7. One tailed and two tailed p value <0.001 *fig.4*).

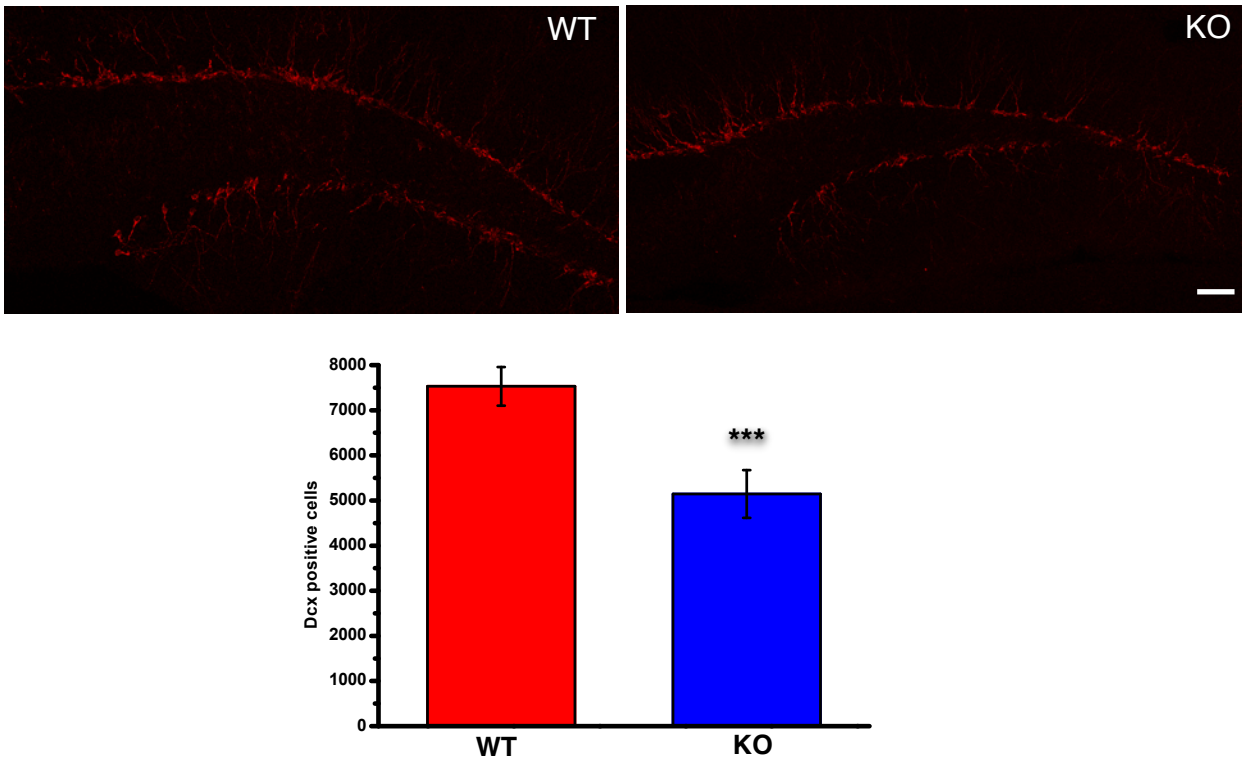


Fig. 4: Number of migrating neuroblasts. It is evident that *Ophn-1*$^{-/y}$ animals display a reduction in Dcx-positive cells. The confocal images report examples of Dcx labeling in KO and wt mice. Scale bar: 80 μm. Histograms represent mean ± SEM. Statistical significance, ***P< 0.001.

3.3.2 Axonal growth by newborn cells in the Dentate Gyrus of hippocampus

We are currently collaborating with Marco Canossa and his team (University of Bologna) to investigate axonal growth and morphological maturation of newly generated neurons in the hippocampus of Ophn-1 KO mice and controls. Specifically, this group has injected into the hippocampus retroviral vectors expressing GFP to label proliferating cells and has assesses axon formation at 21 days. They found that while about 90% of the cells in wt animals have extended an axon at 21 days, this percentage is dramatically reduced in KO mice (about 60%; t-test, $p < 0.05$) (*Ophn-1*$^{+/y}$ cells: with axon= 90.62%; *Ophn-1*$^{-/y}$ cells: with axon= 56.81 T-test p value < 0.05. Thanks to Beatrice Vignoli and Marco Canossa for the results. *fig.5*).

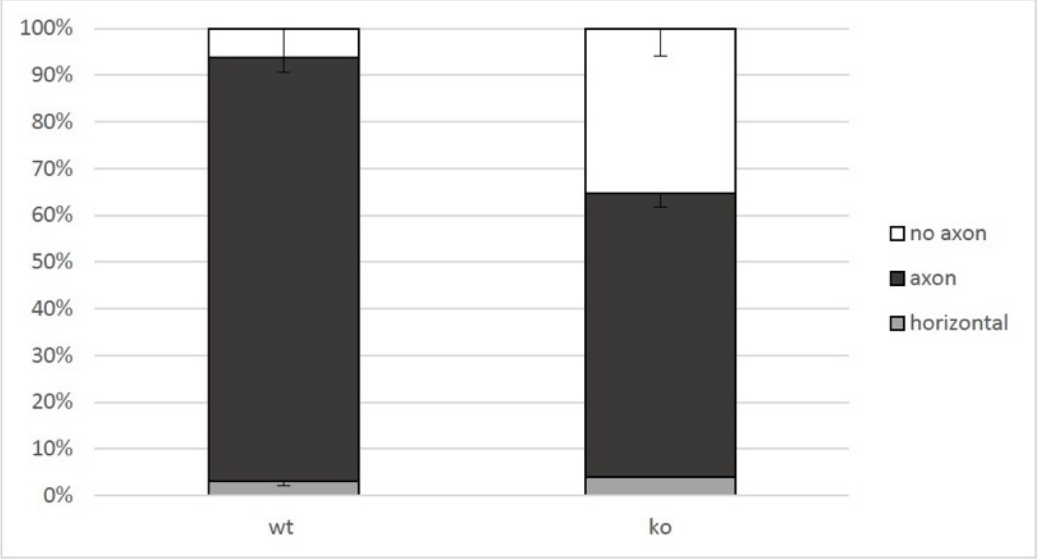


Fig.5: Percentage of the cells which produce a functional axon. It's clear that KO mice present a significant impairment respect to WT animals (Thanks to Beatrice Vignoli and Marco Canossa, University of Bologna).

3.3.3 STATE OF NEWBORN NEURONS IN THE DENTATE GYRUS OF HIPPOCAMPUS

The data described in the previous sections demonstrate a reduction in the number of migrating neuroblasts and a deficit in axon formation by the Ophn-1 KO neurons at 21 days after birth. Lack of the axon is predicted to impede functional integration of the newly generated cells, that could eventually be eliminated via programmed cell death. Accordingly, we quantified the total numbers of early neuronal cells (21-28 days after birth) via Dcx-NeuN double labelling (*fig.6a*). The results indicate that *Ophn-1*$^{-/y}$ animals have a decreased number of doubled marked cells respect to the controls (*Ophn-1*$^{+/y}$ total number of cells= 1161, n= 4; *Ophn-1*$^{-/y}$ total number of cells= 678, n= 3; One tailed and two tailed test p value < 0.01 *fig. 6b*).

After that, the percentage of newborn neurons cells respect to Dcx cells were calculated. The data shows that there is a significant decrease in the percentage of newborn neurons in *Ophn-1*$^{-/y}$ animals respect to *Ophn-1*$^{+/y}$ animals (*Ophn-1*$^{+/y}$ newborn neurons percentage = 17,34%, n= 4; *Ophn-1*$^{-/y}$ newborn neurons percentage= 13,44%, n= 3; One tailed and two tailed test p value < 0.001 *fig. 6c*).

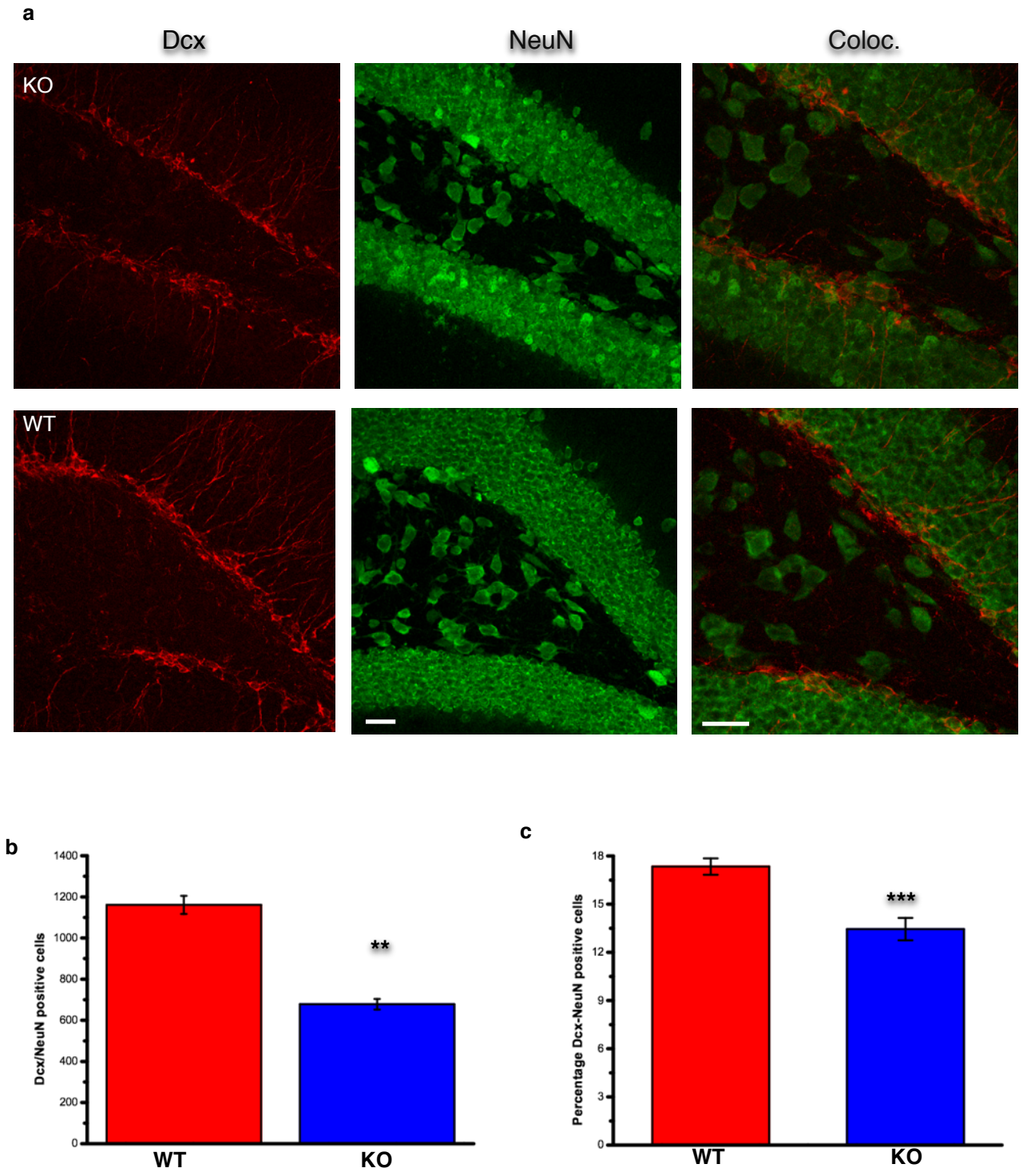


Fig 6: State of newborn neurons in the DG of hippocampus. ***a*)Confocal images that report the state of newborn neurons: (First line: WT. Second Line: KO). Scale bars: 100 μm. *b*) Note the reduction in the total number of Dcx-NeuN double positive cells in KO mice. *c*) Percentage of double stained cells respect to the total number of Dcx cells. The results indicate a decrease of this proportion in *Oplm-1*$^{-/y}$ animals. Histograms represent mean ± SEM. Statistical significance, **P<0.01,***P< 0.001.**

3.4 RESCUE OF THE PHENOTYPE VIA SYSTEMIC FASUDIL ADMINISTRATION

We next treated a group of *Ophn-1*$^{-/y}$ mice with the ROCK inhibitor fasudil, to check whether pharmacological blockade of the hyperactivation of the GTPases could lead to a rescue of the phenotype. In particular, we investigate if fasudil rescued the impairments seen in total number of migrating neuroblasts (Dcx positive cells) and early neuronal cells (Dcx/NeuN positive cells).

3.4.1 NO RESCUE OF MIGRATION DEFECTS BY FASUDIL IN *OPHN-1*$^{-/Y}$ MICE

Analyses of the total number of migrating neuroblasts in *Ophn-1*$^{-/y}$ mice treated with fasudil were performed, comparing the obtained results with the data seen above. This test showed that there was no significant difference in the rescue group with respect to *Ophn-1*$^{-/y}$ mice treated with water. Indeed, the total number of migrating neuroblasts in Ophn-1 KO mice treated with fasudil remained significantly lower than that of WT controls (*Ophn-1*$^{+/y}$ total number of cells= 7531, n=9; *Ophn-1*$^{-/y}$ (H_2O) total number of cells= 5144, n=7; *Ophn-1*$^{-/y}$ (fasudil) total number of cells = 5475, n=4. One way test ANOVA p value < 0,001; WT vs. KO (fasudil) t-test p value < 0.05, KO(H_2O) vs. KO(fasudil) t-test p value > 0.05 *fig.7a-7b*).

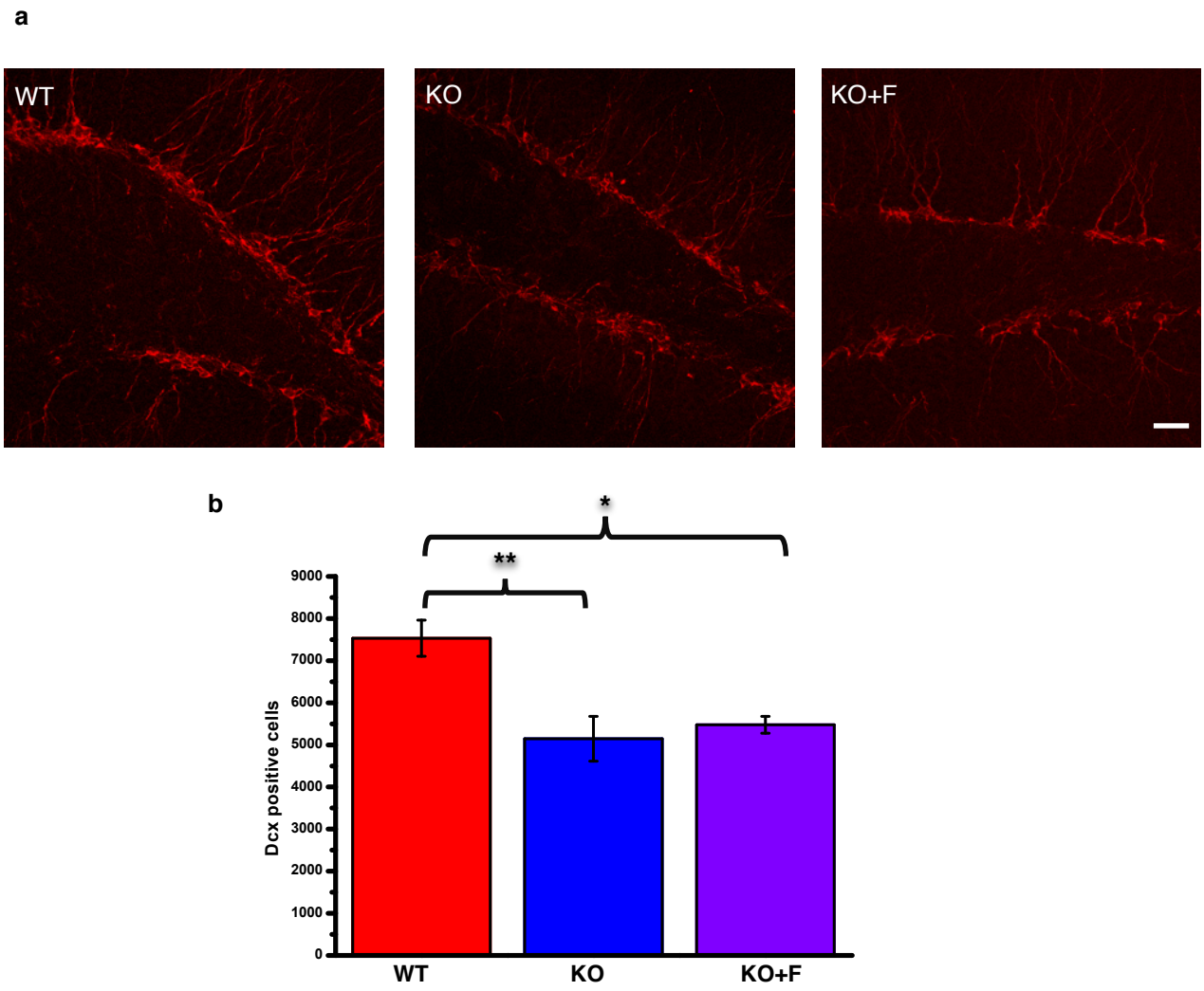


Fig 7: Rescue of the migratory cells phenotype. a) Confocal image showing the state of the migratory cells in KO mice treated with fasudil. Scale bar: 100 µm. b) The diagram shows that there is no significant increase in the total number of migratory cells in the rescue group respect to KO mice. Histograms represent mean ± SEM. Statistical significance, **P<0.05,P< 0.01.**

3.4.2 RESCUE OF THE EARLY NEURONAL CELLS IN *OPHN-1*$^{-/Y}$ MICE

The same fasudil treatment was also used to evaluate if fasudil could lead to a rescue in the population of newborn neurons and their percentage with respect to the total number of migrating cells (*fig.8a*). The data showed that there was a significant rescue in numbers of early neuronal cells (Dcx-NeuN double positive) in *Ophn-1*$^{-/y}$ mice treated with fasudil respect to *Ophn-1*$^{-/y}$ mice treated with water (*Ophn-1*$^{+/y}$ total number of cells= 1161, n=4; *Ophn-1*$^{-/y}_{(H2O)}$ total number of cells= 678, n=7; *Ophn-1*$^{-/}$

$^{y}_{(fasudil)}$ total number of cells = 930, n=4. One way test ANOVA p value < 0,001; *Ophn-1*$^{+/y}$ vs. *Ophn-1*$^{-/y}_{(fasudil)}$ t-test p value > 0.05, *Ophn-1*$^{-/y}_{(H2O)}$ vs. *Ophn-1*$^{-/y}_{(fasudil)}$ t-test p value < 0.05 *fig.8b*.). Interestingly, the percentage of Dcx-NeuN double labelled cells over the total sample of Dcx-stained cells was also rescued by fasudil (*Ophn-1*$^{+/y}$ newborn neurons percentage= 17.34%, n= 4; *Ophn-1*$^{-/y}_{(H2O)}$ newborn neurons percentage= 13.44%, n= 3; *Ophn-1*$^{-/y}_{(fasudil)}$ newborn neurons percentage= 17.1%. One way test ANOVA p value < 0,001; *Ophn-1*$^{+/y}$ vs. *Ophn-1*$^{-/y}_{(fasudil)}$ t-test p value > 0.05, *Ophn-1*$^{-/y}_{(H2O)}$ vs. *Ophn-1*$^{-/y}_{(fasudil)}$ t-test p value < 0.001 *fig. 8c*).

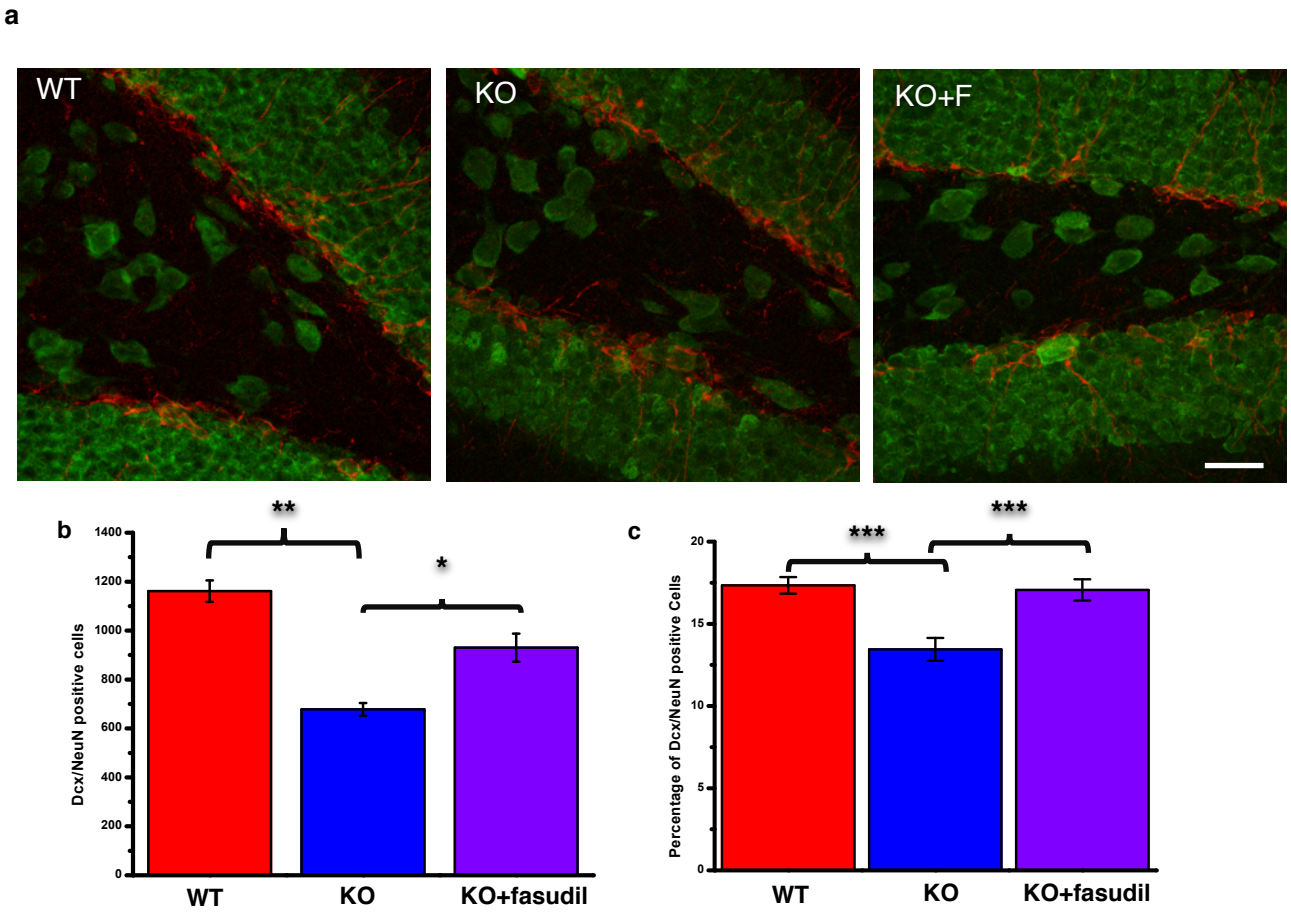


Fig. 8: Rescue of newborn neurons with fasudil. a) Confocal images showing the state of newborn neurons in KO mice treated with fasudil. Scale bars: 100 µm. b) Stereological count of newborn neurons, double marked with Dcx and NeuN. The data show a significant increase of double stained cells in *Ophn-1*$^{-/y}$ mice treated with fasudil. c) Percentage of newborn neurons. It's evident that in *Ophn-1*$^{-/y}$ mice the percentage is similar to WT animals. Histograms represent mean ± SEM. Statistical significance, *P<0.05,*P< 0.001.**

3.5 State of NPY inhibitory interneurons in the Dentate Gyrus of hippocampus

According to several studies (Ring H. ACNR, 2013; Morgan C.L. et alia, Am. J. Ment. Retard. 2003), a significant percentage of patients with ID suffer from epilepsy. These data suggest an impaired excitation/inhibition ratio in Ophn-1 mice. As a first step towards the characterization of inhibitory neurons in ID, we stained hippocampal sections from wt and KO mice with an anti-NPY antibody, and the number of NPY interneurons was quantified. The results indicate that *Ophn-1*$^{-/y}$ animals showed a reduced number of interneurons in the hilus of the DG respect to the *Ophn-1*$^{+/y}$ animals (*Ophn-1*$^{+/y}$ total number of interneurons= 1534, n=4; *Ophn-1*$^{-/y}$ total number of interneurons= 1286, n=3. Two tailed p value < 0.05, one tailed p value < 0.01 *fig.9*).

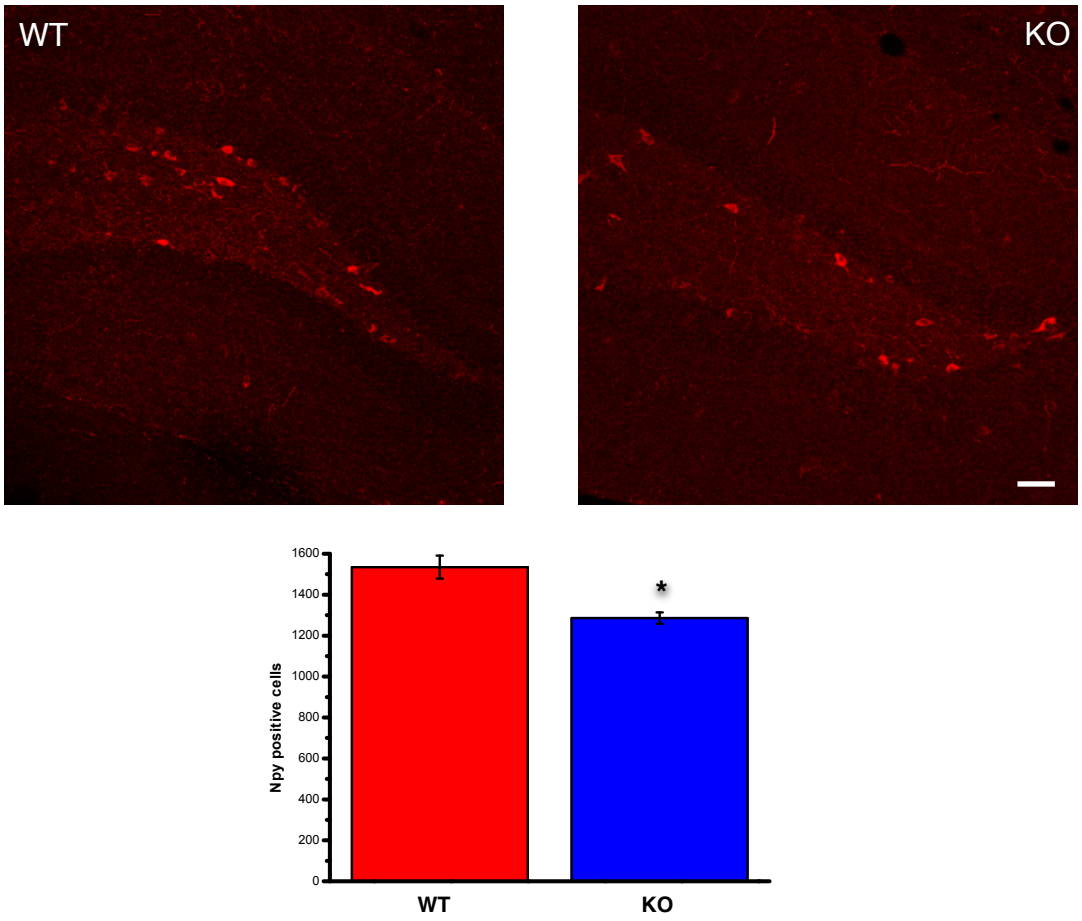


Fig 9: State of inhibitory system in the DG of hippocampus. The data show that there is a significant impairment concerning the number of NPY positive cells in *Ophn-1*$^{-/y}$ mice respect to WT animals. The confocal images report examples of NPY labeling in KO and WT mice. Histograms represent mean ± SEM. Scale Bar: 100 μm. Statistical significance, *P<0.05

4- DISCUSSION

The aim of this thesis was to investigate neurogenesis in the DG of the hippocampus in a mouse model of ID, caused by a loss of function in Ophn-1. Moreover, the state of the NPY positive inhibitory circuitry was analyzed, to verify if the loss of function of Ophn-1 could lead to an epileptic phenotype.

First of all, an analysis of the macroscopic morphological changes in the DG was performed: the measures of the volume of blades and hilus of DG showed that loss of Ophn-1 does not alter dramatically the cytoarchitecture of the hippocampus. In this context, it is interesting to note that a more detailed analysis of pyramidal neurons showed alterations in dendritic architecture in Ophn-1 KO mice. Specifically, spine densities and spine morphology were particularly affected (Khelfaoui et al., *J. Neurosci.* 2007). At the behavioral level, hippocampus-dependent tasks such as the Morris water maze test were significantly impaired in the KO animals (Khelfaoui et al., *J. Neurosci.* 2007).

Next, I focused on the neurogenesis process and analyzed every step of it, with the aim of detecting possible impairments in KO mice.

To study the proliferating phase, I used BrdU as marker for mitotic cells. BrdU label permits to study cell proliferation in the acute phase and the subsequent survival, migration and integration of newborn neurons at different times. Accordingly, I studied BrdU labelling in wt and KO mice in acute conditions (1 day), and at 15 and 50 days post-injection. The results indicate that the impairment is evident at P50, but no in the acute phase and after 15d, in Ophn-1 KO mice. These results indicate that loss of Rho GAP activity has no impact on cell division in the SGZ of the hippocampus, and indicates that later steps of neuronal differentiation are impacted.

Newborn cells differentiate into migrating neuroblasts and migrate short distances to enter the DG of the hippocampus, where they become excitatory neurons and extend axons towards their targets in the CA3 region (Gage F.H., *Science* 2000).

Thus, I studied the state of migrating cells in the DG, using Dcx as marker. The results show that Ophn-1 KO mice have a significant impairment with respect to WT. The reduced number of Dcx-positive cells in Ophn-1 KO mice may be explained by the important role played by Rho GTPases in cell motility. Indeed cells in motion need actin dynamics to attach and detach from the extracellular matrix, and these processes are dependent on actin polymerization and depolymerization, which are potently impacted by Rho GTPases and their effectors.

In collaboration with the group of Marco Canossa in Bologna, we asked whether axon extension (another process potently impacted by Rho GTPases) could by affected by Ophn-1 deficiency. Indeed we found a robust deficit in axon formation by the Ophn-1 neurons. Hyperactivation of the Rho signaling pathway is one of the most powerful inhibitors of axon extension and regeneration, and this is in agreement with the present results where lack of Ophn-1 produces persistent RhoA activation (Khelfaoui et al., 2014). Failure to extend an axon is likely to result in lack of trophic support from target neurons and consequent degeneration. Thus, we could think that the missing development of the axon leads to apoptosis of migrating neuroblasts in Ophn-1 KO mice around 21-28 days after birth, i.e., the time when they start maturing. Indeed, staining the cells 21-28 days after birth with Dcx-NeuN revealed a profound loss of early neuronal cells in the KO animals. Altogether, all these results confirm that Ophn-1 is involved in the correct development and integration of the newborn hippocampal cells.

Because the loss of function of Ophn-1 leads to an hyperactivation of the Rho GTPases activity, I wanted to test if the ROCK inhibitor fasudil could lead to a rescue of the phenotype: then, a rescue group of animals was analyzed, in particular on the state of the migrating cells and early neuronal cells. The results of the migrating cells, marked as before, show that there is a significant increase in the number of Dcx/

NeuN positive cells in the rescue group respect to KO mice treated with water. Moreover, the percentage of newborn neurons respect to the total number of Dcx positive cells were calculated, showing that there is a rescue of the phenotype in KO mice treated with fasudil. All these results show that fasudil rescues the phenotype of ID. Fasudil has recently been proposed as a possible treatment for Ophn-1-dependent ID based on the positive results of a mouse study (Khelfaoui et al., *Phil. Trans. R. Soc. B* 2014).

Finally, because recent studies showed that the ID phenotype is strictly connected to epilepsy, a preliminary study of the inhibitory NPY-positive circuitry was performed. The data show that there is a significant impairment in the state of NPY positive cells in Ophn-1 KO mice: thus, Ophn-1 seems involved, also, in the normal formation of the inhibitory circuitry. Previous data in the literature have shown that Ophn-1 mice have deficits in both excitatory and inhibitory synaptic transmission in the hippocampus (Powell et al., 2012). Interestingly, these alterations can be rescued by acute bath application in slices of a ROCK inhibitor (Khelfaoui et al., *Hum. Molec. Genet.* 2009). We are currently expanding our analysis of inhibitory circuitry in the hippocampus and I have stained sections of wt and KO animals with antibodies that label other GABAergic cell subsets (parvalbumin and somatostatin-positive). The counts of these populations are currently underway. I also plan to perform recordings of electrographic (EEG) activity from the hippocampus of Ophn-1 KO mice to detect evidence for epileptiform activity, and the possible rescue effects of fasudil treatment.

In summary, this study demonstrates that Ophn-1 plays a major role in adult neurogenesis. These data are important since they suggest that specific steps of neuronal development such as axon extension, migration of neuroblasts, and integration of newborn neurons are disrupted in the embryonic brain following loss or mutation of Ophn-1. These alterations may be at the basis of the cognitive deficits observed in Ophn-1-related ID. The deficits in adult neurogenesis could further add to the cognitive impairment (Gage F.H., *Science* 2000). However, it seems that there is a pharmacological way to rescue the phenotype: the ROCK inhibitor fasudil,

blocking signaling downstream of activated Rho, leads to a partial rescue of neurogenesis in KO mice. Interestingly, fasudil appears to be ineffective in correcting the total number of migrating neuroblasts. These data indicate that migration deficits are insensitive to ROCK inhibition and may thus depend on the hyperactivation of other Rho GTPases (such as Rac or Cdc42).

Altogether, this study has revealed how adult neurogenesis is influenced by the loss of function of Ophn-1 protein, permitting to better understand not only the mechanisms that regulate adult neurogenesis, but also the disrupted developmental steps involved in the ID phenotype. Using these results as a start point, it will be possible to focus on more specific molecular mechanisms involved in neurogenesis and ID, permitting a possible way to assist the current therapies for this complex syndrome.

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