The role of Tenascin-R in human neurodevelopmental disorders associated with cerebellar dysfunctions

Mémoire

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Résumé

Environ 500 000 enfants au Canada souffrent de maladies génétiques rares. Chacune de ces pathologies causant divers problèmes de santé et touchant un nombre restreint d'individus, nos connaissances des mécanismes sous-jacents et des possibles approches thérapeutiques sont ainsi limitées. Néanmoins, les progrès actuels des technologies de séquençage de l'ADN permettent désormais de découvrir efficacement de nouveaux gènes impliqués dans les maladies neuronales. Grâce à cette approche, le gène de la Tenascin R (TNR) a récemment été identifié comme étant à l'origine d'une maladie neurologique rare. Jusqu'ici, il a été montré chez un enfant souffrant de troubles du développement neurologique que des mutations de la TNR sont associées à une ataxie cérébelleuse et un retard de développement global. TNR est une glycoprotéine de la matrice extracellulaire exclusivement exprimée dans le système nerveux central. Elle participe à la régulation de l'extension et la régénération de l'axone, mais également à la synaptogenèse, la croissance et la migration neuronales. Néanmoins, nos connaissances du rôle de la TNR dans les processus neurodéveloppementaux se basent sur des travaux réalisés chez des rongeurs, et la fonction de cette protéine au cours du développement du cerveau humain demeure inconnue. L'objectif de mon projet de recherche est d'investiguer le profil développemental de cellules progénitrices neuronales humaines (NPCs) issues du patient mentionné ci-dessus, et de déterminer si les anomalies observées au sein du cerveau humain présentant une mutation de TNR sont liées à une altération de la migration, maturation ou encore intégration fonctionnelle des neurones. Grâce à ces travaux, il sera possible d'acquérir des informations importantes sur la fonction de la TNR dans la migration et la maturation des neurones humains. Ce programme de recherche approfondira également notre compréhension des mécanismes fondamentaux régulant le développement neuronal des NPCs issues de patients, ceci étant essentiel à la conception de stratégies thérapeutiques ainsi qu'à la validation de médicaments.

Abstract

Approximately 500,000 children in Canada are affected by rare genetic disorders. Each specific disorder causes several health problems and affects a small number of individuals, therefore our knowledge about mechanisms underlying the disease and possible therapeutic interventions are strongly limited. However, the progress in DNA sequencing technologies now provides an effective way to discover new genes involved in neuronal diseases. Using this innovative approach, Tenascin R (TNR) gene has been recently identified as novel rare neurological disease-causing gene. So far, it has been showed, in a child affected by neurodevelopmental disorder, that mutations in TNR correlate with cerebellar ataxia and global development delay. TNR is a member of extracellular matrix glycoproteins and is exclusively expressed in the central nervous system. TNR contributes to the regulation of axon extension and regeneration, but also to synaptogenesis, neuronal growth and migration. However, our knowledge about the role of TNR in different neurodevelopmental processes is based on experimental work performed in rodents, and the function of this protein in human brain development remains unknown. The aim of this research project is to study the developmental profile of human neuronal progenitor cells (NPCs) derived from the above-mentioned patient and control subjects and to determine whether abnormalities observed in the human brain with TNR mutation are linked to affected neuronal migration, maturation or functional integration. This work will provide crucial information on TNR function during migration and maturation of human neurons. This research project will also deepen our understanding of fundamental mechanisms regulating neuronal development of patient-derived NPCs which will be crucial for designing treatment strategies and drug testing/validation.

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List of abbreviations

ADHD	Attention deficits hyperactivity disorder
ARID1B	AT-rich interaction domain 1B
ASD	Autism spectrum disorders
BMPs	Bone morphogenetic proteins
BDNF	Brain-derived neurotrophic factor
BLT	bone marrow, liver, thymus
CH	Cerebellar hypoplasia
CHD8	Chromodomain helicase DNA binding protein 8
CNS	Central nervous system
CSPGs	Chondroitin sulfate proteoglycans
DCN	Deep cerebellar nuclei
DG	Dentate gyrus
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGL	External granular layer
EGZ	External germinal zone
FG	Fibrinogen-like
fMRI	Functional magnetic resonance imaging
FNIII	Fibronectin type III
GABA	Gamma- aminobutyric acid
GCL	Granule cell layer
GalNAc-4-SO4	N-acetylgalactosamine-4-SO4
GPI	Glycosyl phosphatidylinositol
GPCs	Glial progenitor cells
GWAS	Genome-wide association study
hESC	Human embryonic stem cell

hiPSC	Human induced pluripotent stem cell
HNK-1	Human natural killer-1
HSCs	Hematopoietic stem cells
ID	Intellectual disability
IGL	Internal granular layer
IsO	Isthmic organizer
MAP2	Microtubule Associated Protein 2
MeCP2	Methyl CpG-binding protein 2
MB	Medulloblastoma
MBP	Myelin basic protein
MCD	Malformations of cortical development
MCL	Molecular cell layer
Mpi	Months post-injection
NDDs	Neurodevelopmental disorders
NGF	Nerve growth factor
NOD	Non-obese diabetic
NPCs	Neural progenitor cells
NRXN	Neurexin
NSCs	Neural stem cells
OB	Olfactory bulb
OPCs	Oligodendrocytes precursor cells
PBMCs	Peripheral blood mononuclear cells
Pcw	Postconceptional weeks
PCL	Purkinje cell layer
PIPs	PAX2-expressing interneuron progenitors
PNNs	Perineuronal Nets
Prkdc	Protein kinase, DNA activated, catalytic polypeptide

PTF1	Pancreas transcription factor 1
Rag1	recombination-activating gene 1
Rag2	recombination-activating gene 2
RG	Radial glia
RL	Rhombic lip
Scid	Severe combined immunodeficiency
SynGAP	Synaptic Ras/RapGTPase-activating protein
SVZ	Subventricular zone
TGF- β	Growth factor-β
TNC	Tenascin C
TNR	Tenascin R
TNX	Tenascin X
TNW	Tenascin W
WES	Whole-exome sequencing
WGS	Whole-genome sequencing
Wpi	Weeks post-injection
VZ	Ventricular zone

To my parents,
for their support
and their love.

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Thanks to my family, for their love, caring and sacrifices for educating me and give me the opportunities for a brilliant future. Thanks to Maxime, for his essential presence in my life. Thanks to all my friends for their support and their love.

Introduction

During development, the initiation, maintenance and modification of cell contacts critically depend on the dynamic interactions between cells and the surrounding matrix which result in signal transduction and regulation of gene expression. Neural cells produce specialized and distinctive extracellular matrix (ECM) molecules that fill the diffuse space between neurons and glial cells, providing physical support and control of tissue homeostasis. The specific composition of the ECM together with the presence of an appropriate repertoire of cell surface receptors, enable cells to engage in initial contacts that lead to different stages of proliferation, migration and differentiation[1]. Throughout the critical period of development, the role of the ECM molecules in the central nervous system (CNS) is essential for modulating synaptic plasticity, learning and memory[2]. The expression of ECM components is developmentally regulated and often spatially restricted within specific CNS matrices, being therefore associated with specific neural functions and with different neurodevelopmental pathologies[3]. The extracellular space between glial cells and neurons contains hyaluronan, as a main component, and a large complexity of glycoproteins, including laminins, tenascins, thrombospondins and proteoglycans[4]. Tenascin R (TNR) is an ECM glycoprotein exclusively expressed in the CNS during postnatal development and adulthood. TNR is a versatile molecule and it has been implicated in a variety of cell-matrix interactions, underlying axon growth inhibition/guidance, myelination and neural cell development[3]. Lately, mutations in TNR gene have been associated with cerebellar dysfunctions, cognitive deficits and general developmental delay[5,6]. In addition, using exome sequencing, the laboratory of our collaborator Dr. Kym Boycott, at The Children's Hospital of Eastern Ontario (CHEO), recently identified a biallelic mutation in TNR gene in a child with cerebellar ataxia, axial hypotonia and global developmental delay. Focusing on this clinical case, we decided to investigate the involvement of TNR in cerebellar development and the role of this gene in the pathogenesis of neurodevelopmental disorders associated with cerebellar dysfunctions. For all these reasons, in the next pages the role of TNR in developmental and adult CNS, as well as the relation between TNR mutations and neurodevelopmental disorders, particularly involving the cerebellum, will be discussed. In addition, it will be showed how to model rare neurodevelopmental diseases and to study the development of human cells in *in vivo* context by using stem cell technology and "*humanized mice*", which consist of mice grafted with human cells.

1. Tenascin R: a versatile molecule in the CNS

TNR modular structure and expression

The tenascin family constitutes a group of ECM proteins with four members so far identified in vertebrates, namely tenascin C, R, W and X. Tenascins are characterized by their unique domain structure: an N-terminus with heptad repeats flanked by cysteine residues, followed by epidermal growth factor-like (EGF) domains, a variable number of fibronectin-type III (FN) repeats, and a fibrinogen-like (FG) domain at the carboxyl-terminal region[7] (*see figure 1*). FN repeats are site of alternative splicing, leading to variable numbers of isoforms of these molecules. Via their N-terminal oligomerization domain, tenascin subunits form disulfide-linked homo-trimers (TNR and TNX) or -hexamers (TNC and TNW). Rather than representing bona fide structural components of the extracellular matrix, tenascins are involved in modifying the interaction of cells with extracellular matrix and growth factors, and hence regulating cell adhesion, migration, growth and differentiation in a context-dependent manner[7,8]. The TNR sequence is phylogenetically highly conserved: the predicted amino acid sequences of chicken and rat TNR reveal a homology of more than 80%, and that of human TNR shows a homology to chicken and rat TNR of 75% and 93%, respectively[1].

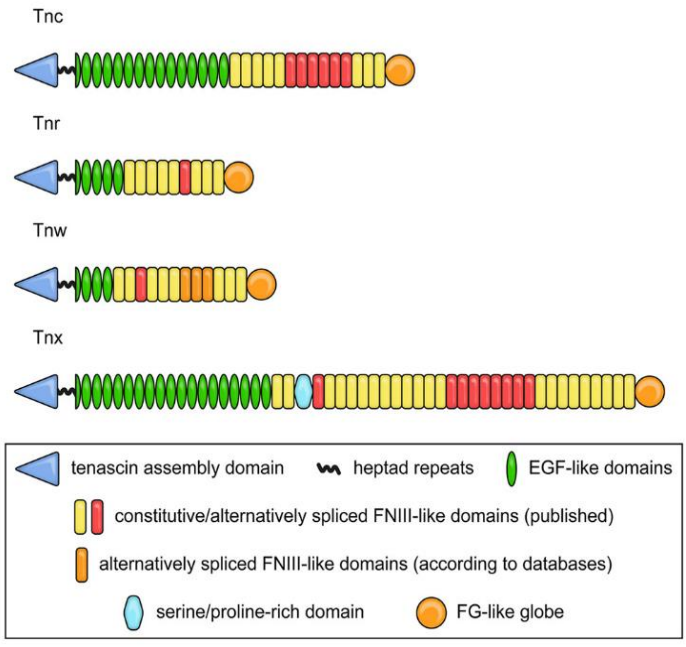


Figure 1. Molecular structure of tenascin glycoproteins in the mouse. All four tenascin molecules have a modular structure: a N-terminal cysteine-rich tenascin assembly domain followed by heptad repeats, epidermal growth factor (EGF)-like domains, fibronectin type III (FNIII)-like domains and a C-terminal fibrinogen (FG)-like domain. All tenascins can be alternatively spliced, which results in a variable number of FNIII-like domains (shown here in red and orange). *(Figure adapted from Roll L. et al. 2019)*

TNR, originally designated as janusin in rodents and restrictin in chicken, is almost exclusively located to the central nervous system, expressed as two major molecular forms of 160 (TNR 160) and 180 kD (TNR 180)[9]. TNR has so far been found exclusively expressed in the developing and adult CNS by oligodendrocytes and small subsets of neurons (mainly interneurons and motoneurons), such as the small inhibitory interneurons of the cerebellar cortex (stellate and basket cells), motoneurons in the spinal cord and the brain, and the horizontal cells of the retina[1,10]. Oligodendrocytes-derived TNR becomes abundant in the white matter of different CNS regions during the phase of active myelination while decreases in the adult brain, where is predominantly associated with the surface of oligodendrocytes, myelinated axons and the nodes of Ranvier. Along with neuronal maturation (first three postnatal weeks in rodents), TNR expression has been shown to accumulate in specialized ECM structures, named Perineuronal Nets (PNNs), surrounding mainly interneurons and motoneurons[1]. The presence of TNR in the PNNs is a common feature of different CNS regions, such as the cortex, hippocampus, cerebellum, retina, brainstem and spinal cord [11]. Despite to what has been shown for oligodendrocytes-released TNR, the expression of TNR by neurons is not downregulated in adulthood in physiological conditions. However, it has been shown that the expression pattern of TNR expression can be changed in functions of different pathological conditions[8,12]. TNR up-regulation has been reported in different forms of brain cancer (pilocytic astrocytoma, oligodendroglioma and ganglioglioma)[13] and in response to spinal cord injury[14]. On the other hand, activation of microglial cells after lesioned facial nucleus have been implicated in the downregulation of TNR expression[15]. Overall, all these studies conducted in murine models show that TNR expression is tightly regulated in the CNS, in a spatial and temporal manner.

TNR interactions and functions

A plethora of interacting molecules reflect the repertoire of TNR functions. As many ECM molecules, TNR can go through extensive post-translational modifications. Characterized by numerous potential sites for N- and O- glycosylation, so far TNR has been linked with three different types of sulfated oligosaccharide structures: human natural killer-1 (HNK-1), O-linked chondroitin sulfate glycosaminoglycans and GalNAc-4-SO4[16]. Each one of this modification is spatial and temporal regulated and contribute to different TNR functions[17].

TNR can mediate different effects either by directly binding cellular receptors or by binding other ECM molecules. For instance, is well known that TNR has an anti-adhesive effect towards various CNS neurons, while is an adhesive substrate for CNS glial cells, astrocytes

and oligodendrocytes[9,10]. Both adhesive and anti-adhesive properties of TNR are F3/F11 receptor-mediated mechanisms[18]. TNR has also been involved in axon growth inhibition/guidance, myelination and neural cell development[10]. Indeed, by interacting with β1 integrin receptor, which is a prominent sensor of signaling from ECM components expressed by neural stem cells (NSCs), TNR can mediate different stages of neural development[19]. Therefore, here we will describe more in detail TNR role during neural proliferation, migration and maturation.

Cell proliferation. TNR can have different effects on neural proliferation, depending on which of its domains interacts with β1 integrin receptor. *In vitro* studies, have shown that the TNR FN6–8 domains inhibit NSCs proliferation, whereas the EGFL domains do not affect NSCs proliferation, but promotes NSCs differentiation mainly into neurons[20]. *In vivo*, as well, TNR mediates hippocampal neurogenesis during development and in the adulthood, by regulating the fate of NSCs[21]. Moreover, has been shown that TNR influences adult, but not developmental neurogenesis in the murine olfactory bulb (OB)[22,23].

Migration. The presence of TNR is also fundamental to guide cell migration as showed in the murine OB, where the presence of TNR is pivotal in initiating neuroblasts radial migration and sufficient to reroute tangentially migrating neuroblasts[22,23]. On the other hand, *in vitro* studies show that TNR, through both EGFL and FN6–8 domains, can also inhibit neural migration from neurospheres[24]. These results show that TNR can have different roles on the migration of neuronal progenitors, depending on the molecular and cellular context.

Maturation. Along with the maturation of the CNS, TNR results to be mainly accumulated in the Perineuronal Nets (PNNs), specialized ECM structures (*see figure 2a*). PNNs appear in the CNS at the end of critical periods and surround cell soma and proximal neurites of neurons in several brain areas (hippocampus, cerebellum, cortex, etc.), participating in signal transduction and in controlling neuronal activity and plasticity[25,26].The importance of TNR for the assembly of PNNs has been demonstrated by several studies in which the lack of TNR postnatally and during adulthood disrupts the molecular scaffolding of PNNs [11,27]. In the PNNs, TNR binds to members of the lectican family of chondroitin sulfate proteoglycans (CSPGs), including aggrecan, versican, neurocan and, with highest affinity, brevican (*see figure 2b*). Interactions with lecticans can result in collaborative or inhibitory action depending on the developmental context[28].

In the mature brain, TNR has been showed to be also associated with nodes of Ranvier, where it controls the localization and the function of voltage-gated sodium channels (Na_v)[29,30]. It has been shown that TNR, together with other adhesion and ECM molecules,

has a crucial role for the clustering and the localization of Na_v channels[29].The interaction between TNR and Na_v channels involves the EGF-L domain of TNR and the β2 subunit of Na_v channels, [30,31]. The mice deficient for TNR have decreased conduction velocity of action potential propagation in CNS axons, but no apparent change in the distribution of Na_v channels at the nodes of Ranvier [32].

TNR roles in the human brain

To date, our knowledge on the role of TNR in the CNS is mostly based on experimental works performed in rodents, and little is known about its functions in human brain. Is known that TNR is highly homologous between species and, compared with rat TNR, the homology of the protein coding region of the human TNR is 93%[33]. Additionally, has been demonstrated that in the human developing cortex TNR expression is spatio-temporally regulated, suggesting a functional role during corticogenesis[34]. Recent studies started to associate TNR mutations with human neurodevelopmental disorders, suggesting an important role of TNR in the development of human CNS[5,6,13]. However, the mechanisms by which TNR could regulate human neurodevelopment remain still poorly understood.

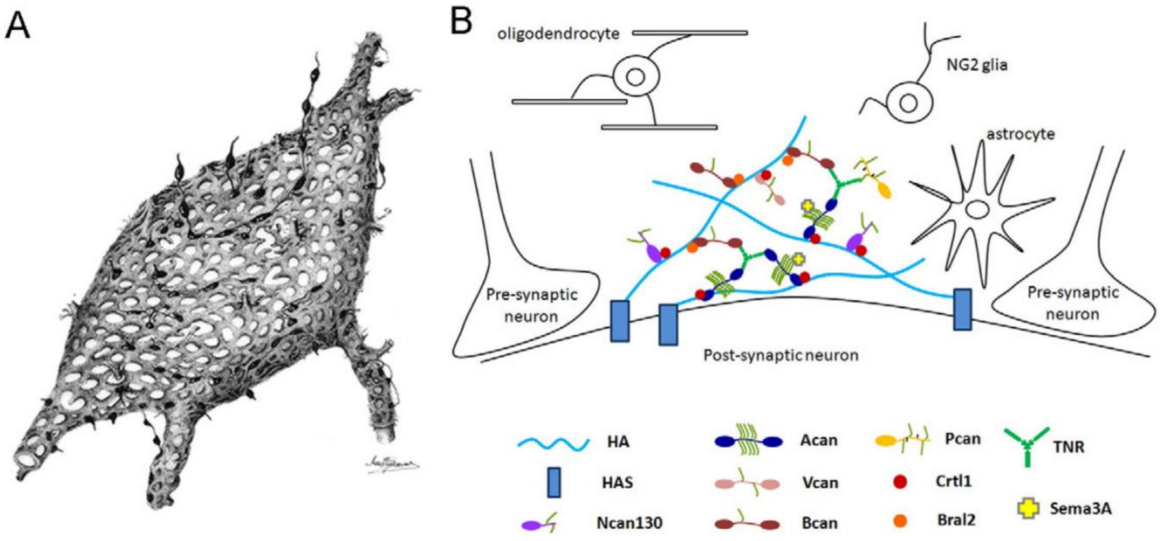


Figure 2. Structure and composition of PNNs.

(A) Scheme of the typical reticular structure of a PNN, with representative synaptic boutons included in PNN holes. (B) Scheme of the known interactions between PNN components, and the cellular types which contribute to their synthesis. *(Figure adapted from Oohashi T. et al. 2015)*

2. Human neurodevelopment

Prenatal and postnatal development

The human CNS is possibly the most complex biological tissue, comprising on average 86.1 billion neurons in the brain and spinal cord, along with a roughly equal number of glial cells[35]. Due to its remarkable complexity, the human CNS takes over several years to build via precisely regulated molecular and cellular processes governed both by genetic and environmental factors[36]. The CNS is one of the earliest organ systems of the human body to begin its development prenatally and among the last to complete it postnatally. The average length of human prenatal development is 38 weeks, which are divided in embryonic and fetal period.

Embryonic period. The early central nervous system begins as a simple neural plate that folds to form a neural groove and then a neural tube[36,37]. When the neural tube is complete, the neural progenitors form a single layer of cells that lines the center of the neural tube, defining the region that will become the ventricles, and therefore called the ventricular zone (VZ)[38]. The neural progenitor cells in the most rostral region of the VZ will give rise to the forebrain, while more caudally positioned cells will give rise to the hindbrain and spinal cord. Over the next month, the embryo undergoes rapid growth, acquiring a basic three-dimensional organization[37]. At this stage, the neural tube is patterned along the rostro-caudal axis into the following three major vesicles of the future brain: forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon)[37]. These three segments further subdivide: the prosencephalon divides into the “telencephalon” and the “diencephalon”, and the rhombencephalon divides into the “metencephalon” and “myelencephalon”. The mesencephalon does not further divide. These five subdivisions establish the primary organization of the central nervous system[39]. In addition, the neural tube is patterned along the dorsal-ventral axis to establish defined compartments of neural progenitor cells that generate specific types of neural cells. These changes mark the beginning of a protracted process of neural patterning within the CNS that begins in the embryonic period and extends for many years.

Fetal period. The fetal period of human development extends from the ninth gestational week through the end of gestation[39]. During this time, dramatic structural and morphological changes take place, reflecting dramatic changes occurring at the cellular level. Indeed, neuron production begins in the embryonic period on E42, but extends through the fetal period in most brain areas. Soon after they are produced, neurons migrate away from the

proliferative regions of the VZ to reach their final destination[39]. For instance, the neurons that will form the neocortex migrate and form the six-layered neocortical mantel. At this stage, cortical neurons begin to differentiate, producing neurotransmitters and extending the dendritic and axonal processes[39]. Guided by the molecular cues present in the extracellular compartment, the neurites reach their target cell and establish synapses. Mature synapses allow the transmission of electrochemical signal between neurons, which is the essential for brain maturation and functioning. During this stage, among the most prominent morphological changes is the massive growth of the cerebral hemispheres with the characteristic formation of gyri and sulci[36].

Postnatal period. By birth, the gross anatomy of the CNS is reminiscent of its adult appearance, however there is striking morphological and functional development of the brain's fiber tracts as well as remodeling of cortical and subcortical structures[40]. Postnatally, development is also characterized by massive outgrowth of dendrites and axons, synaptogenesis and myelination, predominately in the forebrain and cerebellum[41]. The human CNS undergoes remarkably rapid growth until the third postnatal year. During this period the expansion of primary sensory areas continues but more slowly, while association areas in the parietal, frontal and temporal lobes are highly expanded[41]. However, brain development does not only involve generation of neurons and connections, but also regressive phenomena. Indeed, after the third postnatal year, the rate of growth slows down and processes, such as apoptosis and synaptic pruning, take place in the developing CNS (*see figure 3*)[35]. The protracted length of human brain development explains why it takes a certain time before neurodevelopmental disorders become clinically manifest.

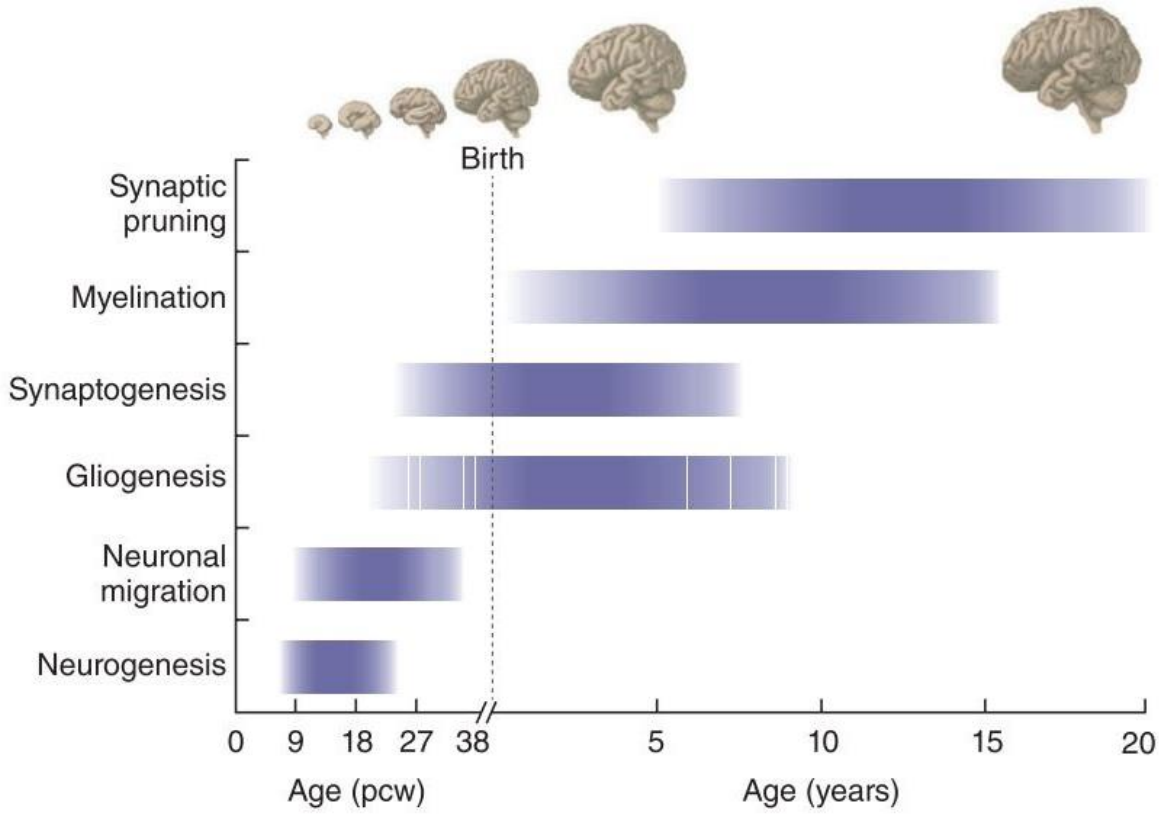


Figure 3. Prenatal and postnatal development of human brain.

The figure provides a timeline of human development during prenatal (in post conception weeks, pcw) and postnatal (in years) periods, in which the horizontal bars represent the approximate timing of key neurobiological processes and developmental milestones. The illustrations show gross anatomical features and the relative size of the brain at different stages. *(Figure adapted from Marin O., 2016)*

Neurogenesis, neuronal migration and maturation

Much of brain development depends on three crucial early development events: the proliferation of neural stem cells, the migration of postmitotic neurons from their birthplaces to appropriate target areas, and the maturation of different types of neurons within functional neural circuits. This section will consider these processes in greater detail.

Neurogenesis. The bulk of neurogenesis in humans and most experimental species occurs during embryonic and fetal development. During these phases, the population of neural progenitor cells divides by what is described as a "symmetrical" mode of cell division, namely a cell divides and produces two identical neural progenitor cells[42]. Over multiple rounds of cell division between E25 and E42, symmetrical cell division provides the means for augmenting the size of the neural progenitor pool[38]. Beginning on E42, the mode of cell division begins to shift from symmetrical to asymmetrical and each neural progenitor division produces one neural progenitor and one neuron[38]. The shift to asymmetrical cell division among the progenitor population is gradual, and initially includes only a small proportion of progenitors, but those numbers increase dramatically by the end of cortical neurogenesis. The vast majority of neurons in the human telencephalon are generated before birth and neocortical excitatory neuron generation ends around within the fetal period[36]. After birth, neurogenesis remains mainly restricted to the subventricular zone (SVZ), where new neurons continue to emerge and migrate to the olfactory bulb (OB)[43], and to the dentate gyrus (DG) of the hippocampus[44]. In addition to these two canonical neurogenic sites, postnatal neurogenesis has been reported in other CNS regions[45]. For instance, the postnatal mammalian cerebellum gives rise to granule cells through a transitory germinative layer localized on its surface (the external granular layer, EGL), which persists until the end of the first postnatal year in humans[46]. However, while being widely studied in other mammals models, adult neurogenesis remains still debated in humans[47].

Migration. Coordinated migration of newly born neurons to their target regions is essential for correct neuronal circuit assembly in the developing brain. During CNS development, neurons utilize mainly two modes of migration: radial migration and tangential migration[48]. In the early stages of mouse developing cortex (E13-E14), the migration distance that neurons must traverse are smaller, therefore they can migrate by somal traslocation[49]. As development proceeds, the brain becomes larger and neurons require radial glia (RG) to support their migration towards the developing cortical plate[49,50]. Within the developing cortex, projection neurons migrate radially along the elongated fiber of RG, while GABAergic cortical interneurons born in the ganglionic eminences migrate tangentially into the

developing cortical wall[51]. Within the CNS, despite differences in migratory pathways and migration modes, most migratory processes are driven by similar cell-intrinsic mechanisms and determined by extracellular cues to a large extent. Neurons contain a heterogenous cytoskeleton, composed of actin, microtubules and neurofilaments, which together have a critical role in orchestrating dynamic changes of cellular morphology. Indeed, neuronal migration is a cyclical multi-step process that consists of collectively interrelated but independent discrete events, including: polarization, protrusion, adhesion, and retraction[52]. Additionally, the ECM plays important roles in the regulation of neuronal migration by defining the timing, the direction and the final destination for the migrating neurons[53].

Maturation. Once they have reached their target region, the young neurons need to become part of information processing networks. Dendritic and axonal outgrowth followed by the formation of synapses and myelination of axons are key cellular features associated with the functional maturation of the CNS[39]. At midgestation, neocortical neurons have initiated a protracted period of axon outgrowth, dendritic arborization, and synaptogenesis that extends into early childhood[36]. However, many of these prenatal synapses and neural circuits are thought to be transient and the bulk of synaptogenesis in the neocortex occurs during the first 2 postnatal years, peaking between 3 and 15 months[54]. More neurons and synapses are present in the brains of children than in those of adults. Indeed, a proper development of the nervous system requires the removal of large numbers of neurons through apoptosis and the elimination of exuberant connections, a process known as synaptic pruning[55]. Both processes reflect nonpathological events that play an essential role in establishing the complex neuronal networks and inducing the maturation of the CNS. Moreover, it has been shown that parts of the brain associated with more basic functions mature early (motor and sensory brain areas), followed by areas involved in spatial orientation, speech and language development, and attention (upper and lower parietal lobes). Later to mature are areas involved in executive function, attention, and motor coordination (frontal lobes)[40]. The mature organization of brain, and especially of the neocortex, emerges over a protracted time during the postnatal period, and it requires diverse forms of input. Some of this input arises from within the organism in the form of molecular signaling, whereas some comes from external experiences. Being essential during the critical periods, these experiences remain still fundamental for learning processes and plasticity of the adult CNS[40].

Cerebellum: a late-developing brain region

From the Latin 'little brain', the cerebellum is a relatively small portion of the brain but contains roughly half of the brain's neurons[56]. The cerebellum is one of the first brain structures to begin to differentiate, yet it is one of the last to achieve maturity, since the cellular organization of the cerebellum continues to change for many months after birth[56,57].The human cerebellum begins to develop 28 days post fertilization and it arises mainly from the rhombencephalon, one of the three main brain vesicles characterizing CNS early developmental structure[39]. The initial cerebellar territory is defined by the antero-posterior and the dorso-ventral expression patterns of different key transcription factors, which define cerebellar boundaries and guide its development[58]. The isthmic organizer (IsO), localized at the mid/hindbrain boundary, has an important morphogenetic activity in orchestrating the complex cellular diversity in the cerebellum[59]. The cerebellar primordium expands rapidly during the embryonic period, relying on two main proliferative areas: ventricular zone (VZ) and rhombic lip (RL)[37]. The VZ gives rise to GABAergic cerebellar nuclei neurons first, followed by Purkinje cell precursors and PAX2-expressing cerebellar inhibitory interneuron progenitors (PIPs), which will eventually produce basket, stellate, Golgi, Lugaro, globular, and candelabrum neurons[60]. Bergmann glia and parenchymal astrocytes are also derived from the cerebellar ventricular zone[61]. On the other end, the RL gives rise to all glutamatergic neurons that populate the cerebellum, including cerebellar nuclei neurons and granule cell layer neurons[62]. The specific identity of cerebellar progenitor cells in the VZ and in the RL depends on the region-specific expression of two basic helix-loop-helix transcription factors: pancreas transcription factor 1a (Ptf1a), expressed in the VZ, and the mouse homolog of Drosophila atonal (Atoh1), present in the RL.

From 7 to 10 weeks the rhombic lip and alar plates expand to form the anlages of the cerebellar hemispheres, fused in the midline[64]. From 11 to 12 weeks cerebellar posterolateral and primary fissures developed in the vermis and granular precursor cells from the RL migrate tangentially over the surface of the cerebellum to form the external germinal zone (EGZ)[64]. At this stage, the EGZ is characterized by an external granular layer, containing the proliferating precursor cells, and an internal granular layer, which is composed of postmitotic migrating neurons[65]. Specifically, from the external granular layer the granule cells migrate radially inward, via the Bergmann radial glia, to the molecular layer and, in particular, to the internal granular layer[66](*see figure 4a-h*). The molecular signals involved in the regulation of granule cell proliferation and migration in the EGZ are mainly BMPs and Sonic hedgehog (SHH)[67]. In particular, SHH, secreted by the Purkinje cells,

activates a cascade of signal transduction, mediated by Gli transcription factors, which controls the mitogenic state of granule progenitor cells[68]. Other regulatory factors, including neurotransmitters (glutamate), tenascins and grow factors (brain derived nerve factor – BDNF), have been identified as they exert a direct stimulatory or inhibitory effect on the migration of interneurons in different cortical layers of the postnatal cerebellum[69–71].

In humans, the external granular layer remains fairly stable until 2 postnatal years, after which it gradually disappears[69]. The process of postnatal proliferation and migration of granule cells is fundamental for the acquisition of normal cerebellar size and foliation, leading to the formation of ten cerebellar lobules (I-X) and various sublobules[72]. The mature cerebellum is composed of two cerebellar hemispheres and the vermis, and is divided into cortex, white matter, and cerebellar nuclei[56]. The adult cerebellar cortex is laminated into three layers (*see figure 4i*). The molecular layer (ML), consisting mainly of parallel fibers, purkinje dendrites, and glial cell processes as well as neurons allocated at superficial and deep zones, such stellate and basket cells. The Purkinje cell layer (PCL) is composed of a monolayer of Calbindin positive PCs, candelabrum cells and Bergmann glia. The final and deepest layer is the granular layer (GL) and is the widest cerebellar layer, mainly composed of granule cells as well as Golgi, Lugaro and unipolar brush cells[56].

The mature cerebellum present two major afferent systems (climbing fibers and mossy fibers), from which the cerebellum receives inputs from peripheral nerves, brain stem and spinal cord[73]. The signal conveyed through mossy and climbing fibers, activates the cerebellar cortical circuitries, where Purkinje cells represent the final decoders of information, sending the final output to the deep cerebellar nuclei (DCN)[74]. From the DCN the signal travels until the cerebral cortex, forming the cerebello-cerebral efferent projections[75]. By projecting to the motor cortex, to the prefrontal cortex and to the striatum, the cerebellum controls, respectively, sensory-motor functions, cognitive functions and emotions[76]. These cerebellar higher functions continue to improve during childhood and adolescence, suggesting that the cerebellum may be undergoing substantial development during this period[77]. Indeed, it has been shown that disruption of brain development in children is most likely to occur in those regions of the brain, such as the cerebellum, that develop last. Therefore, different studies have associated the cerebellum with neurodevelopmental disorders such as attention deficit / hyperactivity disorder, autism, and schizophrenia[78].

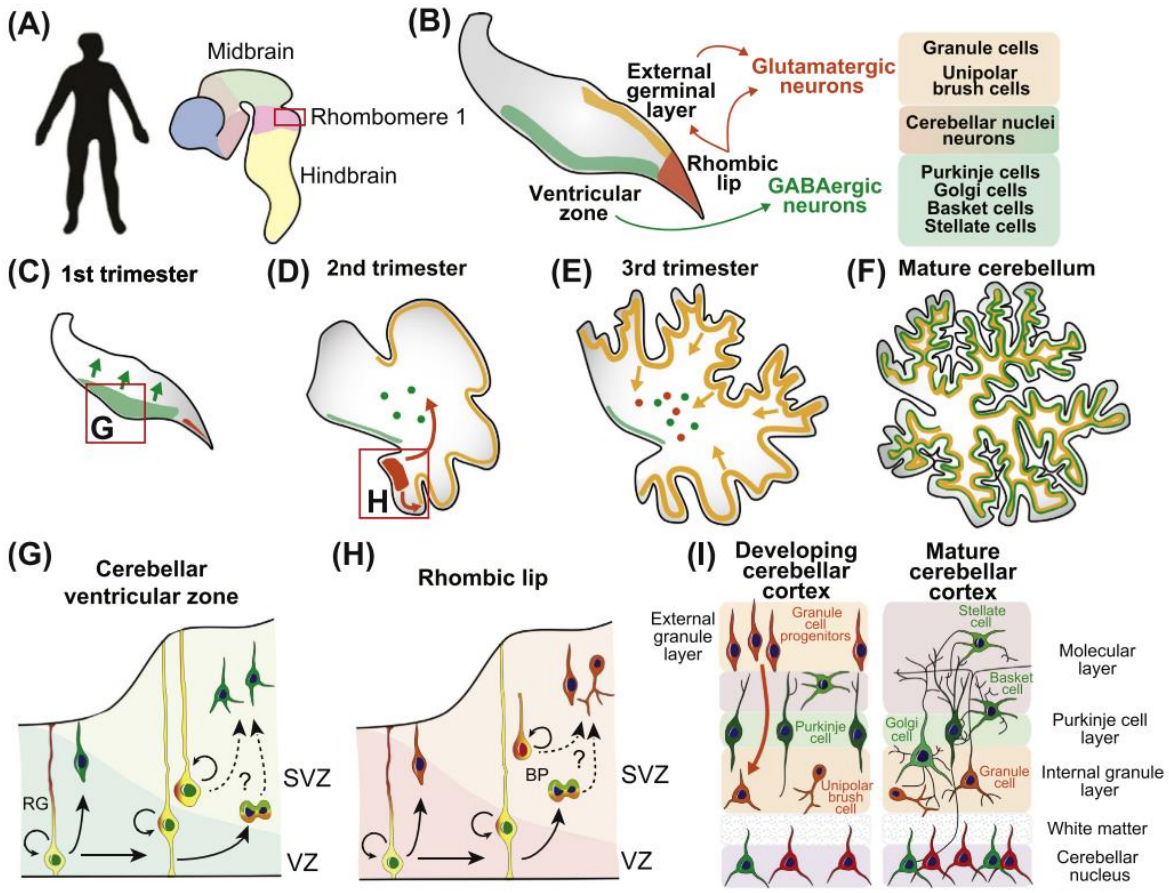


Figure 4. Overview of human cerebellar development across space and time.

(A) Schematic of a developing human embryo CNS. (B) Developmental trajectory showing the three major neurogenic regions of the cerebellum and their cellular output. (C–F) Highlight of the predominant neurogenic zone at different timepoints and the migration of their progeny. (G) Expansion of the cerebellar VZ to form a distinct subventricular zone. (H) Expansion of the rhombic lip to include a proliferative subventricular zone. (I) Connectivity diagram showing organization of cells during development and in the mature cerebellum. (*Figure adapted from Keefe M. et al. 2020*)

3. Neurodevelopmental disorders

Neurodevelopmental disorders (NDDs) are defined as a group of conditions associated with the disruption of the tightly coordinated events that lead to brain development. These are multifaceted conditions characterized by impairments in cognition, communication, behavior and/or motor skills. Since the symptoms and behaviors often overlap between NDDs, there has been a trend to place these disorders within a spectrum more than classify them as discrete entities. Intellectual disability (ID), communication disorders, global developmental delay, autism spectrum disorder (ASD), attention deficit/hyperactivity disorder (ADHD) and schizophrenia are just some examples of NDDs (*see figure 5*)[79]. Turner syndrome, Down syndrome, Rett syndrome and Fragile X syndrome are also among the most prevalent NDDs, marked by significant neurocognitive and neurobehavioral deficits[80–83]. Many genes and mutations have been associated with NDDs, underlying their heterogenous origin[84]. Currently, there are no biomarkers to diagnose NDDs or to differentiate between them. Moreover, many symptoms are not unique to a single NDD, and several NDDs have clusters of symptoms in common. Thus, such overlap of clinical symptoms presents a challenge for nosology and course of treatment.

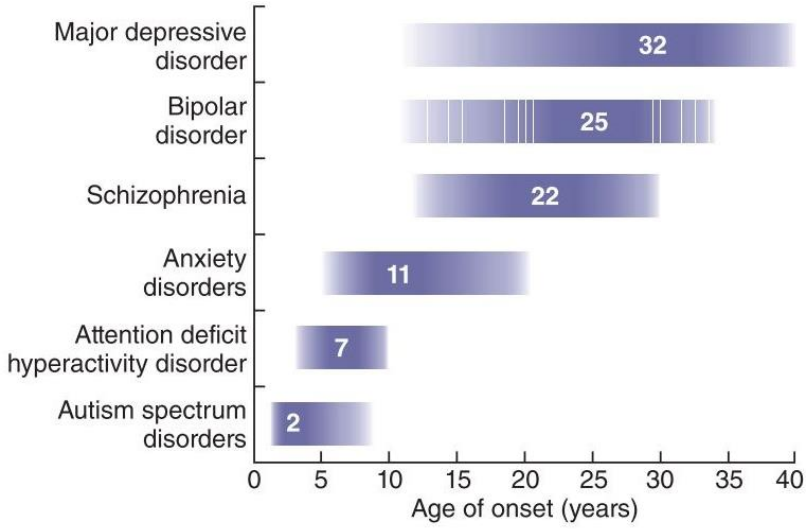


Figure 5. Age of diagnosis for neurodevelopmental disorders.

The age of onset for most NDDs disorders falls during childhood or adolescence. Neurodevelopmental conditions in ASD can be diagnosed shortly after birth, typically before 2 years of age. Most impulse-control disorders (such as attention-deficit hyperactivity disorder, ADHD) and anxiety disorders also begin in childhood, whereas schizophrenia and bipolar disorder are typically diagnosed in late adolescence or early adulthood. *(Figure adapted from Marin O., 2016)*

Genetics of NDDs

In terms of genetics, different types of mutation have been associated with NDDs, including chromosomal rearrangements, copy number variations, small indels, and point mutations. The past decade has seen a rapid development of advanced technologies in genetics and genomics, thus allowing an unprecedented identification of mutations that are involved in complex neurodevelopmental conditions[85]. The advent of whole-genome and whole-exome sequencing (WGS and WES) led to the identification of many inherited and de novo variants that significantly contribute to total NDD risk[86]. Indeed, these new technologies applied on familial NDDs represent a useful paradigm for analyzing the contribution of genetic and nongenetic factors to the pathogenesis of these disorders in the presence of a shared genetic background. This line of research has the enormous potential to establish more accurate genotype–phenotype correlations and has shown that phenotypical outcome essentially depends on *gene vulnerability* and *mutational load* [87]. Gene vulnerability can be defined as the capability of a given gene to tolerate disruptive mutations: the lower the tolerance, the higher the vulnerability. For instance, haploinsufficient genes are highly vulnerable genes and are associated with significant disease risk. However, since mutations affecting these genes are normally subject to a strong negative selective pressure, they represent rare variants associated with significant disease risk[88]. On the other hands there can be genes less sensitive to disruptive mutations and they do not undergo negative selective pressure. Therefore, variants in these genes can be transmitted in families for generations and the additive effects of these mutational events, or mutational load, could result in a disease phenotype[87]. Thus, the current literature suggests that NDDs have a multifactorial and/or polygenic nature, hence confirming the broad heterogeneity of these disorders[89]. Importantly, the clinical outcome might also be influenced at various levels by nongenetic factors, such as environmental factors.

Molecular and cellular pathways involved in NDDs

Recent technological advances have provided new and exciting opportunities for understanding molecular and cellular factors that shape brain development. Functional studies performed during the past decades have shown that most rare and common variants associated with NDDs affect genes that have a role in a few conserved pathways. Some susceptibility genes encode for proteins involved in transcriptional and epigenetic regulation, others NDDs-causing genes are associated with the homeostatic balance of protein synthesis[90]. However, many confirmed or suspected mutations implicated in NDD causation

are in genes encoding for proteins that regulate protein synthesis, transcriptional and epigenetic regulation and synaptic signaling[87]. Homeostasis of these processes can be disturbed during neurogenesis, migration of neurons, and their differentiation in prenatal brain development, or synaptic maturation and proper inhibitory/excitatory balance in postnatal development. For the purposes of this research, this section will be mainly focused on the molecular and cellular basis of NDDs involving different phases of neural development, such as proliferation, migration and synaptic development.

Defects in cell proliferation. Numerous genes associated with NDDs belong to the category of transcriptional factors regulating developmental genes that drive neuronal proliferation. Defects in cell proliferation during brain development can lead to different pathologies, mainly related to brain size modifications and cognitive defects[91]. Classical examples of this kind of defect are megalencephaly and hemimegalencephaly, in which brain size is macroscopically larger than normal and this is usually accompanied by important developmental delay and intellectual disability[92]. The molecular basis of these diseases converges on the mTOR pathway. Indeed, genetic studies on mouse models found that mutations in mTOR itself and in its upstream regulators can lead to the hyperactivation of the mTOR pathway and to an increased progenitor proliferation[93]. On the opposite side, reduced proliferation of neuronal progenitor cells is at the basis of primary microcephaly, in which patients display a substantial decrease in brain size (preserving brain structure and cortical layering) and intellectual disability[91]. Microcephaly is considered to be a disorder of neurogenic mitosis and several mutations have been identified in genes involved in cellular mitosis, such as CDK5RAP2 and CENPJ[94]. Both megalocephaly and microcephaly are examples of malformations of cortical development (MCD)[95].

In addition, cell proliferation impairments are also implicated in the association of NDDs with cancer. For instance, the constitutive growth of granule progenitor cells in the cerebellar EGL has been suggested as the main cause of medulloblastoma (MB), the most common pediatric brain tumor[96]. The current consensus is that MB can be sub-classified, based on genetic, epigenetic, and transcriptomic characteristics, into four distinct subgroups: Wingless-related integration site (WNT), Sonic Hedgehog (SHH), Group 3 and Group 4[97]. The WNT and SHH sub-groups have been associated with constitutive activation of the WNT/β-catenin and SHH pathways, respectively, in the granule cell progenitors of the developing cerebellum[98]. Although the last two groups of MBs remain poorly characterized, it has been shown that Group 3 MBs may arise from cerebellar stem cells and be associated

with the overexpression of MYC, whereas Group 4 MBs are often related to tandem duplication of a-synuclein-interacting protein (SNCAIP)[99].

Defects in cell migration. A fundamental property of the developing brain is that newborn neurons must leave their site of origin to migrate varying distances to their target regions. Defects in this developmental stage, can have consequences in the final cortical network and in the manifestation of pathological conditions, including seizures and cognitive disability[91]. One of the cause of impaired neuronal migration is the alteration of the radial glia scaffold, which normally support and guide the migration of neurons in the developing brain[50]. However, an altered neuronal migration can be caused also by cell-autonomous factors, which is the case in lissencephaly, a pathology characterized by smooth cortex, increased thickness and the production of only 2-4 cortical layers[51]. Similarly, the subcortical band heterotopia is a pathology characterized by the presence of a band of gray matter between the 6-layered cortex and the ventricular wall[91]. The most common genetic cause of these diseases is represented by mutations in LIS1 and DCX genes, which encode for proteins involved in the remodeling of the cytoskeleton in migrating neurons[91]. Lissencephaly and subcortical band heterotopia patients suffer from mental retardation and epilepsy[51]. Indeed, modifications of the correct program of neuronal migration give, as a final outcome, not only an altered cortical structure but also a defective neuronal wiring. In support of this, studies conducted in rodents showed that the silencing of DCX in embryos lead to neurons uncapable to migrate but also with impaired dendritic and synaptic development[100]. Cell migration defects have been also studied in Rett syndrome, whose genetic origin has been traced to mutation in the X-linked MeCP2 (methyl CpG-binding protein 2)[82]. Indeed, recent evidence has demonstrated that mouse NPCs lacking MeCP2 exhibit delayed corticogenesis with respect to migration from the subventricular and ventricular zones into the cortical plate[82,101]. These findings suggest a role of MeCP2 in cortical migration and lamination.

Defects in synaptic signaling. Many NDDs are diagnosed at early stages of life, usually before three years of age, a period when intense synaptogenesis is happening[54]. During this period, the synaptic signaling can be disrupted by mutations in genes encoding for cell adhesion molecules, scaffolding proteins and proteins involved in synaptic transcription, protein synthesis and degradation. Cell-adhesion molecules, such as NRXN1, NRXN2, NRXN3, mediate the bidirectional organization of the pre- and postsynaptic compartments through trans-cellular signaling[87]. Mutations in these genes cause significant synaptic impairment coupled with dysregulated release of the neurotransmitter and have been

associated with autism spectrum disorders (ASD) and also with intellectual disability (ID)[102]. Mutations in genes encoding for scaffolding proteins, located at the postsynaptic density, have been also pinpointed as cause of NDDs. For instance, loss-of-function mutations of SHANK2 have been found to induce either an increase of excitatory synapse or hyperconnectivity of excitatory neurons in ASD[103]. SynGAP, the synaptic Ras/RapGTPase-activating protein, is another critical component of the postsynaptic density associated with scaffolding proteins involved in the regulation of AMPA receptors. Patients with SYNGAP1 loss-of function mutations exhibit ID and ASD[104]. Deficits in synaptic transmission and plasticity during postnatal development have also been investigated in Rett Syndrome, showing that mice lacking of MeCP2 have weaker excitatory synaptic transmission and impaired mechanisms of long-term plasticity[82]. Similarly, abnormalities in synaptic structure and signaling have been found in mouse models of Down syndrome, a neurodevelopmental disorder caused by the presence of an extra copy of chromosome 21 (i.e., trisomy 21)[105,106]. Taken together, these data show that various aspects of synapses can be affected, including synapse formation and elimination, synaptic transmission and plasticity. Moreover, these mutations can often coexist, and the clinical outcome can result from the simultaneous dysregulation of multiple synaptic pathways.

Cerebellum involvement in NDDs

As mentioned in the previous section, the cerebellum is one of the earliest brain regions to develop and it continues to grow until adulthood. Therefore, the cerebellum is particularly vulnerable to insult and it can contribute to the pathogenesis of neurodevelopmental disorders. Although NNDs are often reported as cognitive disorders, stereotypic and repetitive motor behaviors are common features of these disorders. Indeed, deficits in fine and gross motor skills, lack of coordination, and poor performance in postural stability have been reported in 50% and 80% of children with ADHD and ASD, respectively[107]. Moreover, abnormalities in the cerebellum have been reported in more than 95% of post mortem examinations of autistic individuals[108]. Cerebellar volume reduction, also known as cerebellar hypoplasia (CH), is one of the most widely reported neuropathology associated with NDDs[107]. CH refers to underdevelopment of the cerebellum and almost all individuals affected by CH exhibit cognitive and motor impairments[109]. Several genes have been associated with CH and each of them causes developmental defects in a multitude of cerebellar developmental programs, including progenitor proliferation and neuronal migration and even developmental cell survival[110].

Pathologic changes in Purkinje cells (PCs) and a substantial loss of these neurons are another important feature of cerebellar disorders, particularly of cerebellar ataxias. Cerebellar ataxias comprise a heterogeneous group of neurological disorders characterized by gait disturbances, motor incoordination and imbalance[111]. The traditional view of cerebellar ataxias as mainly neurodegenerative disorders, has been challenged by accumulating evidence from cell and animal models that suggest that PCs development and related early changes in PC physiology might contribute to the disease[109].
Numerous cerebellar malformations described in humans have a genetic basis, however inflammation, fetal hemorrhage, and prematurity are often contributing factors[65].

Tenascin R mutations in NDDs

All potential roles of TNR in disorders are mostly derived from experimental evidence from mutant rodents. In some studies, the loss of TNR impairs cognition, synaptic plasticity and motor abilities in mice[112,113]. Despite, other studies show that TNR knock-out mice demonstrates structural and electrophysiological changes, but the only clinical feature is mild behavioral aberration[1]. In TNR-deficient mice, reduced density of perineuronal nets and altered inhibitory synapse formation result in affected inhibitory/excitatory balance and alteration in long-term plasticity[23,27,114].
Although ECM molecules such as laminin, fibronectin, TNC are involved in certain human diseases, the implication of TNR in human diseases remains barely known. Has been shown that TNR is strongly expressed in pediatric brain tumors, arguing in favor of a role of TNR in modulating glioma invasion[13]. On the other hand, a striking loss of TNR was observed in postmortem tissue samples of patients with multiple sclerosis, in accordance with the reduction of the levels of ECM components and PNNs in neurodegenerative diseases[115].
More recent studies started to associate TNR mutations with neurodevelopmental disorders. A Genome-wide association study (GWAS) of a clinical cohort diagnosed for attention deficit hyperactivity disorder (ADHD), reported an intriguing association between DNA variation in the TNR gene and ADHD[116]. Furthermore, homozygous deletion of TNR gene has been associated with global developmental delay, cognitive deficit and transient hyperkinetic movement disorder[5]. Another recent study identified a cohort of 13 individuals with biallelic variants in TNR sharing a phenotype consisting of spastic para- or tetraparesis, axial muscular hypotonia and general developmental delay[6]. All these data are in accordance with the clinical case, subject of this study, in which biallelic mutations TNR have been

associated with cerebellar ataxia, hypotonia, mild atrophy of the cerebellar hemispheres and vermis, and global developmental delay (*confidential data*).

Although, the role of TNR in human brain development remains not well-known because of evident limitations of human studies, these GWAS studies suggest for an important role of TNR during the CNS development. Therefore, the intriguing association between TNR mutations and neurodevelopmental disorders warrant further studies.

4. Modeling human neurodevelopmental disorders

Study models: potentials and limitations

An ideal model of a human disorder has to mimic the genetic insult causing the disease (construct validity), generate a phenotype resembling the human disease (face validity) and, exposed to the same treatment, respond similarly to the patient (predictive validity)[85]. Several systems (cells, rodents, primates) have been used to generate models of NDDs that can partially reproduce disease features and can be of interest for understanding underlying mechanisms. The most favored model organism, the mouse, has been extensively employed for modeling neurological disorders. Even though mice share 95–98% of their genomic information with humans, have a relatively rapid reproduction time and are cost-effective, they present some important limitations[117]. For example, assessment of higher brain functions is difficult in mouse models. Therefore, non-human primates have started to be employed for modelling complex behavior and higher cortical functions[118], whereas zebrafish and invertebrates for genetic screens[119].

Along with animal models, *in vitro* reprograming of stem cells has enabled the generation and study of human neurons. Using either human embryonic stem cell (hESC)-derived or human induced pluripotent stem cell (hiPSC)-derived neurons, researchers have recapitulated several neuronal defects involved in NDDs[103]. The ability to model diseases directly from affected individuals and the unlimited source of cells are just some of the advantages of stem cell-based models. On the contrary, the high heterogeneity of iPSC clones, the immature identity of neurons differentiated *in vitro* and the difficulty related to a 2D system are some of the obvious weaknesses of iPSC-derived disease models. To overcome some of these limitations, recently several researchers have developed protocols for the generation of 3D cortical organoids (mini-brains/spheroids), providing avenues to study features of cortical lamination and brain development *in vitro*[120]. Such major technological breakthroughs contribute additional tools for modelling human development and for studying the mechanisms underlying NDDs. Indeed, all these advances in modeling human disorders and in next-generation sequencing technologies have the final aim to sustain and promote a personalized medicine, in other words provide diagnosis and medical treatments based on each patient's unique characteristics. (*see figure 6*).

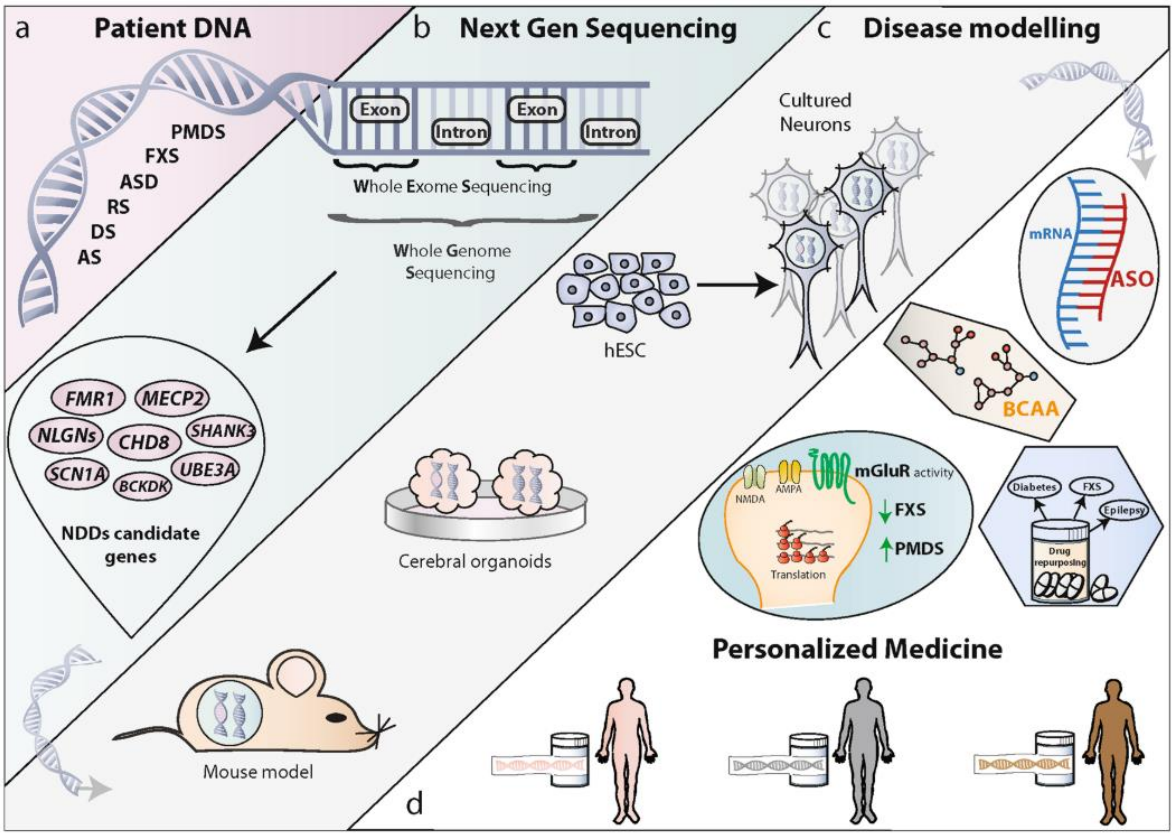


Figure 6. From patient DNA to personalized medicine.

(A) DNA from patients diagnosed with NDDs used for sequencing. (B) Next-generation sequencing can be used to decipher the genetic code within exons (dark blue section; whole-exon sequencing) or throughout the entire genome (dark and light blue section; whole-genome sequencing). Mutations are identified in a series of genes with predisposition to NDDs (pink ovals). (C) The mutations are regenerated in models (mice, organoids, or hESC-derived neurons) to understand their underlying mechanism. (D) Disease modeling reveals targets that enable the implementation of personalized medicine. (*Figure adapted from Tărlungeanu D. et al, 2018*).

iPSC-based disease models

Since the first iPSCs from human fibroblasts in 2007, patient-derived iPSCs have carried great promise for modeling human disease[121]. This approach is particularly appealing for studying NDDs. Indeed, as neural development is substantially more complex and prolonged in humans than in the mouse and other model organisms, animal models often do not recapitulate human disease phenotypes[122]. In addition, for many complex NDDs, both potentially pathogenic mutations and the genetic background can interact to define the final phenotype and these distinct contributions often cannot be exactly mimicked in animals. Nowadays, iPSCs are efficiently obtained by reprogramming a number of somatic cell types from patients, including skin fibroblasts, renal epithelial cells, blood, and dental pulp, via expression of four transcription factors (OCT4, SOX2, KLF4 and c-MYC)[121,123]. Both neural progenitor cells (NPCs) and differentiated neurons (i.e. excitatory glutamatergic projection neurons and inhibitory GABAergic interneurons) can be efficiently generated from iPSCs *in vitro*. Therefore, several NDDs, including ASD/ID, schizophrenia, bipolar disorder, and epilepsy, which appear to frequently involve an imbalance between the excitatory and inhibitory activity in the cerebral cortex, have been studied by using iPSC-based models[124]. Some of these *in vitro* studies focused on the role of some NDD-causing genes in the regulation of synaptic signaling, whereas some others analyzed genes implicated in epigenetic regulatory activities. For instance, iPSCs derived from ASD-affected patient with SHANK2 haploinsufficiency have been used for studying synaptic function of cortical neurons and for showing an altered neuronal connectivity due to SHANK2 mutations[103]. Moreover, one of the most commonly mutated genes in ASD encodes the chromodomain helicase DNA-binding protein 8 (CHD8), a member of the CHD family of ATP-dependent chromatin-remodeling proteins. Several studies have used iPSC-based modeling to define how CHD8 mutations alter neurodevelopment by disrupting the expression of several transcription factors and important regulators of neurodevelopment[125]. Therefore, developing patient-specific models for NDDs is advantageous to model key cellular and molecular features of the underlying mechanisms.

Humanized mice models

Although iPSC-based models are considered a useful tool for studying the mechanism underlying human NDDs, some complex biological process often require *in vivo* analysis. However, the study of human biology *in vivo* is severely limited by ethical and technical constraints. Thus, there is a growing need for animal models to carry out *in vivo* studies of human cells, tissues and organs, without putting humans at risk. Humanized mice, have been developed to overcome these limitations and are now an important research tool for the *in vivo* study of human cells[126]. Humanized mice are immunodeficient mice that have been engrafted with human cells or tissues. In these models, the immunodeficiency is necessary for the graft tolerance and it can be induced either by the injection of immunosuppressive drugs or by genetic manipulation of specific mouse strains. In the first case, immunosuppressive drugs, such as Cyclosporine A, act as suppressors of T-cell-mediated immunity and natural killer cell activity and therefore have been introduced to improve the survival of transplanted cells[127,128]. However, most of the immunosuppressors require life-long administration, which increases the risk of multiple side effects, including increased susceptibility to infection[129–131].

Thus, the study of genetically modified immunodeficient mice has been progressed in last decades, giving promising results. The development of these mice started with the discover that mutations in Prkdc^{scid} (protein kinase, DNA activated, catalytic polypeptide) was causing severe combined immunodeficiency, abbreviated scid) in CB17 mice and that these mice were supporting the engraft of human cells[132]. However, further studies showed that the CB17-scid mice were undergoing spontaneous generation of mouse T and B cells during aging (known as leakiness), limiting the engraftment of the human cells. Later on, was shown that targeted mutations at the recombination-activating gene 1 (Rag1) and Rag2 loci prevented mature T- and B-cell development in the mice but do not cause leakiness, providing a good model for human cells engraftment[133]. In the following years, another efficient model of immunodeficient mice was developed: non-obese diabetic (NOD)-scid mice[134]. Crossing the scid mutation onto different strain backgrounds led to the observation that NOD-scid mice supported higher levels of engraftment with human cells than any of the other strains that were tested[134].

Nowadays, various humanized mouse models, which are engrafted with human transplants, including peripheral blood mononuclear cells (PBMCs), a combination of bone marrow, liver, and thymus (BLT), and hematopoietic stem cells (HSCs), are widely used for the biomedical research[135]. Immunodeficient mice are widely used for engraftment of a patient-derived

tumors, providing an efficient *in vivo* platform to investigate genomic profiling and drug efficacy studies in individual patients[135]. Humanized mice, engrafted with human glial progenitor cells (GPCs) derived from patient, have also been used to investigate whether intrinsic glial dysfunction contributes to the pathogenesis of schizophrenia[136].

Improvements in humanized mouse technology have facilitated research and preclinical studies in various fields. Although next-generation humanized mice can recapitulate human diseases, several limitations remain. For example, several types of human hematopoietic cells are not fully differentiated from HSCs in any humanized mouse strain[126].

Further improvement and refinement of immunodeficient mice for engraftment and differentiation of some subsets of human cells will enable the development of more accurate models of human diseases.

Chapter 1. Rationale and hypothesis of the research project

Rare neurodevelopmental disorders affect approximately 500,000 children in Canada. These disorders cause a broad spectrum of health problems, embracing birth defects, intellectual disability, developmental difficulty and organ failure. Since each specific disorder affects a small number of individuals, our knowledge about mechanisms underlying the disease and possible therapeutic interventions are limited. During the last years, the progress in DNA sequencing technologies has allowed to analyze the entire human genome. Using exome sequencing, the laboratory of our collaborator Dr. Kym Boycott at The Children's Hospital of Eastern Ontario (CHEO) identified a biallelic mutation in Tenascin R (TNR) gene in a child with cerebellar ataxia, axial hypotonia, cerebellar atrophy and global developmental delay. This clinical case is in line with a previous case-report showing an overlapping clinical profile in a child with an homozygous deletion of TNR gene[5]. Furthermore, an additional cohort of patients with TNR mutations has been recently discovered suggesting that TNR is a novel rare neurodevelopmental disease-causing gene[6]. As mentioned above, TNR is a member of extracellular matrix glycoproteins and is exclusively expressed in the central nervous system during postnatal development and adulthood. TNR is implicated in a variety of cell-matrix interactions, underlying axon growth inhibition/guidance, myelination, and neural cell development in different brain regions, as the cerebellum. However, our knowledge about the role of TNR in different neurodevelopmental processes is based exclusively on experimental work performed in rodents. The function of this protein in human brain remains still unknown. Overall, the clinical phenotype of children carrying TNR mutations is mainly characterized by neurodevelopmental delay and cerebellar-related dysfunctions, both motor and cognitive. Therefore, ***we hypothesized that TNR could have a pivotal role in human neurodevelopment, especially during the cerebellar postnatal development***. To investigate this hypothesis, we took advantage of patient-derived iPSCs carrying TNR mutation and of humanized mice in order to assess, *in vitro* and *in vivo*, the consequences of a lack of TNR during human neurodevelopment (*see figure 7*).

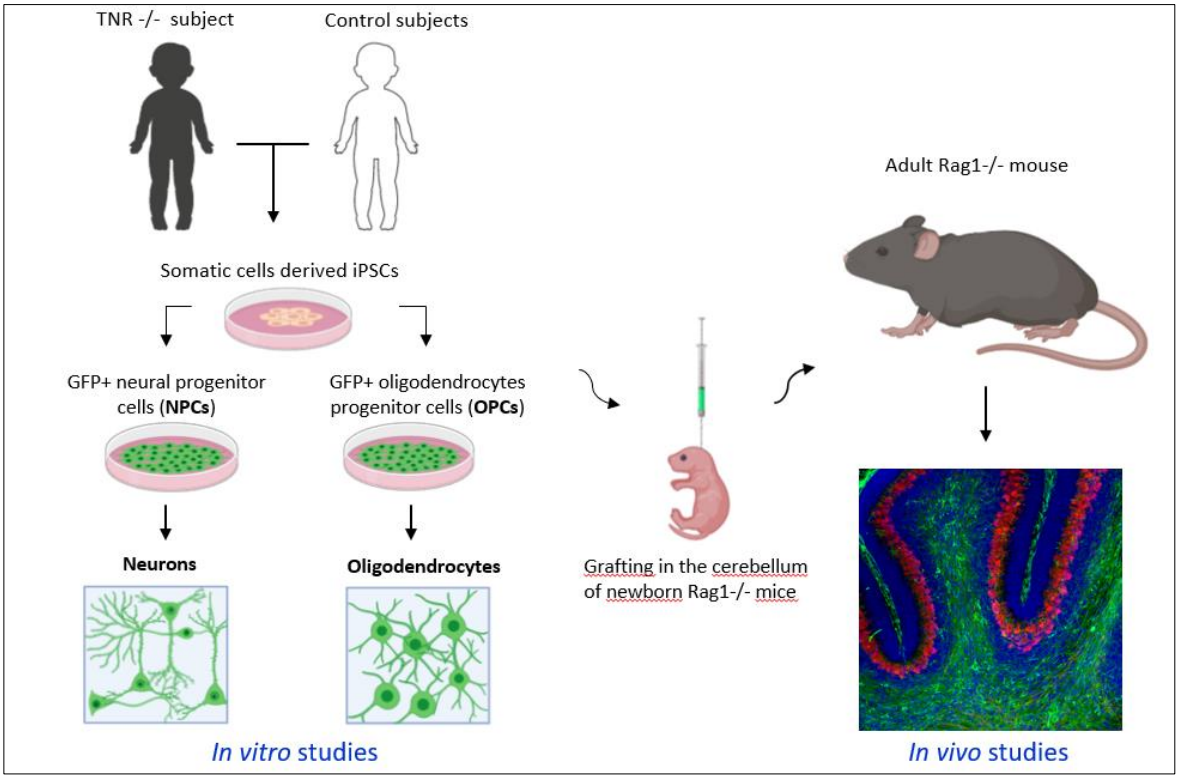


Figure 7. Experimental design.

Somatic cells from patient, carrying TNR deletion, or from control subjects were converted into induced pluripotent stem cells (iPSCs), via the viral expression of the pluripotency transcriptional factors (OCT4, SOX2, KLF4 and c-MYC). The human iPSCs were used in turns for deriving either Neuronal Progenitor Cells (NPCs) or alternatively Oligodendrocyte Progenitors Cells (OPCs). The development of progenitor cells, control or TNR-/-, have been studied *in vitro*, investigating cell proliferation as well as the neuronal differentiation in dish. In parallel, *in vivo* studies were performed to better understand the biological pathways underlying TNR role in neurodevelopmental disorders. To this end, we took advantage of humanized mice models. hNPCs were engrafted in the cerebellum of newborn immunodeficient Rag1-/- mice and NPCs developmental was investigated in a more complex environment, such as the mouse brain. The rate of proliferation of hNPCs as well as their maturation and dendritic arborization was evaluated *in vivo*.

Chapter 2. Objectives of the research project

2.1 Assess TNR expression in mouse and human cerebellums

Since the clinical conditions of the patients carrying TNR mutations suggested a strong impairment of cerebellar functions, the first objective of this project was to investigate TNR expression and distribution in mouse and human cerebellums. Immunolabeling was used to detect TNR protein in the human postmortem samples and mouse cerebellar samples, taken from different postnatal stages. In addition, RNAscope in situ hybridization was performed to detect TNR mRNA, identifying the cellular sources of this molecule within the human and mouse cerebellums.

2.2 Tracking the neuronal development and function of human neuronal progenitor cells (hNPCs), TNR-/- or controls, *in vivo* and *in vitro*.

The second objective of this project was to study, *in vitro* and *in vivo*, the developmental profile of hNPCs derived from the above-mentioned patient and control subjects. For the *in vitro* study, the proliferation, differentiation and Ca^{2+} activity of patient-derived and control hNPCs were monitored in culture. Instead, for the *in vivo* studies, hNPCs were engrafted in the cerebellum of immunodeficient Rag1 mice and their development was studied within the brain physiological environment. The role of TNR *in vivo* was investigated in different developmental processes, such as cell proliferation and neuronal maturation.

Chapter 3. Materials and methods

3.1 iPSC lines derivation and production of hNPCs

Skin biopsies were obtained from patient with TNR mutations and controls. In this study, 2 control subjects were used: CTRL line 1 (E188A-AF-IRS; 3-year-old female); CTRL line 2 (CH3242; 19-year-old male); while 1 TNR KO patient was used: TNR KO line (CH3197; 3-year-old-female). Somatic cells from these subjects were reprogrammed to induced pluripotent stem cells (iPSCs) using ReproRNA™-OKSGM, a non-integrating, self-replicating RNA-based reprogramming vector[137]. Human PSCs (hPSCs) were cultured on matrigel matrix (Corning, #354277) in mTeSR Plus medium (STEMCELL technologies, #05825), following the STEMCELL Technologies technical manual (Document #10000005507). hPSCs were directed to differentiate into neural progenitor cells (NPCs) using STEMdiff™ SMADi Neural Induction Kit (STEM CELL Technologies, #08581). NPCs were generated using a monolayer culture protocol and kept in culture with complete STEMdiff™ Neural Progenitor Medium (STEM CELL technologies, Document #28782). hNPCs were further differentiated into active mature neurons by culturing them on Poly-L-Ornithine (PLO)/Laminin-coated plates using BrainPhys™ Neuronal Medium[138]. Differentiated neurons were cultured for a maximum of 45 days by following the BrainPhys Neuronal Differentiation manual (STEM CELL Technologies, Document #DX20519).

3.2 hNPCs viral transfection

hNPCs stably expressing GFP (GFP+ hNPCs) were generated using LV-CMV-GFP-Puro (SignaGen, #SL100268). hNPCs were incubated with growth medium containing the desired amount of lentivirus: 0.5µL of virus (>10^9 TU/mL) for 5x10^5 cells at Multiplicity of infection (MOI)=1. The optimal MOI was calculated by incubating the target cells with a range of MOI for GFP lentivirus transduction and by calculating the percentage of transduced cells at the various MOI. The optimal MOI was also chosen taking into account the possible cytotoxicity by using high MOI. After adding the viral supernatant, the plate was centrifuged at 2200g and 37℃ for 60 min, using rotor for plate centrifugation and then left overnight in CO^2 incubator. To create a stable cell line, during the 3 days following the transduction, the cells were culture in growth media containing Puromycin (Gibco #A11138-03; 10 mg/ml).

3.3 Animals

Two-, 7-, 15- and 64- old CD1 (Jackson laboratories, strain code: 022), mice were used for immunohistochemical analysis and RNAscope in situ hybridization. In addition, Rag1-/- immunodeficient mice (Jackson Laboratory, B6.129S7-Rag1tm1Mom/J) were bred in our animal facilities and used as hosts for hNPCs transplantation. All experiments were approved by the Université Laval animal protection committee. The mice were kept on a 12 h light/ dark cycle at a constant temperature (22°C) with food and water available *ad libitum*.

3.4 hNPCs engraftments

GFP+ TNR KO or control hNPCs, were engrafted in the cerebellum of Rag1-/- mice on postnatal day 3 or 4 (P3, P4). TNR KO and control hNPCs were cultured as cell lines, but only cells with passage (p) number between p4 and p10 were used for transplantation. Indeed, although hNPCs have an unlimited lifespan and may continue to proliferate for an extended period, over time their phenotype and genotype can change. All the procedures were performed under a biosafety cabinet, in sterile conditions and respecting the confinement procedures for biological hazards. The pups to be injected were transferred in a new sterile cage and wrapped in a sterile glove to prevent direct contact with ice, then cryo-anesthetized for 5 to 10 min, depending on their size. The pups were then removed from ice and cleaned alternatively with isopropyl alcohol and chlorohexidine. For the injection, the pups were placed on a customized support with bended head to have easier access to the site of injection, the cerebellum. The skin was slightly cut in correspondence of the cerebellum and pups were injected directly into the cerebellum, using two sites of injection per hemisphere: at lambda, 1) mediolateral (ML) ±0.5 mm, dorsoventral (DV) -1.00 mm and antero-posterior (AP) -3.00 mm; 2) ML ± 0.8 mm, DV -1.50 mm and AP -3.50 mm. Per each site of injection, 0.75µl of a suspension of single hNPCs were spun down with a density of 50,000 cells/µl. Overall, 150,000 hNPCs were grafted in the host cerebellum. Following injection, the wounds were sealed by applying Vetbond and the pups were returned to a heating pad for recovery. Upon recovery, the litter was returned to the dam. Pups were weaned at 21 days and then used at different time points: 1- and 3-week post injection (wpi) or 3- and 5-month post injection (mpi). It is worth to mention that, for the purposes of this study, only cell engraftments withing the granule and molecular layers of the cerebellum were taken in consideration. Thus, all Rag1-/- animals presenting cell engraftments outside the target regions were discarded of this study.

3.5 Immunohistochemistry and immunocytochemistry

For immunohistochemistry experiments, animals were deeply anesthetized with sodium pentobarbital (12 mg/mL; 0.1 mL per 10 g of body weight) and were perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde (PFA) (Sigma-Aldrich, #P6148). Brains were collected and post-fixed in 4% PFA at 4°C overnight. Cerebellums were cut in free-floating, 50-µm-thick, sagittal sections using a vibratome (VT 1000S; Leica). Cerebellar sections were incubated with the following antibodies: mouse/rat anti-TNR (1:50; #MAB1624, R&D Systems Inc.), rabbit anti-Ki67 (1:1000; #ab15580, Abcam), mouse anti-human Nuclei (1:100; #MAB1281, Millipore Sigma), chicken anti-GFP (1:1000; #GFP-1010, Aves Labs). For immunocytochemistry experiments, cells were fixed with 4% PFA for 15 minutes on a shaker at Room Temperature (RT). Fixed cells were rinsed with PBS 1x solution and then incubated with the following primary antibodies: human/mouse Oct3/4 (1:1000, # MAB1759, R&D Systems Inc.), rabbit anti-Pax-6 (1:500; #60094, STEM CELL technologies), rabbit anti-Ki67 (1:1000; #ab15580, Abcam), rabbit anti-Olig2 (1:1000, # AB9610, Millipore). All the primary antibodies were diluted in 0.2% Triton X-100, 4% milk PBS solution and the incubation with the primary antibody was performed overnight at 4°C on a shaker. Cerebellar sections or cells were incubated with the following secondary antibodies: Alexa Fluor-conjugated anti-mouse or anti-chicken 488 (1: 1000; Life Technologies), Alexa Fluor-conjugated anti-rabbit 568 (1: 1000; Life Technologies), Alexa Fluor-conjugated anti-mouse 633 (1: 1000; Life Technologies). The secondary antibodies were diluted in PBS 1x solution and the incubation was performed at RT on a shaker for 3hours. Images were acquired using FV1000 confocal microscope (Olympus) equipped with argon 488 nm, helium-neon 543 nm, and helium-neon 633 nm lasers.

3.6 In situ hybridization

Two-, 7-, 15-, and 64- days old CD1 mice were used for RNAscope in situ hybridization. Animals were deeply anesthetized and transcardially perfused with 0.9% NaCl followed by 4% PFA. The brains were then post fixed overnight in 4% PFA at 4°C, then rinsed with PBS 1x solution and submerged in 30% sucrose-PBS solution until they were sunk at the bottom. The cerebellums were then embedded in a block of OCT compound (Sakura #4583), quickly frozen in liquid nitrogen and kept at -80°C. The day of use, the frozen blocks were gradually brought at -20°C and kept in the cryostat cold chamber for at least one hour before cutting. The cerebellums were cut in sagittal sections 15-µm thick using a cryostat (Leica CM 1900). The cryosections were directly collected on pre-warmed SuperFrost glass slides, let them

stand for one hour at -20°C and then stored at -80°C until the day of use. The fixed frozen sections were used to perform RNAscope Fluorescent Multiplex Assay (ACD biotechnology, #320851) with the following probes: anti-TNR, anti-Olig2 and anti-NeuN (ACD biotechnology, #570701, #447091-C2, #313311-C3). The RNAscope Fluorescent Multiplex Assay uses a novel method of *in situ* hybridization (ISH) to simultaneously visualize up to three different RNA targets per cell in samples mounted on slides. After several steps of signal amplification, each single RNA transcript of the genes of interest can be visualized as a fluorescent dot. For the detailed procedures consult the user manual doc. no 320535-TN and 320293-USM, ACD biotechnology. Images of the sections stained for the RNAs of interest were acquired using 60x oil emersion objective (NA: 1.42) on a FV1000 confocal inverted microscope (Olympus). Cells expressing Olig2 or NeuN RNAs in colocalization with TNR RNAs were quantified for each cerebellar layer: white matter (WM), granular layer (GL) and molecular layer (ML). Quantifications were performed using the CellCounter tool of ImageJ and expressed as means ± SEM.

3.7 Morphological analysis of dendrites

For studying neural morphology, we take advantage of the Bonfire method. For the in vitro part, GFP-labeled hNPCs were cultured on Poly-L-Ornithine (PLO)/Laminin-coated coverslips using BrainPhys™ Neuronal Medium[138]. At day 40 *in vitro* (DIV40) the cells were fixed with 4% PFA for 15 minutes at RT and stained with anti-GFP antibody (1:1000; #GFP-1010, Aves Labs) and then with Alexa Fluor-conjugated anti-chicken 488 (1: 1000; Life Technologies). For the *in vivo* counterpart, Rag1-/- mice were deeply anesthetized and transcardially perfused with 4% PFA at different time points (3wpi, 3mpi or 5mpi). The brains were then post fixed in 4% PFA at 4°C overnight. Cerebellar, 50-µm-thick, sagittal sections were cut and stained for GFP, as previously described, to boost the signal of engrafted human GFP+ neurons. Images of single neurons were acquired using 40x air emersion objective on a FV1000 confocal inverted microscope (Olympus). The Bonfire method is a series of custom scripts written in MATLAB (MathWorks) described by Langhammer et al.[139]. According to this method, neuronal morphology is digitized in three stages based on the initial 8-bit images. In the first stage, the semi-automated tools available through the NeuronJ plugin[140]to ImageJ (NIH, Bethesda MD) were used to define positions of all neurites. The data for each neurite were exported using NeuronJ in the form of a series of nodes with defined positions in the X-Y plane. In the second stage, the Bonfire program was used to convert the strings of nodes provided by NeuronJ into SWC format for further

manipulation. NeuronStudio[141] was then used to define the pattern of connectivity between neurite segments using the data into SWC format. After linking is complete, another component of the Bonfire program checks the resulting structure for errors and non-linkages. These two steps fully determine the structure of each cell's neuritic arbor in 2-dimensional space and encode it in a digital format. Using these digitized neuritic arbors, a second component of the Bonfire program was then used to perform process identification and extract the following metrics: number of primary neurites, number of secondary neurites, number of branch points per cell, number of terminal neurite tips per cell, and Sholl analysis performed with a 6.0 µm ring interval. Bonfire parameters customized for this study: N, the number of Sholl rings to use; pix_conv, the number of µm per pixel; r_inc, the increment (in pixel) between Sholl rings. For images acquired at 40x magnification with x2 zoom: N=50, pix_conv=1/(3.22), r_inc=19.32.

3.8 Ca^{2+} activity: *in vitro* time-lapse imaging

For assessing the spontaneous calcium activity, *in vitro* differentiated human neurons (DIV30) were incubated for 1hour (37°C, 5% CO2) with 2µM Cal590-AM (#20510, AAT Bioquest), diluted in the differentiation media. CAL590-AM is a sensitive dye for detecting the intracellular Ca^{2+} changes, which can be recorded as differences in the amplitude of cells' fluorescence. After incubation, cells were rinsed with the differentiation media to remove excess of indicator and then the cell dish was transferred to the confocal microscope for the live imaging (ZEISS LSM 700, AxioObserver). Time-lapse imaging was performed using ZEISS incubator chamber and maintaining the temperature at 37°C and the CO2 at 5%. The images were acquired every 15 seconds for 30 minutes. Each recorded video was analyzed on ImageJ, where the regions of interest (ROIs) were traced around the cell bodies and the fluorescence intensity per each ROIs and per each time point of the video were extracted into an excel file (.csv). The data were then analyzed through a MATLAB script (MathWorks), created in our laboratory, able to detect the Ca^{2+} events per each cell and the relative amplitude and frequency. Ca^{2+} events with an amplitude higher than a threshold, equal to the standard deviation (SD) of the mean fluorescence of the trace, were detected. The code analyzed the trace and the relative peaks three times. Each time the already detected peaks and the values above the SD threshold were deleted from the traces. This allowed the detection of peaks with smaller amplitude. In this way, the mean amplitude and frequency of calcium events were obtained per each active cell. Lastly, the relative amplitude of the calcium events was calculated as $\Delta F/F_0 = (F - F_0)/ F_0$, where F_0 was the total

background, calculated as the mean of three background ROIs. The Ca^{2+} frequency was calculated as the number of peaks over the total duration (s) of the video (time points: 120; duration: 1785 seconds).

3.9 Statistical analysis

Data are expressed as means ± SEM. Statistical significance was determined using an unpaired two-sided Student's t-test. Equality of variance for the unpaired t-test was verified using the F-test. The levels of significance were as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Chapter 4. Results

4.1 TNR expression in mouse and human cerebellums

The clinical conditions of the patient carrying TNR mutation suggested a strong involvement of the cerebellum in the disease. Indeed, functional magnetic resonance imaging (fMRIs) of the same patient four years apart, performed at 3- and 7-year-old, show a significant progressive increase in degree of diffuse atrophy of cerebellar hemispheres and vermis (figure 8A-B). Therefore, the first step was to assess TNR protein expression and distribution in both mouse and human cerebella in physiological conditions. TNR expression in mouse cerebellum was analyzed at different developmental stages (postnatal days 2, 7, 15, 40, 64), using cerebellar sagittal sections immunolabeled with anti-TNR antibody. We observed that in the early developmental stages (P2 and P7) TNR was mainly expressed in the cerebellar white matter (WM; figure 8C) and barely present in the outer layers (EGL, IGL and PCL). In contrast, at the later developmental stages (P15, P40 and P64) this pattern was reverted, and TNR was mainly expressed in the molecular and in the granular layers (respectively ML and GL; figure 8D). In parallel, in collaboration with the Douglas Brain Bank (McGill University), TNR labeling was performed on post-mortem cerebellar samples of adolescent-aged human and TNR was detected in both GL and ML (figure 8D). This expression pattern, found both in human and murine cerebella, suggests that TNR expression is tightly regulated in a spatial and temporal manner.

TNR is an ECM protein and it is synthetized by oligodendrocytes and by a subset of interneurons in several brain areas, including the cerebellum. Therefore, we hypothesized that the switch in TNR expression pattern observed in the cerebellum could have been linked to the involvement of different TNR-releasing cell types. To confirm this, we performed RNAscope in situ hybridization on mouse cerebellar sections using specific probes to detect TNR mRNA together with Olig2 and NeuN mRNAs (figure 9A-B). For this experiment, four developmental stages have been considered (P2, P7, P15, P64) and for each two animals have been used. Cells co-expressing TNR and Olig2 mRNAs or TNR and NeuN mRNAs have been quantified in the white matter (WM), molecular layer (ML) and granular layer (GL) of the murine cerebellum (figure 9C). We were able to confirm a switch in TNR expression also at RNA level: during early development TNR mRNA was colocalizing with Olig2 mRNA in the cerebellar WM and later on TNR mRNA was co-expressed with NeuN mRNA in both ML and GL. It is worth to mention that no TNR-expressing cell was observed in the ML during P2 and P7 stages, when the ML is still thin compared with the other cerebellar layers.

However, to consider the in situ hybridization data statistically significant, further replicates will be needed since so far the experiment was conducted on just two animals per condition. Same analysis was performed on human cerebellar samples of different stages: 2-, 18- and 26-year-old (figure 9D) and the expression of TNR was detectable mainly in the ML, showing an age-related decrease. Further analysis, combining TNR probes with Olig2 and NeuN probes, will provide more detailed data regarding the cellular sources of TNR during human cerebellar development. Overall, we can conclude that in mouse cerebellum TNR is differently expressed by neurons and oligodendrocytes, depending on the cerebellar areas and on the developmental stage. Further studies will be needed to confirm the same pattern of expression of TNR in human cerebellums.

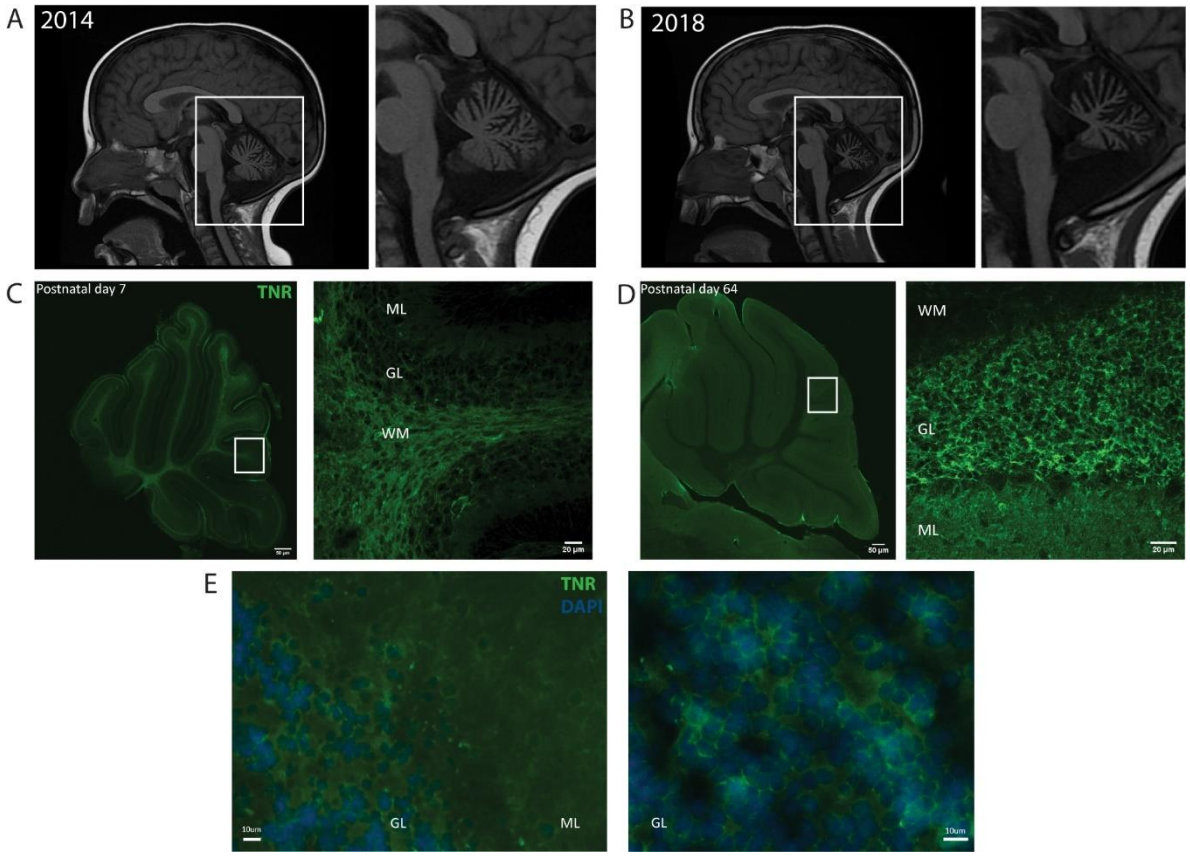


Figure 8. TNR protein expression and distribution in mouse and human cerebellums. (A) A fMRI of the patient carrying TNR deletion in 2014, the year of the diagnosis. (B) fMRI of the same patient four years later the diagnosis, in 2018. Four years apart, the cerebellum of the patient undergoes a progressive and rapid cerebellar atrophy. (C) In the murine cerebellum, TNR expression is tightly regulated and at postnatal day 7 is mostly present in the white matter, WM. (D) In adult murine cerebellum (postnatal day 64), TNR expression pattern changes and it is mostly present in the molecular, ML, and in the granular layers, GL. (E) Levels of TNR expression have been evaluated also in adolescent-aged samples of human cerebellum revealing TNR expression in both GL and ML.

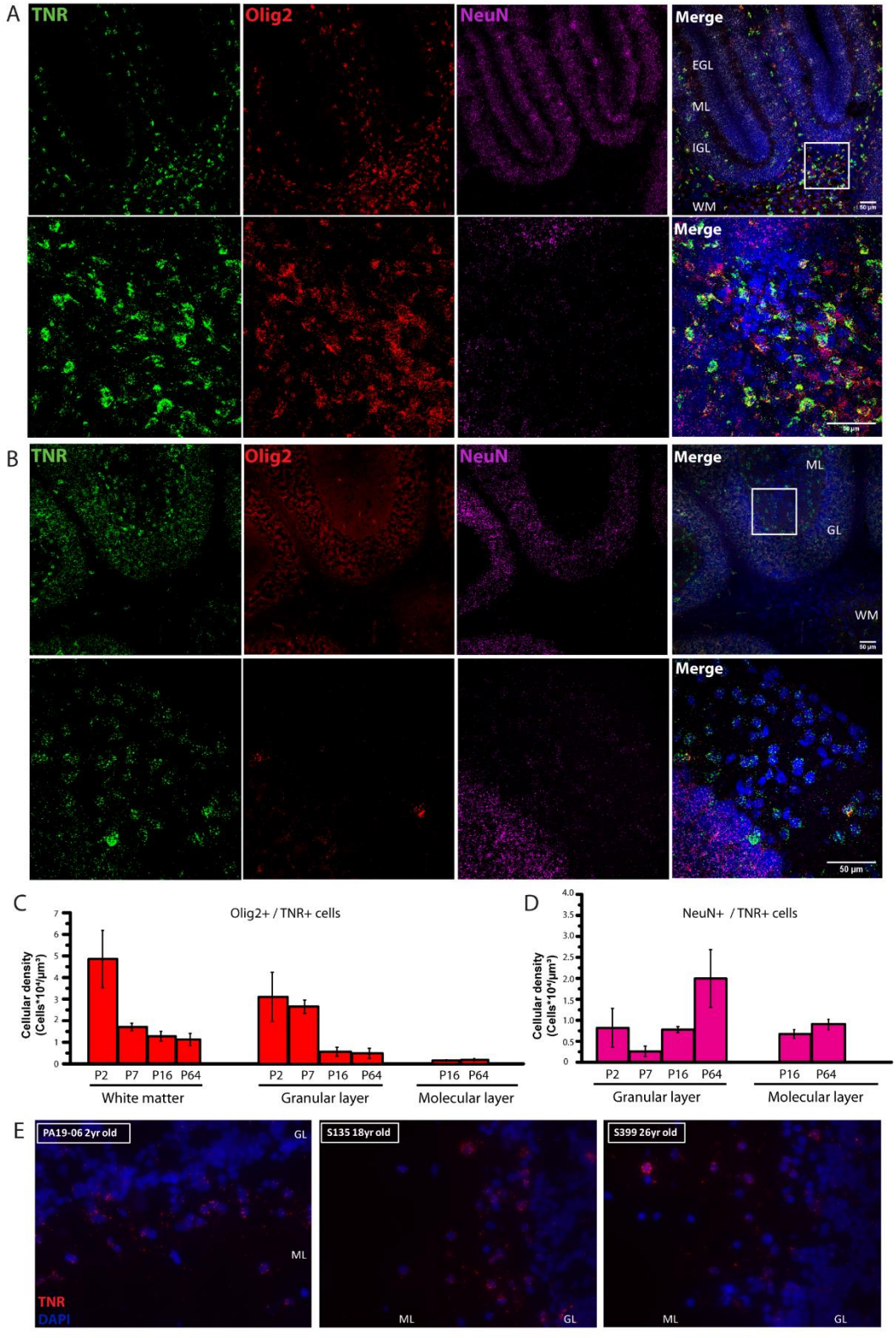

A
TNR
Olig2
NeuN
Merge
EGL
ML
IGL
WM
Merge
B
TNR
Olig2
NeuN
Merge
ML
GL
WM
50 µm
Merge
50 µm
C
Olig2+ / TNR+ cells
Cellular density (Cells*10⁴/µm³)
P2 P7 P16 P64
White matter
P2 P7 P16 P64
Granular layer
P16 P64
Molecular layer
D
NeuN+ / TNR+ cells
Cellular density (Cells*10⁴/µm³)
P2 P7 P16 P64
Granular layer
P16 P64
Molecular layer
E
PA19-06 2yr old
GL
ML
TNR
DAPI
S135 18yr old
ML
GL
S399 26yr old
ML
GL

Figure 9. RNAscope in situ hybridization in mouse and human cerebellums.

RNAscope in situ hybridization has been used to localize TNR mRNA (green) co-expressed with NeuN (magenta) or Olig2 (red) mRNAs in mouse and human cerebellums during development. (A) At postnatal day 7, TNR mRNA is mostly present in the white matter of mouse cerebellum, colocalizing mostly with Olig2 mRNA. Images at the bottom are showing high magnification of RNAscope in situ hybridization in the WM at P7. (B) In the adult cerebellum, specifically at postnatal day 64, TNR mRNA is mainly co-expressed with NeuN in ML and GL. Images at the bottom are showing high magnification of RNAscope in situ hybridization in the ML at P64. (C) Cellular densities of cells co-expressing Olig2 and TNR mRNA for three cerebellar areas and for four different developmental stages (n=2 per each time point). (D) Cellular densities of cells co-expressing NeuN and TNR mRNA (n=2 per each time point). (E) TNR mRNA expression has been confirmed as well in human samples of different developmental stages: 2-, 18- and 26-year-old. Means ± s.e.m. plotted.

4.2 Study of hNPCs developmental profile in *vitro* and *in vivo*

Patient-derived iPSCs, carrying a mutation for TNR, and control iPSCs were first characterized *in vitro* and then used for the generation of human neural progenitor cell (hNPCs). To easily track hNPCs they were infected with GFP-expressing lentivirus and thus, hNPCs were stably expressing GFP. As already mentioned, hNPCs development was investigated both *in vitro* and *in vivo*. Newborn Rag1-/- mice, engrafted with TNR KO or CTRL cells, were used for studying hNPCs developmental profile *in vivo*. In this study, the cerebellum represented the most suitable brain region for the engraftment of hNPCs for these three main reasons: 1. the possible involvement of TNR in cerebellar development and dysfunctions, as suggested by TNR expression pattern (*see figures 8 and 9*); 2. its well-established cytostructure; 3. the presence of postnatal neurogenesis in the cerebellar external granular layer, which creates a perfect micro-environment for the viability and the integration of the engrafted cells. In this context, the developmental profile of TNR mutated and CTRL hNPCs grafted into the cerebellum was studied to assess whether the lack of TNR affects proliferation, maturation and neuronal activity.

4.2.1 hNPCs proliferation

The proliferation rate of hNPCs was evaluated *in vitro* as well as *in vivo*, after engraftment. In both cases, CTRL and TNR KO hNPCs were considered in a proliferating state when they were expressing Ki67, a proliferation marker. *In vitro*, hNPCs were stained with anti-Ki67 antibody and cells expressing Ki67 were quantified among the total of DAPI cells (figure 10A). No significant differences were found in the proliferation rate of control and mutated hNPCs *in vitro* (TNR KO: 68.58% ± 5.31 Ki67+/DAPI cells, n=3; CTRL 1: 74.83% ± 3.15 Ki67+/DAPI cells, n=3; CTRL 2: 78.35% ± 7.07 Ki67+/DAPI cells, n=3; figure 10C). For *in vivo* quantification, GFP+ hNPCs were transplanted in Rag1-/- mice cerebellum and cell proliferation was evaluated one-week post injection (wpi; figure 10B). Cells co-expressing GFP and Ki67 were quantified over the total of GFP+ cells and the results showed not significant differences between TNR KO and CTRL cells in terms of proliferation (TNR KO: 13.25% ± 0.75 Ki67+/GFP+ cells, n=3; CTRL 2: 9.29% ± 1.33 Ki67+/GFP+ cells, n=3; figure 10D). So far, the *in vivo* quantification has been performed on grafted TNR KO cells and CTRL line 2 cells. Thus, further engraftment and analysis of CTRL line 1 cells will be needed to confirm these data *in vivo*. The evaluation of TNR role in cell proliferation both *in vitro* and *in vivo* experiments, has allowed us to have more robust data. Indeed, supporting the *in vitro* proliferation analysis with data obtained *in vivo*, we confirmed that even in a micro-environment more complex, like the mouse cerebellum, where the hNPCs are exposed to multiple and different stimuli, the absence of TNR was not influencing the proliferation of the hNPCs. Taken together, these results do not show any differences between CTRL and TNR KO hNPCs in terms of proliferation, underlying that TNR lack does not affect this specific developmental stage.

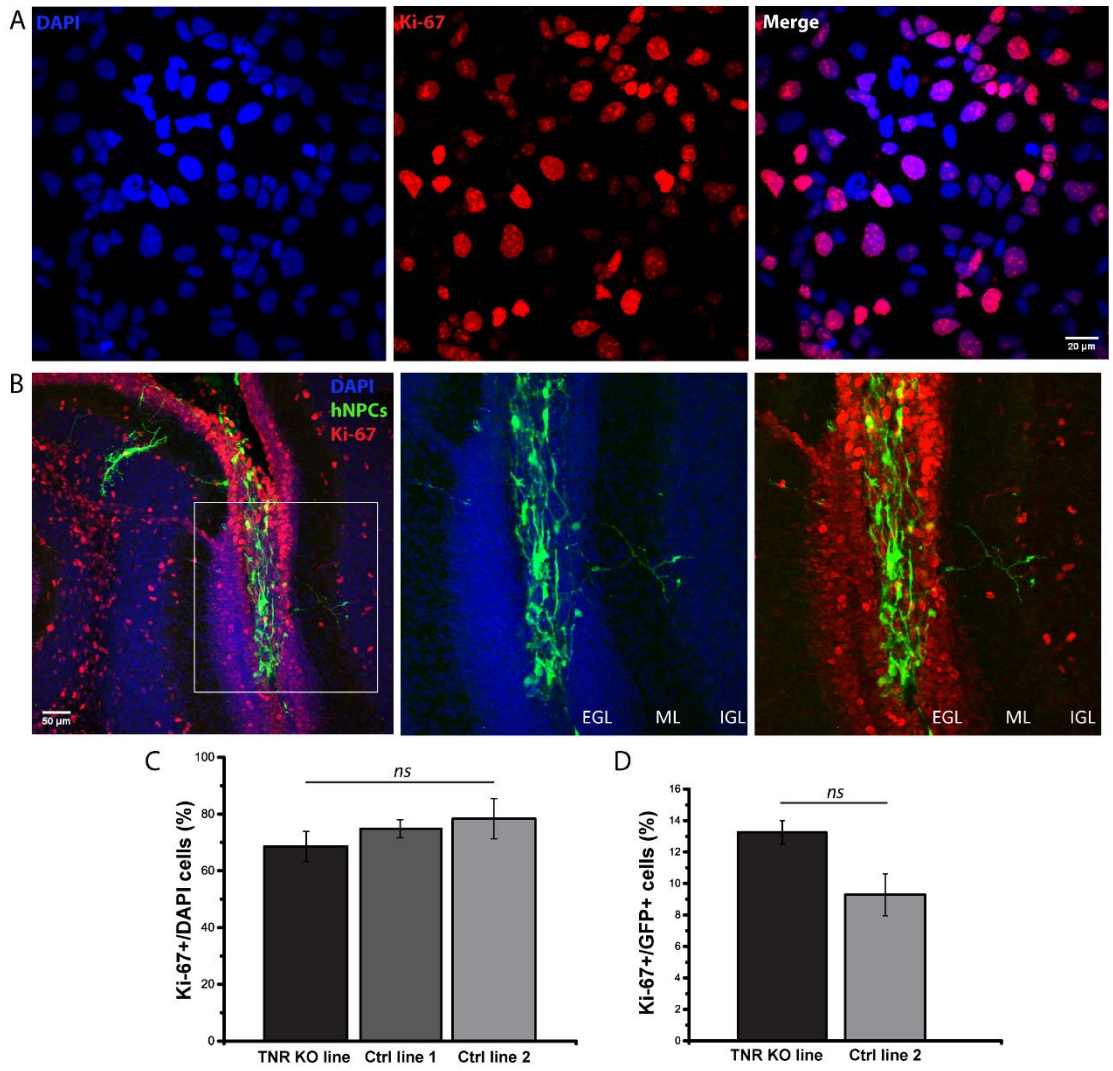


Figure 10. *In vitro* and *in vivo* analysis of hNPCs proliferation.

(A) Confocal images of *in vitro* hNPCs immunolabeled for anti-Ki67 antibody (red). (B) Confocal images of GFP+ and Ki67+ TNR KO hNPCs in the cerebellum of Rag1-/- mice, sacrificed one-week post injection. (C) *In vitro* Ki67+ cells were quantified over the total number of DAPI stained cells in order to assess their proliferation rate. (D) *In vivo* hNPCs proliferation rate was determined by quantifying Ki67+/GFP+ cells. Means ± s.e.m. plotted; *P < 0.05, **P < 0.01, ***P < 0.005.

4.2.2 hNPCs maturation *in vitro* and *in vivo*

Alterations in neuronal morphology and branching patterns have been observed in a wide range of neurodevelopmental disorders. Moreover, one of the multiple roles of TNR is the regulation of neurite outgrowth during neuronal maturation[142,143]. Thus, we decided to analyze the morphological features of mature TNR KO and CTRL neurons.

In vitro hNPCs were first differentiated in mature neurons, using the BrainPhys protocol[138]. After one week in differentiating medium, the TNR KO and CTRL induced neurons were showing mature neuronal morphology (figure 11A) and were expressing Map2, a marker of mature neurons (figure 11B). Then, the Bonfire method was used for analyzing the neuronal morphology of TNR KO and CTRL neurons. This method, built upon other available open-source morphological analysis tools, was developed to facilitate and optimize digitization of neurite morphology and subsequent Sholl analysis[139]. Images of single differentiated neurons, after 40 days *in vitro*, were taken and analyzed with the Bonfire method (figure 11C). *In vitro*, no significant differences were observed in terms of number and average length of processes per cell between TNR KO and CTRL neurons (figure 11D-E). Similarly, the Sholl analysis did not reveal any significant differences between TNR KO and CTRL line 1 neurons, whereas a slight difference between TNR and CTRL line 2 neurons was observed (figure 11F). These data indicated that *in vitro*, the morphological maturation of neurons, lacking TNR, was not dramatically impaired if compared with the control ones.

Since, the micro-environment in a dish cannot recapitulate the variety of molecules and cells present in the brain, we decided to follow the development of hNPCs, TNR KO or CTRL, *in vivo*. As already mentioned, hNPCs were grafted in the cerebellum of newborn Rag1-/- mice and their development was evaluated at three different time points: 3wpi, 3mpi and 5mpi. For the *in vivo* analysis, we took in consideration that the specific cerebellar layer, in which the grafted hNPCs migrated and maturated, could influence their neural fate and consequently their morphology. Therefore, human neurons integrated in the molecular and/or in the granular layer of the mouse cerebellums were considered as two different experimental groups.

At 3wpi, GFP+ hNPCs resulted spread in the host cerebellum and expressed the human nuclear marker (huNu; figure 12A). Images of single differentiated neurons, 3wpi, were taken and analyzed with the Bonfire method (figure 12B). At 3wpi, TNR KO and CTRL neurons in the molecular and in the granular layers showed significant differences in the process number and length (figure 12C-D). However, these data need further confirmation since they were quite controversial, showing significant difference also between CTRL1 and CTRL 2

neurons. Intriguingly, the Sholl analysis for 3wpi neurons, in both molecular and granular layers, showed an interesting trend. Indeed, the number of intersections for TNR KO neurons seemed to be high until 70um-distance from the soma and then dropped compared with CTRL neurons (figure 12E-F).

At 3mpi, TNR KO and CTRL neurons in the molecular layer did not show any significant differences in the number and/or length of processes (figure 13C). Instead, the Sholl analysis for neurons in the molecular layer showed that both TNR KO and CTRL neurons undergo a more complex morphological modification, if compared with the 3wpi time point (figure 13D). In addition, we observed a significant difference in terms of process number and Sholl analysis, but not in terms of process length, between TNR KO and control neurons in the granular layer (figure 13E-F). Comparable data of the CTRL 2 neurons are missing for this time point.

At 5mpi, the last time point evaluated in this study, TNR KO and CTRL neurons in the molecular layer did not show any significant differences in the number and/or length of processes (figure 14B). Instead, the Sholl analysis for neurons in the molecular layer showed that CTRL neurons undergo a more complex morphological organization while TNR KO remain quite unchanged (figure 14C). Similarly, 5mpi TNR KO and CTRL neurons in the granular layer showed significant difference in terms of process number and Sholl analysis, but not in terms of process length (figure 14D-E).

All together, these data showed that at later time points the morphological complexity of CTRL neurons increases, while the one of TNR KO neurons remains mainly unchanged.

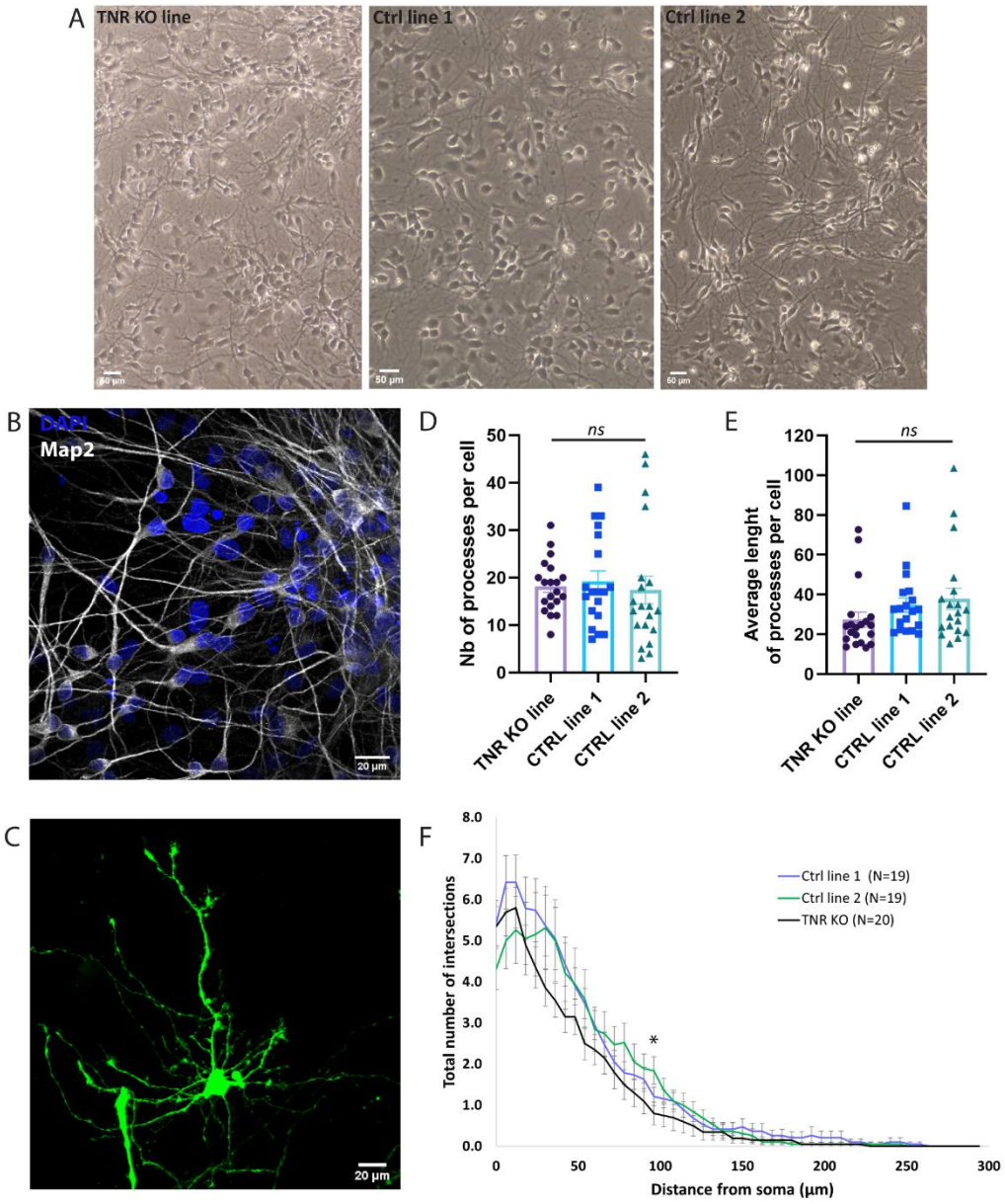


Figure 11. *In vitro* analysis of hNPCs maturation.

(A) Phase-contrast images of the mature neurons at day 7 of differentiation. (B) *In vitro* neurons at day10 express markers of neuronal differentiation, as MAP2. (C) Confocal image of a single neuron in *vitro* at day 40 traced and used for morphological analysis (Bonfire Method). (D-E) Single neurons for each cell line were traced and the number of processes and the average length of processes per cell was calculated. (F) *In vitro* Sholl analysis performed for both control and TNR KO lines, the significance is between Ctrl line 2 and TNR KO line (TNR KO n=20; Ctrl 1 n=19; Ctrl 2 n=19). Means ± s.e.m. plotted; *P < 0.05, **P < 0.01, ***P < 0.005.

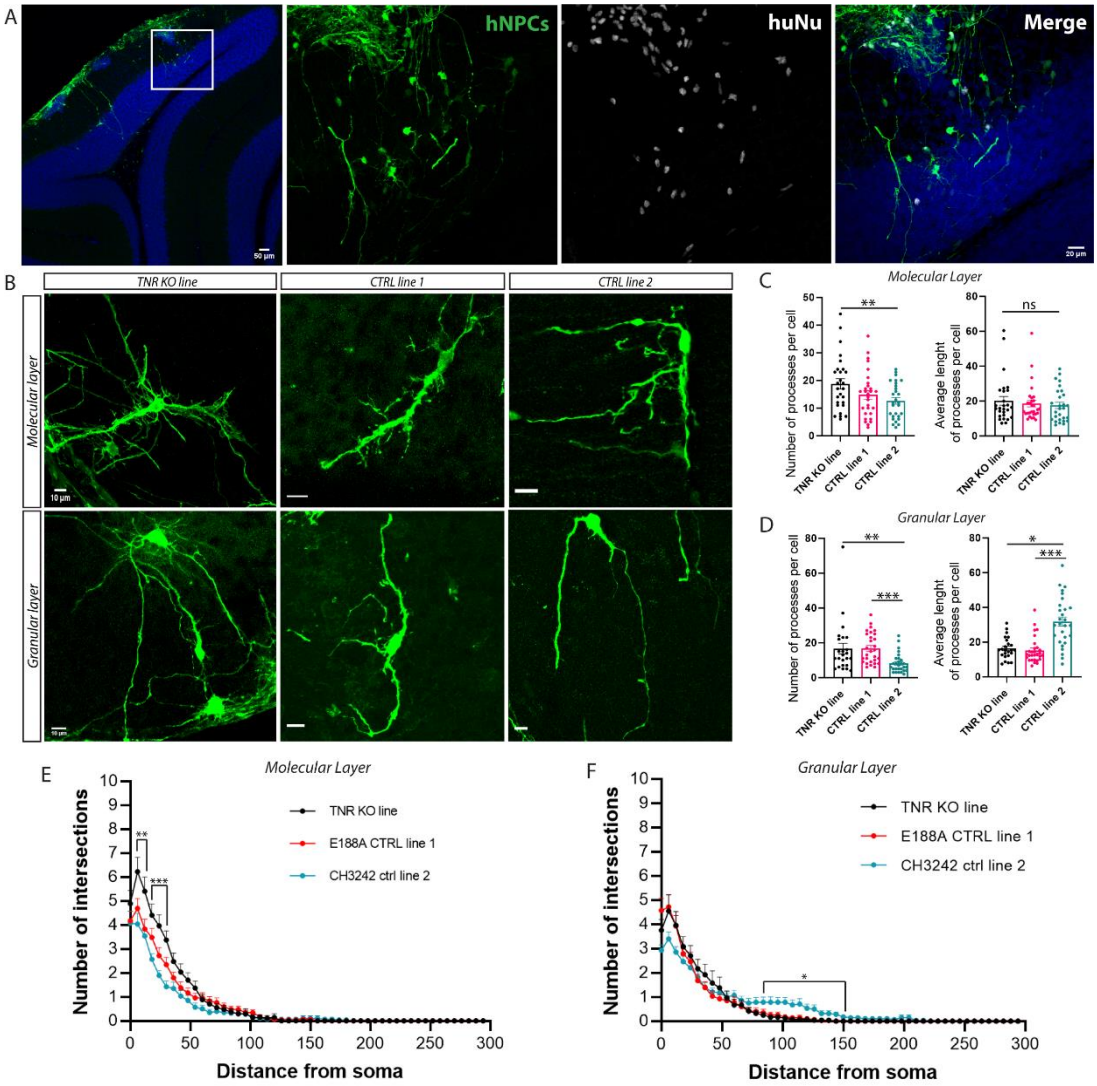


Figure 12. *In vivo* analysis of hNPCs maturation at 3wpi.

(A) Confocal images of hNPCs 3 weeks post-injection in the cerebellum of Rag1-/- immunodeficient mice. hNPCs were stained for anti-human nuclei (huNu, gray).

(B) Confocal pictures of human neurons in the molecular and granular layer of the host cerebellum at 3wpi, which were used for morphological studies (Bonfire method).

(C) Number and average length of processes per cell for TNR KO or CTRL neurons in the molecular layer. (D) Number and average length of processes per cell for TNR KO or CTRL neurons in the granular layer. (E) Sholl analysis for neurons in the molecular layer. (F) Sholl analysis for neurons in the granular layer. In the Sholl analysis graphs E-F the significance is relative to differences between TNR KO line and CTRL line 2. (TNR KO: n=27, CTRL 1: n=29, CTRL 2: n=28 in the molecular layer and TNR KO: n=24, CTRL 1: n=29, CTRL 2: n=28 in the granular layer). Means ± s.e.m. plotted; *P < 0.05, **P < 0.01, ***P < 0.005.

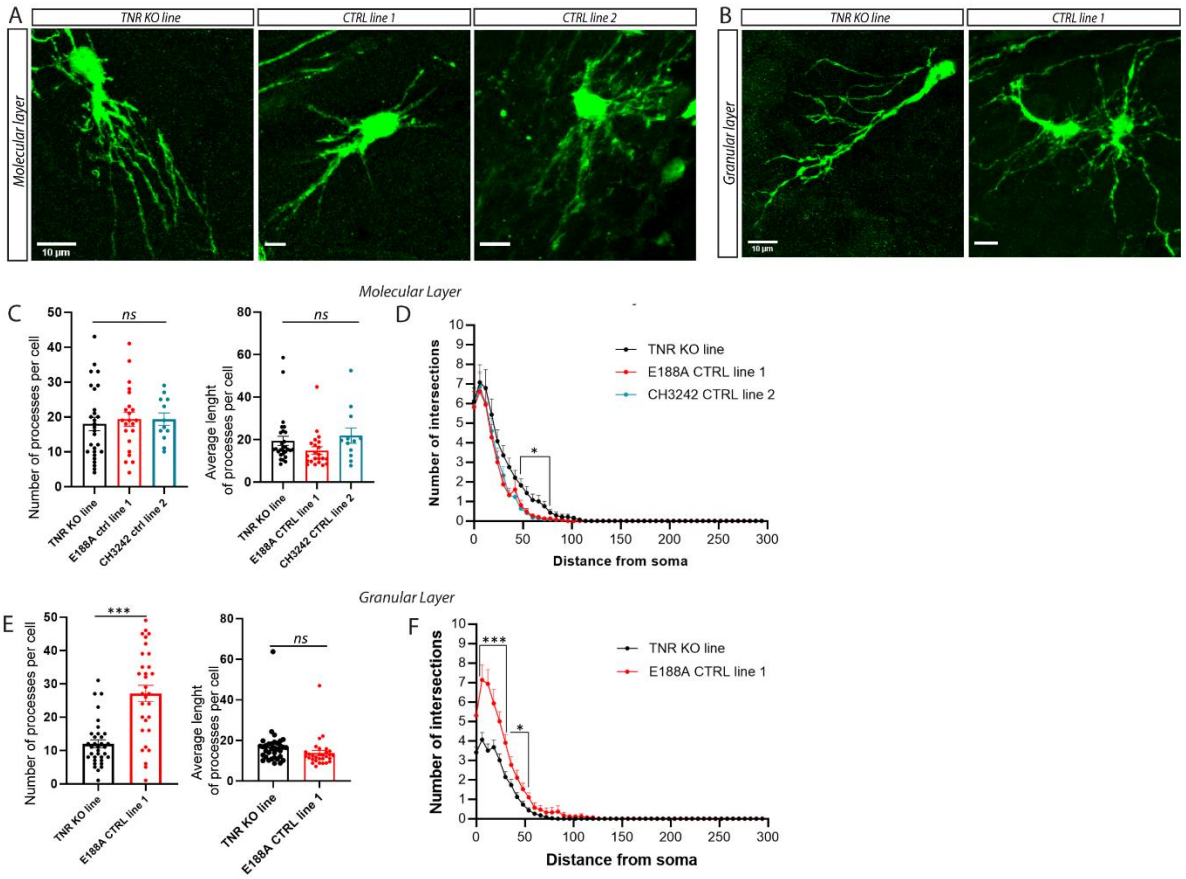


Figure 13. *In vivo* analysis of hNPCs maturation at 3mpi.

(A-B) Confocal pictures of human neurons in the molecular and granular layer of the host cerebellum at 3mpi, which were used for morphological studies (Bonfire method).

(C-D) Neurite number and length, and Sholl analysis of neurons in the ML at 3mpi (TNR: n=28; CTRL1: n=22; CTRL2: n=12). (E-F) Neurite number and length and Sholl analysis of neurons in the GL at 3mpi (TNR: n=34; CTRL1: n=31). In the Sholl analysis, the statistic is related to the comparison between TNR KO and CTRL 1 lines. Means ± s.e.m. plotted; *P < 0.05, **P < 0.01, ***P < 0.005.

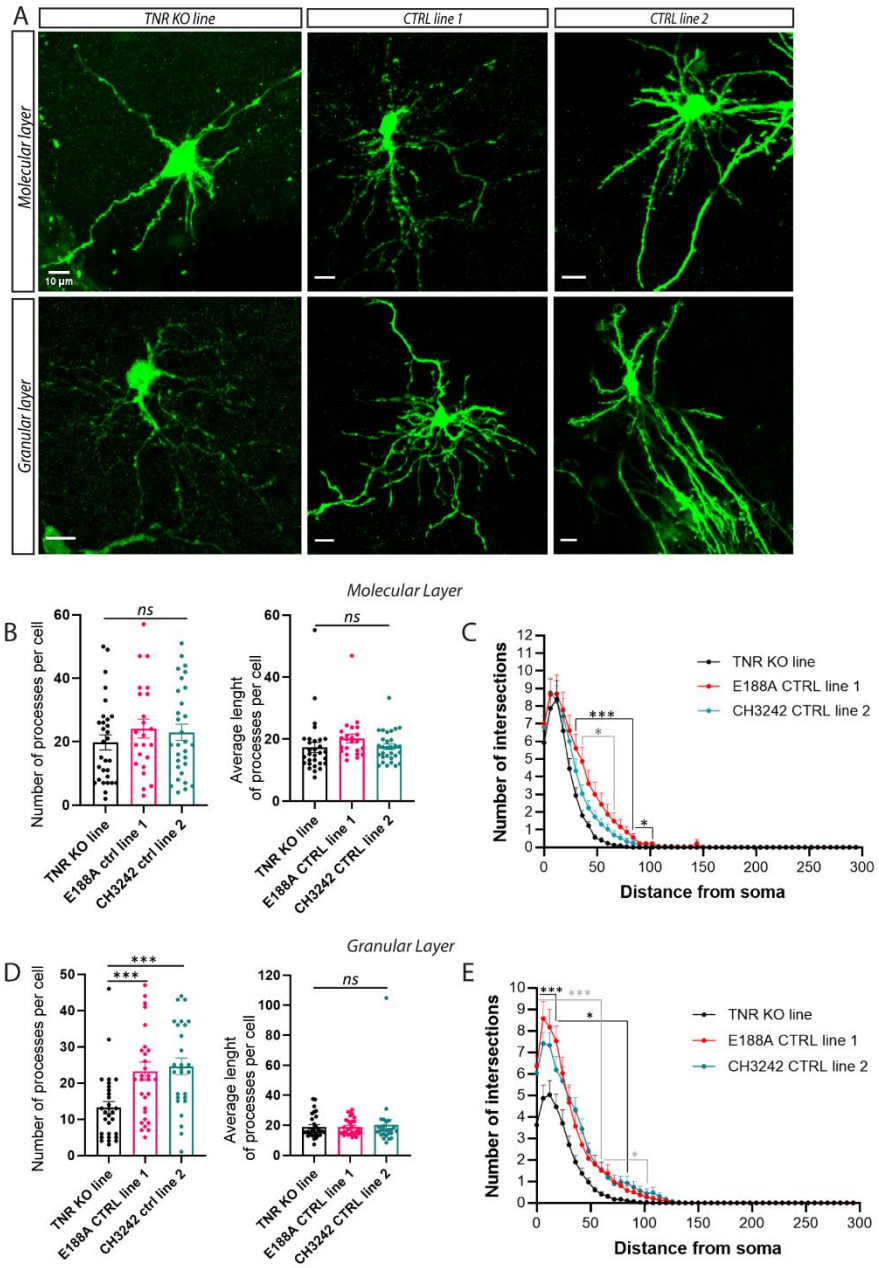


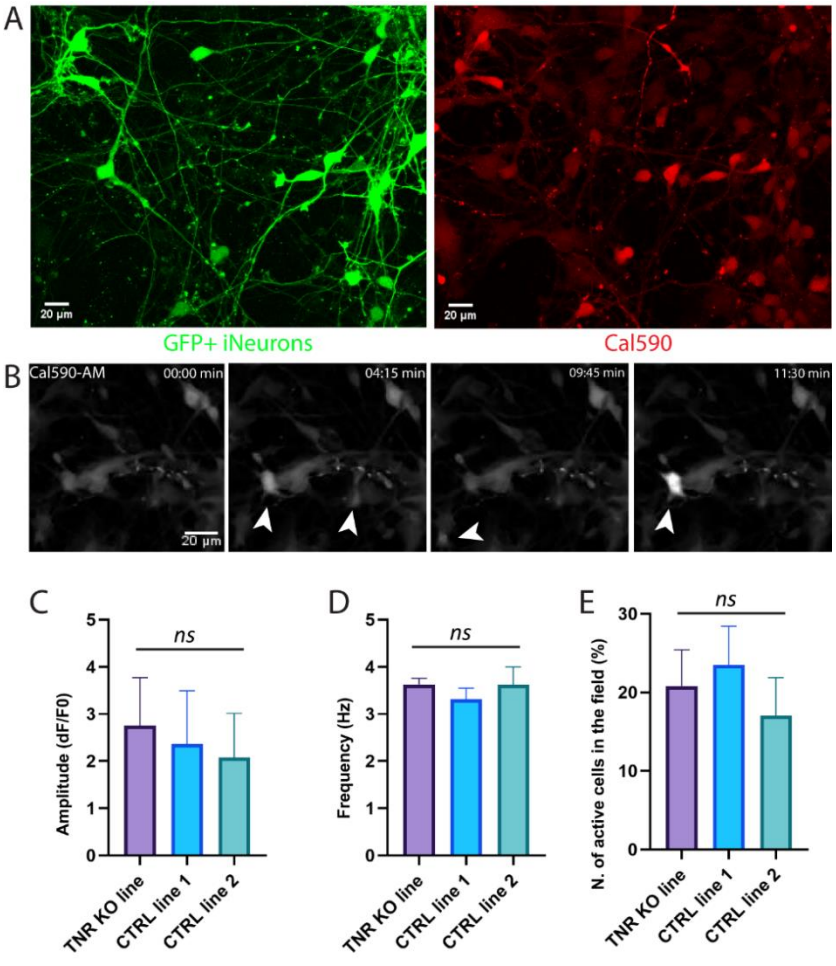
Figure 14. *In vivo* analysis of hNPCs maturation at 5mpi.

(A) Confocal pictures of human neurons in the molecular and granular layer of the host cerebellum at 5mpi, which were used for morphological studies (Bonfire method). (B-C) Neurite number and length and Sholl analysis of neurons in the ML at 5mpi (TNR: n=30; CTRL1: n=23; CTRL2: n=30). (D-E) Neurite number and length and Sholl analysis of neurons in the GL at 5mpi (TNR: n=30; CTRL1: n=30; CTRL2: n=27). In the Sholl analysis, the statistics indicated in black are related to TNR KO and CTRL 1 comparison, while the ones in gray to TNR KO and CTRL 2 comparison. Means ± s.e.m. plotted; *P < 0.05, **P < 0.01, ***P < 0.005.

4.2.3 hNPCs functional activity *in vitro*

To assess the role of TNR in the functional activity of differentiated human neurons, the calcium activity of TNR KO and control neurons was assessed *in vitro*. Neurons were incubated for 1hour with 2µM Cal590-AM, a sensitive dye for detecting the intracellular Ca^{2+} changes (figure 15A). After incubation, neurons were imaged using time-lapse imaging and spontaneous Ca^{2+} activity was detected as changes in the fluorescence activity. We were able to detect the Ca^{2+} activity of several neurons in both TNR KO and control conditions (figure 15B). However, no significant differences were observed in terms of amplitude (figure 15C), frequency (figure 15D) and percentage of active cells (figure 15E) between TNR KO and control neurons.

Figure 15. *In vitro* analysis of hNPCs Ca^{2+} activity.



(A) GFP+ CTRL line 2 neurons, at day 35, loaded with Cal590 dye. (B) Snapshots of spontaneous Ca^{2+} activity of neurons recorded over 30 minutes at 37°C and 5% CO_2. The arrowheads indicate neurons with Ca^{2+} activity during the period of imaging. (C) Amplitude (dF/F0) of the Ca^{2+} activity recorded for CTRL and TNR KO neurons. (D) Frequency of the Ca^{2+} events was quantified per cell as number of peaks during the time of recording. (E) Percentage of number of active cells in the field for TNR KO and CTRL lines. Means ± s.e.m. plotted; *P < 0.05, **P < 0.01, ***P < 0.005.

Chapter 5: Discussion

The number of clinical cases in which TNR mutations have been associated with neurodevelopmental disorders increased in the last few years, thus affirming the need of understanding more about the role and the mechanisms of TNR during human development[5,6]. In this study, we combined the use of humanized mouse models together with iPSCs derived from a clinical case carrying TNR mutations, in order to improve the current knowledge on TNR functions. The cognitive and motor dysfunctions as well as the cerebellar atrophy, observed in the patient, were suggesting an important role of TNR during cerebellar development. Moreover, has been shown that human cerebellar development can be extended until many years after birth, thus cerebellar-related dysfunctions appear often postnatally. Consistently, the clinical subject of this study reported her first symptoms at 1year of age (confidential data).

TNR expression in the cerebellum

The first aim of this project was to analyze TNR expression in both mouse and human cerebellums. We have shown that TNR protein and mRNA expression pattern is tightly regulated in spatial as well as temporal manner during cerebellar development. This expression pattern is related to different cell types involved in the expression and secretion of the protein in the ECM compartment. Indeed, during early postnatal development, oligodendrocytes are the main cell type implicated in the synthesis of TNR, mostly in the cerebellar white matter. Accordingly, the first two postnatal weeks correspond to the phase of oligodendrocyte precursor migration and active myelination in the mouse brain[144]. During this time window, TNR has been shown to be abundant also in the white matter of other CNS regions[1]. Once cerebellar myelination has ceased, both mRNA and protein levels were downregulated in the white matter. On the contrary, TNR mRNA and protein was increasing in the cerebellar ML and GL along with neuronal maturation and their expression levels were high during adulthood (mice at P64). Consistently, a similar switch in TNR expression by oligodendrocytes and neurons has been shown in the mouse optic nerve[1]. Little is known about TNR in the human brain and our data showed TNR expression in human cerebellum at different developmental stages. The age-regulated expression of TNR in human cerebellum is in accordance with the expression of TNR during human corticogenesis, when TNR distribution is spatial and temporal regulated[34]. In this context, should be considered that TNR functions are not simply related to its distribution but also to the interaction that TNR can have with other ECM molecules. For instance, TNR functions could be regulated

by the binding to carbohydrates present in the ECM space. Interestingly, it has been showed that during cerebellar development HNK-1, a carbohydrate carried by TNR, is temporally and spatially regulated[17]. Moreover, it has been demonstrated that in the cerebellar molecular layer TNR is uniquely linked with GalNac-4-SO_4, a sulfated carbohydrate whose expression is dramatically increases between postnatal days 14 and 21, corresponding to a period of Purkinje cell dendrite extension and synaptogenesis[17]. To summarize, TNR can be differentially expressed in the mouse cerebellum and once released in the ECM space, it can interact with different carbohydrates and proteins, whose expression is spatially and temporally regulated as well. Regarding the human cerebellum, we have observed an age-related expression pattern of TNR, however it remains still unknown which cells are releasing TNR during human development and which type of post-translational modifications can change the human TNR properties.

Study of control and TNR KO hNPCs

Due to the obvious ethical limitations related to the study of human developmental disorders *in vivo*, in this study we combined patient-derived iPSCs and humanized mouse models to investigate TNR functions during *in vitro* and *in vivo* neural development.

We showed that the lack of TNR does not influence hNPCs proliferation either *in vitro* or *in vivo*, after their engraftment into mouse cerebellum. Therefore, the cerebellar dysfunctions observed in the clinical subject in study cannot be linked to affected proliferation of neuronal precursors. In support of these results, there are data showing that *in vitro* TNR is not detectable in cultured neural stem cells (NSCs), whereas is expressed by differentiating or differentiated cells[21]. Similarly, *in vivo* studies have demonstrated that TNR is not expressed in the neurogenic niche of the SVZ in the murine brain[23].

Later, hNPCs maturation was investigated *in vitro* and *in vivo* by evaluating the neural morphology. Neurite branching affects how single neurons integrate synaptic inputs and how they communicate as networks. Alterations in neuronal morphology and branching patterns have been observed in a wide range of developmental or acquired disorders in which it is thought that altered arbor structure plays a role in the pathogenesis of the disorder[145]. To assess the morphological maturation of TNR KO and control neurons, we took advantage of the Bonfire method, an automated method that provides, per each cell traced, the number and the length of neurites as well as Sholl analysis data. By using this morphological analysis *in vitro,* we did not observe any dramatic differences between TNR KO and CTRL neurons cultured over 40 days in differentiation medium.

Since, the micro-environment in a dish cannot recapitulate the variety of molecular and cellular cues present in the brain, we investigated the morphological maturation of hNPCs, TNR KO or CTRL, *in vivo* at three different time points: 3wpi, 3mpi and 5mpi. For this *in vivo* analysis, we considered that hNPCs could mature as different neuronal subpopulation and by consequence its morphology could have been affected by the different cerebellar layer in which hNPCs were integrated. Thus, neurons in the GL and in the ML were considered as two different experimental groups per each time point evaluated in this study. Overall, our results showed that neurons lacking TNR showed a smaller number of process and less dendritic arborization, as shown by the decreased number of intersections in the Sholl analysis. However, this effect become visible just in the late developmental stages, whereas in the early developmental stages TNR KO neurons seem to develop as well as control neurons. Over the development periods, the branching pattern of TNR KO neurons remains steady and does not complexifies over the time. This is in contrast to CTRLs cells that continue to develop their dendritic arbor over the period of 5 months. These data are particularly interesting since TNR has been implicated in the regulation of neurites outgrowth[142,143]. A possible explanation to this, could be that human neurons have longer developmental dynamics, and they reach their mature state within longer time points as compared to mouse neurons. In line with this are recent data showing prolonged developmental dynamics of xenografted human cortical neurons in the visual cortex[146].

Our data could be also explained as a balanced result between the intrinsic properties of the hNPCs and the extrinsic properties of the host murine environment. The micro-environment of the host cerebellum can be an important player in the maturation of hNPCs. Indeed, it is possible that the presence of particular molecular and cellular cues in the host brain contributed to the final morphological aspect of the engrafted human neurons. In this context, should be considered the high affinity of TNR with different carbohydrates, whose levels in the molecular and granular layer of the cerebellum could influence the neuronal maturation of TNR KO and control hNPCs. In addition, it should be taken into account that Rag1-/- mice cerebellums, used as site of engraft, express functional murine TNR which might influence the developmental profile of hNPCs. This hypothesis could be tested by using Rag1-/- x TNR-/- mice as host for the hNPCs engraft.

It should also be considered that in some cases significant differences were also observed between the control lines. This observation leads us to consider that some of these differences could be related to the origin of the control iPSCs used in this study. Indeed, while the control line 1 derived from a 3-year-old female, the control line 2 derived from a

19-year-old male. Although these two control donors were healthy subjects, the differences in non-genetic factors, such as age and sex, could explain the variability observed in this study. Therefore, it is important to understand how age and sex influence the epigenomes and genomes of iPSCs. To this regard, recent evidence has demonstrated that age can reduce the efficiency of cell reprogramming and that iPSCs retain an epigenetic signature of age that diminishes with passaging[147,148]. Moreover, iPSCs heterogeneity can be linked to the donor sex and it has been shown that X chromosome gene dosage as well as Y chromosome can drive sex-specific differences in the growth and differentiation of iPSCs[149–151]. Thus, for further studies, it will be important to consider the use of isogenic lines, namely use the patient-derived line and correct the mutation using CRISPR/Cas9 technique. This will provide control lines with the same genetic background of the mutated line, thus decreasing the subject-related variability.

In light of this, it must be considered that in this study hNPCs were engrafted into male and female pups and that no distinction has been made according to the host sex. Therefore, we cannot exclude that the sex of the host influenced the results obtained.

In this study, we also showed that the lack of TNR seems to do not affect the spontaneous calcium activity of human neurons *in vitro*. However, must be considered that calcium analysis was performed by using neurons at DIV=40 and that, as mentioned above, the degree of maturation and differentiation of the neurons was maybe not enough to observe significant differences between control and TNR KO neurons. Therefore, the role of TNR as modulator of neuronal activity must be assessed also *in vivo*, in a more complex brain environment, and by evaluating not only the spontaneous calcium activity but also the electrophysiological properties of neurons lacking TNR. Indeed, has been reported that TNR, together with other extracellular molecules, regulates the localization of Na_v channels at the nodes of Ranvier[30]. Given the central role of Na_v channels in electrical excitability, it is not surprising that neurons lacking TNR showed also decreased conduction velocities of action potential along the axons[32]. Moreover, the activity of Na_v channels has long been linked to disorders of neuronal excitability and to NDDs, such as autism[152]. To this purpose, TNR has also been described as an essential molecule in the formation of PNNs, specialized extracellular structures that appear in some CNS areas postnatally and that have a key role in the modulation of synaptic signaling[32,153]. The cerebellum is one of the brain regions more enriched of PNNs[154].

Taking all together, it might be possible that TNR plays a main role in neuronal plasticity and that the clinical phenotype of the patient carrying TNR mutations would be more linked to deficiency in synaptic structure and transmission, as seen in many other NDDs[107,108].
Moreover, the clinical case reported in this study shows cognitive and motor deficits as well as cerebellar atrophy, strongly associating TNR mutations with dysfunctions in cerebellar development. In this perspective, further studies are needed to unravel the functional roles of TNR and their association with a normal cerebellar development.

Conclusion

The clinical phenotype of the patient evaluated in this study, and all the data collected so far in human and murine brains, suggest a potential role of TNR in the pathogenesis of neurodevelopmental disorders. In this study we provided data on TNR expression and distribution in the mouse and human cerebellums. We demonstrated that TNR is expressed postnatally and in the adulthood with a specific temporal and spatial pattern, which reflects its differential release by neurons or oligodendrocytes. However, the mechanisms through which TNR mutations lead to pathological conditions remain still unknown. In this study we tried to decipher how TNR can be involved in some important developmental phases, such as cell proliferation, maturation and functional activity, focusing mostly on TNR role in neuronal fate. However, it has been shown that TNR is an important autocrine signal for glial development[155]. Thus, we cannot exclude that cerebellar dysfunctions, observed in the patient in study, could be also related to impairments in oligodendrocytes precursor cells (OPCs) development in the absence of TNR. For this reason, further studies are warranted in order to investigate this hypothesis.

Overall, this study provides an example of modeling human NDDs through *in vitro* differentiation of patient-specific iPSCs into neurons and humanized mice models. The biggest advantage of such studies is to offer clues about the underlying neurobiology of NDDs and to transfer those clues into clinical practice, bridging the gap between research and the clinic. In addition, studies employing animal and human cell models allow the design of novel and personalized therapeutic strategies. Due to the very complex nature of NDDs, interdisciplinary approaches combining genetics, robust biological models as well as the capability of researchers and clinicians to work side by side, will be essential.

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