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# Low intensity rTMS to the cerebellum : age dependent effects and mechanisms underlying neural circuit plasticity

Tom Dufor

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# Université Pierre et Marie Curie

Ecole doctorale 158 « Cerveau Cognition Comportement »

*Laboratory of UMR8256 Biological Adaptation and Ageing*

*Team : Brain Development, Repair and Ageing (DRVC)*

## **Low intensity rTMS to the cerebellum : age dependent effects and mechanisms underlying neural circuit plasticity**

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Thèse de doctorat de Neurosciences

Dirigée par Rachel Mary Sherrard

Présentée et soutenue publiquement le 24 Octobre 2016 devant un jury composé de :

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*A mes parents*

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## **Abstract**

Neuroplasticity is essential for the establishment and strengthening of neural circuits during the critical period of development, and are required for the brain to adapt to its environment. The mechanisms of plasticity vary throughout life, are generally more difficult to induce in the adult brain, and decrease with advancing age. Repetitive transcranial magnetic stimulation (rTMS) is commonly used to modulate cortical excitability and shows promise in the treatment of some neurological disorders. Low intensity magnetic stimulation (LI-rTMS), which does not directly elicit action potentials in the stimulated neurons, have also shown some therapeutic effects, and it is important to determine the biological mechanisms underlying the effects of these low intensity magnetic fields, such as would occur in the regions surrounding the central high-intensity focus of rTMS. We have used a focal low-intensity magnetic stimulation (10mT) to address some of these issues in the mouse cerebellum and olivocerebellar path. The cerebellum model is particularly useful as its development, structure, ageing and function are well described which allows us to easily detect eventual modifications. We assessed effects of *in vivo* or *in vitro* LI-rTMS on neuronal morphology, behavior, and post-lesion plasticity.

We first showed that LI-rTMS treatment *in vivo* alters dendritic spines and dendritic morphology, in association with improved spatial memory. These effects were age dependent. To optimize stimulation parameters in order to induce post-lesion reinnervation we used our *in vitro* model of post-lesion repair to systematically investigate the effects of different LI-rTMS stimulation patterns and frequencies. We showed that the pattern of stimulation is critical for allowing repair, rather than the total number of stimulation pulses. Finally, we looked for potential underlying mechanisms participating in the effects of the LI-rTMS, using mouse mutants *in vivo* or *in vitro*. We found that the cryptochromes, which have magnetoreceptor properties, must be present for the response to magnetic stimulation to be transduced into biological effects.

The ensemble of our results indicate that the effects of LI-rTMS depend upon the presence of magnetoreceptors, the stimulation protocol, and the age of the animal suggesting that future therapeutic strategies must be adapted to the neuronal context in each individual person.

## Résumé

Les mécanismes de neuroplasticité sont essentiels pour la mise en place et le renforcement des circuits neuronaux lors de périodes critiques du développement, et permettent au cerveau de s'adapter au cours des différentes étapes de la vie. Ces mécanismes varient avec l'âge, sont généralement plus difficile à activer chez l'adulte, et diminuent dans le cerveau âgé. La stimulation magnétique transcrânienne répétée (rTMS) est actuellement utilisée pour moduler l'excitabilité corticale et est décrite comme prometteuse dans le traitement de certains troubles neurologiques. La rTMS de faible intensité (LI-rTMS), ne déclenchant pas directement de potentiels d'action dans les neurones stimulés, a aussi montré des effets thérapeutiques, il est donc important de comprendre les effets biologiques de ces champs magnétiques d'intensités similaires à celles présentes dans les régions adjacentes à la région ciblée par la rTMS de haute intensité. Nous avons utilisé une stimulation magnétique focale de faible intensité (10 mT), ciblant le cervelet ainsi que la voie olivocérébelleuse chez la souris, afin d'aborder certaines de ces questions. Le cervelet est un modèle pertinent, en effet son développement, sa structure, son vieillissement et ses fonctions sont bien décrits, facilitant la détection d'éventuelles modifications dans cette région. Nous avons étudié les effets de LI-rTMS, *in vivo* ou *in vitro*, sur la morphologie neuronale, le comportement, et la plasticité post-lésionnelle.

Dans une première étude nous avons montré que la LI-rTMS *in vivo* modifie les épines et la morphologie dendritique des cellules de Purkinje, ces modifications sont associées à une amélioration de la mémoire spatiale et dépendent de l'âge de l'animal. Afin d'optimiser les paramètres de stimulation pour induire de la réinnervation post-lésionnelle et d'identifier des mécanismes sous-jacents, nous avons utilisé notre modèle *in vitro* de réparation post-lésionnelle et étudié de manière systématique les effets de différents patrons de LI-rTMS. Nous avons montré que le patron de stimulation, plutôt que le nombre de pulses, est essentiel afin d'induire de la réinnervation. Afin d'identifier des mécanismes participant aux effets de la LI-rTMS, nous avons utilisé des souris mutantes, *in vivo* et *in vitro*. Nous avons montré pour la première fois que les cryptochromes, ayant des propriétés de magnétoreception, sont nécessaires afin d'observer des effets biologiques induits par la LI-rTMS. Nos résultats indiquent que les effets de la LI-rTMS dépendent de la présence de magnétorecepteur, du patron de stimulation et de l'âge de l'animal, suggérant une adaptation des stratégies thérapeutiques en fonction du contexte neuronal de chaque individu.

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## **Abbreviations**

<b>aAPs</b>	Anterograde action potentials
<b>AMPA</b>	$\alpha$ -amino-3- hydroxy-5-methyl-4 isoxazolepropionic acid
<b>AP</b>	Action potential
<b>baAPs</b>	Backward propagating anterograde action potentials
<b>BDNF</b>	Brain derived neurotrophic factor
<b>CABP</b>	Calcium binding protein
<b>cAMP</b>	Cycline adenosine monophosphate
<b>CB</b>	Calbindin
<b>CF</b>	Climbing fibers
<b>CNS</b>	Central nervous system
<b>CREB</b>	cAMP responsive element binding protein
<b>CRY</b>	Cryptochrome
<b>CS</b>	Complex spikes
<b>CSF</b>	Cerebrospinal fluid
<b>CSP</b>	Cortical silent period
<b>CST</b>	Cortico spinal tract
<b>cTBS</b>	Continuous theta burst stimulation
<b>DCN</b>	Deep cerebellar nuclei
<b>DLPFC</b>	Dorso lateral DFC
<b>DTI</b>	Diffusion tensor imaging
<b>EEG</b>	Electroencephalogram
<b>ELF-MF</b>	Extremely low frequency magnetic fields
<b>EMF</b>	Electromagnetic field
<b>EPSP</b>	Excitatory post synaptic potential
<b>FAD</b>	Flavin adenine dinucleotide
<b>fMRI</b>	Functional magnetic resonance imaging
<b>FSIs</b>	Fast spiking interneurons
<b>GABA</b>	Gamma-aminobutyric acid
<b>GAD</b>	Glutamic acid decarboxylase
<b>GAP43</b>	Growth associated protein
<b>GC</b>	Granular cells
<b>H<sub>2</sub>O<sub>2</sub></b>	Hydrogen peroxide
<b>HF-rTMS</b>	High frequency repetitive transcranial magnetic stimulation
<b>ICF</b>	Intracortical facilitation
<b>IEGs</b>	Immediate early genes
<b>ION</b>	Inferior olive neurons
<b>iTBS</b>	Intermittent theta burst stimulation
<b>KO</b>	Knock-out
<b>LFMS</b>	Low field magnetic stimulation
<b>LF-rTMS</b>	Low frequency repetitive transcranial magnetic stimulation
<b>LI-rTMS</b>	Low intensity repetitive magnetic stimulation
<b>LTD</b>	Long term depression
<b>LTP</b>	Long term potentiation
<b>L-VGCC</b>	L- voltage gated calcium thermal
<b>M1</b>	Primary motor cortex
<b>MEP</b>	Motor evoked potential

<b>MP</b>	Motor threshold
<b>MRI</b>	Magnetic resonance imaging
<b>NGF</b>	Nerve growth factor
<b>NMDA</b>	N-methyl-D-ascorbic acid
<b>NO</b>	Nitric oxide
<b>NT3</b>	Neurotrophin-3
<b>O<sub>2</sub><sup>•-</sup></b>	Superoxide anion
<b>OCP</b>	Olivo cerebellar pathway
<b>PC</b>	Purkinje cells
<b>PD</b>	Post-natal day
<b>PEMF</b>	Pulsed electromagnetic fields
<b>PET</b>	Positron emission tomography
<b>PSD95</b>	Post synaptic density
<b>PV</b>	Parvalbumin
<b>Px</b>	Pedunculotomy
<b>RGCs</b>	Retinal ganglion cells
<b>RMT</b>	Resting motor threshold
<b>ROR<math>\alpha</math></b>	RAR-related orphan receptor alpha
<b>ROS</b>	Reactive oxygen species
<b>rTMS</b>	Repetitive transcranial magnetic stimulation
<b>SICI</b>	Short interval intracortical inhibition
<b>SYP</b>	Synaptophysin
<b>T</b>	Tesla
<b>TBS</b>	Theta burst stimulation
<b>TMS</b>	Transcranial magnetic stimulation
<b>TrK B</b>	Tyrosine kinase receptor B
<b>VDCC</b>	Voltage dependent calcium channel
<b>VESCs</b>	Voltage gated sodium channels
<b>VSD</b>	Voltage sensitive dye

# **Chapter I - Introduction**

## **I) Magnetic stimulation: a non-invasive approach to enhance neuroplasticity?**

### **I.1) What is neuroplasticity?**

Neuroplasticity is the ability of the brain to reorganize neural connections at the structural and functional level in response to extrinsic or intrinsic factors. This is a fundamental process during development of the central nervous system and its physiological functioning in the adult. Neuroplasticity allows the nervous system to adapt to environmental pressure, experience (psychological or somatic) and injury/lesion, beyond its genetically-determined structure (Pascual-Leone et al., 2005).

Neuroplasticity can be observed at the whole-brain level, when spatiotemporal patterns of activation occur in distinct brain regions; at the circuit level, as modulation of long-range or local contacts between different neuronal types; or at the synaptic level, where dynamic modifications adjust the strength of a preexisting connection and thus a neuron's response to an input (Ganguly and Poo, 2013). These short-term processes may be followed by structural change, e.g. dendritic growth and arborization leading to the formation of new synapses. Plasticity is thought to be the neural basis for learning and memory formation (Hübener and Bonhoeffer, 2010; Johansen et al., 2011; Squire et al., 2004), through diverse mechanisms such as cellular long-term potentiation (LTP) in the hippocampus (Bliss and Lomo, 1973), long-term depression (LTD) in the cerebellum (Ito and Kano, 1982), and synaptic formation or dissolution.

Neuroplasticity also contributes to functional recovery after brain injury by modulation of neurogenesis, dendritic morphology, axon sprouting, and neural circuits in the peri-lesion area (Cramer, 2008; Taub et al., 2002). It is essential to understand which of the local and distant changes are optimal for recovery because these modified patterns of neural activation may themselves lead to maladaptive neuroplasticity and abnormal behavior. This adverse aspect of plasticity is illustrated in developmental, acquired and degenerative neuropathologies, involving either excessive (Naro et al., 2016; Saab, 2012; Sheehy and Marsden, 1982) or impaired plasticity (Cramer et al., 2011; Elbert et al., 1998; Parihar and Brewer, 2010). Impaired plasticity would not allow the brain to adjust to changing environmental situations, and an inappropriately-high level of plasticity would make the

structural connections unstable and therefore adversely affect functional systems required for cognition and behavior (Pascual-Leone et al., 2011).

To find ways of modulating cerebral plasticity to provide functional benefits, it is crucial to understand neuroplasticity mechanisms, and the relationships between these mechanisms, neuronal activity, and behavior. The aim is to suppress neural changes resulting in unwanted behavior and to increase those leading to improved behavior beneficial for a healthy subject or a patient.

## **I.2) Neuroplasticity over the lifespan**

The brain can be seen as a structure constantly adapting to its environment and therefore it is essential to understand its plasticity over the lifespan. During development a highly plastic state has been characterized, termed the critical period. Plasticity during this critical period is essential for the physiological establishment of neural circuitry and function. The developmental critical period has been clearly demonstrated to interact with postnatal experience and activity-dependent plasticity in both the visual system (Anderson et al., 2011; Chapman and Stryker, 1993; Espinosa and Stryker, 2012) and in the descending corticospinal tract (CST) of the motor system (Anderson et al., 2011; Martin, 2005). Monocular deprivation on the one hand, or silencing of the CST on the other, during this critical period results in respectively a dramatic permanent loss of the response to the deprived eye (Hubel and Wiesel, 1963, 1998) and in an altered topography and axon terminal morphology of the CST leading to long-term motor disorders (Martin, 2005). This critical-period plasticity might partly explain the Kennard Principle which notes that lesions in childhood are characterized by better recovery than equivalent lesions in adults (Dennis, 2010). Conversely, this plasticity can also lead to devastating effects of some early brain injuries (Kolb et al., 2000). Investigation of the time window and factors that balance these opposite outcomes is required in order to find optimal interventions for functional recovery in the immature brain (Anderson et al., 2011).

In contrast, in the adult brain plasticity phenomena are different, occurring at different levels, in response to environmental factors (sensory input, motor act, learning, injury...). Functional short-term synaptic modifications (Abbott et al., 1997; Zucker and Regehr, 2002) allow immediate adaptation of signal-processing in neural circuits. Long-term synaptic

plasticity mechanism, such as LTP and LTD (Malenka and Nicoll, 1993), underlie more prolonged changes, e.g. learning and memory. These functional synaptic plasticity mechanisms are accompanied by structural modifications such as synapse turnover (Grutzendler et al., 2002; Trachtenberg et al., 2002) and formation of stable new dendritic spines (Hofer et al., 2009; Xu et al., 2005; Yang and Zhou, 2009). On a more cellular level, morphological plasticity of the neuronal dendritic tree was observed following sensorimotor learning (Gonzalez et al., 2005) or following peripheral activity (Churchill et al., 2004) such as exposure to an enriched environment (Jacobs et al., 1993; Volkmar and Greenough, 1972). At a larger scale, cortical map reorganization in response to sensory deprivation occurs in the mature brain (Buonomano and Merzenich, 1998; Feldman and Brecht, 2005). Adult brain neuroplasticity is nonetheless region dependent and is often described only in restricted areas such as the hippocampus, cerebellum or sensorimotor cortex. Although the mature brain can sometimes overcome injury through the creation of new connections between surviving neurons, its repair capacity is limited by a growth-inhibitory cellular environment, poor growth potential in mature neurons, and the restriction of stem cells necessary for neuronal replacement to specific brain areas (Fuchs and Flügge, 2014).

Although adult neurogenesis in mammalian brain is now well accepted in the subventricular zone (Doetsch et al., 1997; Saha et al., 2012, 2013), which migrate to the olfactory bulb (Lois and Alvarez-Buylla, 1994; Luskin, 1993) and subgranular zone of the hippocampus (Altman and Das, 1965; Gage et al., 1998; Saha et al., 2013), their capacity to repair cerebral lesions is unclear. Recently it was shown that motor cortical lesion in adult mice induces the proliferation of neural progenitors in the subventricular zone, which migrate to the lesion and differentiate mainly into glial cells and to a lesser degree into neurons (Saha et al., 2013). However, evidence of neurogenesis in areas such as neocortex, amygdala, striatum and substantia nigra lacks reproducibility and requires clarification (Gould, 2007; Saha et al., 2012). Also, the re-growth ability of axons in the mature central nervous system (CNS) sharply decreases after the critical period. The ability of the CNS to regenerate injured axons becomes restricted by the lack of intrinsic neuronal growth capacity and an extrinsic inhibitory molecular environment (Chen and Zheng, 2014). Axonal sprouting of uninjured axons is a natural repair mechanism after injury in the adult CNS. Sprouting from remaining axons, rather than regenerating the whole path, requires growth over shorter distances to make functional connections on appropriated targets (Geoffroy and Zheng, 2014). Therefore

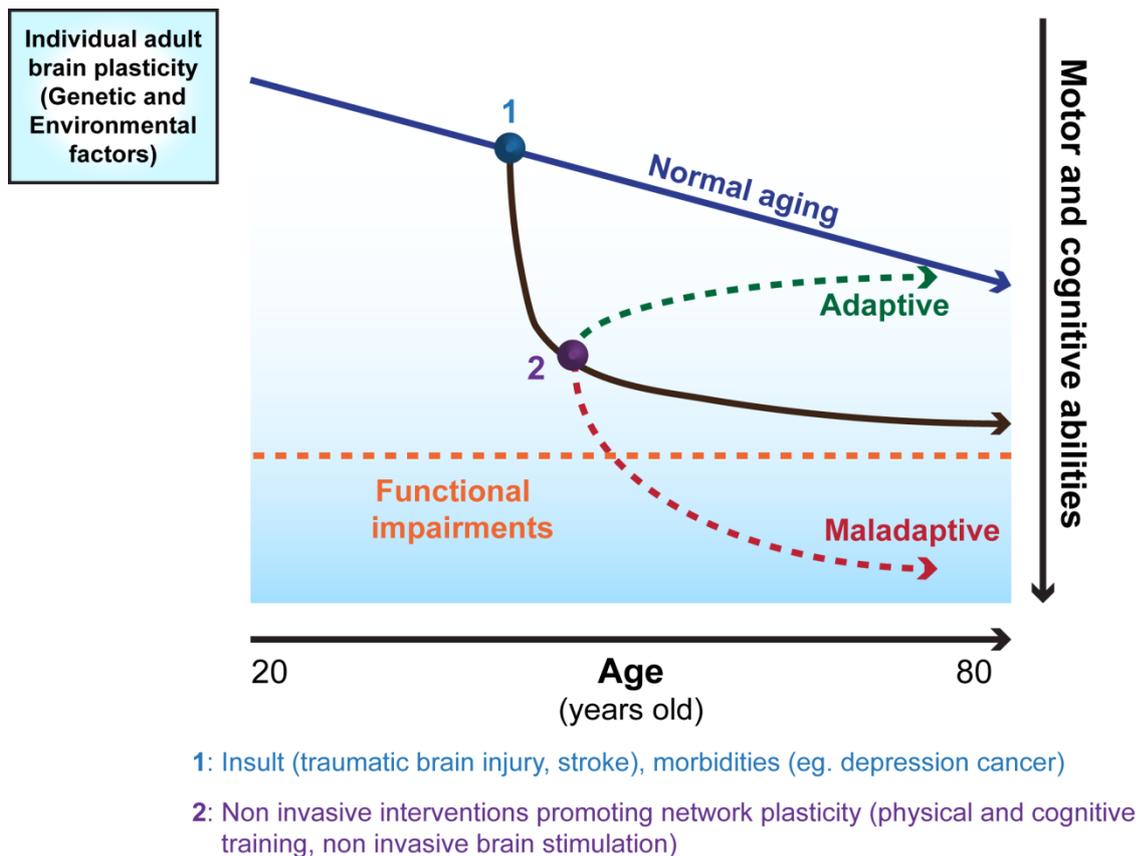
modulation of axonal sprouting may represent a more realistic therapeutic goal to promote functional recovery, than trying to induce regeneration. Axonal sprouting can be promoted by extrinsic factors such as application of neurotrophins or genetic manipulation (Geoffroy and Zheng, 2014; Giger et al., 2010; Willson et al., 2008) but this is invasive and therefore not yet clinically applicable. There is an evident medical need to find non-invasive ways to promote neuroplasticity in the mature brain.

Later in life, age-related decline of synaptic plasticity correlated with neurocognitive alterations (Rosenzweig and Barnes, 2003). For example, hippocampal LTP declines faster after its induction in aged rats, and this is linked to altered memory (Barnes and McNaughton, 1980; Kelly et al., 2006). In humans, however, there is generally only indirect evidence of decreasing neuroplasticity as aging progresses (Fotinos et al., 2005; Scahill et al., 2003) through the assessment of cognitive and motor decline combined with imaging techniques that show structural (magnetic resonance imaging (MRI), diffusion tensor imaging (DTI) and functional functional MRI (fMRI), positron emission tomography (PET) deficits (Pascual-Leone et al., 2011). Neuroplasticity over the lifespan shows a downward trend in all individuals but it does so from variable “baseline” levels and with different slopes according to genetic factors and environmental influences (Figure 1)

In contrast, direct experimental evidence of decreasing corticomotor plasticity over human lifespan can be obtained through studies combining transcranial magnetic stimulation (TMS) together with electroencephalogram (EEG) and fMRI (Freitas et al., 2011a, 2011b). TMS can be used as a non-invasive exploration tool to understand and assess adult brain plasticity, or in its repetitive form (rTMS) as a non-invasive way to modulate cortical excitability and therefore to enhance plasticity. By using the same technique, these two aims are particularly complementary, since a better understanding of the underlying mechanism of the neuroplasticity is essential for the optimization of the parameters of the non-invasive way to induce it. In the scope of this thesis I will focus on the rTMS as a tool to induce neuroplasticity. It appears essential to consider the stage of brain development, the age of the subject, and if it is a healthy or injured brain, since the mechanisms and level of plasticity are not identical, in order to optimally induce neuroplasticity with rTMS.

In summary, brain plasticity has a central role from development to ageing that allows the brain to adapt to intrinsic or extrinsic factors and therefore optimizes function in healthy

subjects. The plasticity mechanisms are reduced in the mature and ageing brain so that there is an absence of appropriate compensatory changes to brain pathology. As plasticity is closely linked to neuronal activity, modulating activity, and thus plasticity, by non-invasive approach such as the rTMS represent an interesting strategy to reduce or even treat neurological dysfunction.



**Figure 1: Schematic representation of individual plasticity across the lifespan.**

Neuroplasticity over the lifespan shows a downward trend in all individuals but it does so from variable “baseline” levels and with different slopes according to genetic factors and environmental influences. Alteration to local plasticity by brain injury, morbidity or unhealthy lifestyle will trigger secondary adaptive responses across neural networks, and they may be that adaptive or maladaptive for the individual. Non invasive interventions to promote adaptive network plasticity and suppress maladaptive plasticity mechanism are essential therapeutic goals. Modified from (Pascuale-Leone et al; 2011)

### 1.3) What is rTMS?

Using electric and magnetic field to stimulate the human brain and muscle has a long history. In 1791, Galvani was the first to show that electric focal stimulation can induce responses in isolated frog nerves and muscles (Galvani, 1791). This attracted broad interest

into the electrical excitability of biological tissues, and led to the discovery of muscle activation by electric stimulation of various motor cortex areas (Rothwell et al., 1991).

Since then, electrical stimulation has shown considerable efficiency in various clinical applications but has some technical limitations that require clear application guidelines (Antal et al., 2017). Stimulation can be painful due to activation of pain fibers in the scalp (Hallett, 2000; Merton and Morton, 1980), and deep structures are very difficult to stimulate non-invasively due to the high electrical resistance of the skull. An alternative approach is to apply time-varying magnetic fields.

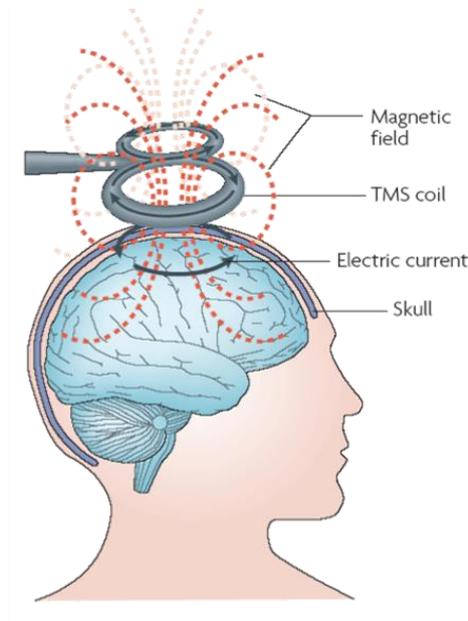
The mechanism underlying electromagnetic induction was discovered by Michael Faraday in 1831. Faraday's law of induction is a law of electromagnetism predicting how a magnetic field will interact with an electric circuit to generate an electromotive force, a phenomenon called electromagnetic induction (Walsh 2003). The first magnetic activation of isolated frog muscle *in vitro* by Kollin and colleagues (Walsh 2003) paved the way to stimulation of the human brain and muscle via electromagnetic induction.

At that time the reliable generation of powerful and rapidly alternating electromagnetic fields was limited by the technology advancement. Therefore it was only in 1985 that Barker and colleagues were able to show for the first time hand muscle activation as a result of human motor cortex stimulation with alternating magnetic fields (Barker et al., 1985; Hallett, 2007). The technique has generated particular interest since, unlike electrical stimulation, rTMS does not produce pain and is not attenuated by hair, tissue, or skull (Hallett, 2007). Thus the intensity of the secondary electric field generated by the time-varying magnetic field in the underlying brain tissue is only affected by distance and time from the initial stimulation point (Pascual-Leone et al., 2000; Wagner et al., 2007). It allows the stimulation of discrete brain regions and increases the accuracy of the stimulation. Since its first application, the use of TMS in various domains such as neurology, neuroscience and psychiatry has grown widely, as an explorative tool in research applications. With technological advances, TMS devices were rapidly able to deliver multiple pulses within a short time period (10-20ms) (Pascual-Leone et al., 1991; Rossini and Caramia, 1992). This form of TMS, called repetitive TMS (rTMS), modifies a range of measures of brain function including cortical excitability for up to few weeks after the last stimulation. Thus it logically became an important research tool due to its therapeutic potential in the treatment of a variety of neurological and psychiatric disorders (Hallett, 2000; Rossini et al., 2015).

#### **I.4) Basic principle of rTMS**

Transcranial magnetic stimulation (TMS) is based on Faraday's principles of electromagnetic induction. In TMS studies, the stimulating wire coil is held over a subject's head, a brief pulse of current flows through the coil, generates a magnetic field which passes through the subject's scalp and skull with negligible attenuation (the field only decays by the square of the distance from the source) (Hallett, 2007). This time-varying magnetic field induces a secondary perpendicular electric field which can create an electric current of opposite direction in any adjacent conductor and thus stimulates the neural tissue (Pascual-Leone et al., 2000; Walsh and Rushworth, 1999) (Figure 2). The field's rate of change determines the size of the induced current (Maxwell-Faraday equation; Jackson 1962). This secondary electric current is thought to modify the membrane potential and therefore the excitability of the neurons underneath the coil. It requires a flow of electric charge through the excitable neuronal membrane and creates a transmembrane potential that propagate along the nerve. TMS can therefore stimulate both output and input connections of any cortical areas (Siebner et al., 2009). At the system level, the effects of the TMS are thus not restricted to the targeted region but produce altered activity in distant interconnected cortical, subcortical and spinal regions (Kobayashi and Pascual-Leone, 2003; Naro et al., 2016). The direction and strength of the electric field as well as the biological structure's intrinsic conductivity influence the amount of charge going through the neuronal membrane. Change in neuronal membrane properties is the result of a gradient change of electric charge induced by the electric field at a specific location of the neuron (Di Lazzaro et al., 2003; Radman et al., 2009; Siebner et al., 2009). Some studies suggest that TMS stimulates axons more easily than the soma because axons are most efficiently activated by a brief electrical current, as in the TMS, whereas somata require longer pulses. Exactly which axons are activated is not clear and factors like the degree of myelination, neuronal type and geometry of the axons must be considered (Siebner et al., 2009). However it has also been suggested that excitation occurs in the cortical grey matter rather than in the subcortical white matter because of its location (just below the scalp surface) and lower electrical resistance (Edgley et al., 1997).

Various types of excitatory and inhibitory neurons from interconnected regions are activated by the TMS, thus it seems more rigorous to consider the TMS mechanism of action in terms of neural circuits rather than local excitability changes. Since neurons have different thresholds of electrical activation, lower stimulation intensities activates a much smaller number of neurons. Therefore if one wants to have focal stimulation it appears more logical to use low intensity stimulation to avoid unwanted activation of distant structures.



**Figure 2: Overview of electromagnetic induction in TMS.**

A brief pulsed (time varying) primary current flows through the coil and generates a changing (time varying) magnetic field. The magnetic field passes through the skull and induces a perpendicular electric field within the brain. This secondary electric field can create secondary electric currents, of opposite direction, in any conductive tissue, and thus stimulate the brain. The magnetic field decreases rapidly with distance from the coil, thus cortex and subcortical white matter are assumed to be the principal neural elements stimulated. Image adapted from Ridding & Rothwell (2007)

## **I.5) TMS and rTMS applications**

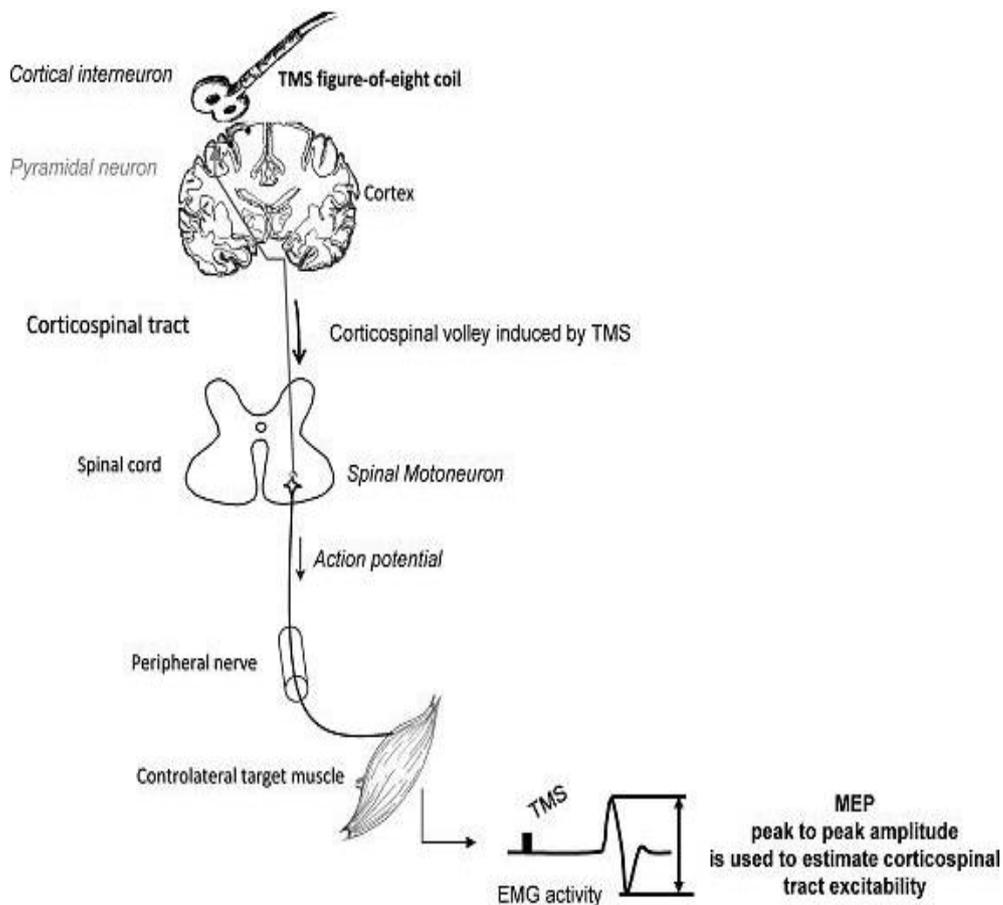
In clinical neurophysiology, TMS of the motor cortex is widely used as an exploratory tool to assess the excitability of descending cortico-nuclear and cortico-spinal circuits (Rossini et al., 2015). The motor cortex is a widely used region since modulation of motor cortex activation and excitability can be readily evaluated via recordings of motor evoked potentials (MEPs) (Figure 3). A wide variety of neurophysiological measures made with TMS are possible. Among the most commonly used are: corticomotor threshold (MT), MEP amplitude and latency, cortical silent period (CSP), cortical mapping of motor representations and, following paired TMS pulses, short interval intracortical inhibition (SICI) and intracortical

facilitation (ICF). They all provide evidence of pathology-related alteration of motor function in patients, or neuroplasticity in the motor cortex of healthy subjects (Rossini et al., 2015). Combination of neuroimaging techniques and EEG during TMS allows for non-invasive assessment of cortical excitability and good time resolution of connectivity in regions outside the motor cortex (Ilmoniemi and Kicić, 2010; Siebner et al., 2009; Ziemann, 2011).

This thesis will focus on the potential of rTMS to induce neuroplasticity and to treat neurological and psychiatric disorders. Its modulatory effect on cortical activity of various motor and non-motor areas that outlasts the stimulation period makes it a very interesting research tool. Studies of rTMS effects on cortical activity have been mostly done with stimulation of the primary motor cortex (M1), since the motor outputs can be easily measured. In studies of corticospinal motor output it is generally accepted that low frequency stimulation ( $\leq 1\text{Hz}$ ) is mainly inhibitory, while high frequency stimulations ( $\geq 5\text{Hz}$ ) are mainly facilitatory (Fitzgerald et al., 2006). More recent patterned protocols such as the popular “theta burst stimulation” (TBS) delivered as continuous (cTBS) or intermittent (iTBS) trains are described to be inhibitory and excitatory respectively. They have been suggested to have longer lasting effects on cortical excitability than classic low or high frequency rTMS protocols (Iezzi et al., 2011; Di Lazzaro et al., 2011). The stimulation frequency is thought to be the main parameter that influences the direction of the modulation. However the effect of a defined rTMS or TBS protocol do not always follow this rule (“high frequency and iTBS = excitatory”; “low frequency and cTBS=inhibitory”)(Fitzgerald et al., 2006; Gamboa et al., 2010; Houdayer et al., 2008). The influence of the stimulation frequency will be discussed in more detailed in the following section addressing stimulation parameters.

The long term-modulatory action of rTMS on cortical reactivity has driven interest in the use of rTMS to facilitate recovery from a broad range of neurological diseases (stroke, multiple sclerosis, epilepsy, Parkinson’s disease and tinnitus), psychiatric disorders (depression, anxiety, schizophrenia and obsessive-compulsive disorders) and pain syndromes (migraine and chronic pain). A recent evidence-based review demonstrated level “A” efficacy (definite efficiency) in the antidepressant effect of high-frequency rTMS (HF-rTMS) targeting the dorsolateral prefrontal cortex (DLPFC), and the analgesic effect of HF rTMS targeting M1, contralateral to the pain (Lefaucheur et al., 2014). Specific rTMS devices received FDA approval for the treatment of these two pathologies in 2008 and 2014 respectively (O’Reardon et al., 2007). Treatment of the negative symptoms of schizophrenia and chronic

motor stroke by HF-rTMS of the left DLPFC and by low-frequency rTMS (LF-rTMS) of contralesional M1 respectively, received level B recommendation (probable efficiency) (Lefaucheur et al., 2014). Some studies report improvements in neurological and psychological disorders after rTMS applications that last up to few months (Wassermann and Zimmermann, 2012).



**Figure 3: Simplified scheme of TMS mechanism of action on the motor cortex**

Interneurons oriented in a plane parallel to the brain surface are preferentially activated by the TMS applied over the motor cortex. This leads to a transynaptic activation of pyramidal cells evoking descending volleys in the pyramidal axons projecting on spinal motoneurons (corticospinal tract). Motoneuron activation in response to corticospinal volleys induced by TMS leads to a contraction in the target muscle evoking a motor-evoked potential (MEP) on electromyography (EMG). Surface electrodes applied over the muscle belly are used to record the MEP. Its peak-to-peak amplitude is measured to estimate excitability of the corticospinal tract. MEP is the most common read-out of human cortical excitability.

From Klomjai et al 2015

However a great discrepancy in outcomes, with studies failing to reproduce reported beneficial effects of rTMS, represent an obstacle for the acceptance of rTMS as a viable therapeutic tool (Héroux et al., 2015; Platz and Rothwell, 2010; Ridding and Rothwell, 2007;

Wassermann and Zimmermann, 2012). This variability can be partially explained by several factors (Figure 4); among the most important:

1) Inter- and intra-individual variability of rTMS effects is one of the major sources of discrepancies between and within studies (Goldsworthy et al., 2014; Hannah et al., 2016; López-Alonso et al., 2014; Vallence et al., 2015). Each subject's individual baseline level of cortical excitability, prior to stimulation, strongly influences the direction of modulation produced (Daskalakis et al., 2006; Siebner and Rothwell, 2003). The susceptibility to induce neuroplasticity is generally influenced by previous neuronal activity, relying on mechanisms of homeostatic plasticity and metaplasticity (Abraham and Tate, 1997; Bienenstock et al., 1982; Turrigiano and Nelson, 2004). Other biological factors such as age, gender, genetics, physical fitness, hormone levels and brain anatomy also affect the rTMS effects and thus represent another cause of inter-individual variability of rTMS clinical outcomes (Hoogendam et al., 2010; Ridding and Ziemann, 2010).

2) rTMS parameters, including total duration of stimulation, pulse width, coil type, magnetic field orientation and, very importantly, the intensity and frequency pattern of the stimulation dramatically influence the neural and behavioral effects of the stimulation. (Chen et al., 1997; Di Lazzaro et al., 2011; Rothkegel et al., 2010; Rubens and Zanto, 2012) High variability in the stimulation parameters applied in different studies probably explains much of the lack of reproducibility between studies and even contradictory results. While consideration of the biological factors involved in inter-individual variability is essential, rTMS parameters are the most convenient and accessible factors of influence to carefully control.

## **I.6) rTMS parameters**

A large number of stimulation parameters influence the neural and behavioral outcomes of rTMS. I will briefly describe the influence of different pulse shapes and coil types, with particular attention to the effects of different stimulation intensities and frequency patterns.

### **I.6.1) Pulse shapes and coil types**

Direction and amplitude of the secondary electric field are determined by the rate of change of the magnetic field over time, typically occurring during the rise-time and fall-time of the magnetic pulse as represented in the Maxwell-Faraday equation:  $\nabla \times E = - \frac{\partial B}{\partial t}$

Where  $\nabla$  is the curl operator, E is the electric field, B is the magnetic field and t the time.

Therefore the amplitude of the induced electric field is a function of the amplitude of the magnetic field and how fast it changes over time. Since axons have various strength-duration properties, it is possible to target specific sets of peripheral axons by changing the pulse width of the stimulation (Mogyoros et al., 1996). However all commercially-available stimulators deliver sinusoidal pulses that cannot be modified and therefore limit the possibility to target specific sets of neurons (Goetz et al., 2013; Hannah et al., 2016).

Stimulators can generate either a monophasic pulse or a biphasic/polyphasic pulse. Generally these two types of pulse have different neuromodulatory effects, with bi/polyphasic pulses suggested to be more efficient in single stimulus application and monophasic pulses thought to have greater neuromodulatory effects in rTMS application (Arai et al., 2005; Hamada et al., 2013; Sommer et al., 2006). Because of lower energy requirements, classical rTMS devices deliver high-frequency biphasic stimuli that activate different cortical circuits and induce a variety of effects that could partly explain inter-individual variability in outcomes (Hamada et al., 2013). However the implementation of a novel modifiable device, which can deliver a nearly-triangular monophasic pulse during high frequency rTMS (controllable TMS; (Peterchev et al., 2014), is thought to produce stronger and more reproducible effects on MEP than classical rTMS devices (Goetz et al., 2016).

Various wire coil designs are available for TMS clinical applications, the two most popular being the classic round coil and the figure-of-eight-shaped coil (Cohen et al., 1990; Hallett, 2007) The shape of the magnetic field produced depends on the coil type, which will determine the area with the highest intensity stimulation (hotspot), the total area stimulated and the stimulation depth (Deng et al., 2013; Lang et al., 2006a). Commercially available TMS coils used in the clinic usually deliver a maximum magnetic field of 1.25-2 Tesla (T). Intensity of the magnetic field decreases with increasing the distance from the coil and the central focal hotspot, leaving surrounding cortical and sub-cortical regions to be stimulated albeit at lower intensities (Cohen et al., 1990; Deng et al., 2013).

## **I.6.2) Stimulation intensity**

The rTMS as described to this point delivers focal high-intensity stimulation which depolarizes neurons of the targeted cortical regions sufficiently to induce action potential firing. Another area of research called low field magnetic stimulation (LFMS), pulsed electromagnetic field (PEMF) stimulation or extremely low frequency magnetic field (ELF-MF) stimulation use diffuse subthreshold low-intensity magnetic fields that induce a generalised electric current throughout the brain but do not *directly* depolarize neurons sufficiently to induce action potential firing. However these low intensity magnetic fields have wide effects on brain function that will be extensively described in chapter III.

### **I.6.2.1) High intensity rTMS**

The intensity of an individual's rTMS is usually determined by the cortical motor threshold (MT), defined as the minimal intensity at which TMS over the M1 induces a reliable electromyography (EMG) response around 100 $\mu$ V or a visible muscle twitch response in the target muscle (Rossini et al., 1994; Westin et al., 2014) Although twitch-based MT evaluation is easier to carry out, it is related to a high intra- and inter-rater variability and MTs assessed visually are about 10% higher than EMG-recorded MTs (Westin et al., 2014). Suprathreshold stimulations inducing action potential firing in stimulated neurons are required to induce such EMG or muscle twitch responses (Pell et al., 2011). MEP amplitude is commonly used to assess modulation of cortical excitability promoted by rTMS, and increases with increasing stimulation intensity (Rothwell et al., 1987). Intrinsic differences in cortical excitability induce inter and intra-subject variability in the MEP amplitude. While physiological fluctuations cannot be avoided, other physiological and technical parameters should be kept constant, including baseline activity of the target muscle, arousal and attention levels, environmental noise, and coil position/orientation (Cuypers et al., 2014; Rossini et al., 2015). The stimulation intensity needed to induce a response in a resting muscle is often expressed relative to the resting motor threshold (RMT) and represents the percentage of the stimulator output to reproducibly induce MEPs (Fitzgerald et al., 2006; Rothwell et al., 1987). Stimulation intensity in human rTMS is generally applied around 80-120% of the RMT and therefore is only characterized as a percentage of the stimulator output. This represents another factor of variability between studies since identical devices are not always used.

### **1.6.2.2) Low intensity magnetic stimulation**

As for high intensity rTMS, low intensity magnetic fields are delivered by one or more coils in which time-variable current flows. Parameters of stimulation are usually in the microtesla to millitesla ( $\mu\text{T}$ -mT) range and generally 10-100 Hz (0-300Hz) frequency (Di Lazzaro et al., 2013). Waveforms also can be mono-phasic or biphasic. Extremely low frequency magnetic field (ELF-MF) studies use various waveforms that can be asymmetric, biphasic, quasi-rectangular, or quasi triangular (Bassett, 1989) although most ELF-MF sources of stimulation produce sinusoidal waveforms (Juutilainen and Lang, 1997). Pulsed electromagnetic fields (PEMF), a subset of ELF-MF, usually induces greater electrical current than sinusoidal waveforms due to their faster magnetic field rate of change (Tesla/seconds) (Di Lazzaro et al., 2013). Unlike rTMS which delivers central focal high intensity stimulation of a targeted brain region, ELF-MF generates a diffuse homogeneous magnetic field within the whole brain. Helmholtz coil-based exposure systems are the most commonly used and are made of two identical circular magnetic coils that deliver a nearly uniform magnetic field. ELF-MF experiments use a wide variety of stimulation devices and coils which induce a broad range of waveforms (sinusoidal or pulsed), intensities ( $\mu\text{T}$ -mT) and frequencies (0-300Hz) of stimulation, making comparisons between studies very difficult.

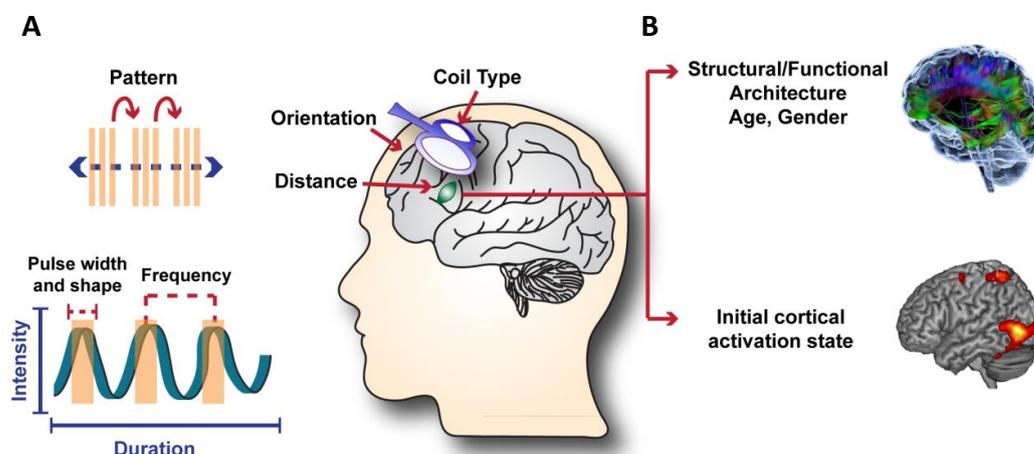
### **1.6.3) rTMS frequency**

The temporal spacing between repeated magnetic stimuli (rTMS frequency) is the most widely studied and manipulated parameter of magnetic stimulation because of its crucial influence on the direction of cortical excitability modulation. As discussed above (Section 1.5), the general consensus is that high frequencies  $\geq 3$  Hz and iTBS increase cortical excitability, while low frequencies  $\leq 1$  Hz and cTBS decrease cortical excitability (Fitzgerald et al., 2006; Huang et al., 2005; Rossini et al., 2015). (Huang et al., 2005), motivated by the limited efficiency of classical rTMS frequencies (1Hz and 10Hz) to modulate cortical excitability, took advantage of the high-efficiency of electrical theta burst stimulation to induce LTP in hippocampal slices (Hernandez et al., 2005), to design an alternative approach of rTMS in which a three-pulse 50-Hz burst is applied at 5 Hz (theta burst). In intermittent TBS (iTBS), trains of theta bursts are delivered intermittently, for 2s every 10s for about 190s (600pulses in total), while in continuous TBS (cTBS), theta bursts are delivered continuously

for 40s (600 pulses in total) (Huang et al., 2005). Both iTBS and cTBS were able to produce the most consistent and sustained effects on cortical excitability in human studies, while using lower intensity and shorter duration of stimulation than classical high- and low-frequency rTMS (Hoogendam et al., 2010; Huang et al., 2005). Thus these patterned stimulation frequencies represent promising tools for induction of neuroplasticity.

This strict distinction of excitatory and inhibitory rTMS may be a simplistic vision, and both types of rTMS might have more mixed inhibitory and excitatory effects (Houdayer et al., 2008; Matheson et al., 2016). It seems more appropriate to consider the effects of different “excitatory” or “inhibitory” rTMS protocols as the result of stimulation and modulation of various cortical circuits (Di Lazzaro et al., 2010, 2011). For example, the increased MEP following HF rTMS could be the consequence of decreased gamma-aminobutyric acid (GABA)-mediated intracortical inhibition rather than a direct increase of motor cortex excitability (Hamada et al., 2013; Di Lazzaro et al., 2001; Wu et al., 2000; Ziemann, 2004). Hamada and colleagues showed that the great variability between individuals in iTBS “excitatory” effects and cTBS “inhibitory” effects on MEP size is related to different interneuronal cortical networks recruited by the TMS (Hamada et al., 2013). Therefore comparison between experiments using different protocols described as “excitatory” or “inhibitory” should be made cautiously, especially with theta-burst patterns.

Similar distinctions between different types of modulation induced by different ELF-MF frequencies have not been described. A large number of studies have assessed the effects of sinusoidal 50-60Hz ELF-MF on the brain, since electromagnetic fields of this type are present in our environment.



**Figure 4: Factors influencing the effects of transcranial magnetic stimulation (TMS).** A) TMS mechanical parameters that can be tailored for a desired outcome. B) Individual brain characteristics that also influence the outcomes of a given TMS protocol and potentially contribute to the discrepancy between results of rTMS studies. The different colors in the upper brain represent connections between different brain regions.

## **II) Potential mechanisms underlying high-intensity rTMS**

As described above variability in the outcomes between studies on the effects of rTMS are partly due to different applied protocols (pulse shape, intensity, frequency pattern...) as well as intra and inter-individual variability due to different baseline level of cortical excitability and other individual biological factors. Altogether this will in turn specifically modify the intracellular pathways activated by the magnetic field and therefore the subsequent long-term changes. It is thus of major importance to search for neurophysiological mechanisms responsible for these long-term effects. An understanding of these mechanisms will help us to fine-tune our rTMS protocols in order to optimally activate those mechanisms, depending on the type of individual subject, the neural network targeted and the pathology implicated.

### **II.1) Synaptic plasticity: evidence from human studies**

rTMS has the appealing potential to modulate cortical excitability beyond the stimulation period (Pell et al., 2011) and in both directions, either excitatory or inhibitory. These after-effects are dependent on several parameters which were presented in the previous chapter. A main approach has been to explain those effects on the brain through LTP/LTD like mechanisms.

Long-term changes in synaptic strength like LTP (long-term potentiation) and LTD (long-term depression) are durable changes in synaptic efficacy (Malenka and Bear, 2004; Raymond, 2007). LTP results in potentiation of synaptic strength that may last for days, weeks or months. Brief high-frequency stimulations are used to induce this potentiation. LTD results in a long-lasting reduction in synaptic strength (Duffau, 2006). Synaptic plasticity obeys to key rules that are described in detail in different experimental models (Abraham, 2008; Cooke and Bliss, 2006; Malenka and Bear, 2004) Studies on rTMS and their effects on human brain have used several key concepts shared with classic synaptic plasticity which are summarized in figure 5. However it is essential to specify that LTP- and LTD-like mechanisms induced by rTMS differ from their classic form of synaptic plasticity, which use direct electric

stimulation of synapses *in vivo* and *in vitro* (Malenka and Bear, 2004; Pell et al., 2011). A main difference is related to the different conditions of stimulation: TMS activates a wide number of axons at presynaptic and postsynaptic terminals simultaneously (Funke and Benali, 2011) This could explain the significantly lower stimulation frequencies that induce LTP in rTMS studies compared to classic electrophysiology synaptic plasticity protocols (10Hz vs 100Hz) which induce facilitation (Vlachos et al., 2012).

Cortical excitability is modulated by magnetic stimulation in a frequency dependent manner. Brain activity is also affected by the stimulation as indicated by regional cerebral blood flow (Lee et al., 2003; Rounis et al., 2005), EEG responses (Esser et al., 2006; Huber et al., 2007; Litvak et al., 2007), and blood-oxygen level-dependent (BOLD) activation patterns (Hubl et al., 2008). High frequency rTMS for 10-20 minutes preceding a task produces prolonged increases in attentional control, consolidation of new motor skills, and tactile discrimination (Boyd and Lindsell, 2009; Hwang et al., 2010; Tegenthoff et al., 2005). All of these effects outlasted the stimulation period, which is an important component of synaptic and network plasticity.

The temporal pattern of stimulation is another key determinant of synaptic plasticity. In classic LTP/LTD studies the direction and strength of synaptic plasticity are generally frequency-dependent. High-frequency stimulation allows evoked post synaptic potential EPSP-summation, release of the N-methyl-D-aspartate receptor NMDA-receptor magnesium block, allowing calcium influx and triggering LTP. Low-frequency stimulation might allow for a lower calcium influx, leading to LTD (Collingridge, 2003; Malenka and Bear, 2004).

As discussed in section 1.6.3, similarly to the synaptic plasticity mechanism elicited by electrical stimulations, rTMS stimulation frequency and pattern have a critical influence on the direction of the cortical excitability modulation induced by the rTMS (Cooke and Bliss, 2006; Ziemann, 2004)

Metaplasticity changes in synaptic plasticity over time is another key concept in synaptic plasticity which takes into account the previous history of activation of the synapse and can facilitate or inhibit synaptic plasticity, stabilize synapses or adjust cellular activity (Abraham, 2008). It allows homeostatic synaptic plasticity which allows adjustment of synaptic strengths to maintain stability in neural activity. Several rTMS studies have shown that the history of activity of the stimulated network influences the observed outcomes (Valero-Cabré et al., 2008). Studies on the after-effects of rTMS showed that prior stimulation would

"prime" subsequent stimulation effects and influence their direction (Karabanov et al., 2015; Todd et al., 2009). Physiological activity such as motor action or training is able to modify/reverse the rTMS effects on the motor cortex (Huang et al., 2008b). Thus rTMS effects are influenced by prior activation of a neural circuit.

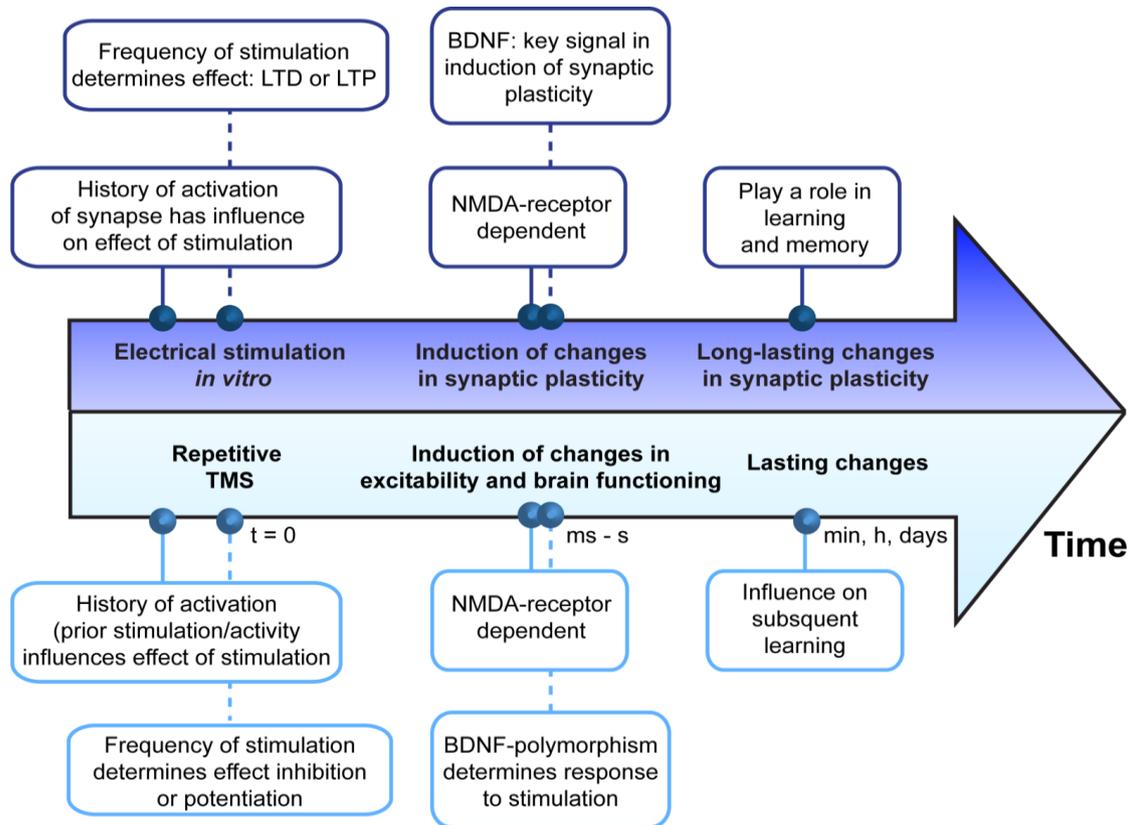
The NMDA glutamate receptor has been extensively described as essential for LTP induction at most glutamatergic synapses (Collingridge et al., 1983; Morris et al., 1986). In support of this notion, studies in humans showed that the effects of 10 Hz rTMS were blocked by administration of the NMDA antagonists ketamine (de Andrade et al., 2014) or memantine (Huang et al., 2008b).

Brain-derived neurotrophic factor gene (BDNF) also modulates LTP (Aicardi et al., 2004; Figueroa et al., 1996; Woo et al., 2005) and LTD (Woo et al., 2005) in the adult central nervous system. A common single nucleotide polymorphism (called "BDNF Val66Met") is present in 35% of the Caucasian population, and is associated with differences in hippocampal volume and episodic memory (Pezawas et al., 2004) and decreased experience-dependent plasticity in the motor cortex (Kleim et al., 2006). Effects of iTBS and cTBS on the excitability and plasticity of the motor cortex were reduced in subjects carrying this BDNF Val66Met allele (Cheeran et al., 2008); the authors suggest that this is due to the role of BDNF on the susceptibility of synapses to undergo LTP/LTD. These results reinforce the hypothesis that rTMS is acting in part through mechanisms similar to synaptic plasticity. Given that a specific BDNF gene allele can influence the response to rTMS it is possible that other genetic mutations could be an explanation for the great inter-individual variability in rTMS studies.

LTP and LTD have been demonstrated in several animal experiments to be the neural substrate of learning (Rioult-Pedotti et al., 2000). Therefore to make a link between rTMS and synaptic plasticity it seems logical to test if the rTMS affects learning. Several studies showed that rTMS influence learning (Baraduc et al., 2004; Muellbacher et al., 2000) and suggests that rTMS has the potential to alter synaptic plasticity.

We have seen that there are several similarities between rTMS induced plasticity and classic synaptic plasticity (Figure 5), but no direct links can be demonstrated from only human studies. Animal studies allow more direct evidence of the underlying molecular and cellular mechanisms of synaptic plasticity induced by rTMS. A fundamental advantage of using appropriate animal or in vitro experiments to study mechanisms underlying rTMS is the fact that various forms of plasticity (synaptic plasticity of excitatory or inhibitory synapses,

metaplasticity, structural and functional plasticity) have been thoroughly described. We can therefore compare results of rTMS-induced neural plasticity with these data, to try to identify underlying cellular and molecular mechanisms of rTMS. Outcomes of these studies will be very useful to optimize rTMS therapies, and could provide key information for “personalized” rTMS-treatments of patients with neurological or neuropsychiatric diseases.



**Figure 5: Common key concepts between the induction of classic synaptic plasticity in brain slices and the induction of rTMS outcomes in the normal human brain.** Time is shown by the two bars. The boxes represent the different processes influencing the lasting changes induced by either electrical stimulation *in vitro* or rTMS, and the time windows at which they are important. Modified from Hoogendam et al 2010.

## II.2) Direct evidence for synaptic plasticity induced by rTMS in animal models

### II.2.1) rMS induced synaptic plasticity at excitatory synapses

In vitro studies of rMS on organotypic rodent hippocampal slice cultures have provided evidence to support the synaptic plasticity thought to occur after human rTMS. (rMS indicates that the magnetic field does not go through the cranium). Direct evidence of LTP induced by high-frequency rMS (10Hz) has been found (Lenz et al., 2015; Tokay et al., 2009; Vlachos et al., 2012). Those studies provide pivotal insight into underlying mechanisms of

rMS which are consistent with long-term potentiation of synaptic transmission. A durable increase (2-6 hours post stimulation) in synaptic transmission mediated by the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid AMPA receptor was demonstrated, along with remodeling of small dendritic spine on CA1 pyramidal neurons (Vlachos et al., 2012). This was associated with increased AMPA receptor cluster size and number in an NMDA receptor dependent manner, consistent with modifications observed after classical LTP protocols. Another recent study from this group (Lenz et al., 2015) showed that this induced potentiation of excitatory synapses is located on proximal dendrites, and that it requires voltage-gated channels (sodium and calcium), NMDA receptors, and calcium.

Using a computational approach to test the possible cooperative depolarization of pre- and postsynaptic structures, Lenz and colleagues showed that rMS-induced anterograde action potentials (aAPs) will provoke release of glutamate from the presynaptic ending. Backward propagating action potentials (bAPs) will depolarize dendrites of the post-synaptic neuron, favoring removal of the magnesium block from NMDA-R (Lenz et al., 2015). They propose that at the proximal dendrite of CA1 pyramidal neurons these two phenomenon are additive and therefore induce accumulation of AMPA-R on the post synaptic cell resulting in a facilitated potentiation (Lenz et al., 2015). This hypothesis of rMS-induced bAP-aAP mediated cooperative plasticity could account for the induction of rMS induced LTP-like effects with frequencies much lower (i.e., 10Hz) than the 100Hz needed with direct electrical stimulation in classic LTP experiments. However, direct evidence for this “bAP-aAP theory” is currently missing. It has to be noted that there is no direct evidence of rMS induced LTP in cortical neurons.

### **II.2.2) rTMS modulation of inhibitory networks**

A considerable number of recent studies have demonstrated a crucial role for inhibitory synaptic transmission in cortical network function (Letzkus et al., 2011; Lovett-Barron et al., 2014; Pecka et al., 2014) and suggested that alterations in excitation and inhibition (E/I) balance could underlie behavioral and cognitive dysfunction in brain disease (Rojas et al., 2014; Rubenstein and Merzenich, 2003; Yizhar et al., 2011). LTD-like effects induced by rTMS have been thought to be the result of the modulation of excitatory neuron activity. However a growing base of evidence is showing that rTMS can also act on inhibitory

interneurons (Funke and Benali, 2011; Labedi et al., 2014; Trippe et al., 2009). It is therefore essential to study the effect of rTMS on inhibitory circuitry.

The study of rTMS effects on inhibitory neurons was begun by the group of Pr Klaus Funke. A series of experiments on rats showed that rTMS modulates the expression of immediate early genes (cfos and zif 268) (Aydin-Abidin et al., 2008; Funke and Benali, 2011), GABA-synthesizing enzymes GAD65 and GAD67 (Funke and Benali, 2011; Trippe et al., 2009; Volz et al., 2013), and calcium binding proteins of inhibitory cortical interneurons (PV,CABP,CB). (Labedi et al., 2014; Mix et al., 2014; Volz et al., 2013). They also showed that different patterns of stimulation will affect distinct populations of interneurons: iTBS reduces parvalbumin (PV)-expression in fast-spiking interneurons of the rat cortex, while cTBS and 1 Hz rTMS predominantly affect calbindin-expression (Benali et al., 2011; Trippe et al., 2009; Volz et al., 2013). As PV-expressing interneurons and calbindin-expressing interneurons are important for somatic and dendritic inhibition, respectively, (Markram et al., 2004) these results suggest that different rTMS paradigms may affect distinct aspects of inhibition. In other studies from the group, modulation of inhibitory neuron activation induced by rTMS occurred with functional alterations such as tactile learning performance (Mix et al., 2010) and sensory responses in rat barrel cortex (Thimm and Funke, 2015). However, these are only indirect measures for changes in GABAergic neurotransmission.

In contrast the Vlachos group directly assessed long lasting rTMS-induced changes in inhibitory neurotransmission in the rat hippocampus, using entorhino-hippocampal slices cultures. They showed that a 10Hz rTMS protocol induces a calcium-dependant reduction in strength of GABAergic synapses onto CA1 pyramidal neurons 2–4 h after stimulation. In addition, the size and stability of gephyrin-aggregates, which anchor GABA receptors to the postsynaptic cytoskeleton, were significantly decreased after rTMS (Lenz et al., 2016). This reduction of inhibitory synaptic strength required  $Ca^{2+}$ -dependent signaling pathways (activation of NMDA-receptors and L-Voltage Gated Calcium Channel (L-VGCC)) during stimulation, pathways which are also needed to induce LTP with rTMS (Huang et al., 2008b; Labedi et al., 2014; Lenz et al., 2015, 2016; Vlachos et al., 2012)

### **II.2.3) Neurobiological effects of rTMS underlying induced plasticity**

Given the wide range of cell populations with specific excitability properties affected by rTMS (excitatory/inhibitory neurons, glial cells...) and the heterogeneity of outcomes depending on the brain area stimulated (cortex, hippocampus, cerebellum...) it is too reductive to only investigate mechanisms of rTMS-induced LTP/LTD. Animal studies have shed light on an abundant number of neurobiological processes modulated by rTMS that could underlie neural plasticity.

### **II.2.3.1) Acute effects on cellular properties**

The evaluation of acute rTMS effects in human studies has traditionally been assessed through physiological or behavioral parameters. The overall status of the cortex is inferred from a handful of physiological indicators, including the resting motor threshold (RMT), motor evoked potentials (MEPs), cortical silent period (CSP) and the phosphene threshold (PT). However the use of animal models allows more direct assessment of the acute changes induced by rTMS. With current electrophysiology and imaging techniques it is now possible to study the acute effects of rTMS on intrinsic neuronal excitability, Ca<sup>2+</sup> dynamics and immediate early gene expression that represent potential short-term neural bases for the initiation of plasticity mechanisms.

#### **II.2.3.1.i- Membrane potential and spontaneous activity**

Synaptic plasticity is not the only potential mechanism for excitability changes. High intensity rTMS can cause depolarization of targeted neurons either directly, or indirectly via depolarization of interneurons (Pell et al., 2011), which may modify neural excitability. Alteration of membrane excitability induced by modification of the resting membrane potential, the action potential threshold (AP) and ion channels properties could be an alternative mechanism leading to modulation of excitability (Wagner et al., 2009)

Whole-cell patch-clamp recordings in acute rat brain slices showed that at voltages ranging from -65 mV to -25 mV each magnetic pulse led to transient influx of current into neurons (Banerjee et al., 2017). This current flow is sensitive to the membrane voltage and requires the activation of Voltage Gated Sodium Channels (VGSCs) in the soma of cortical neurons, thereby leading to an increased steady state current of the neurons, induction of action potentials, and modulation of spike timing in these neurons. 10s of stimulation increased the

intracellular calcium concentration 70s after the stimulation, potentially due to the cumulative effect of rTMS induced depolarization of the neuron (Banerjee et al., 2017).

Combining optical imaging with voltage-sensitive dye (VSD), researchers were able to record gradual changes of membrane potential induced by each TMS pulse in cat neurons, providing crucial information on neuronal population dynamics (Kozyrev et al., 2014) A single pulse of high intensity TMS induced a brief peak of activity in cortical neurons followed immediately by decreased activity below baseline, characteristic of suppression (Kozyrev et al., 2014). The authors suggest that TMS preferentially affects inhibitory (Werhahn et al., 1999) parvalbumin positive neurons (Benali et al., 2011; Funke and Benali, 2011), potentially because of their axonal and dendritic morphology (Chung et al., 2013; McAllister et al., 2009), and that it results in strong synchronized inhibition of the targeted cortical network (Pascual-Leone et al., 2000). When TMS pulses were applied at 10Hz the first pulse induced suppression and the consecutive pulses produces a progressive increase of activity in a large population of neurons reaching a level concordant with high spiking activity, weakening inhibitory action and stimulating excitatory circuits through NMDA receptors (Huang et al., 2008b). Similarly, spontaneous single-unit spiking activity in cerebellar slices showed that short term inhibition was induced by 1Hz rTMS whereas 20Hz stimulation produces excitation and although the intensity of stimulation regulated the level of modulation it did not influence the direction of this modulation (ie inhibition or excitation) (Tang et al., 2016c). In the rat brain, stimulation with an iTBS pattern depolarized neuronal membrane potential, increased sEPSCs and the rate of action potential firing, of fast spiking interneurons (FSIs) at postnatal day (PD) 29-38, but neither before nor after these ages (Hoppenrath et al., 2016). In accordance with previous finding showing that the reduction of PV expression could not be induced before PD 30 but gradually increased between PD32 and PD37 (Mix et al., 2014) these results suggest that FSIs are particularly sensitive to iTBS during critical cortical development time windows.

It is known that neuronal excitability decreases during normal aging (Disterhoft and Oh, 2007; Randall et al., 2012). This is partially due to enhanced after-hyperpolarization (AHP) (Burgdorf et al., 2011; Disterhoft et al., 1996) and a hyperpolarized resting membrane potential (Moyer et al., 1992; Power et al., 2002). These phenomena are thought to play a crucial role in impaired cognitive function associated with age (Disterhoft and Oh, 2007; Landfield and Pitler, 1984; Oh et al., 2010). High-frequency rTMS significantly reduced these

deficits in neuronal excitability and cognitive functions (Wang et al., 2015) by reducing voltage-dependent  $\text{Ca}^{2+}$  currents (Deyo et al., 1989; Rose et al., 2007). It has been shown in aged neurons that blockade of VGCCs increases different forms of synaptic plasticity, so modulation of VGCC could heighten neuronal excitability and lead to an improvement of the cognitive impairments in neurodegenerative diseases (Deyo et al., 1989; Rose et al., 2007)

#### **II.2.3.1.ii- Intracellular $\text{Ca}^{2+}$ concentration and further signaling pathway**

Changes in intracellular calcium concentration have been suggested to be one of the underlying mechanisms responsible for the effects of magnetic stimulation on neural activity. This is based on the fact that increased intracellular calcium activates signaling pathways and gene expression changes linked to molecular, anatomical or functional plasticity (Fung and Robinson, 2013; Thickbroom, 2007). These events can be activated by calcium influx from the extracellular medium or from intracellular stores. Because high intensity rTMS can sufficiently depolarize the membrane to trigger action potentials, and thus open voltage-gated calcium channels (Alberts et al., 2002), calcium influx from extracellular milieu is the favored mechanism (Gersner et al., 2011). The increased intracellular calcium directly activates various protein kinase signaling pathways (Neves et al., 2002) and cAMP signaling that can induce the activation of the nuclear transcription factor CREB (cAMP responsive element binding protein) (Kornhauser et al., 2002). A study testing the effects of a 1Hz stimulation on human-derived neuron-like cells showed increased intracellular cAMP and phosphorylation of CREB (Hellmann et al., 2012). CREB plays an important role in neuroplasticity, notably by regulating expression of early response genes and growth factors, such as c-fos and BDNF (Ginty et al., 1994; Kornhauser et al., 2002; Tao et al., 1998)

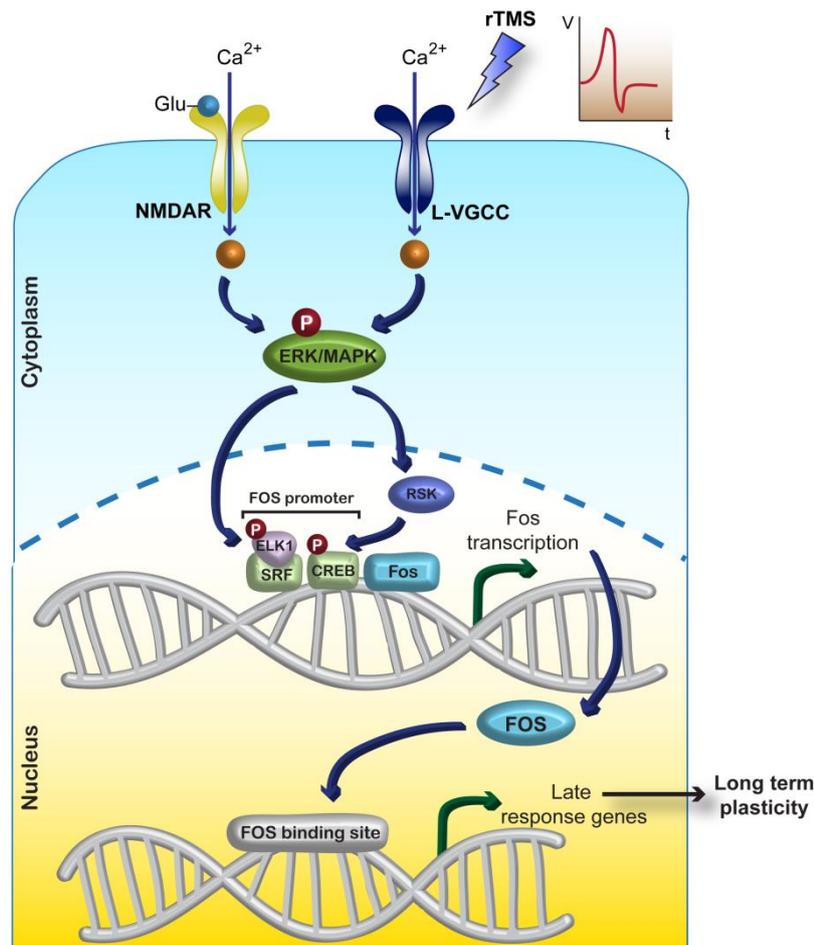
Membrane potential changes and calcium dynamics are probably involved at the early stage of rTMS-induced plasticity. This is often accompanied by changes in immediate early genes (IEGs) expression which, in turn, activate downstream target genes, leading to long-term functional and structural modifications of neurons (Jones et al., 2001) (Figure6).

#### **II.2.3.1.iii- Immediate early gene expression and type of neuron activated**

Immediate early genes (IEG) are rapidly-induced but transiently-expressed genes which are activated directly by intracellular signaling cascades and alter patterns of gene expression

(Sng et al., 2004). Among these genes, c-fos and zif268 expression induction by rTMS have been the most studied. Both genes are suggested to be involved in synaptic and neuronal plasticity (Fleischmann et al., 2003; Knapska and Kaczmarek, 2004). A study of regional IEG expression revealed that they are modulated in a specific frequency/pattern dependent manner. c-Fos protein expression was increased in all cortical areas by 1Hz and 10Hz rTMS , while iTBS was effective only in limbic cortices (Aydin-Abidin et al., 2008). Zif268 expression was increased in almost all cortical areas after iTBS, but 10 Hz rTMS was effective only in the primary motor and sensory cortices (Aydin-Abidin et al., 2008).

(Hoppenrath and Funke (2013) investigated the time course of gene expression changes induced by a single block (20 trains of 10 bursts) of iTBS. The expression of c-Fos and zif268 is thought to be a marker of excitatory activity, and glutamic acid decarboxylase isoforms, GAD65 and GAD67, CB and PV are considered markers of inhibitory activity. An early phase (20min) of increased c-Fos and GAD65 expression was observed, indicating strong activation of excitatory and inhibitory neurons. This was followed by a phase (20-80min) of reduced inhibitory neuronal activity, as reflected by decreased GAD67, PV and CB expression; then finally (160min) there is an overall decrease in c-Fos expression (Hoppenrath and Funke, 2013). However, when stimulation is increased to five blocks of iTBS (same blocks as in Hoppenrath and Funke, 2013 spaced by 15min), Trippe et al. (2009) found a greater increase in zif268 compared to c-fos expression, suggesting that zif268 activation is more sensitive to theta-burst stimulation than c-fos . A similar study also found a greater decrease in GAD67, CB and PV expression, showing that the repeated application of iTBS could have cumulative effects (Benali et al., 2011). Depending on the number of pulses delivered and the interval between blocks, the effects of the TBS on cortical excitability could be increased, decreased or reversed (Gamboa et al., 2011; Gentner et al., 2008), underscoring the importance of the prior activation history in response to rTMS. Overall, these findings add some potential neural substrates of rTMS effects and suggest that induction of IEG expression varies with the pattern of neuronal activation, according to the area studied and the time window of observation. These variations must be considered in order to identify optimal cortical region to target and interval of application for a specific clinical goal.



**Figure 6: Schematic representation of the potential CREB-Fos pathway activated by the rTMS.** Membrane depolarisation and neurotransmitter release induced by rTMS lead to a rapid flows of calcium ions into the neurons through NMDA receptors and VGCC. This in turn activates several calcium-dependent kinase cascades, which lead to the phosphorylation of CREB. Activation of CREB induces transcription of immediate early genes (IEGs) such as *cfos* which in turn might regulate the expression of late response genes leading to long term functional and structural plasticity in the neuron (Jones et al 2001).

### II.2.3.2) Long lasting plasticity: Neurotrophic factors

Neurotrophins (NTs) are a thoroughly-studied family of neurotrophic factors with essential functions in neuronal development, survival, axon and dendritic growth, synaptic plasticity and apoptosis (Lewin and Barde, 1996; Waterhouse and Xu, 2009). Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5) are the four neurotrophins expressed in the mammalian brain (Reichardt, 2006). They activate specific tyrosine kinase receptors (Trk): NGF activates TrkA, BDNF and NT4/5 activate TrkB and NT3 activates TrkC. They all bind to the common p75

receptor to induce different biological functions such as pro-apoptosis cascades (Chao, 2003).

BDNF is probably the most-studied neurotrophin, especially in the rTMS field. BDNF is widely expressed in the CNS and is essential for normal brain development, synaptic transmission, plasticity and repair (Lu et al., 2013) through the TrkB receptor which activates MAPK/ERK and PKC protein kinase pathways (Bramham and Messaoudi, 2005; Harvey et al., 2012). Through a  $Ca^{2+}$ -dependent mechanism and the activation of the immediate early gene encoding Arc, BDNF is important in the induction of LTP (Binder and Scharfman, 2004; Kovalchuk et al., 2002). BDNF is generally involved in a wide range of functions such as neurogenesis, dendritic and axon growth, synapse formation and neuronal survival after a CNS lesion (Baquet et al., 2004) making it a highly appropriate candidate to mediate brain repair after injury, especially in the adult CNS (Lu et al., 2013). Human studies showing a lack of responsiveness to TBS in BDNF Val66Met allele carriers (Cheeran et al., 2008) can explain the great interest granted to BDNF in the clinical context, providing a potential biomarker and giving it a central role in the potential mechanism underlying rTMS induced plasticity.

Increase of BDNF in human serum and cerebrospinal fluid (CSF) after rTMS is still controversial. Indeed a number of studies have found that rTMS increases serum BDNF (Yukimasa et al., 2006; Zanardini et al., 2006) whereas other studies did not (Gedge et al., 2012; Lang et al., 2006b). Again this can be explained by the fact that increased BDNF expression is probably a long term effect (Cirillo et al., 2017). Animal studies are in line with this hypothesis: 20Hz rTMS applied five days per week for 11 weeks increased the expression of BDNF mRNA and protein in specific rat brain areas (Müller et al., 2000).

Animal studies reveal that the effects of the rTMS on BDNF expression are also frequency-, intensity- and state-dependent. Increased expression of BDNF was observed after a single 20 Hz rTMS session in awake rats but not after 1 Hz. In contrast a single high frequency stimulation in anesthetized rats reduced the expression of BDNF, highlighting the importance of the neural activity during rTMS treatment (Gersner et al., 2011). Repeated sessions of low frequency stimulation (0.5 Hz) in cultured hippocampal neurons did increase BDNF expression after 5 days (Ma et al., 2013). A similar effect was seen after 7-21 days of rTMS in rats with cerebral infarction, as well as a functional improvement in the neurological severity score (NSS) (Zhang et al., 2007); and after 6 weeks' treatment in a rat model of

vascular dementia, along with an rTMS-induced improvement of cognitive function (Wang et al., 2010).

A recent study showed that rTMS (1.14T; 1Hz) in aged mice upregulated BDNF and TrkB receptor expression (Ma et al., 2014). In agreement with the literature, expression of neuroplasticity markers were modified in the same direction as the BDNF-TrkB signaling pathway (Li and Keifer, 2012). rTMS increased dendritic and axonal arborization, synapse density, synaptophysin (SYN), growth associated protein 43 (GAP43) and post-synaptic density 95 (PSD95). It was accompanied by an improvement of spatial cognition in aged mice. Surprisingly, in the same study, they tested the effects of a slightly higher intensity rTMS (1.55T; 1Hz) also on aged mice and it decreased BDNF and TrkB receptor expression increased neuronal apoptosis, reduced dendritic and axonal arborization, altered synapse number and structure together with and worsened spatial cognition of the aged mice (Ma et al., 2014). Results of recent studies suggest that rTMS effects do not show a simple linear and cumulative effect. Longer durations and higher intensity is not necessarily better and could even results in detrimental outcomes (Fitzgerald, 2017; Ma et al., 2014). These findings reinforce the importance of very careful control of the stimulation parameters (ie the intensity) in the future development of clinical treatment protocols, especially in the aged population.

BDNF is known to have roles in development of neural circuits. In the visual system, for example, light modulates cortical development (Hensch, 2005), and visual deprivation in young rats during the critical period delays maturation (Berardi et al., 2000). In this context, cortical activation induced by 5 days of iTBS alleviated the effects of visual deprivation on cortical development. A likely mechanism would be through the stimulation of BDNF synthesis and release (Castillo-Padilla and Funke, 2015). All together these results suggest that the regulation of the BDNF-TrkB signaling pathway might be a neurological basis of the beneficial mechanisms of rTMS in neuronal repair and neuroprotection.

In summary, the mechanisms underlying the effects of high-intensity magnetic stimulation identified from animal studies on rTMS have given us crucial information that potentially explain the effects observed in clinical studies; from synaptic activity through molecular signaling up to the network or behavioral levels. Understanding these mechanisms will help

scientists and clinicians to work toward a more tailored use of rTMS in neurological and psychiatric diseases. It is now accepted that rTMS does not only directly trigger LTP- or LTD-like mechanisms but can also modulate the cellular homeostatic background to influence the strength and direction of plasticity (Cirillo et al., 2017).

### **III) Low intensity magnetic stimulation**

As indicated above under stimulation parameters (section I.6), pulsed magnetic stimulation can also be delivered at low, sub-stimulation threshold intensities. Understanding the effects and underlying mechanisms of the low intensity magnetic field is important for two main reasons. The first reason is safety since high intensity magnetic fields need large electric currents for their induction and in turn generate large currents which can induce seizures, pain etc. High intensity stimulation has been established based on threshold measurements of cortical depolarisation, such as MEP, RMT etc. Thus stimulation equipment can only be used in locations with a high voltage electricity source (ex: hospital) and coils are designed to produce a central focal hot spot of maximal high intensity in order to activate the underlying neurons (Deng *et al.*, 2013; Trillenber *et al.*, 2012). However, this hot spot is surrounded by a weaker magnetic field that stimulates a large but undefined area of cortical and sub-cortical structures albeit at lower intensities (Cohen *et al.*, 1990; Deng *et al.*, 2013). These lesser magnetic fields induce electric currents of subthreshold intensities and therefore are often regarded as negligible. However there is a growing body of evidence showing that magnetic fields in the  $\mu\text{T}$ -mT range modify human cortical excitability (Capone *et al.*, 2009; Robertson *et al.*, 2010), brain oscillations (Cook *et al.*, 2004), and cognitive function (Barth *et al.*, 2010), as well as induce beneficial effects in the treatment of depression (Jin and Phillips, 2014; Martiny *et al.*, 2010; Rohan *et al.*, 2014).

Thus the application of low intensity magnetic stimulation could avoid the potential hazards of high intensity rTMS and freedom from high voltage electric sources would open the rTMS field to wider clinical applications. Potential clinical application is the second important reason for understanding the effects of low-intensity magnetic fields. If these fields show some efficacy in alleviating the symptoms of neurological and neuropsychiatric disorders, LI-rTMS could be used with more flexibility, at a wider range of stimulation parameters than

high-intensity stimulation, with fewer safety and technical limitations (Rossi *et al.*, 2009; Shafi *et al.*, 2014). Like in high intensity rTMS literature, but worse, this area is characterized by the use of highly variable terminology (PEMF, ELF-MF, non-ionizing radiation...), frequencies (0 Hz-300 kHz) and intensities ( $\mu$ T-mT) and stimulation devices, which consequently lead to a lack of reproducibility among the experiments. Therefore it is too early to envisage the routine application of such magnetic fields in the clinic. In addition, relatively little information about their actual biological effects limits any systematic evaluation of low intensity stimulation. This observation is even more prominent than in the classic high intensity rTMS literature. Thus the following section will start with a clear standardization of the terminology employed and define criteria to characterize each type of magnetic stimulation.

### **III.1) A need for a defined terminology and reproducible parameters**

As defined by our criteria in the previous chapter, a low intensity magnetic field is a stimulation that induces an electric current but does not *directly* depolarize the neuron sufficiently to induce action potential firing. I will first discuss ELF-MF and PEMF stimulations, which usually use parameters in the 50-60Hz and  $\mu$ T-mT range and apply a diffuse magnetic field to the whole brain (Figure 7), as opposed to the focal high intensity stimulation in rTMS. Non ionizing radiation refers to any type of electromagnetic radiation that does not carry enough photon energy to ionize atoms or molecules. Among others it pools extremely low frequency, radio frequency and microwave frequency electromagnetic radiation. ELF-MFs are present in our environment and are sinusoidal magnetic fields, while PEMF is a technology employed to deliver specific monophasic pulse with faster magnetic field rate of change. ELF-MF and PEMF use fairly similar intensity and frequency range of stimulation therefore I will discuss both of them in this part but I will try to specify when the magnetic fields are delivered by pulses (PEMF). Like high intensity rTMS, ELF-MF and PEMF experiments use a wide range of different, often home-made, stimulation devices and outcome measures, thus making comparison between studies very difficult.

The influence of genetic factors on subject response to rTMS is now well accepted. For example subjects carrying the Va166Met polymorphism in BDNF gene show a reduced

response to rTMS (Cheeran *et al.*, 2008). However the genetic influence on the response to ELF-MF has never been studied preventing any stratification of the sampled population. This could also participate to the variability of the results in studies on the effects of ELF-MF in humans. Future research should be designed using comparable exposure conditions and standardized test both *in vitro* and *in vivo* to facilitate comparisons between studies and decrease variability.

## **III.2) ELF-MF effects on the human brain**

### **III.2.1) Effects on cortical excitability and brain oscillations**

Motor evoked potentials (MEPs) are the most frequently-used measurement of modulation of motor cortex excitability in the rTMS field. However it is not the case for low intensity so there is little information about the effect of low magnetic field on cortical excitability. However a study showed that the intracortical facilitation, related to cortical glutamatergic activity, was significantly enhanced by ELF-MF (75 Hz; 1.8 mT; 45 min) in 22 healthy volunteers compared to sham stimulation. As this was the only parameter of cortical excitability to be affected, the results suggest that ELF-MF may selectively enhance cortical excitatory neurotransmission (Capone *et al.*, 2009).

Interestingly like rTMS, low intensity magnetic stimulation modulates brain oscillations. In contrast to MEP, the electroencephalogram (EEG) is often used to assess the neurophysiological effects of weak electromagnetic field (EMF) exposure. In a comparison study between the effects of 1.5 and 10Hz EMF (0.02-0.04 mT), 10Hz induced a greater increase in EEG power (amplitude of the oscillations). More notably, specific EMF frequencies influenced EEG power in the same specific frequency range (Bell *et al.*, 1994). Another study analyzing the effects of multiple frequencies (50, 16.66, 13, 10, 8.33 and 4 Hz) of ELF-MF exposure (at 20 $\mu$ T) partially confirmed the entrainment of alpha and beta EEG bands by correspondent specific ELF-MF frequencies (Cvetkovic and Cosic, 2009). This is consistent with findings that showed entrainment of EEG in specific frequency bands by corresponding rTMS frequencies (Romei *et al.*, 2016; Thut *et al.*, 2011). Among the numerous studies using EEG to assess the effects of ELF-MF on brain activity, results are not very consistent and sometimes contradictory. The most consistent result is the change in the alpha band (8–13 Hz) over occipital–parietal regions, but even there the direction of the

modulation is not clearly defined. Some studies show an increase in EEG alpha band by EMF (Heusser *et al.*, 1997; Lyskov *et al.*, 1993) and others show reduced alpha spectral power (Cook *et al.*, 2004). This could be partly explained by the variability of magnetic field intensity and frequency, but other factors such as individual differences and previous exposure to magnetic fields should be taken into account. As in the rTMS literature, data from ELF-MF studies suggest it induces metaplasticity. Indeed Cook and colleagues (2009) showed that the initial exposure of subjects to magnetic fields determined their subsequent responses (Cook *et al.*, 2009). They propose that individual differences and previous exposure to weak magnetic fields might play a role in the subject's response to ELF-MF.

### **III.2.2) Effects on human brain functions and potential therapeutic applications**

ELF-MF was shown to modulate pain perception in the intact human brain, but the direction of this modulation is dependent on magnetic exposure parameters and the type of stimulation used to induce pain (Ghione *et al.*, 2005; Di Lazzaro *et al.*, 2013; Prato, 2015). The motor system is also sensitive to ELF-MF. A significant improvement of standing balance was induced by specific pulsed EMFs (200 $\mu$ T; 2min) and interestingly this EMF-induced improvement was dependent on the light intensity conditions (Prato *et al.*, 2001) suggesting that magnetic field detection could be influenced by light as shown in birds and drosophila (Giachello *et al.*, 2016; Rodgers and Hore, 2009). Also, application of a EMF (50Hz; 1mT) decreased the excitability of human motor cortex and resulted in a attenuation of postural tremor intensity, although between-subject responsiveness was variable (Legros *et al.*, 2006; Pavlov *et al.*, 2007). Neurophysiological sleep parameters (sleep time, efficiency, slow wave sleep and activity) were decreased by exposure to a EMF (50Hz; 1 $\mu$ T) (Akerstedt *et al.*, 1999). Another study showed that only intermittent exposure to magnetic fields impaired sleep parameters (Graham and Cook, 1999).

The effects of ELF-MF on human cognition are a good example of the great variability in outcomes between studies due to the different exposure systems on distinct cognitive functions (Cook *et al.*, 2002). A meta-analysis attempted to overcome this discrepancy, by including nine studies using 50Hz ELF-MF, and revealed a small effect only on the most difficult level of a visual discrimination task and on the flexibility dimension of correct

answers (Barth *et al.*, 2010). Due to the small number of studies in this field, results should be interpreted with caution.

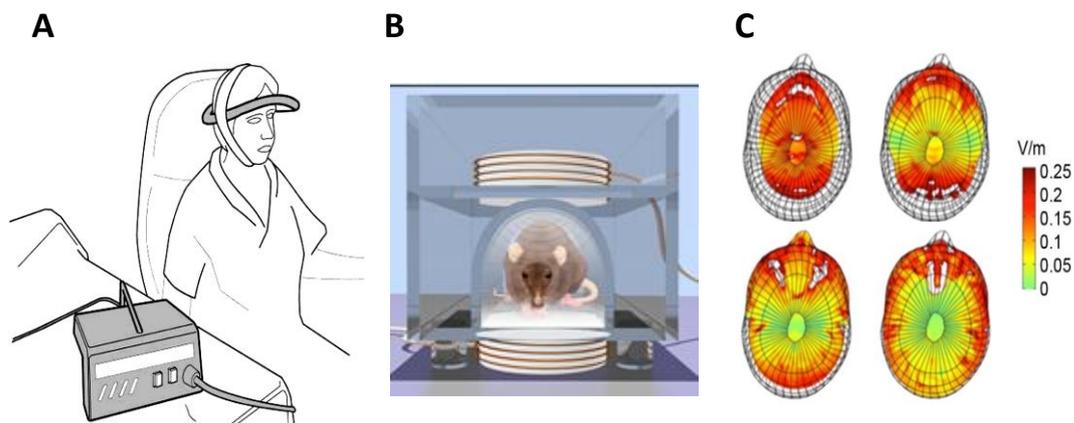
Despite this variability, some encouraging results have emerged from recent studies on the beneficial effects of ELF-MF, to alleviate depression symptoms. A first study showed the effectiveness of PEMF, with a specific complex biomimetic pattern, in the treatment of drug-resistant depression in a sham-controlled double-blind study on 50 patients (Martiny *et al.*, 2010). Alternative approaches are also being explored including electroencephalogram-synchronized low intensity TMS (sTMS). The hypothesis is that rTMS delivered at the subject's individual alpha frequency would be efficient with lower stimulation intensity. A device was developed with three rotating neodymium magnets at three brain locations to deliver a subthreshold low-intensity sinusoidal waveform magnetic brain stimulation. A pilot study showed that subjects with either fixed (individual alpha) or random (8-13Hz) frequency sTMS had significantly greater reductions in depression symptoms than those receiving sham stimulation (Jin and Phillips, 2014). A recent study using a portable tabletop device that delivers oscillating low intensity magnetic fields at 1 kHz found some immediate improvement across several mood scales in patients with depression (Rohan *et al.*, 2014). In order to optimize stimulation parameters for potential clinical applications, animal studies on the underlying mechanisms of the ELF-MF are essential.

### **III.3) ELF-MF effects on animal models and underlying mechanisms**

A wide number of studies have described effects of ELF-MF on animal brain functions or circuitry at the system level. Functional improvement and increased neuronal survival were observed with the application of ELF-MF following lateral fluid percussion brain injury (20 Hz, 0.1-0.5 mT) (Yang *et al.*, 2012) and in a model of Huntington's disease (60 Hz, 0.6 mT) (Tasset *et al.*, 2012). In the rat, many studies have found increased peripheral nerve regeneration and function induced by ELF-MF (Markov, 2007; De Pedro *et al.*, 2005; Siebner and Rothwell, 2003; Walker *et al.*, 1994) but intriguingly this result was not found in the mouse (Baptista *et al.*, 2009). Repeated magnetic field (50Hz; 2mT) exposure has anxiogenic effect on rats, but beneficial effects on spatial learning and long-term retention of spatial memory (He *et al.*, 2011). Both protective and deleterious effects on cognitive functions have been observed in studies of ELF-MF on AD. One study suggested that intermittent occupational exposure to

various EMF (from 200 $\mu$ T to 10mT) might increase peripheral A $\beta$  deposition and A $\beta$  production in the brain through modulation of Ca<sup>2+</sup> channels (Sobel and Davanipour, 1996). In contrast 60d ELF-MF (50 Hz, 400-500  $\mu$ T) exposure improved cognitive function in AD rats model (Liu *et al.*, 2015), and in 3xTg mice together with reduced tau hyperphosphorylation (Hu *et al.*, 2016). These studies indicate that the effects of ELF-MF exposure on preclinical models of neurological or neuropsychological disease require further investigation to identify underlying mechanisms and optimize the stimulation parameters in order to consistently have beneficial outcomes.

In the next section, I will give particular attention to the potential underlying mechanisms of these effects observed after low intensity magnetic stimulation. These mechanisms would be the key processes to target in order to induce the desired biological effects, and can be operationally separated into two levels: 1) the direct interaction mechanisms between external MF and the biological system; and 2) the cascade of biological events leading to the behavioral/physiological effect.



**Figure 7: ELF-MF devices and magnetic field propagation in the human brain.** (A) Schema of a PEMF exposure system composed of a custom coil made of 1400 turns of copper wire (0.2 mm) wrapped around a flexible plastic support positioned to orient the positive pole of the magnetic field toward the top of the skull and connected to a pulse generator (B-01; IGEA, Carpi, Italy) (Capone *et al* 2009) (B) Schematic representing the problem of the coil size/head size ratio in animal ELF-MF studies. Coil size are disproportionately large and thus stimulate the whole animal. (C) Electric field penetration of low field magnetic stimulation (LFMS) into the cortex. The magnitude of electric field, induced by LFMS, in four transverse slices of the human brain were calculated with the use of the finite element method (FEM) (Deng *et al* 2014) with a magnetic resonance imaging based model of the human head positioned inside a longitudinally symmetric and periodic model of the LFMS coil. A) From Di Lazzaro *et al* 2013, B) From Machado *et al* 2012 C) Adapted from Rohan *et al* 2014.

### III.3.1) Magnetoreception: the cryptochrome radical-pair mechanism hypothesis

The direct interaction between magnetic fields and biological tissues, magnetoreception, is well described in different species and allows orientation to the ambient geomagnetic field (GMF). This phenomenon is established in a wide range of different species including, insects (Gegebar *et al.*, 2010; Kirschvink, 1981), fish (Kavaliers *et al.*, 1984; Molteno and Kennedy, 2009; Takebe *et al.*, 2012), rodents (Kavaliers *et al.*, 1984) and birds (Ritz *et al.*, 2004; Wiltschko and Wiltschko, 2012). In this literature the most prominent theory suggest that photoreceptors might have a central role through their radical pairs which are generated by biochemical reactions and become sensitive to EMFs (Dodson *et al.*, 2013; Hore and Mouritsen, 2016; Jones, 2016; Ritz *et al.*, 2000; Rodgers and Hore, 2009). Cryptochrome (Cry), a blue light photoreceptor, meets the requirements of a radical pair mechanism (Dodson *et al.*, 2013) and is thought to transduce the signal of EMF in *Arabidopsis* through blue light activation of the flavin adenine dinucleotide (FAD) cofactor followed by electron transfer (ET) from a conserved triad of tryptophan residues (Ahmad *et al.*, 2007; Kattinig *et al.*, 2016; Maeda *et al.*, 2012; Solov'yov *et al.*, 2007). Cryptochrome is currently the only candidate radical-pair magnetoreceptor (Dodson *et al.*, 2013; Liedvogel and Mouritsen, 2010). The essential role of cryptochromes in the ability of insects and birds to detect magnetic fields is now well accepted. Advances in molecular biology and especially transgenic approaches in *Drosophila*, have allowed identification of various cryptochrome-mediated static magnetic field-dependent behaviors, including: circadian timing (Yoshii *et al.*, 2009), conditioning assay in a T-Maze (Foley *et al.*, 2011; Gegebar *et al.*, 2008, 2010), negative geotaxis (Fedele *et al.*, 2014) and seizure response through alteration of the level of neuronal excitation (Marley *et al.*, 2014). Effects on neural excitation were confirmed in a recent study using electrophysiological recordings from fly motoneurons over expressing *drosophila* CRY. They showed that blue light-activated Cry-dependent membrane potential depolarization was increased by a 100mT EMF. Membrane excitability analysis revealed that these effects of EMF exposure resulted in increased action potential firing (Giachello *et al.*, 2016). Equally, researchers very recently performed transgenic insertion of mammalian cryptochrome 2 into the eye of two cockroach species and showed that ocular cryptochrome 2 mediates directional magnetic responses in these species (Bazalova *et al.*, 2016).

Historically the animal orientation literature was limited to the study of the static GMF and static magnetic fields delivered in the laboratory, aiming to assess behavioral orientation responses when the direction of a static magnetic field is manipulated. These studies

assumed that since the ELF-MF signal produced by natural and anthropogenic activity were much weaker than the GMF (25-65 $\mu$ T), they would be undetectable by animals (Prato, 2015). This vision is now challenged since the animal orientation literature has provided some evidence of disruption of orientation to static GMF by exposure to ELF-MF (50Hz; 0.1 $\mu$ T) in ruminants (Burda *et al.*, 2009). A number of other studies reported similar animal orientation disruption by ELF-MF (Beason, 2005; Mora *et al.*, 2014) even as weak as 100 nT and at a broad range of frequencies (between 2 kHz and 5MHz) in the migratory European robin (Engels *et al.*, 2014). Therefore it appears reasonable to consider that ELF-MF, rather than just static magnetic fields, may affect animal orientation. These observations might be relevant for non-orientation effects of ELF-MF and it is possible that they share the same initial transduction mechanism. However the possible identity of the magnetically sensitive radical pair in CRY is currently a matter of considerable debate (Hogben *et al.*, 2009; Nießner *et al.*, 2014; Solov'yov and Schulten, 2009). A recent study done on migratory birds and using pulsed magnetic fields even proposed that magnetoreception is possible in the dark and therefore involves the radical pair formed during flavin re-oxidation. The current widely-accepted model of cryptochrome signaling in the presence of light and based on the radical pair generated during photo-reduction is thus challenged by these results (Wiltschko *et al.*, 2016). Therefore, the pending issue is **how** a time-variable magnetic field could produce changes in neuronal signals and alter brain function and **whether** mammalian CRY is involved in mediating such signals.

### **III.3.2) Biological events induced by ELF-MF**

Many signaling cascades involve a variety of potential cellular targets which are closely interrelated, such as Ca<sup>2+</sup>, kinases, nitric oxide, ROS and G-protein receptor couplings (Del Seppia *et al.*, 2007). The following sections will therefore summarize the effects of ELF-MF on these pathways.

#### **III.3.2.1- Calcium signaling and downstream intracellular pathways induced by ELF-MF**

The common pathway in different biological effects of ELF-MF involves modulation of intracellular calcium levels and downstream signalling cascades (Kavet *et al.*, 2001; Morabito *et al.*, 2010; Saunders and Jefferys, 2007; Zhang *et al.*, 2017). As suggested in the high

intensity rTMS literature, the calcium-dependent activation hypothesis is thus potentially applicable to ELF-MF (Fung and Robinson, 2013). Changes in intracellular calcium arising from voltage-gated calcium channels (VGCC; 50 Hz, 0.05-1 mT) have been described in response to ELF-MF (Barbier *et al.*, 1996; Grassi *et al.*, 2004; Piacentini *et al.*, 2008), while another group found that neural differentiation and neurite growth induced by ELF-MF (60 Hz, 0.7 mT) required L-type calcium channels (Morgado-Valle *et al.*, 1998).

Another mechanism for regulating neuronal intracellular  $\text{Ca}^{2+}$  concentration is G protein signalling. A recent study observed a higher expression of  $\text{G}_i$  protein and IP3 induced by ELF-MF (50Hz; 8mT) in mouse hippocampus. The authors suggest that increased  $\text{G}_i$  protein expression may activate PLC, producing IP3 and DAG from PIP2. Increased  $\text{Ca}^{2+}$  concentration and higher PP2B activity were also induced by ELF-MF, this is in accordance with lower CaMK II expression and CREB phosphorylation (Zhang *et al.*, 2017). Another group showed calcium release from intracellular stores, rather than influx from the extracellular milieu, after magnetic stimulation (50 Hz, 3 mT) (Aldinucci *et al.*, 2000; Pessina *et al.*, 2001). Mitochondria have a central role in storage and regulation of intracellular calcium (Berridge *et al.*, 2000), and thus the decreased mitochondrial membrane potential following ELF-MF exposure is a potential mechanism mediating the simultaneous changes in free cytoplasmic calcium levels (Morabito *et al.*, 2010).

Modulation of cell proliferation, differentiation and death are often described as effects of ELF-MF stimulation (Lacy-Hulbert *et al.*, 1998; Nikolova *et al.*, 2005; Oda and Koike, 2004; Wolf *et al.*, 2005), which are thought to be due to ELF-MF induced changes in gene expression (Lupke *et al.*, 2006; Olivares-Bañuelos *et al.*, 2004) and are often associated with altered calcium signaling (Fanelli *et al.*, 1999; Grassi *et al.*, 2004; Zhou *et al.*, 2002). ELF-MF has been shown to increase the percentage of cells expressing phosphorylated CREB mediated by increased  $\text{Ca}^{2+}$  influx through VGCC (Piacentini *et al.*, 2008). This  $\text{Ca}^{2+}$ -dependent increase in CREB phosphorylation is a decisive step allowing fine-tuning of the expression of genes involved in proliferation of neural stem cells and their neuronal differentiation induced by ELF-MF (Cuccurazzu *et al.*, 2010).

Intracellular  $\text{Ca}^{2+}$  also regulates Nitric oxide (NO) through calmodulin (CAM) binding. NO is an important intercellular messenger with several physiological functions including modulation of neurotransmitter release (Toda *et al.*, 2009). In the CNS, NO derived from

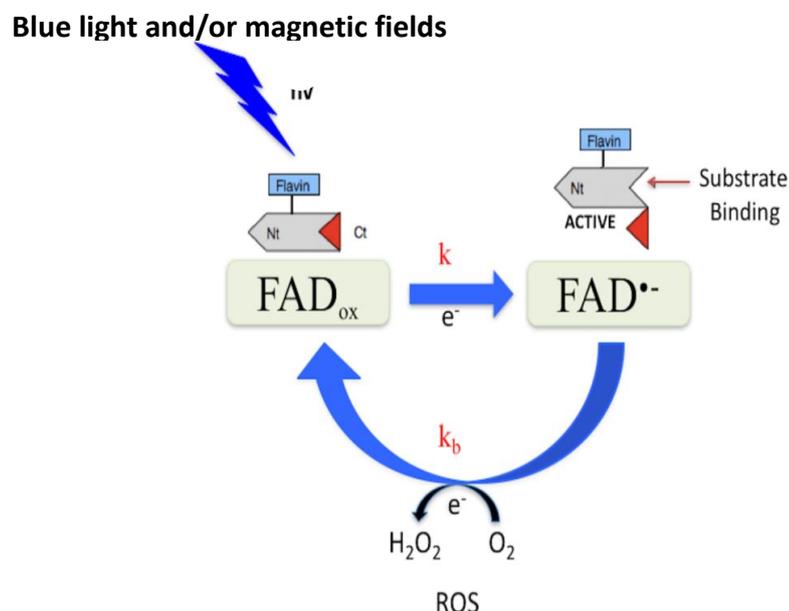
neuronal nitric oxide synthase (nNOS) acts as a neuromodulator or neurotransmitter for the regulation of synaptic plasticity, sleep-wake cycles and hormone secretion. NO effects can be physiologic or cytotoxic (Brookes et al., 2000; Huang et al., 2008a; Jeynes and Provias, 2009) depending on NO levels and the cell type studied. A study in the rat demonstrated that ELF-MF exposure increased the number of nNOS positive cells and NO levels, together with an elevated cGMP concentration; this finding is coherent with the hypothesis that higher NO levels could have physiological relevance (Cho *et al.*, 2012). Our group showed that low intensity magnetic stimulation with complex biomimetic high frequency (8mT; BHFS) could correct abnormal connections in the retinocollicular circuitry of ephrin A2A5 KO mice along with an increase nNOS (Rodger et al., 2012). NO induces long-term depression (LTD) in ectopic connections, weakening synaptic strength and targeting those connections for removal (Mu and Poo, 2006). Therefore the increased nNOS could represent a mechanism by which low intensity magnetic stimulations eliminate ectopic projections (Rodger et al., 2012).

### **III.3.2.2 ROS production**

Calcium interacts with several other systems and pathways, including reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ), superoxide anion ( $O_2^{\bullet-}$ ), and hydroxyl radicals ( $HO^{\bullet}$ ). ROS were originally characterized as having harmful effects on cells but accumulating evidence shows that at moderate levels they participate in signal transduction pathways. ROS-dependent reversible oxidation and reduction of specific amino acids, proteins, and lipids can lead to physiological responses such as gene expression, cell proliferation, and apoptosis (Brookes *et al.*, 2002; Ermak and Davies, 2002; Holmström and Finkel, 2014). Moreover ROS and  $Ca^{2+}$  interact, which has important implications for fine tuning cellular signaling networks (Görlach *et al.*, 2015). Additionally ROS production, like  $Ca^{2+}$  signaling, is modulated by ELF-MF (Di Loreto *et al.*, 2009; Mattsson and Simkó, 2014; Simkó, 2007). Indeed almost all studies that tested the effects of EMFs above 1mT found modifications of ROS, which is proposed as an early response of the exposure to ELF-MF (Mattsson and Simkó, 2014). Magnetic stimulation of cortical neurons (50Hz; 1mT) for 7 days increased cell viability, decreased apoptosis, and protected the cells from increased ROS levels. The authors suggest that it is through modulation of survival-promoting genes

including BDNF, TrkB, IL1 and the antioxidant enzyme Glutathione (GSH) (Di Loreto *et al.*, 2009). ELF-MF effects on ROS production showed modulation in both directions that are of small amplitudes (30-60%) compared to those obtained with positive controls. This « low level » oxidative response could be compatible with both the protective effects of ELF-MF exposure (Mattsson and Simkó, 2014) and the inhibitory effect of ROS on the AKT pathway and Foxp3 expression to enhance ELF-MF antitumor effect in metastatic melanoma (Tang *et al.*, 2016d).

In addition to its mutual interplay with Ca<sup>2+</sup>, ROS is also a product of cryptochrome activation (Consentino *et al.*, 2015; Jourdan *et al.*, 2015). A recent study extended these results and demonstrated the transient formation of O<sub>2</sub><sup>•-</sup> and accumulation of H<sub>2</sub>O<sub>2</sub><sup>-</sup> in the nucleus of cultured drosophila cells following Cry activation. Hence light-dependent formation of ROS is a byproduct of the cryptochrome photocycle and may contribute to its signaling role (Arthaut *et al.*, 2017) (Figure 8). It remains speculative to make a direct link between magnetoreception through Cryptochrome, ROS production and calcium signaling, but since they interact and are all involved in the biological effects of magnetic fields and their subsequent biological events, it would be very interesting to find a potential common pathway between these central actors of cellular signaling.



**Figure 8: Diagram of the potential common mechanism leading to the production of ROS triggered by blue light and magnetic field activation of Cryptochrome.**

In the dark, the protein-bound cofactor (FAD<sub>ox</sub>) is shown in the oxidized redox state. Blue light triggers cryptochrome flavin photoreduction (Berdnt *et al.* 2007) at a rate constant *k*. Spontaneous reoxidation to the FAD<sub>ox</sub> state occurs in the dark at a rate constant *k<sub>b</sub>* leading to the production of ROS (Jourdan *et al.* 2017). In the presence of magnetic fields, decreased O<sub>2</sub><sup>•-</sup> and increased H<sub>2</sub>O<sub>2</sub> concentration is observed and is thought to occur through singlet-triplet modulation of semiquinone flavin (FADH<sup>•</sup>) enzymes and O<sub>2</sub><sup>•-</sup> spin-correlated radical pairs (Usselman *et al.* 2014).

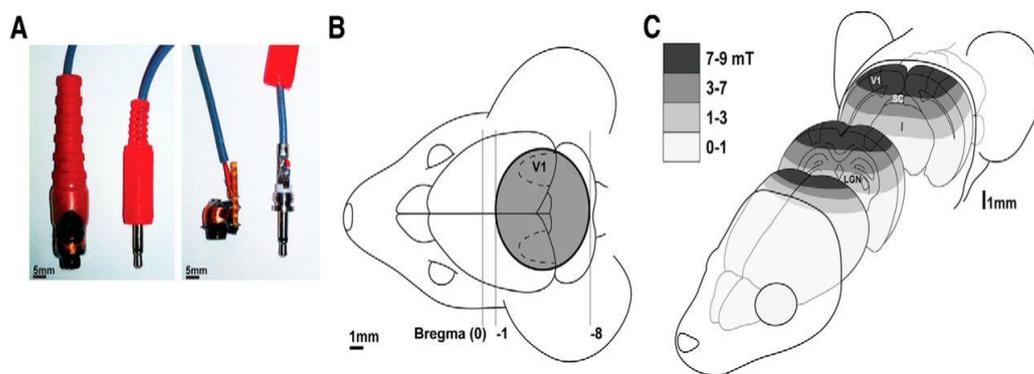
Image modified from Jourdan *et al.* 2017.

### III.4) LI-rTMS effects on the rodent brain

Our laboratory has combined the focal approach of high intensity rTMS and the lower intensities of ELF-MF or PEMF and created a coil that allows delivery of a focal pulsed low-intensity (6-15mT) magnetic stimulation of the mouse brain (Figure 9). In ephrin A<sup>-/-</sup> KO mice, which lack a key axon guidance cue and as a result have disrupted topographic organization of the primary visual projection (Haustead *et al.*, 2008; Wilks *et al.*, 2010), LI-rTMS (12mT; BHFS) decreased the number of abnormal projections in subcortical (Rodger *et al.*, 2012) and cortical visual circuits (Makowiecki *et al.*, 2014). Interestingly, the abnormal visual tracking behavior of these mice was also restored to normal wild type levels (Haustead *et al.*, 2008; Rodger *et al.*, 2012). BDNF expression and synthesis was upregulated by this stimulation, which is coherent with a plastic environment conducive to beneficial reorganization of abnormal cortical circuits (Makowiecki *et al.*, 2014). However, another study found no neuroprotective or regenerative effects of the LI-rTMS in a mouse model of complete optic nerve crush, and no significant increase of BDNF in the retina or optic nerve (Tang *et al.*, 2015). It is possible that the lack of BDNF up-regulation and the absence of positive effects is explained by the very rapid death of retinal ganglion cells (RGCs) ( $\leq 10\%$  RGCs survival) and/or the complete discontinuity between the retina and brain targets in this model, preventing effects of LI-rTMS. In fact, studies demonstrating beneficial reorganization promoted by LI-rTMS were done on *existing* although abnormal connections (Makowiecki *et al.*, 2014; Rodger *et al.*, 2012). These data suggest LI-rTMS might be efficient in less severe abnormalities but unable to correct extensive disruptions and neural connectivity in neurotrauma models (Tang *et al.*, 2015).

When LI-rTMS (12mT; 1Hz and BHFS) was applied in a unilateral penetrating stab injury model of glial scarring in young adult and aged male and female mice, no change was found in the total volume of tissue containing reactive glia. Nonetheless ipsilateral 1Hz and BHFS stimulations decreased the density of astrocytes and microglia cells neighboring the injury in young and aged females but increased it in young and aged males. The outcomes were generally more marked in the aged mice (Clarke *et al.*, 2017). It is not yet known if such sex and age specific effects occurs in human, but these observations suggest that rTMS

protocols should be tailored by taking into account the characteristics of the individual receiving the stimulation. Another study used a new custom-built coil for mice which approximates the brain-size ratio used in humans and that delivers a quite focal 100mT field to the mouse brain. 10Hz stimulation of the rat motor cortex with this coil increased their motor evoked potential (MEP) immediately after the stimulation (Tang et al., 2016a). Although it is an order of magnitude stronger than what we classified as low intensity stimulation, it is not eliciting action potentials in stimulated neurons, in contrast with high intensity rTMS (Lenz et al., 2016; Tang et al., 2016b). LI-rTMS (100mT; 1Hz) applied with this coil on a validated mouse model of tinnitus (Mulders et al., 2014; Robertson et al., 2013) induced a significant reduction of tinnitus without significant effect on BDNF expression or on tinnitus-associated hyperactivity of inferior colliculus neurons (Mulders et al., 2016). These results suggest that LI-rTMS can alleviate symptoms of tinnitus through a pathway independent from inferior colliculus activity and BDNF expression. LI-rMS delivered with the same coil (100mT; 10Hz) in a slice preparation showed that it does not modify the resting membrane potential but increases neural excitability by altering the AP threshold and increasing evoked spike firing rate in layer 5 pyramidal cortical neurons (Tang et al., 2016b). The altered AP threshold occurred immediately and was observed 20 minutes after the stimulation but not at 10 minutes after the stimulation. This cyclical nature of AP threshold modulation might be the result of LI-rTMS altering the voltage-sensing mechanism through two different pathways; the rapid alteration of AP threshold may just be the result of a direct interaction between the neuron and the electric field whereas the delayed one (20min post stimulation) might come from the activation of a signaling pathway with a longer onset (Tang et al 2016). Since another study from our group showed that LI-rTMS (12mT ; 10Hz, cTBS, BHFS) increased intracellular calcium release, from intracellular stores, in cortical neurons (Grehl et al., 2015), a potential mechanism responsible for the late onset change of AP threshold may be through calcium signaling/calmodulin known to alter function of voltage-gated sodium channels (Herzog et al., 2003). The same study (Grehl et al., 2015) also found a pattern-specific change in expression of genes involved in apoptosis and neurite outgrowth, consistent with their observation on cell survival and neurite branching .



**Figure 9: Methods for focal LI-rTMS delivery in mice.** (A) Custom-built stimulation coil and 3.5 mm jack used to plug into the pulse generator with (left) and without (right) protective plastic coating. (B) Diagram of the stimulation coil in relation to the mouse's head with stereotaxic coordinates. To ensure bilateral stimulation the coil was held along the midline 1mm above the mouse head. (C) Schematic diagram of magnetic field intensity (in milli-teslas) in the mouse head, measured by a Hall Device, showing approximate range of field intensities in different brain areas. The magnetic field is more focal to the targeted region than the wide spread stimulation induced by human coils in mice (Salvador and Miranda, 2009) but remains less focal compared to stimulation of the human brain by butterfly-shaped coils (Deng et al., 2013). Images from Makowiecky et al 2014

### III.5) Conclusion

Taken together, these results show that diffuse or focal low intensity magnetic fields in the milli-Tesla range have biological effects. This low intensity stimulation has the potential to alter human brain activity and many cerebral functions, including motor control, sensory perception, cognition and mood. Animal and *in vitro* studies suggest that it does so through effects on cellular signaling pathway (Ca<sup>2+</sup>, NO, ROS, G protein receptor and potentially Cryptochrome activation).

Nonetheless the reproducibility and specificity of the effects of low-intensity magnetic fields is still quite poor due, among other reasons, to a wide variability in the parameters applied in different studies (Di Lazzaro et al., 2013). This is an essential point to take into account, given that effects vary depending on cell type, brain area, previous activity of the stimulated neurons or network (metaplasticity), as well as stimulation intensity and frequency. The relevance of this information to human high intensity rTMS is that, the variable intensities of rTMS delivered are associated with lower (but still variable) intensity stimulation to perifocal cortex and subcortical centers (Deng et al., 2013), whose contribution to overall outcome is poorly defined (Ellaway et al., 2014). Indeed, these low-intensity stimulations are very likely to contribute to the effects of high-intensity rTMS. Nevertheless, low intensity

magnetic stimulation, *per se*, may be a promising therapeutic tool (Shafi et al., 2014), with increasing evidence showing positive effects in the treatment of depression disorders (Jin and Phillips, 2014; Martiny et al., 2010; Rohan et al., 2014). The study of their effects on specific brain functions and biological mechanisms therefore merit investigation. In order to optimize the stimulation parameters for a potential tailored application of LI-rTMS in a clinical context, we first need to understand the underlying cellular and molecular mechanisms. Systematic investigation of the effects of precisely controlled stimulation parameters on biological mechanisms at the system, circuit, or cellular level is critical. This will help scientists to make a link between behavioral outcomes in humans and the changes in cellular physiology identified in fundamental studies, and will lead us toward a more rational and reliable use of LI-rTMS in clinic.

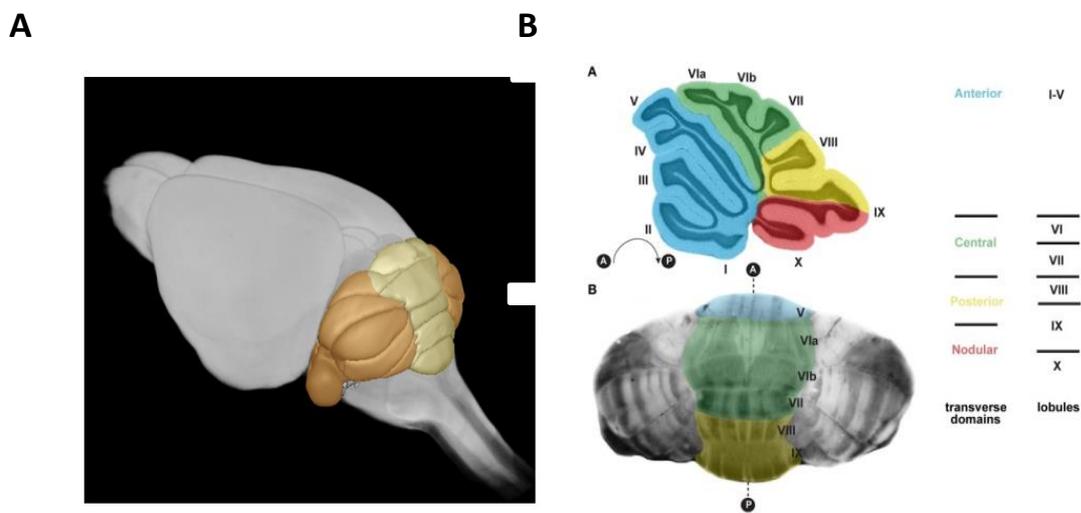
This systematic investigation into the actions of different LI-rTMS parameters requires a well-defined reproducible experimental model, for which experimental parameters and read-out can be easily controlled. We therefore used the mouse cerebellum as a model to study LI-rTMS induced plasticity in the central nervous system and the potential underlying mechanisms; the cerebellar structure, development, function, and plasticity are all well-described, so changes in these characteristics due to magnetic stimulation are easy to detect. Furthermore using this system, the laboratory has developed preclinical models of ageing and neural circuit repair, which increase the possibility of potential translational approaches (Jarvis et al., 2002; Letellier et al., 2009; Willson et al., 2008).

## **IV) The cerebellum as a model to study LI-rTMS induced plasticity**

### **IV.1) Cerebellum**

The cerebellar organization, circuitry and functions are very well described in the literature (Apps and Garwicz, 2005; Buckner, 2013; D'Angelo and Casali, 2012). The mouse cerebellum is a highly-foliated structure located dorsal to the brainstem. It is connected to the brainstem by three pairs of cerebellar peduncles: the inferior peduncles containing afferents from the spinal cord and medulla oblongata; the middle peduncles with afferents from the pontine nuclei; and the superior peduncles, which are almost entirely efferent projecting to the brainstem (Ruigrok et al., 2015). The cerebellum consists of two symmetric hemispheres connected by a central longitudinal vermis. In the anteroposterior axis the cerebellum is

divided into three main lobes: the anterior, posterior and flocculonodular lobes (Figure 10). The cerebellar cortex is the superficial grey matter of the cerebellum and is composed of three distinct layers (see below). The inner white matter is formed by nerve fibers and 4 pairs of cerebellar nuclei. The cerebellum receives three main afferents: climbing fibers (CF), originating from inferior olivary neurons (ION), projecting to Purkinje cells (PCs); mossy fibers, axons from the brainstem and spinal nuclei, contacting granule cells (GCs) and providing indirect input onto PCs via their parallel fiber axons; catecholaminergic afferents from the brainstem noradrenergic locus coeruleus and serotonergic raphe nuclei (Ruigrok et al., 2015)



**Figure 10: Morphology and organisation of the adult mouse cerebellum.**

(A) Dorsolateral view of the mouse brain using magnetic resonance imaging. The cerebellum is highlighted with the vermis in yellow surrounded by the hemispheres in light brown. Modified from Ullmann et al 2012 (B) The top image is a schema of a sagittal section cut within the highly foliated cerebellar vermis, which consists of 10 lobules, that can be divided along the anterior–posterior axis into anterior, central, posterior and nodular domains (Ozol et al., 1999). The transverse zones are color coded according to panel. Adapted from Reeber et al 2013

#### IV.1.1) Cerebellar circuitry

The cerebellar cortex is a uniform structure composed of seven neuronal cell types organized in three layers: the granular cell layer, the Purkinje cell layer and the molecular layer (Figure 11).

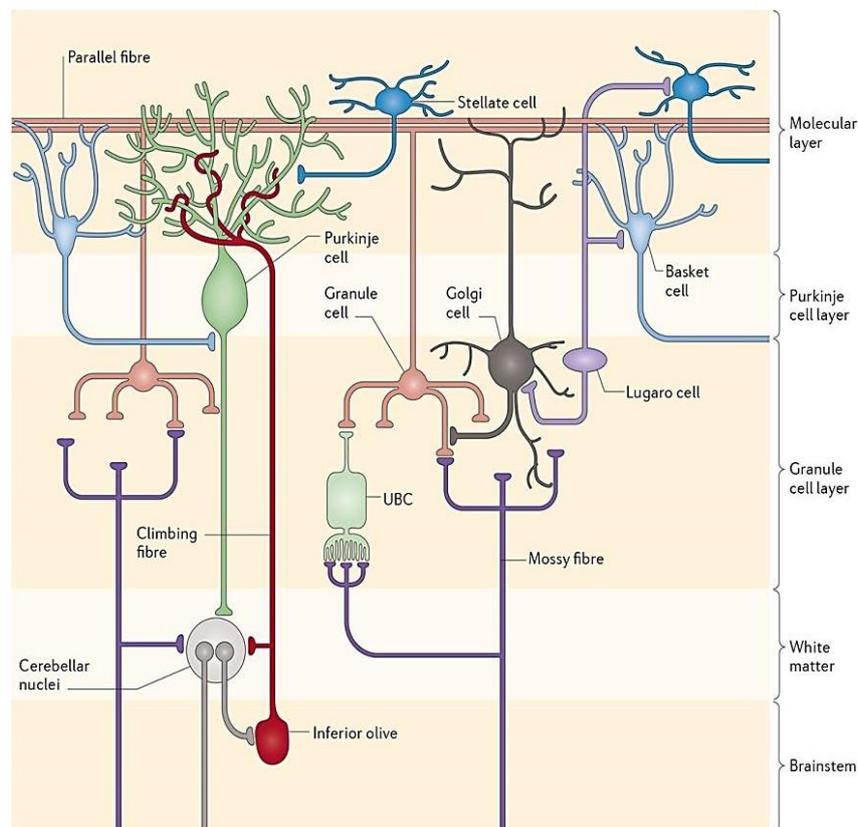
The granular layer is the innermost layer and contains a high number of small densely packed granule cells (5-8  $\mu\text{m}$ ). These glutamatergic neurons relay the input from mossy fibers to PCs via their unmyelinated parallel fibers. Three types of interneurons are present

in this layer: Golgi and Lugaro cells (GABAergic) and unipolar brush cells (glutamatergic). (Cerinara et al., 2015; Mugnaini et al., 2011).

The Purkinje cell layer is composed of a monolayer of PC somata lying between the granular layer and the molecular layer into which they project their highly-arborized dendritic tree. The dendritic tree is flat in a plane perpendicular to the long axis of the transverse folia being highly branched only in the orthogonal parasagittal plane (Palay and Chan-Palay, 1974). PCs form the sole output from the cerebellar cortex and the excitatory afferents (CF and parallel fibers) converge onto them thereby forming discrete, topographical, neuronal circuits (Apps and Garwicz, 2005). These circuits have a central role in the integration and processing of information within this structure (Apps and Garwicz, 2005). Each PC only receives input from one CF (De Zeeuw et al., 1998a) synapsing on short stubby spines of the lower part of the dendritic tree (Sotelo and Rossi, 2013). In contrast, up to 200 000 synaptic contacts between parallel fibers and PCs take place on long necked spines (Ramnani, 2006) in the distal part of the dendritic tree (Leto et al., 2006). The Purkinje cell axons emerge from the inferior pole of the cell and are immediately myelinated. These PC axons project to the DCN, and extend granular layer collaterals that contact other Purkinje, Golgi and Lugaro cells generating surround inhibition to increase signal-to-noise ratio (De Camilli et al., 1984; Lainé and Axelrad, 2002; Palay and Chan-Palay, 1974). PCs are GABAergic cells (Oertel et al., 1981) and therefore inhibit their axon targets in the deep cerebellar nuclei, located in the inner white matter of the cerebellum (Ito and Yoshida, 1966). Deep cerebellar nuclei (DCN) are the sole output of the cerebellum to other regions of the central nervous system (with the exception of few PCs in the flocculonodular lobe that project directly to the vestibular nuclei (Ruigrok et al., 2015). The deep cerebellar nuclei form a non-homogeneous population with excitatory (glutamatergic) neurons projecting essentially to the thalamus, red nucleus and other nuclei of the brainstem, and GABAergic neurons that project back to the ION. They also include a special population of glycinergic neurons co-expressing GABA which are potential inhibitory interneurons. (Chen and Hillman, 1993; Sultan et al., 2003). In addition to the PCs afferents, the deep cerebellar nuclei neurons also receive collaterals from olivary axons and mossy fibers.

The molecular layer is the outermost layer of the cerebellar cortex and it contains the major components of cerebellar cortical neural circuit. As previously described, the main cerebellar circuit is based on the convergence of climbing fibers and mossy fiber-granule cell-parallel

fiber projections to the Purkinje cells that takes place in this layer. Additionally, there is a large population of GABAergic interneurons: the stellate cells in the upper part of the molecular layer, and basket cells in the deeper part. Axons of stellate cells make synapses onto PC dendrites whereas the axons of basket cells target the PC soma and axon initial segment. Together with the granular layer interneurons, basket and stellate cells are believed to play a crucial role in timing and consolidation of functional interactions in this circuit (Crowley et al., 2009; D'Angelo and De Zeeuw, 2009; Wulff et al., 2009).



**Figure 11: Cerebellar cytoarchitecture:** Schematic diagram of the cerebellar cortical circuit. The cortex is composed of three layers: the granular cell layer, the Purkinje cell layer and the molecular layer. The cellular types present in the cerebellar cortex are the Purkinje cells, granule cells, Golgi cells, Lugaro cells, unipolar brush cells (UBCs), stellate cells and basket cell interneurons. Climbing fibres, which synapse directly with Purkinje cells and a subset of neurons in the cerebellar nuclei, and the mossy fibres, which synapse with granule cells are the two main afferents to the cerebellum.

From Cerminara et al 2015

#### IV.1.2) Cerebellar afferents

The cerebellar cortex receives three types of afferents, mostly originating from extra cerebellar regions.

1) The mossy fibers: these fibers have various origins, from the deep cerebellar nuclei, brainstem nuclei and spinal cord grey matter, but share a certain number of morphological and functional features. These myelinated axons traverse the cerebellar peduncles into the deep cerebellar white matter, where they project collaterals to the deep cerebellar nuclei, and continue into the granular layer. In the granular layer they form excitatory glutamatergic synapses with the dendrites of granule and Golgi cells at the level of a specific synaptic complex called the glomerulus. In contrast, mossy fibers arising from deep cerebellar nuclei are GABAergic inhibitory fibers.

2) The catecholaminergic fibers: axons originating in the *locus coeruleus*, are noradrenergic fibers which project through the superior cerebellar peduncle to the cerebellar cortex where they establish contacts with granule, basket and stellate cells but more importantly with the Purkinje cells. Recently it has been shown that this network also contains serotonergic axons, from the raphe nuclei, and cholinergic fibers and it is considered to form a modulatory system (Ito, 2006; Ruigrok et al., 2015).

3) The climbing fibers: These are the second major cerebellar afferent and originate exclusively from inferior olivary neurons (ION) (Desclin and Escubi, 1974); these will be discussed in more detail in the following section.

## **IV.2) The olivocerebellar pathway (OCP)**

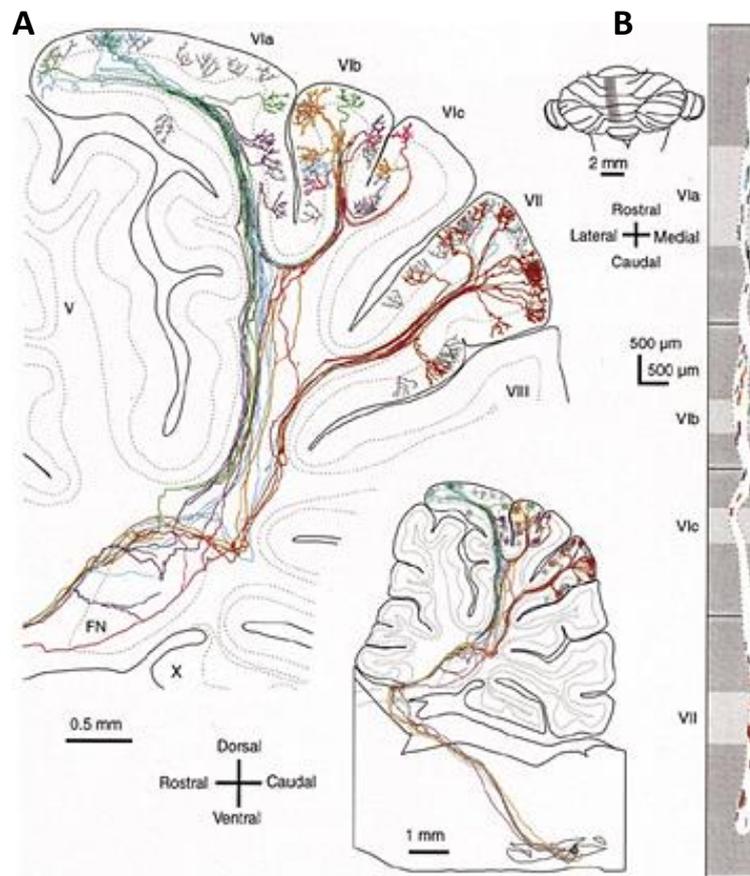
The olivocerebellar circuit is composed of the ION, the cerebellar cortex and the deep cerebellar nuclei. The ION axons innervate PCs and deep cerebellar nuclei neurons. PCs send their axons to the same deep cerebellar nuclei which in turn project their axons back onto the same afferent inferior olivary neurons.

After a succinct description of the OCP, I will focus on the development and the plasticity of this pathway since it is the experimental model used to study LI-rTMS induced plasticity and the potential underlying mechanisms.

### **IV.2.1) The inferior olivary nucleus**

The inferior olivary nucleus is a bilateral structure present in all vertebrate species. It is located in the ventral brainstem, each side of the midline and is subdivided into three parts, the principal olive and the medial and dorsal accessory olive (Voogd, 2014) that are each

separated into subnuclei. Each subnucleus projects to one or more distinct longitudinal zones in the cerebellar cortex (Ruigrok and Voogd, 2000), which forms the base of a highly specific topographical organization of the OCP into longitudinal zones and microzones. The detail of this olivocerebellar microzonal topography lies outside the area of study of this thesis, but is summarized in Figure 12.



**Figure 12 Topographical organization of the OCP into longitudinal zones and microzones.**

A) Lateral view of the anatomical reconstruction of six olivocerebellar axons (colored) and the climbing fibers that arises from them. These axons all originate from the same olivary sub nucleus.

B) Climbing fibers, following the same color code as in A) are distributed in narrow longitudinal bands thus defining functional and anatomical microzones. From Sugihara et al 2001

There are two distinct types of inferior olivary neurons with unique morphological features are present. The first type has highly branched dendrites surrounding the soma; the second type has a simple dendritic arborization that radiates far from the soma (De Gruijl et al., 2013). ION neurons are organized into structures called glomeruli containing the dendritic spines from a cluster of approximately 6 neurons as well as synaptic terminals grouped together within a glial sheet (De Zeeuw et al., 2011). The dendrites in a glomerulus are electronically coupled via gap junctions formed by connexin-36 (Llinás, 2013; De Zeeuw et

al., 2003), which is thought to synchronize oscillations of adjacent olivary neurons (Schweighofer et al., 2013) resulting in synchronous activity of their target PCs (Lang et al., 1999). However this hypothesis requires further investigation since one study found no changes in PCs synchrony in connexin-36 KO mice (Kistler et al., 2002).

The afferents to the ION come from various parts of the central nervous system. However the origin of the afferents to a particular subdivision seems to be quite restricted and to match with the modular organization of climbing fiber projections. Excitatory afferents from brainstem and spinal cord bring sensory and motor signals (De Zeeuw and Ruigrok, 1994; De Zeeuw et al., 2011). GABAergic afferents from the DCN connect to olivary neurons and modulate electronic coupling (Medina et al., 2002; Sotelo et al., 1986) and therefore secondarily the synchronous activity of PCs (Lang, 2002), generating a direct negative feedback loop.

#### **IV.2.2) Climbing fibers and the OCP**

The inferior olive axons project to the contralateral hemocerebellum. It is well accepted that the ION is the only source of cerebellar CFs (Desclin and Escubi, 1974; Sotelo and Chédotal, 2005). Olivary axons cross the midline and enter the cerebellum through the inferior peduncle (De Gruijl et al., 2013; Sotelo and Chédotal, 2005), then curve in a rostradorsal direction around the cerebellar nuclei and ramify into thick branches and thin collaterals. In the mature cerebellum, each axon splits into an average of 6-7 thick branches that go through the granular layer and contact PCs as CF arbors in a one-to-one relationship (Sugihara, 2005; Sugihara et al., 1999). Thin collaterals of these same axons enter the cerebellar nuclei where they establish contacts on neurons that receive inputs from PCs that are in the same sagittal zone and thus contacted by the same olivary neurons (Ruigrok and Voogd, 1990). A subset of these DCN neurons, especially the GABAergic ones, in turn send their axons into the corresponding cluster of inferior olivary neurons (Fredette and Mugnaini, 1991). This establishes a topographically organized olivo-cerebellar-nucleo-olivary feedback loop (Sotelo and Chédotal, 2005; De Zeeuw et al., 1998b).

Each CF forms about 150-300 synapses onto the proximal dendrites of the innervated PC (Fujita and Sugihara, 2013). These synapses trigger complex spikes (CS) in the PC at a notably low frequency ( $\approx$  1Hz) in contrast to the high frequency of the simple spikes triggered by

parallel fibers (10-200Hz; (Warnaar et al., 2015). The CS results from a large calcium influx concomitant with multiple somatic Na<sup>+</sup> spikes, and they are followed by a ≈20ms long pause in the simple spike trains (Bell and Grimm, 1969; McDevitt et al., 1982). These pauses are thought to be involved in the transfer of downstream information through a synchronized disinhibition of the cerebellar nuclei (Maiz et al., 2012; De Schutter and Steuber, 2009).

Without synaptic activation, Purkinje cells are tonically active (Cerminara and Rawson, 2004; Gähwiler, 1975; Llinás and Sugimori, 1980; Woodward et al., 1974) and fire at an average rate of 44 Hz (Armstrong and Rawson, 1979). Although the direct effect of CF activation PC depolarization, it is well known that increasing CF firing leads to decreased or silenced PC activity (Cerminara and Rawson, 2004; Colin et al., 1980; Rawson and Tilokskulchai, 1981) and can even block the cerebellum-dependent conditioned blink response (Zucca et al., 2016). CFs can induce LTD of the PF-PC synapses, which is proposed to be involved in cerebellar learning (Ito, 2001; Steuber et al., 2007; Zucca et al., 2016). The precise functional role of this plasticity mechanism remains incompletely described, but it seems to operate in a spatially and temporally distributed manner within the cerebellum, in order to create optimal output for behavior (Gao et al., 2012).

### **IV.3) Development of the OCP**

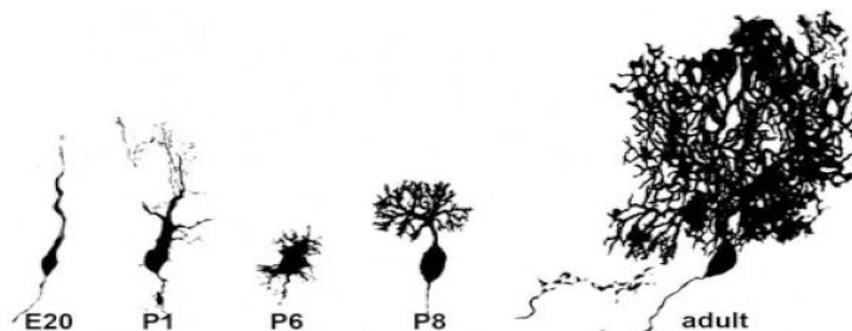
#### **IV.3.1) Morphological PC differentiation**

Since the first observations by Ramon y Cajal in 1911, several studies have described and Purkinje cell differentiation during development. Like many cells in migration, PCs have a bipolar organization until they reach the cerebellar cortex. Then they differentiate progressively to acquire polarity, elaborate their dendritic tree, and project their axons.

In 1991 Armengol and Sotelo combined retrograde tracing and electron microscopy and described several stage of PCs differentiation in the rat (Figure 13). From the end of the migration (E18-E19) until the day of birth (P0) PCs have a bipolar form with a simple apical dendritic extension and a basal axon that Ramon y Cajal termed the “simple-fusiform” state (Armengol and Sotelo, 1991; McKay and Turner, 2005; Ramón y Cajal 1911). At this stage, CFs make their first transient non-synaptic contacts with PCs (Chedotal and Sotelo, 1992; Morara et al., 2001). From P0 to P2 PCs present a branched apical dendrite, which characterizes the “complex fusiform” state. These long primary extensions then regress and

PCs adopt their non-polarized “stellate cells with disorientate dendrite” morphology, characterized by short perisomatic protrusions. At P5 these cells become organized in a monolayer. From this stage they progressively elaborate a polarized and complex dendritic tree that reaches its adult width together with a synaptically mature soma at P15 (Leto et al., 2016). Subsequently the dendritic tree grows in a vertical plane, increasing its height in parallel with an increase in PF deposition until P30 (Sotelo, 2004).

The signals involved in PC differentiation are still under debate and some studies are contradictory, but it seems accepted that there are some mechanisms (Baptista et al., 1994; Boukhtouche et al., 2006a; Sotelo and Dusart, 2009) that depend on extracellular signals which promote the elaboration of the dendritic tree during distinct developmental period. Among these signals are neurotrophic factors (NT-3, NT-4 and BDNF) (Fukudome et al., 2003; Joo et al., 2014; Schwartz et al., 1997), and thyroid and sex hormones (Sakamoto et al., 2002, 2003; Vincent et al., 1982). In addition, the later differentiation stages are linked to the synaptic input from the granule cells (PF) (Altman and Anderson, 1972; Hirai and Launey, 2000; Lohof et al., 2005; Sotelo and Dusart, 2009). The morphology of the final dendritic arbors depends on synaptic interaction with the CFs and PFs afferents (Sotelo, 2004).



**Figure 13: Morphological differentiation of Purkinje cells.**

Dendritic remodeling of Purkinje cells pursue continuously during development. In rodents it starts just before the birth and is achieved by the end of the post natal second week.

Modified from Armengo and Sotelo 1991

#### **IV.3.2) Synaptogenesis of the olivocerebellar pathway and refinement of CF projections**

The first axonal contacts with PCs are observed at the end of the embryonic period, where PCs have their fusiform morphology. The first clear morphological synaptic interactions between CFs and PCs were described in the rat at P0 (Chedotal and Sotelo, 1992), when

parvalbumin-expressing CFs establish synaptic contact on the dendritic elongation of the “fusiform” PCs: this is termed the “creeper” stage of CF development. These first contacts are followed by extensive remodeling that allows the execution of two simultaneous but distinct processes: (1) the subcellular targeting of CF terminals from the soma toward the proximal PC dendritic tree of the PCs (Chedotal and Sotelo, 1992; Mason et al., 1990; Scelfo et al., 2003) and (2) transient PC multi-innervation by CFs followed by a selective synapse elimination to reach the mature one-to-one relationship (Mariani and Changeux, 1981).

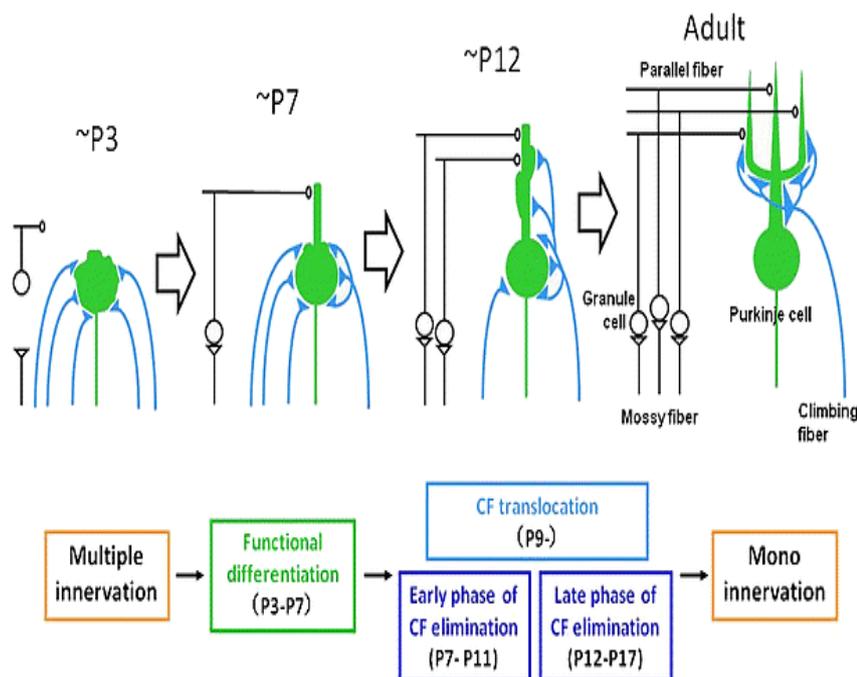
During these 2 processes, the “creeper” CFs at P3 form a small number of functional type-2 vesicular glutamate transporter (Vglut2)-positive synaptic connections on the surface of immature PC dendrites (Watanabe and Kano, 2011). At this stage, each CF that innervates the PC induces EPSCs with longer duration and smaller amplitude than in the mature PC (Hashimoto and Kano 2003). By P5 the number of climbing fibers per olivocerebellar axons sharply decreases from around 100 to about 10 due to the potential retraction of collaterals branches (Sugihara, 2006). These remaining CFs are now structured into a nest-like structure surrounding the PC somata aligned in a monolayer, the “pericellular nest” stage (Santiago Ramón y Cajal). At this stage up to 6 CFs (average 3.5) synapse onto perisomatic protrusions of each PC (Crepel et al., 1976; Larramendi and Victor, 1967; Mariani and Changeux, 1981)

The CF terminals then move from their perisomatic position towards the main stem of the PC dendritic tree to form the peridendritic “capuchon” stage ( $\approx$  P9) (Ramón y Cajal, 1911) (Watanabe and Kano, 2011). At the same time, the number of multi-innervated PCs sharply decreases, with only 50% of the PCs being multi-innervated, and mostly by only 2 CFs (Kano et al., 1995).

Finally, during the last phase (P10-P15) the somatic protrusions are removed and the CF terminals move from the soma, climb along the principal branches of the developing dendritic tree and become stable (“dendritic” stage; (Ramón y Cajal 1911; Sugihara, 2005)). During this last crucial refinement step, the elimination of the last weak CFs occurs to reach the mature one-to-one relationship with only one strong winning CF contacting each PC (Crepel et al., 1976; Hashimoto and Kano, 2003).

This process of CF refinement during development is essential for the OCP to function appropriately. Some studies show that transgenic or mutant animals with impaired CF-PC synapse elimination present striking motor behavioral deficits. These studies on mutant mice have also defined the critical role of GCs (*weaver* and *reeler* mutant mice; (Crepel and

Mariani, 1976; Mariani et al., 1977; Puro and Woodward, 1977) and PF-PCs synapses (*staggerer* mutant mice; (Mariani, 1982; Mariani and Changeux, 1980) in selective CF elimination. In addition we know that OCP synaptic maturation also requires molecules involved in PC post-synaptic signaling: the synaptic receptors GluRdelta2 (Kashiwabuchi et al., 1995; Lalouette et al., 2001), mGluR1 (Hashimoto et al., 2001) and NMDA (Kakizawa et al., 2000; Rabacchi et al., 1992) ; voltage-dependent calcium channels and PKCgamma (Kano et al., 1995). More recently the TrkB receptor (Choo et al., 2017; Johnson et al., 2007), the secreted protein cerebellin1 (Cbln1; (Hirai et al., 2005) and Semaphorin3A (Uesaka et al., 2014) have also been shown to regulate CF synapse elimination. The presynaptic protein C1ql1 receptor for Cbln1 has been shown to act as an essential anterograde signal to determine and maintain the single-winner CF (Kakegawa et al., 2015; Sigoillot et al., 2015). It is important to note that none of these molecules alone are sufficient to explain the whole process of CF-PC synaptogenesis, suggesting the interplay of different signaling pathways.



**Figure 14: Postnatal development of CF-PC synapses.**

Until P3: relatively uniform synaptic strength of multiply innervating CFs. P3-P7: “functional differentiation” with the selective strengthening of one CF. From P9, the strengthened (“winning”) CF translocate its synapses onto the growing dendrites of the PC while the other CFs remain around the soma and are eventually eliminated in “early” and “late” phases of CF elimination (Watanabe and Kano 2011)

Diagram from Leto et al 2015

#### IV.4) Plasticity of the OCP following lesion

Unilateral transection of the OCP establishes a well-defined system where CF plasticity and targeted re-innervation can be assessed reproducibly in different conditions and with easily interpretable anatomical read-out thanks to the characteristic morphology and synaptic interaction between CF and PCs. In addition to the structural reorganization during development, olivocerebellar axons at CF terminals are capable of remodeling post lesion to reinnervate denervated PCs.

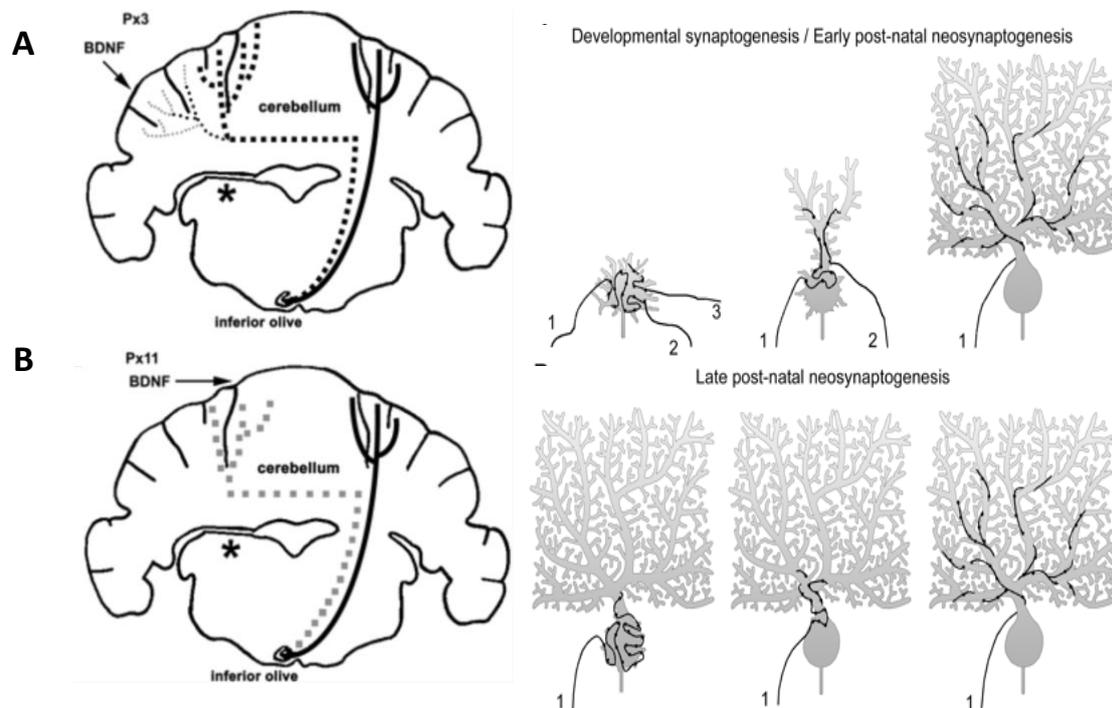
#### **IV.4.1) Developmental plasticity of the OCP**

The capacity of olivary axons to form collaterals that will make new synapses onto denervated PCs was found following a unilateral transection of the inferior cerebellar peduncle, termed pedunculotomy (Px) (Angaut et al., 1982). PCs of the lesioned hemocerebellum lose their CF synaptic contacts and the axotomized ION degenerates (Angaut et al., 1985; Sherrard and Bower, 1986). The uninjured contralateral OCP reacts by sprouting thin branches, which cross the midline and reach the lesioned hemocerebellum as soon as 18 hours post lesion (Sugihara et al., 2003; Willson et al., 2007; Zagrebelsky et al., 1997) (Figure 15A). In this lesion model the transcommissural CFs are distributed in longitudinal bands coherent with zebrin2 expression in the reinnervated hemocerebellum, suggesting that the topographical organization of the OCP is restored (Zagrebelsky et al., 1997). The reinnervating CFs form functional microzones in the reinnervated hemocerebellum, which are similar to those observed in the intact side. The new CF arbors are appropriately distributed along the PC proximal dendrites in the molecular layer, and form electrophysiologically normal functional synapses (Sugihara et al., 2003). Interestingly, the reinnervation of PCs after pedunculotomy during development follows the developmental refinement step, going through a transient multi-innervation which then regresses and leads to normal monoinnervation (Lohof et al., 2005) (Figure 15A). About 80% of the PCs are reinnervated in the vermis and around 50% in more lateral regions (Sugihara et al., 2003). These newly formed specific synapses could explain the restoration of motor and cognitive functions in these animals (Dixon and Sherrard, 2006; Dixon et al., 2005; Willson et al., 2007, 2008).

#### **IV.4.2) Plasticity in the mature OCP**

The capacity of the developmental OCP to spontaneously form of a compensatory path following a pedunculotomy disappears between P7 and P10 in the rat (Sherrard et al., 1986). The critical period of the OCP plasticity can be further extended, even in adult animals, by injecting into the denervated hemocerebellum certain trophic factors, including insulin growth factor 1 (IGF-1; (Sherrard and Bower, 1997, 2003), neurotrophin-3 (NT3; (Sherrard and Bower, 2001) or most efficiently BDNF (Dixon and Sherrard, 2006; Sherrard and Bower, 2001; Willson et al., 2008). The diffusion and the stability of these factors as well as the signaling pathway they induce in the cerebellum are yet to be discovered. Cellular targets in the cerebellum are multiple and include PCs, GCs and the CFs themselves (Sherrard and Bower, 2002). BDNF injection 24 hours post-pedunculotomy at P15 induces transcomissural olivocerebellar reinnervation (Dixon and Sherrard, 2006). The distribution of reinnervating CFs in that study spread throughout the denervated hemivermis and to some extent into the hemisphere (Figure 15B). They show a topographic organization in parasagittal stripes that are almost symmetrical to their branches in the intact hemocerebellum. Reinnervating CFs contact the denervated PCs as expected onto their proximal dendrites (Dixon and Sherrard, 2006) and form functional synapses (Letellier et al., 2007). Interestingly, mature PCs are reinnervated by only one CF, contrasting with the developmental initial multi-innervation step (Letellier et al., 2007) but appropriate for the maturation stage of the PC. It is important to note that the BDNF-induced plasticity in the mature OCP promotes partial functional recovery of motor and spatial learning (Willson et al., 2008).

In addition to transcomissural reinnervation, CFs retain a degree of plasticity within the non-myelinated molecular layer. The injection of the neurotoxin, 3-acetylpyrimidine (3-AP), in the rat induces the majority of the ION to degenerate, leaving a few surviving CFs synapsing normally on PCs (Benedetti et al., 1983). These surviving CFs are able to form thick collateral sprouts in the molecular layer to reinnervate neighboring PCs primarily within a few  $\mu\text{m}$  (Benedetti et al 1983; Rossi et al 1991 a, b), although the stem branch spreads several millimeters and will synapse on distant PCs (Dhar et al., 2016). Synaptic contacts are generally formed on the proximal dendrite of the denervated PCs, are functional with the induction of complex spikes (Benedetti et al., 1983). These CFs only reinnervate PCs located within the same zebrin II parasagittal band (Zagrebelsky et al., 1996; Dhar et al., 2016)) suggesting that the mechanisms involved in the establishment of the olivocerebellar topographical longitudinal zones are conserved in the adult.



**Figure 15: Developmental and mature post lesion plasticity of the olivocerebellar pathway.** A) On the left are schematic diagrams of the normal olivocerebellar pathway (filled-line). If a unilateral transection of the inferior cerebellar peduncle (\*) is made on P3, the axotomised contralateral ION degenerates. The uninjured ION spontaneously reacts by growing transcommissural fibres to reinnervate Purkinje cells densely in the vermis (bold dotted line). The uninjured ION spontaneously reacts by growing transcommissural fibres to reinnervate Purkinje cells densely in the vermis (bold dotted line). BDNF injection into the intermediate/lateral hemicerebellum allow to partially reinnervate further hemispheric region of the lesioned hemicerebellum (grey dotted line). On the left developmental CF-PC synaptogenesis and neosynaptogenesis recapitulate similar mechanisms (multi innervation, translocation of CF terminal , synaptic refinement leading to mono innervation). (B) On the left schematic diagram of the BDNF induced compensatory reinnervation of the mature hemicerebellum by transcommissural projection. If the peduncle transection is made at P11 spontaneous transcommissural reinnervation does not occur, but BDNF injection into the left medial/intermediate hemicerebellum 24h after the peduncle transection can extend the plasticity time window and induce transcommissural reinnervation (Sherrard and Bower, 2001; Willson et al 2008). On the right: A direct mono-innervation of PCs by reinnervating CF is observed during late postnatal

#### IV.5) Ageing of the cerebellum

Additionally the cerebellum is also a structure that is markedly affected by ageing. Given the implication of the cerebellum in a wide variety of functions (Koziol et al., 2014; Leiner et al., 1989; Stoodley and Schmahmann, 2009; Stoodley et al., 2012), it is not surprising that age-related morphological changes in the cerebellum are linked to motor and cognitive altered

performance (MacLulich et al., 2004; Miller et al., 2013; Paul et al., 2009; Woodruff-Pak et al., 2001). Indeed, cerebellar morphology presents a very good predictor, equal to if not better than prefrontal cortex morphology, of behavioural performance decline in elderly subjects (Eckert et al., 2010; Hogan et al., 2011; Lee et al., 2005). The volume of the cerebellum and cerebellar white matter structural integrity decrease with age (Fjell et al., 2013; Giorgio et al., 2010; Hoogendam et al., 2012; Lee et al., 2009). In addition cerebellar functional activation and resting state functional connectivity are also affected by ageing. All of this alters the cerebellar connectivity with cortical regions, reducing efficient transmission of updates resulting from performance monitoring, and thus leading to behavioural deficits (Bernard and Seidler, 2013; Ito, 2008). As previously described, PCs are the centre of the cerebellar circuitry, and a significant proportion of these cells die during cerebellar ageing in both humans and rodents (Andersen et al., 2003; Rogers et al., 1984; Zhang et al., 2010) and this PC death is at a relatively early stage compared with the hippocampus for example (Woodruff-Pak et al., 2010). Additionally, remaining PC dendritic spines lose about 33% of their synapses, although whether this reflects age-related afferent impairments and/or dendritic spine vulnerability is unknown (Glick and Bondareff, 1979).

As PCs age their electrophysiological features change leading to a deficit in PC activation and increased inhibitory threshold (Rogers et al., 1981) that are thought to be the result of either altered receptor response (Parfitt, 1988), reduced postsynaptic sensitivity to neurotransmitters (Marwaha et al., 1980) and/or functional modification in several ion channels (Chung et al., 2001a, 2001b, 2003). In mice, deficits of cerebellar LTD appear between 4 and 8 months and are associated with motor and cognitive decline (Thouvaracq et al., 2001; Woodruff-Pak et al., 2010). This is followed by atrophy of the height, width and distal branchlets of the PC dendritic tree (Hadj-Sahraoui et al., 2001; Quackenbush et al., 1990; Zhang et al., 2006). Hence aged PCs receive fewer synaptic inputs and therefore have less information integration and signal transmission leading to dysfunction in the old cerebellum (Zhang et al., 2010). The cause of this PCs dendritic atrophy is still unclear but hypotheses include: decreased inputs from PF to PCs (Dlugos and Pentney, 1994; Huang et al., 2006, 1999); and/or reduced cerebellar expression of trophic factors, such as IGF-1 and BDNF, which promote dendritic growth (Erickson et al., 2010; Markowska et al., 1998). All this fine structural and/or biochemical changes might precede the actual PC death (Caston et al., 2003, 2004; Woodruff-Pak et al., 2010).

Different models of premature ageing of the cerebellum are available. The use of Lurcher and Staggerer mutant mice have helped to correlate reduced PC numbers with direct cerebellar dysfunction (Caston et al., 2003; Hilber and Caston, 2001; Porrás-García et al., 2010). They present a premature and aberrant apoptosis of PCs and GCs in the cerebellum associated with deficits in motor skills and learning, spatial orientation, associative learning and working memory (Caston et al., 2003; Hilber and Caston, 2001; Porrás-García et al., 2010); and are considered a good model of age-related progressive neurodegeneration (Hilber and Caston, 2001). Staggerer mice carry a spontaneous mutation on the gene coding for the ROR $\alpha$  protein. ROR $\alpha$  is a transcription factor that binds to DNA response elements and is essential for the development and survival of PCs (Boukhtouche et al., 2006b; Chen et al., 2013) but is also implicated in the protection against several other factors related to ageing such as osteoporosis, atherosclerosis (Mamontova et al., 1998; Meyer et al., 2000) and oxidative stress (Boukhtouche et al., 2006c). At 12-13 months dendritic tree atrophy and PC death in ROR $\alpha$ <sup>+/*Sg*</sup> mice is as severe as at 24 months in WT (Hadj-Sahraoui et al., 2001). This heterozygous mutant could therefore represent a model of precocious ageing.

## **V) Summary and aims**

Neuroplasticity mechanisms are essential for the establishment and strengthening of neural circuits during the critical period of development and are required for the brain to adapt to its environment during life. Over lifespan these plasticity mechanisms vary and are generally more difficult to induce in the adult brain and decrease with advance in age (Freitas et al., 2011a; Fuchs and Flügge, 2014; Ganguly and Poo, 2013) Thus there is a clear medical need to find non-invasive ways to induce neural plasticity in the adult brain. rTMS is commonly used to modulate cortical excitability and shows promising results to treat some neurological disorders (Pell et al., 2011; Wassermann and Zimmermann, 2012). Central high intensity magnetic fields delivered during rTMS are surrounded by areas of lower intensity stimulation that are very likely to contribute to the effects of high intensity rTMS (Deng et al., 2013; Ellaway et al., 2014). Nonetheless low intensity magnetic stimulation *per se* are described as a promising therapeutic tool and notably in the treatment of depression (Jin and Phillips, 2014; Martiny et al., 2010; Shafi et al., 2014). It is therefore important to determine whether these low intensity magnetic fields can alter neuronal morphology, connectivity and functioning and/or engender negative or positive outcomes. Our group has combined the

approaches of the focal high intensity stimulation (rTMS) and diffuse low intensity magnetic stimulation (ELF-MF) to deliver a focal low-intensity magnetic stimulation (LI-rTMS, 10mT) (Grehl et al., 2016; Makowiecki et al., 2014; Rodger et al., 2012). The three main aims of this thesis are:

1) To evaluate if LI-rTMS can promote neural plasticity of the CNS and alter behavior at different stages of life.

We used the cerebellum as a model to study the effects of LI-rTMS, because its development, structure, ageing and function are well described which facilitate us to assess changes in these characteristics (Apps and Garwicz, 2005; Ruigrok et al., 2015; Sotelo and Dusart, 2009; Woodruff-Pak et al., 2010). Furthermore transgenic mice model with altered cerebellar development and ageing are available and extend the experimental possibilities (Boukhtouche et al., 2006a; Doulazmi et al., 2006; Jarvis et al., 2002) To assess the effects of the LI-rTMS on neural plasticity over the lifespan, we applied *in vivo* stimulation of the mouse cerebellum at different ages, assessed the animals' behaviour, and measured PC dendritic morphology.

2) To optimize stimulation parameters for enhancing neural plasticity, and specifically to induce post-lesion neural circuit repair in the CNS.

Neural rehabilitation using non-invasive treatments to promote post-lesion neural circuit repair is as yet extremely limited. More fundamental research is required to optimize the parameter of existing treatments and to better define some mechanisms by which they facilitate this neurorehabilitation (Ellaway et al., 2014; Lefaucheur et al., 2014). Therefore we systematically investigated the actions of different LI-rTMS parameters on post-lesion repair of the mature OCP. Unilateral transection of the OCP is a well-described system where CF plasticity and targeted re-innervation can be assessed thanks to the characteristic morphology and synaptic interaction between CF and PCs (Dixon and Sherrard, 2006; Letellier et al., 2007; Willson et al., 2008). The optimization of the lesion model in organotypic explant culture (Letellier et al., 2009) allows us to easily control our stimulation parameters and to reproducibly evaluate reinnervation in different conditions.

3) To find potential underlying mechanisms that may participate to the effects of LI-rTMS on neural plasticity.

Finally we looked for potential mechanisms underlying the observed effects of LI-rTMS through a combination of transgenic mice, transcriptome analyses and cell activation within our *in vivo* and *in vitro* studies.

First we studied gene expression changes 6h following 3 daily sessions of LI-rTMS to identify molecules that may contribute to the effects on neuronal morphology and neural circuit repair.

We also examined which cells were activated by LI-rTMS through *cfos* expression in our explants 2.5h after a single LI-rTMS session. Co-labelling with markers for different cell populations revealed those which were involved.

The intensity of our LI-rTMS is too low to directly induce action potentials, suggesting a direct effect of the magnetic field. We tested this in our explant model assessing LI-rTMS induced reinnervation in *Cry1::Cry2*<sup>-/-</sup> mice, knockout for the two magnetoreceptor genes.

To facilitate our interpretation of the effects of LI-rTMS at different lifespan stages, we used heterozygote *RORα*<sup>+/-</sup> transgenic mice, which present altered cerebellar development and ageing (Boukhtouche et al., 2006a; Doulazmi et al., 2006; Jarvis et al., 2002). We tested the effects of LI-rTMS on *RORα*<sup>+/-</sup> animals' behaviour and PC dendritic morphology *in vivo* and on olivocerebellar reinnervation in *RORα*<sup>+/-</sup> explants.



## **Chapter II- Article 1**

### **Low intensity rTMS to the mouse cerebellum induces neuronal structural plasticity and behavioural improvements in an age dependent manner.**

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UMR 8256 Biological Adaptation and Ageing

Brain Development, Repair and Aging (BDRA)

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## Abstract

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive brain stimulation technique gaining popularity as a treatment for neurological dysfunction. Low intensity rTMS (LI-rTMS) does not directly stimulate action potentials but can nonetheless modify neural function. Although there has been an increase in studies trying to understand the effects and mechanisms of action of low field magnetic stimulation, few *in vivo* studies have been conducted looking at its long-term effects on morphology of the stimulated cells and specific function associated with these cells. We used the cerebellum as a model because of its well-described structure, changes during development and aging, and its behavioural functions.

In this study we investigated the short-term and long-term effects of low intensity magnetic stimulation on dendritic morphology and spine density of cerebellar Purkinje cells, effects on their morphological and synaptic development in early post-natal life, and long-term effects on cerebellar behavioural function. We used animals of different ages to determine to what extent the same stimulation patterns would produce similar effects.

We show that LI-rTMS treatment changes spine density after acute stimulation and alters dendritic complexity with longer treatment. These changes were paralleled by improved spatial learning and memory. Importantly, the effects of LI-rTMS were weaker in the aged animal and, unexpectedly, in very young animals. These results emphasize that multiple factors must be taken into consideration when planning therapeutic treatments involving non-invasive brain stimulation with low-intensity magnetic fields.

**Keywords:** LI-rTMS, development, ageing, cerebellum, Purkinje cells, spatial memory, dendrites, magnetic fields

## Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive brain stimulation technique gaining popularity as a therapeutic treatment for neurological dysfunction. Although generally the intensity of rTMS stimulation used is high enough to stimulate action potentials in the underlying brain tissue (Hallett, 2007; Pell et al., 2011), low intensity magnetic stimulation does not directly stimulate action potentials but can nonetheless modify cortical excitability (Capone et al., 2009), alter pain perception (Robertson et al., 2010) and alleviate symptoms of depression (Martiny et al., 2010; Rohan et al., 2014) in humans. In addition, animal studies show that LI-rTMS increases neuronal survival (Yang et al., 2012), alters structure and function of neural pathways as well as alters gene expression of markers of neural plasticity (Makowiecki et al., 2014; Rodger et al., 2012). Although there has been an increase in studies trying to understand the effects and mechanisms of action of low field magnetic stimulation, few *in vivo* studies have been conducted looking at its long term effects on morphology of the stimulated cells and specific function associated with these cells.

We have chosen the mouse cerebellum as a model because of its clearly defined structure and known behavioural functions, the characteristic morphology of the cerebellar Purkinje cells, and its well described development, ageing and involvement in pathology (Cierpka et al., 2017; D’Mello and Stoodley, 2015; Sun et al., 2016). The cerebellum is involved in coordination, adaptation and learning of motor behaviour (Ruigrok et al., 2015) spatial navigation (Rochefort et al., 2013), and cognitive and emotional control (Hoche et al., 2016; Stoodley et al., 2016). The Purkinje cells (PCs) form the centre of the cerebellar cortical circuit and integrate multiple afferents to transmit the combined signals to the deep cerebellar nuclei (Apps and Garwicz, 2005; Palay and Chan-Palay, 1974; Sotelo and Rossi, 2013) and therefore cerebellar outflow to other brain regions. PCs have a distinct morphology with an extensive dendritic tree, with spines which receive synapses from either parallel fibres of granule cells or climbing fibres from inferior olivary nucleus axons (Leto et al., 2006; Ramnani, 2006; Sotelo and Rossi, 2013; De Zeeuw et al., 1998). This PC dendritic tree develops postnatally in close association with its climbing and parallel fibre afferents (Lohof et al., 1996). While the number of PF synapses gradually increases from P7 (Scelfo et

al., 2003), several climbing fibres initially innervate each PC and these regress to the adult mono-innervation in the first 2 postnatal weeks (Hashimoto and Kano, 2013). PCs are also vulnerable to ageing (Rogers et al., 1984; Zhang et al., 2010), undergoing synaptic dysfunction (Rogers et al., 1980; Woodruff-Pak et al., 2010) associated with motor and cognitive decline (Woodruff-Pak, 2006), reductions in spine density (Zhang et al., 2010), dendritic atrophy (Quackenbush et al., 1990; Woodruff-Pak et al., 2010; Zhang et al., 2006) and cell death (Andersen et al., 2003; Woodruff-Pak et al., 2010; Zhang et al., 2010).

As is clearly indicated by PCs, neurons change considerably during their lifespan. This is not just in terms of morphology and circuitry, but also in terms of plasticity, which decreases with time (Freitas et al., 2011a, 2013; Pascual-Leone et al., 2011). For example, young immature PCs are highly vulnerable to insult, e.g. alcohol (Pierce et al., 2006), loss of input (e.g. staggerer and weaver mutant mice; Vogel et al., 1989; Zanjani et al., 1992) and hypoxia (Campanille et al., 2015), yet are able to effectively regenerate their axon, a plastic phenomenon which is lost during maturation (Dusart et al., 1997, 2005; Rossi et al., 2006). During adulthood, PCs retain some plastic capacity, particularly in their dendritic spines which can be influenced by environmental factors (De Bartolo et al., 2015) and activity (Lee et al., 2007). Following periods of motor learning or environmental enrichment in rats, an increase PC dendritic tree size and spine density as well as changes in spine shape can be observed (De Bartolo et al., 2015; González-Burgos et al., 2011; Lee et al., 2007, 2013), associated with changes in synaptic efficacy (Lee et al., 2005). In contrast during ageing, PCs undergo spine and dendritic atrophy (Rogers et al., 1984) (Quackenbush et al., 1990; Woodruff-Pak et al., 2010; Zhang et al., 2006) and their capacity to mount a plastic response to ameliorate this decline is unknown.

Research shows that neuroplasticity is induced by activation of the brain, such as through enriched environments or psychomotor activity (Redolat and Mesa-Gresa, 2012). Some effects of psychomotor activity may be mimicked by rTMS, by providing activation of neuronal circuits. Recent studies have shown that high-intensity rTMS of the cerebellum in humans, for example, modulates neuronal circuits between the cerebellum and the primary motor cortex, changing cerebellar excitability to alter motor and cognitive function (Casula et al., 2016; Grimaldi et al., 2014; Popa et al., 2013). However, the effects on the cerebellum of magnetic stimulation (of either high or low-intensity) has not been studied in detail. Since

low intensity stimulation has shown promise as a neuromodulatory, and therefore potentially therapeutic, tool (Jin and Phillips, 2014; Martiny et al., 2010; Shafi et al., 2014) , it is important to understand its effects on the neurons being stimulated at different life stages to determine whether treatment based on this non-invasive stimulation is likely to provide a therapeutic benefit without negative effects on normal cells and circuits, possibly reverse some of the deleterious effects of ageing, and/or alter neuronal development in the immature brain.

In this study we investigated the short-term and long-term effects of low intensity magnetic stimulation on PC dendritic morphology and spine density, effects on morphological and synaptic development in early post-natal life, and long-term effects on cerebellar behavioural function.

## **Materials and Methods**

### **Animals**

C57Bl/6J and Swiss wild-type mice were bred at Université Pierre et Marie Curie, Paris, or obtained from Elevage Janvier, France. They were housed in single-sex, mixed genotype cages on a 12 h light-dark cycle and with water and food *ad libitum*.

For studies of early post-natal PC morphological and synaptic development, pups were randomly assigned to LI-rTMS or sham groups at P1 and their paws tattooed accordingly. Pups were stimulated daily from P2-P14 and care was taken to limit their separation from the dam.

To determine the effects of short term LI-rTMS on PC morphology, mice aged 3-7 months (adult) and 14-17 months (aged) were stimulated for 10 minutes per day for 3 days. At 24 hours following the last stimulation, animals were euthanized for PC visualization.

To determine the effects of long term LI-rTMS on motor function and PC morphology, mice aged between 3-5months (adult) and 14-16 months (aged), were transferred to the Centre de Recherche et Développement, Institut de la Longévité, Ivry, France, 2-4 weeks prior to the start of experimental procedures. Mice were treated with LI-rTMS for 4 weeks, followed by 2 weeks of behavioural testing. The same animals were then used for visualization of Purkinje cells.

All animal procedures were carried out under the guidelines established by 'le comité national d'éthique pour les science de la vie et de la santé' 2010/63/EU and approved by the "Comité d'éthique en expérimentation animale Charles Darwin, N° 5.

### **Administration of Magnetic Stimulation**

Low intensity magnetic stimulation was applied to the cerebellum, using a previously described method (Rodger et al., 2012; Makowiecki et al., 2014)). Focal cerebellar stimulation was induced with a custom made magnetic coil designed for use on mice (copper wire, 300 windings, 16Ωm, inner diameter 5mm, outer diameter 8mm), and powered by an electromagnetic pulse generator (EC10701; Global Energy Medicine Pty Ltd, Perth, Western Australia). The frequency used for stimulation was a high frequency, complex pattern, comprised of 59.9ms trains of 20 pulses at 3 different frequencies as follows; 1 min at 6.71Hz, 8 min treatment at 10.1Hz and 1 min at 6.26Hz (Rodger et al., 2012; Makowiecki et al., 2014), which is based on the patent PCT/AU2007/000454 (Global Energy Medicine Pty Ltd, Perth, Western Australia). The intensity of the magnetic field was 9mT at the tissue and was measured using a Hall device magnetic field detector. As this stimulation has similar focality to high-intensity rTMS, we call it low intensity rTMS (LI-rTMS)

For LI-rTMS treatment, animals we placed in a small box and the stimulation coil held directly above the scalp, without touching it, over the cerebellar region, for 10 minutes each day. To minimize stress, adult or aged mice were handled and habituated to the researcher and to the small box for 5 days prior to the start of stimulation. The mice showed no signs of discomfort or stress during or after the stimulation, and explored the box or groomed during the procedure. Control mice received sham stimulation: they were handled identically to those that received the LI-rTMS, but the stimulator was turned off. The sound emitted by the coil was tested previously (Rodger et al., 2012) and was shown to be below the audible threshold of mice. Previous tests have shown that this low-intensity coil does not change the coil temperature (Makowiecki et al., 2014).

### **Behavioural analysis**

#### ***Open field - General motor activity***

To assess general motor activity and anxiety, animals were placed in an open field arena (square 500cm x500cm) and recorded for 5 minutes using Ethovision X10 software (Noldus

Information Technology, France). Parameters measured were the total distance moved, time spent moving, and times spent in the centre and the periphery of the box.

### ***Grip strength***

Grip strength of the two front paws was measured using a Grips strength meter (Ugo Basile). The animals were picked up by the tail and the 2 front paws placed on the bar of the grip meter and the tail pulled gently backward until the animals lost their grip. The test was repeated three times and the average grip strength was used and divided by weight of the animals to correct for differences in animal size.

### ***Catwalk - Analysis of gait***

The gait of each animal was analysed using the CatWalkXT (Noldus Information Technology, France). The catwalk consisted of a 7 cm x 2m corridor with a lit-up glass runway floor, through which mouse gait was filmed by a camera placed under the walkway. Two-three trials, where the animal walked in a straight trajectory, with constant speed between 15- 30 cm/s, were analysed. Parameters measured were stride length, stride width (distance between hind paws), paw angle (from the axis of the walking direction) of the hind paws and regularity index (% of regular step patterns). Results were averaged for each animal.

### ***Rotarod - motor coordination***

To test cerebellar motor function and coordination, we used an accelerating rotarod (TSE Systems, Bad Homburg, Germany) with a 3 cm diameter rod. The accelerating protocol consisted of an initial speed of 4 rpm and an increase of 4rpm every 30 seconds for a maximum of 5 minutes (40rpm). Animals were placed on a rotating rod and the latency to fall was measured. Mice received 3 trials per day for 5 days and the average latency to fall was calculated for each day.

### ***Morris Water Maze - spatial learning and memory***

#### ***Learning phase***

To test spatial learning, mice were placed in a circular water tank (120 cm diameter) filled with water (23°C) and were trained to find an invisible clear Plexiglas platform (10cm diameter) hidden 1 cm under the surface of the water. External cues were placed on each wall in the room and were kept constant for all experiments. The animals were given 6 trials per day for 5 consecutive days, with the platform always in the same (northwest) quadrant. In each trial, the mice were released from one of 4 randomly-selected starting points (north, east, south or west). Mice were released facing the tank wall and were given 60 seconds to

locate and climb onto the platform. If the mouse did not find the platform in that period, it was retrieved by the experimenter, placed on the platform, and left there for 15 seconds. Mice were tracked using with EthoVision XT software (Noldus Information Technology, France). Parameters that were measured were latency to platform, path length, time spent in the northwest quadrant, swim velocity, mean distance from the platform during the swim and the percentage of direct swims. For each day, the average for the six trials for each animal was calculated.

#### *Probe test*

To assess spatial memory, two hours following the final trial on the final day, a probe trials was performed. The platform was removed and the animals were released from the south or east positions. Parameters measured were the latency to the platform, frequency of crossings and duration over the platform, time spent in the northwest quadrant, mean distance from the platform and number of direct swims.

#### *Visual Platform test*

To ensure that any navigation deficit was not caused by a decreased ability to orientate and swim to the platform, on the final day, we performed two visible platform tests. The platform was moved to the south east quadrant and was raised above the surface with a highly visible object marking its position and with a light shining directly onto it. Parameters measured were distance moved, latency to platform and swim velocity.

### **Electrophysiological recordings and biocytin filling**

Sagittal cerebellar slices were prepared from LlrTMS-treated animals and sham-treated controls. Mice were sacrificed by rapid decapitation and the cerebellum removed. Sagittal vibratome slices (250  $\mu$ m) were cut from the cerebellar vermis at 4°C in an oxygenated external solution (125 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM Glucose). The slices closest to the midline were used for patch clamping, while another slice was fixed in 4% paraformaldehyde (PFA) for later immunostaining.

In some experiments, electrophysiological recordings in whole-cell current-clamp mode were made to observe the spontaneous action potential frequency.

In some cases, electrophysiological recordings of evoked climbing fibre currents were made from Purkinje cells in whole-cell patch-clamp configuration, in the same external solution, at room temperature. PCs were identified in lobules VI and VII, with an attempt to record from an equal number of cells at the external surface of the lobule and deeper on the bank of the lobule. Patch pipettes were filled with an internal solution containing 120 mM Cs-D-gluconate, 13 mM biocytin, 10 mM HEPES, 10 mM BAPTA, 3 mM TEACl, 2 mM Na<sub>2</sub>ATP, 2 mM MgATP, 0.2 mM NaGTP, pH 7.3, 290–300 mOsm. Climbing fibres were stimulated with a saline-filled glass pipette positioned near the PC soma.

To evaluate the state of the synaptic competition between multiple CF synapses, the amplitudes of individual CF-EPSCs in a multiply innervated PC were measured and numbered in the order of their amplitudes ( $A_1, A_2, \dots, A_N$ ,  $N \geq 2$ ,  $A_N$  represents the largest CF-EPSC). We then calculated the disparity ratio, which is given by  $(A_1/A_N + A_2/A_N + \dots + A_{N-1}/A_N)/(N - 1)$ ; ( $i = 1, 2, 3, \dots, N$ ;  $N \geq 2$ ), where  $A_i$  is the EPSC amplitude for the  $CF_i$ , and  $N$  is the number of CFs for a given PC.

### **Morphological analysis**

After filling several PCs, slices were fixed in 4% paraformaldehyde overnight at 4°C. To visualize the biocytin-filled cells, slices were washed in phosphate buffered saline (PBS) containing 0.25% Triton X-100 (PBS-T 0.25%). In order to reduce background staining, sections were incubated for 30 min in 70% methanol at room temperature. Sections were then washed in PBS-T 0.25%, and incubated for 2 hours at room temperature with Streptavidin AF488 conjugate (1:400; Invitrogen, Molecular Probes) diluted in PBS-containing 0.25% Triton X, 0.2% gelatine, and 0.1M lysine (PBS-TG). Following washes with PBS-T 0.25%, slices were mounted on slides with Mowiol (Sigma) mounting medium.

Image stacks of Purkinje cells were acquired using a confocal laser-scanning microscope (Leica TCS SP5, Leica), at 40x (NA=1.25) or 63x (NA=1.4) objective under oil. Images were acquired with an argon 488 laser in 570-620nm emission range, with a step size of 0.5  $\mu$ m. For analysis of PC morphology, the image stacks were flattened with the Z stack projection function of image J using the maximum intensity for each pixel, and the resulting image was used for analysis. The properties of the PCs measured were total area of the dendritic tree, height (measured from the soma along a straight line perpendicular to the edge of the

dendritic tree closest to the edge of the molecular layer), apical width, basal width, and width halfway between the basal and apical ends. The average width of the dendritic tree was calculated. The length and width of the primary dendrite was also measured, as well as the soma area. The image was also thresholded, and the fraction of the area containing any labelling was measured, allowing calculation of the total area occupied by dendritic branchlets. The level of thresholding was established for each cell, at a point where dendritic spines could still be seen but where as much background as possible was eliminated. Results were expressed as the average  $\pm$  the SEM for each group.

### **Spine density and morphology**

To analyse spines, two separate distal dendrites of one PC located in lobule VI and VII were imaged (3-5 PCs per animal) using a confocal laser-scanning microscope (Leica TCS SP5, Leica). Images were taken with an objective of 63x (NA= 1.4) under oil, with an optical zoom of 4 (final magnification = 252), and a pinhole of 1 airy unit, at a step size of 0.13mm. Images were deconvoluted using Huygens 3.7 software (Scientific Volume Imaging). Theoretical PSF was used and the Maximum Likelihood Estimation algorithm was performed in classical mode with 150 iterations. Background intensity was averaged from the voxels with lowest intensity, and signal to noise ratio values was set to 20. For each neuron, a tertiary distal dendritic segment of 20-40  $\mu\text{m}$  in length was measured. Dendritic spines were analysed with Neuronstudio software (Rodriguez et al., 2008); version 9.92; <http://research.mssm.edu/cnic/tools.html>). The dendrite was reconstructed and the spines were automatically detected and categorized as thin, stubby or mushroom spines. Manual correction was required for a minority of spines. The number of spines for each category and the total number for each cell was counted and expressed as the density of spines per 10 $\mu\text{m}$  of dendrite. The head diameter and spine length were automatically calculated by the software. Spine head diameter corresponds to the rayburst diameter (i.e. the minimal diameter of the ellipse describing the spine head, calculated in the xy axis). Data generated by Neuronstudio were saved in text file format, which was imported into graph pad prism to create frequency distribution of spine head diameter and length for each group. (Heck et al., 2012)

Developing PCs were reconstructed in the same manner but spines were not quantified as the developing dendrites have a mix of growth cones and transient spiny processes that are not necessarily related to synapses.

### **Sholl analysis**

To measure dendritic branching complexity, we performed Sholl method on the z-stacks of biocytin labelled PCs in lobules VI and VII. First, the NIH ImageJ plugin tubeness ( $\sigma$  value of 0.8; <http://rsb.info.nih.gov/ij/index.html>) was applied to the image stack to reinforce tubular structures (i.e., dendrites). Images were then converted to 8-bit grayscale and flattened. For each neuron, the NIH Image J plugin Sholl analysis (Ferreira et al., 2014) was used to count the number of intersections of dendrites with a series of concentric spheres at 10 $\mu$ m intervals from the centre of the cell body, with a starting radius of 10 $\mu$ m and the ending radius the length of the dendritic arbor.

### **Statistical analysis**

All data were tested for normal distribution and homogeneity of variance and square-root or  $\log^e$  transformation were applied if needed. When appropriate, two-way ANOVA and Bonferroni post hoc tests were used, with treatment and age as between-subject factors. If interactions between factors were observed one-way ANOVA and pairwise comparisons with Tukey post hoc tests were performed. When data did not fulfil the requirements for parametric tests, Kruskal-Wallis with Dunn-Bonferroni correction and Mann-Whitney U tests were used.

For the learning phase of Morris Water Maze two-way ANOVA repeated measures tests were done. For the rotarod task, Friedman non-parametric test followed by Wilcoxon ranked tests were performed. For spine morphology analysis Kolmogorov-Smirnoff distribution tests were employed. Statistical analyses were performed using GraphPad Prism version 7 and SPSS version 22 software.

## **RESULTS:**

In this study we used the mouse cerebellum to evaluate the effects of LI-rTMS on the central nervous system at different stages of life: during development (2-14 days), in adulthood (3-6 months) and then aged (15 months) mice. We examined synaptic refinement in early postnatal development, the PC dendritic tree morphology and cerebellar mediated behaviours following LI-rTMS.

### **Early postnatal PC dendritic development and synaptic maturation are not altered by LI-rTMS**

We began by investigating whether very young PCs undergoing rapid development would be particularly susceptible to LI-rTMS, and specifically whether normal dendritic and synaptic development would be perturbed by this treatment. We targeted stimulation to the mouse pup cerebellum; 10 minutes per day from P2 to P14, then studied PC morphology and synaptic development between P8 and P14.

Morphological analysis of the maturing dendritic trees from LI-rTMS-treated or sham-treated mice aged 8-14 days showed that the dendritic area increases over this period, as expected for this stage of development (Kruskal-Wallis and Dunn-Bonferroni pairwise comparisons: effect of age  $p=0.000$  for dendritic density, height and width; all ages different from each other  $p<0.05$  except P10 vs P12 ). Some differences were found between treated and sham groups for tree width and occupied area (Mann-Whitney tests; at P10 LI-rTMS > sham,  $p=0.019$ ; at P12 LI-rTMS < sham,  $p=0.032$ ), but these were not maintained at P14 (Fig 1). In addition, Sholl analysis showed no consistent alterations in branching complexity between groups (Mann-Whitney tests,  $p>0.05$  for most branchpoint distances at all ages) (Fig 2A). Thus dendritic development appears not to be affected by LI-rTMS treatment during this period.

We also examined the morphology of adult PC dendritic arbors from 3 animals stimulated during the P2-P14 period. The height and width of these arborisations were not different between LI-rTMS and sham-stimulated groups (t-tests,  $p>0.05$ )(data not shown).

In addition to dendritic morphological development, maturation of the climbing fibre (CF) extra-cerebellar afferents takes place during the first 2 postnatal weeks, the time of LI-rTMS

stimulation. To examine the effects of LI-rTMS stimulation on CF maturation we recorded CF synaptic currents between P8 and P14 in whole-cell patch clamped PCs from animals treated with LI-rTMS (or sham) from postnatal day 2.

Early in development, up to 5 CFs contact each PC; these CF synapses compete and progressively eliminate until only 1 CF per PC is left in the mature system. In these young mice, we found no perturbation of the CF synapse elimination process; the number of CFs/PC fell during the experimental period, globally in the same manner for the stimulated mice as for their sham-treated littermates (Mann-Whitney tests,  $p > 0.05$  except P12  $p=0.012$ ) (Fig 2A). In addition, we measured the disparity ratio during this period, which measures the progression of the competition between CF synapses, one CF becoming stronger and the others weaker (Hashimoto and Kano, 2003). Although the disparity ratio increases with age, as expected (2-way ANOVA, effect of age  $F_{3,102}= 4.152$   $p=0.008$ ), there were no differences between the LI-rTMS and sham-treated groups  $p>0.05$  (Fig 2C).

### **Mature Purkinje cell dendrites change little with age**

Before studying whether LI-rTMS altered PC dendritic morphology in adult and aged mice we wanted to see whether important changes occur during aging without treatment. We thus compared the morphology of biocytin-filled PC dendritic trees from mice aged 3-months ("adult") and 15-months ("aged"). We measured the height, width, and occupied area of the dendritic trees and found no differences between any of these measures between groups (1-way ANOVA, Bonferroni post-hoc tests: Height  $F_{1,57}=2.272$   $p=0.137$ ; Width  $F_{1,57}=1.271$   $p=0.264$ ; Area  $F_{1,57}=1.095$   $p=0.300$ ), nor in dendritic complexity following Sholl analysis (Mann Whitney U,  $p > 0.05$ ) (data not shown). We also measured the density of spines on the distal dendrites, including the subpopulations of stubby and thin spines. We found no differences in density between adult and aged groups (Student t-test,  $p>0.05$ ) (data not shown). Head diameter and spine length were measured for the thin/mushroom spines; the aged animals were found to have shorter spines (Kolmogorov-Smirnoff test for cumulative distribution,  $p=0.0335$ ) (data not shown). We conclude that the mature PC dendritic arbor does not change much during aging to 15 months; these observations served as a point of comparison for the subsequent analysis of stimulated and sham-treated animals.

### **Short-term treatment with LI-rTMS increases PC spine density and alters spine morphology**

To see early effects of LI-rTMS, we applied 3 daily sessions of stimulation to the cerebellum of adult and aged mice and compared dendritic tree morphology and spine density with sham-treated controls. We also recorded spontaneous action potential activity in PCs from treated and sham-treated adult mice.

Although no differences were found in the global dendritic tree height, width, area, or complexity (Kruskal-Wallis tests,  $p > 0.05$ ) (Fig 3) LI-rTMS treated PCs had increased dendritic spine density at both ages, with a more pronounced effect in the adult group than in the aged (One way ANOVA and Tukey post hoc pairwise comparisons:  $F_{1,83} = 13.451$   $p = 0.000$ ; adult: sham vs LI-rTMS,  $p = 0.000$ ; Aged: sham vs LI-rTMS  $p = 0.034$ ) (Fig 4A). When analysing spine subtypes, stubby-spines confirmed this increased density (1-way ANOVA and Tukey post hoc pairwise comparisons:  $F_{1,83} = 17.5$   $p = 0.000$ ; adult: sham vs. LI-rTMS,  $p = 0.000$ ; aged: sham vs LI-rTMS,  $p = 0.016$ ), while LI-rTMS significantly increased thin-spines only in adult animals with only a trend to increased thin-spine density in aged animals (1-way ANOVA and Tukey post hoc pairwise comparisons:  $F_{1,83} = 5.838$ ,  $p = 0.001$ ; adult: sham vs. LI-rTMS,  $p = 0.0446$ ; aged: sham vs. LI-rTMS,  $p = 0.0569$ ) (Fig 4 B and C). The increase in total spine density induced by LI-rTMS is thus primarily due to a greater increase in stubby-spines compared to thin spines (especially in the adult). Additionally, LI-rTMS treatment modified the morphology of thin/mushroom-spines, reducing their length (Kolmogorov-Smirnoff test;  $p < 0.0001$ ) (Fig 4F) and head diameter (Kolmogorov-Smirnoff test;  $p = 0.002$ ) (Fig 4E) in adult animals; whereas in aged animals only thin/mushroom-spine length was reduced (Kolmogorov-Smirnoff test;  $p = 0.0003$ ) (Fig 4F). These morphological changes suggest more newly-formed or retracting spines, which is consistent with a plasticity state (Lai and Ip, 2013).

To determine whether LI-rTMS treatment significantly altered PC activity, we made current-clamp recordings from PCs from adult LI-rTMS-treated, sham-treated, and noted the resting potential, the action potential threshold, and the action potential frequency. No differences for any of these measures were observed between the groups (data not shown).

We conclude that 3x10 minute sessions of LI-rTMS increases spine density and alters spine morphology of adult PCs, without overtly altering the basal electrophysiological state of the cell. These morphological effects also occur, but are less pronounced, in the aged animal.

### **Long-term treatment with LI-rTMS alters PC dendritic morphology**

These initial data show that only 3 short sessions of LI-rTMS modify PC spines, we next examined whether longer stimulation (4 weeks) induced greater morphological effects. Therefore, after 4 weeks of LI-rTMS or sham, we carried out morphological analysis of biocytin-filled PC dendritic trees and tested associated behaviours, in adult and aged mice.

Aged animals have greater dendritic density compared to adult animals (2-way ANOVA  $F_{1,109}=15.880$   $p=0.000$ ). No effect of treatment or age on PC dendritic height or width were observed (2-way ANOVA  $p>0.05$ ) (Fig 5 A and B) but LI-rTMS significantly increased PC dendritic density of adult mice compared to sham stimulation (1-way ANOVA pairwise comparison with Bonferroni *post hoc*;  $p=0.003$ ) (Fig 5C), although not in aged animals  $p>0.05$ . Sholl analysis showed that this was due to increased dendritic branching complexity in the middle section of the dendritic tree, in the adult LI-rTMS treated PCs (Kruskal-Wallis and pairwise comparisons, Dunn-Bonferroni correction,  $p<0.02$ ) (Fig5 D).

Analysis of total spine density showed a complex series of changes following LI-rTMS (2-way ANOVA and Bonferroni post-hoc tests: effect of age,  $F_{1,109}=7.981$ ,  $p=0.006$ ; interaction LI-rTMS x age  $F_{1,109}=3.645$ ,  $p=0.059$ ). In non-stimulated sham groups, adult and aged animals had similar spine density overall, in keeping with our baseline naive animals (above). However, while LI-rTMS did not change spine density in adult animals, there was a non-significant reduction of total spine density in the aged group ( $p=0.06$ ) so that aged LI-rTMS dendrites had a lower spine density than adult LI-rTMS-treated dendrites ( $p=0.008$ ) (Fig 6A); a change most evident for thin/mushroom-spines ( $p=0.019$ ) (Fig 6C). In addition, LI-rTMS treatment decreased the head diameter of thin/mushroom-spines (Kolmogorov-Smirnoff tests) in both adult ( $p=0.0007$ ) and aged mice ( $p<0.0001$ ) (Fig 6D).

We conclude that LI-rTMS continues to modulate PCs, but to a lesser extent in aged vs adult cells. LI-rTMS modifies the PC dendritic arbor; increasing branch complexity in adult, but not in aged, animals. It also shows persisting effects on spine plasticity by decreasing head diameter of thin-spines in both ages.

### **Handling and behavioural testing alone did not change dendritic morphology but did alter spine density**

Given that these long-term treated animals had undergone daily handling prior to (habituation), during and after (behavioural testing; see below) the 4 week LI-rTMS protocol, it was necessary to verify that the stimulation provided by handling and behaviour testing alone did not alter PC dendritic morphology. We thus compared sham-treated animals from the short-term (3-day) treatment group with the long-term (4 week) sham-treated animals. Analysis of dendritic tree height, width, dendritic density, and branching complexity showed no differences between the two sham-treated groups for either adult or aged mice (Kruskal-Wallis tests,  $p > 0.05$ ) (data not shown).

However prolonged handling and behavioural testing did modify the dendritic spines: decreasing the density of stubby-spines (Two way ANOVA with Bonferroni *post hoc*,  $F_{1,94}$  19.303  $p = 0.000$ ; Fig 7A) and increasing the density of thin-spines ( $F_{1,94}$  9.302  $p = 0.003$ ; Fig 7B), consistent with spine morphology changes associated with learning and memory. Interaction between handling and age ( $F_{1,94}$  3.864  $p = 0.052$ ) revealed that only adult animals demonstrated decreased stubby-spine density (adult  $p = 0.000$ , aged  $p > 0.05$ ; pairwise comparison with Tukey *post hoc*; Fig 7A) and thin/mushroom-spine head diameter (Kolmogorov-Smirnoff tests; adult  $p < 0.0001$ , aged  $p > 0.05$ ; Fig 7C), while thin/mushroom-spine length increased in both adult ( $p = 0.0007$ ) and aged ( $p < 0.0001$ ) PCs (Fig 7D). Consistent with the effects of LI-rTMS, morphology of spines seems to be more plastic in adult than aged mice.

### **Chronic LI-rTMS treatment does not alter motor function, but improves spatial memory**

We have shown that PC dendritic morphology is altered by long-term LI-rTMS treatment (and that spines are altered within a few days' treatment), and that adult animals responded more than aged animals. We next tested whether these morphological changes were accompanied by detectable alterations of associated behavioural functions. We carried out a series of tests of activity and strength, and then motor coordination and spatial learning and memory, to which PC function contributes.

In an open field, aged animals moved less than adults (2 way ANOVA;  $F_{1,45} = 19.661$   $p = 0.000$ ) but there were no effects of LI-rTMS treatment. Similarly, aged mice had lower muscle

strength that adults ( $F_{1,39}=11.817$ ,  $p=0.001$ ), but LI-rTMS treated animals did not differ from shams (data not shown). Gait analysis showed no difference between groups (data not shown), and the accelerating rotarod test showed that all groups learned the task during the 5-day test period (Friedman nonparametric RMANOVA and Wilcoxon ranked test,  $P=0.000$ ; Fig 8), but there were no consistent differences between LI-rTMS and sham treated groups. The aged mice, whether treated or sham, generally fell from the rotarod sooner than adult mice (2-way ANOVA, on day 1  $F_{1,34}=12.575$ ,  $p=0.001$ ; day 2  $F_{1,34}=17.937$ ,  $p=0.001$ ; day 3  $F_{1,34}=6.225$ ,  $p=0.018$  and day 5  $F_{1,34}=6.808$ ,  $p=0.013$ ; Fig 8).

Spatial learning and memory was tested with the Morris Water Maze. To ensure the animals had the ability to orientate themselves and swim to a platform, at the end of the learning and memory experiments we conducted two trials where the platform was visible to the animal. There were no significant differences between any groups for swim speed, distance travelled or the latency to the platform (Kruskal-Wallis test,  $p>0.05$ ) (data not shown).

During the learning phase all groups improved their performance (Repeated measures ANOVA), as measured by the reduced distance travelled ( $p<0.03$  between subsequent days; Fig 9B), latency to reach the platform ( $p<0.001$  between subsequent days) and average distance from the platform ( $p<0.02$  between subsequent days; Fig 9C), as well as greater time spent in the correct quadrant ( $p<0.02$  between subsequent days; Fig 9A), although aged animals swam more slowly ( $F_{1,44}=4.746$ ,  $p=0.035$ ). In addition, an interaction was found between treatment and age, revealing different effects of LI-rTMS on adult versus aged animals for several measures: time spent in the correct quadrant on day 3 ( $F_{1,44}=6.991$ ,  $p=0.011$ ) and day 5 ( $F_{1,44}=4.176$ ,  $p=0.047$ ); and escape latency on day 5 ( $F_{1,44}=5.549$ ,  $p=0.023$ ) suggesting that LI-rTMS tended to improve learning in adult compared to aged animals (Fig 9).

The probe test for spatial memory, which followed the learning phase, showed that adult mice treated with LI-rTMS had improved memory of the platform's location (1-way ANOVA and Tukey post-hoc pairwise comparisons). LI-rTMS treatment increased the number of times the adult animals crossed the platform location ( $p=0.0166$ ; Fig 10A), and entered the NW quadrant ( $p=0.02$ ; Fig 10B). In contrast, none of these measures of spatial memory were

different in the aged LI-rTMS group compared to their sham controls. We conclude that only the adult mice responded to LI-rTMS treatment with improved spatial memory, much as the aged mice showed fewer PC morphological changes in response to LI-rTMS.

## **DISCUSSION :**

We have investigated the effects of LI-rTMS on cerebellar Purkinje cells at different stages of development and during aging to better describe the specific effects *in vivo* of this low intensity stimulation and to see if whether some ages are more susceptible than others to these effects. An understanding of these differences will be particularly important clinically to adapt specific patterns of treatment according to the age of the individual.

### *Early postnatal PC morphological and synaptic development is not disrupted*

We first studied whether PCs during a period of intense morphological and synaptic development would be adversely affected by LI-rTMS. We found no evidence for altered refinement of the climbing fibre synapses, which compete for PC targets in an activity related manner (Hashimoto and Kano, 2003, 2013; Lohof et al., 1996) during this period. We did see some variability in morphological development but these changes were temporary, lasting only a few days and disappearing by P14. This is important for potential clinical applications because it suggests that the developing cerebellum, and perhaps the whole nervous system, may not undergo gross perturbations as a result of LI-rTMS treatment and that more subtle therapeutic changes, if they occur, will not be disrupted. One possible way to evaluate this would have been by behavioural assessment. While it is unlikely that the motor tests for immature cerebellar function, which are essentially reflex responses (righting reflex, cliff avoidance and negative geotaxis) would be sufficiently sensitive, measuring changes in ultrasonic vocalization may have revealed some differences. Although vocalization is not strictly a cerebellar task, speech is perturbed in cerebellar dysfunction (Stoodley et al., 2016) and ultrasonic vocalizations are abnormal in FoxP2 knockout mice in association with cerebellar abnormalities and ataxia (Shu et al., 2005).

However temporary, the alterations to PC dendritic morphology and number of CFs by LI-rTMS reinforce the need for caution that has been advocated for clinical rTMS in children

(Davis and van Koningsbruggen, 2013); especially since sub-threshold stimulation would occur in brain regions adjacent to the structures targeted by high-intensity rTMS. Further systematic evaluation using a variety of different stimulation parameters in different regions of the brain would be important to determine whether LI-rTMS can nonetheless have unwanted effects on some immature brain structures. Conversely, activity and neuroplasticity during the developmental critical period is essential for the establishment and refinement of neural circuits and their function (Ganguly and Poo 2013), being extensively demonstrated in both the visual system (Anderson et al., 2011; Espinosa and Stryker, 2012) and corticospinal tract (CST) (Anderson et al., 2011; Martin, 2005). As both rTMS and LI-rTMS modulate cortical network activity (Di Lazzaro et al., 2013; Pell et al., 2011), their application to promote adaptive neural plasticity in young patients during critical periods of development may allow better recovery from specific brain lesions (Dennis, 2010; Anderson et al., 2011). However it is important to note that maladaptive plasticity can also take place leading to devastating effects of some early brain injuries (Kolb et al., 2000). Our data suggest that normal developing neural circuits are barely modified by our stimulation program, which may reflect phenomena of the cerebellum or immature neurons, or just the short stimulation time (11d to P12). However this result is consistent with our previous work indicating that abnormal neural circuits can be induced to undergo considerable remodelling while normal ones appear unchanged (Makowiecki et al., 2014; Rodger et al., 2012; Sykes et al., 2013). Clearly, further investigation is required, particularly studying abnormal neural circuits, before (LI-)rTMS parameters for functional recovery may be available as therapeutic application for young patients with neurodevelopmental pathologies such as autism spectrum disorders or cerebral palsy.

#### *Mature PCs change little during aging but respond differently to acute LI-rTMS treatment*

There were few morphological differences between PCs from non-treated (naive) animals in adult compared to aged groups, but these two populations responded differently to 3 days of LI-rTMS treatment.

In adult animals this short treatment increased spine density, particularly of stubby-spines, consistent with a state of morphological and possibly synaptic plasticity (Knott et al., 2006; Lai and Ip, 2013). The appearance of new spines continues in the adult brain (Holtmaat and

Svoboda, 2009; Kolb et al., 2008; Lai and Ip, 2013) underlying memory formation (Fu et al., 2012; Segal, 2005) and adaptive brain changes to environmental input (Hofer et al., 2009; Holtmaat et al., 2006). The current study suggests that sub-threshold LI-rTMS can also induce morphological synaptic plasticity without necessarily directly eliciting action potentials in the post-synaptic neuron. Further investigation on the molecular and cellular mechanisms underlying LI-rTMS-induced new spine formation could identify stimulation parameters, which optimally activate those mechanisms. This would be of particular interest since the impairment of activity-dependent spine morphogenesis underlies many neurological disorders (Lai and Ip, 2013; Penzes et al., 2011).

These acute effects of the LI-rTMS on spine structural plasticity were present in both adult and aged animals but were more pronounced in the adult group, suggesting a lower level of synaptic structural plasticity induced by LI-rTMS with age. Poorer acute responses to rTMS of aged people have been previously demonstrated (Huang et al., 2008; Rossi et al., 2004; Tatti et al., 2016; Todd et al., 2010; Zimmerman and Hummel, 2010) and this might be partly attributed to the need to recruit more cortical areas to accomplish the same task (Cabeza et al., 1997; Rossi et al., 2004; Stebbins et al., 2002). However our data may also indicate that aged neurons themselves are less responsive, as indeed is known electrophysiologically (Branch et al., 2014; Hara et al., 2012; Matthews et al., 2009) and consistent with reduced neuronal plasticity during ageing (Freitas et al., 2011b; Pascual-Leone et al., 2011; Zimmerman and Hummel, 2010).

Thus, in summary, these results suggest that even 3x10min cerebellar LI-rTMS is able to increase the number of synaptic inputs onto PCs (Arellano et al., 2007; Hering and Sheng, 2001; Lai and Ip, 2013) reshaping connectivity within cerebellar neuronal circuits and therefore potentially modifying cerebellar function.

#### *Chronic LI-rTMS changes both PC dendritic morphology and spatial memory*

Handling alone, as associated with sham treatment and behavioural testing, did not alter dendritic morphology, although spine density and shape were affected, particularly in adult animals. In contrast, 4 weeks of daily LI-rTMS treatment increased PCs dendritic density/complexity, only in adult animals and not in aged animals. It is tempting to

speculate that the rapid increase in spine density after 3 days of treatment becomes consolidated as increased dendritic complexity to accommodate new synapses.

Increases in spine density and PC dendritic tree size and branching complexity induced by LI-rTMS are consistent with morphological PC changes observed following periods of motor learning or environmental enrichment in rats (De Bartolo et al., 2015; González-Burgos et al., 2011; Lee et al., 2007, 2013) which modulate synaptic efficacy (Lee et al., 2005). Indeed, dendritic branching complexity influences the efficacy of action potential propagation along dendrites (Vetter et al., 2001) and thus impacts upon integration of synaptic input and induction of synaptic plasticity such as LTP and LTD (Johnston et al., 1996; Linden, 1999).

In addition, spatial memory was improved in the LI-rTMS-treated adult animals, and there was a tendency for improved learning as well, providing a correlation between morphological and behavioural effects but these effects were not seen in the aged animals. Although our stimulation targeted the cerebellum, and the cerebellum regulates the egocentric component of spatial learning and memory (Lefort et al., 2015; Rochefort et al., 2011, 2013), it is possible that stimulation of the hippocampus and parietal cortex, which are also involved in spatial behaviour, may have contributed to improved spatial memory performance. According to modelling of stimulation intensity, our 10mT cerebellar stimulation would deliver a magnetic field of 0.5mT in the hippocampus, the effects of which are unknown. However, the interpretation that the LI-rTMS-induced changes to the PC dendritic tree and spines could explain the improvement in spatial memory is supported by the data from the aged animals. In this group LI-rTMS did not induce either structural PC change or improved spatial learning and memory, although the hippocampus will have been equally exposed to the stimulation.

Taken together, these data show that long-term LI-rTMS modifies the structure, and therefore probably the function, of neural circuits, as illustrated in this study by increases to the number of synaptic inputs to PCs. Further electrophysiological analyses will be required to identify whether this is balanced (i.e. equal increase in excitatory and inhibitory input), or whether the PC function is facilitated.

## Conclusions

These studies show that LI-rTMS, which induces an electric field too weak to directly elicit action potentials in the underlying neurons, nonetheless changes spine density after only 30 (3x10) minutes stimulation and alters dendritic complexity with longer treatment: i.e. it alters neural circuit structure. Moreover, these changes were paralleled by improved spatial learning and memory, behaviours requiring normal PC function. Importantly, the effects of LI-rTMS were weaker in the aged and, unexpectedly, very young animals; therefore revealing the scale of factors needing consideration when planning therapeutic treatments such as non-invasive brain stimulation.

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## Figure legends

**Figure 1: LI-rTMS during development induces slight temporary changes of PC morphological maturation that are not maintained at P14.**

**A.** PC dendritic density increased consistently in all animals between P8 and P14 (Kruskal-Wallis and Dunn-Bonferroni pairwise comparison  $p=0.000$ ;  $n=4-5$  and  $N=16-24$ ) except between P10 and P12 ( $p>0.05$ ). PC dendritic density from LI-rTMS stimulated pups is bigger at P10 and smaller at P12 compared to sham-stimulated age-matched controls (Mann-Whitney tests;  $p=0.019$  and  $p=0.032$  respectively) but no differences between groups were observed at P14.  $n$ = number of animals;  $N$ = number of cells.)

**B and C.** No differences were observed between sham and LI-rTMS stimulated mice for PC dendritic height (B) and width (C) (Mann-Whitney tests; all  $p\leq 0.05$ ). PC dendritic height and width increased consistently in all animals between P8 and P14 (Kruskal-Wallis and Dunn-Bonferroni pairwise comparison  $p=0.000$ ), except between P10 and P12 ( $p>0.05$ ).

\* $p\leq 0.05$ ; \*\*\* $p\leq 0.001$

**Figure 2: LI-rTMS during development does not alter PC dendritic complexity or climbing fibre - Purkinje cell synaptic maturation.**

**A.** No differences between groups were observed in the number of branches as a function of distance from the soma between P8-P14 (Sholl analysis; Kruskal-Wallis tests, all  $p>0.05$ ;  $n=4-5$  and  $N=16-24$ ).

**B.** The number of climbing fibre synapses per Purkinje cell decreased between P8 and P14, globally in the same manner for the LI-rTMS and sham stimulated mice littermates (Mann-Whitney tests,  $p> 0.05$  except P12  $p=0.012$ ;  $n=4-5$  and  $N=25-30$ ).

**C.** The disparity ratio increases with age (2-way ANOVA,  $p=0.008$ ) but there were no differences between the LI-rTMS and sham-treated groups ( $p>0.05$ ).

\* $p\leq 0.05$ , \*\* $p\leq 0.001$

**Figure 3: Acute LI-rTMS (3 days) does not alter PC dendritic size or complexity in adult or aged mice.**

**A,B,C.** No differences in dendritic height (A), width (B) or occupied area (C) were observed in the different groups (Kruskal-Wallis tests, all  $p > 0.05$ ;  $n=5$ ;  $N=12-14$ )

**D.** No differences between groups were observed in the Sholl analysis quantifying the number of branch points as a function of distance from the soma (Kruskal-Wallis tests, all  $p > 0.05$ ).

**Figure 4: Acute LI-rTMS (3 days) increases dendritic spine density and modifies spine morphology in both adult and aged mice.**

**A.** Acute LI-rTMS increases total spine density in both adult  $p=0.000$  and aged mice  $p=0.034$ . Aged animals have significantly fewer spines than adult animals (Two way ANOVA  $F_{1,80}=11.057$   $p=0.001$ ;  $n=5$ ;  $N=19-24$ ).  $n$ = number of animals;  $N$ = number of terminal dendrites analysed. \* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ;

**B.** Acute LI-rTMS increase stubby spine density in both adult  $p=0.000$  and aged mice  $p=0.016$ . Aged animals have significantly fewer stubby spines than adult animals (Two way ANOVA  $F_{1,80}=8.316$   $p=0.005$ ).

**C.** Acute LI-rTMS increases thin/mushroom spine density in adult ( $p=0.0446$ ) but not in aged mice ( $p > 0.05$ ). Aged animals have significantly fewer thin/mushroom spines than adult animal (Two way ANOVA  $F_{1,80}=7.187$   $p=0.009$ ).

**D.** Example of PCs terminal dendrites, showing the increased number of dendritic spines induced by LI-rTMS in adult and aged mice (right) compared to sham stimulation in adult and aged mice (left).

**E.** Adult mice stimulated with acute LI-rTMS have smaller thin/mushroom spine head diameter than age-matched sham controls (Kolmogorov-Smirnoff test;  $p=0.002$ ) but no difference due to the stimulation were observed in aged animals ( $p > 0.05$ ).  $n=5$ ;  $N=1575-1896$ .  $n$ = number of animals;  $N$ =number of spines measured.

**F.** Both adult and aged mice stimulated with acute LI-rTMS have shorter thin/mushroom spines than their age-matching sham controls (Kolmogorov-Smirnoff test;  $p \leq 0.0001$  and  $p=0.003$  respectively).

\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$ ; \*\*\*\* $p \leq 0.0001$

**Figure 5: Long term LI-rTMS (4 weeks) increases dendritic density and complexity only in adult mice.**

**A and B.** No difference in dendritic height (A) and width (B) were observed in the different groups (Two way ANOVA; all  $p > 0.05$ ; adult sham  $n=16$ , adult LI-rTMS  $n=15$ , aged sham  $n=9$ , aged LI-rTMS  $n=10$ ; adult sham  $N=50$ , adult LI-rTMS  $N=33$ , aged sham  $N=27$ , aged LI-rTMS  $N=22$ )  $n$ = number of animals;  $N$ = number of cells.

**C.** Aged mice have greater dendritic density compared to adult mice (2-way ANOVA  $p=0.000$ ). Long-term LI-rTMS (4 weeks) increases dendritic density in adult (1-way ANOVA pairwise comparison with Bonferroni *post hoc*;  $p=0.003$ ), but not in aged animals  $p > 0.05$ .

**D.** Example of PCs showing the increased dendritic density induced by LI-rTMS in adult mice (right) compared to sham stimulation in adult mice (left).

**E.** Sholl analysis, quantifying the number of branching as function of distance from the soma, shows that long-term LI-rTMS (4 weeks) increases PC dendritic complexity in the middle section (150-190 $\mu$ m from the soma) in adult animals (Kruskal-Wallis and pairwise comparisons, Dunn-Bonferroni correction, all  $p \leq 0.02$ ).

\* $p \leq 0.05$ ; \*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Figure 6: Long term LI-rTMS (4 weeks) tends to reduce spines density in aged animals and modifies spine morphology in both adult and aged animals.**

**A.** Long term LI-rTMS tends to decrease total spine density only in aged mice (1-way ANOVA pairwise comparison with Bonferroni *post hoc*;  $p=0.06$ ;  $N=19-41$ ), and this non-significant tendency is not present in adult mice  $p > 0.05$ . This differential effect of LI-rTMS depending on the age results in lower total spine density in aged mice stimulated with LI-rTMS compared to adult animals stimulated with LI-rTMS (1-way ANOVA pairwise comparison with Bonferroni *post hoc*;  $p=0.08$ ).

**B.** No difference between groups was observed for stubby spine density (2-way ANOVA  $p > 0.05$ ).

**C.** Lower density of thin/mushroom spines in aged mice stimulated by LI-rTMS compared to adult animals stimulated by LI-rTMS (1-way ANOVA pairwise comparison with Bonferroni *post hoc*;  $p=0.019$ ).

**D.** LI-rTMS treatment decreases head diameter of thin/mushroom spines in both adult and aged mice (Kolmogorov-Smirnoff tests;  $p=0.0007$  and  $p\leq 0.0001$  respectively; adult sham  $N=3084$ , adult LI-rTMS  $N=3045$ ; aged sham  $N=915$ ; aged LI-rTMS  $N=707$ ). Adult sham stimulated animals have smaller thin/mushroom spine head diameter than adult sham stimulated mice,  $p=0.0000$ .  $N$ = number of spines measured.

\* $p\leq 0.05$ ; \*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$ ; \*\*\*\* $p\leq 0.0001$

**Figure 7: Handling and behavioural testing alters dendritic spine density and morphology**

**A.** Handling decreases density of stubby spines in adult mice (1-way ANOVA pairwise comparison with Bonferroni *post hoc*;  $p=0.000$ ) but not in aged mice  $p>0.05$ .

**B.** Handling increases density of stubby spines (2-way ANOVA;  $p=0.003$ ).

**C.** Handling decreases the head diameter of thin/mushroom spines in adult mice (Kolmogorov-Smirnoff tests;  $p\leq 0.0001$ ) but not in aged mice  $p>0.05$ .

**D.** Handling increases the length of thin/mushroom spines in both adult and aged mice (Kolmogorov-Smirnoff tests;  $p=0.0007$  and  $p\leq 0.0001$  respectively).

\*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$ ; \*\*\*\* $p\leq 0.0001$

**Figure 8 : LI-rTMS does not alter motor coordination or learning.**

**A.** All groups learned the accelerating rotarod task during a 5-day testing period (Friedman non-parametric RMANOVA and Wilcoxon ranked test,  $p=0.000$ ; adult sham  $n=16$ , adult LI-rTMS  $n=15$ , aged sham  $n=9$ , aged LI-rTMS  $n=10$ ).

**B.** No differences were found in the accelerating rotarod task between LI-rTMS and sham treated groups (2-way ANOVA; all  $p>0.05$ ). The aged mice, whether treated or sham, generally fell from the rotarod sooner than adult mice (2-way ANOVA, on day 1  $p=0.001$ ; day 2  $p=0.001$ ; day 3  $p=0.018$  and day 5  $p=0.013$ ).

\* $p\leq 0.05$ ; \*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$

**Figure 9: LI-rTMS does not significantly alter spatial learning.**

**A,B,C.** In the learning phase of the Morris Water Maze (MWM) all groups improved their performance as measured by greater time spent in the correct quadrant (A), reduced distance travelled (B), and reduced average distance from the platform (C). (Repeated measures ANOVA; all  $p\leq 0.03$  between subsequent days). \*\*\* $p\leq 0.001$

**Figure 10: LI-rTMS improves spatial memory in adult but not in aged mice.**

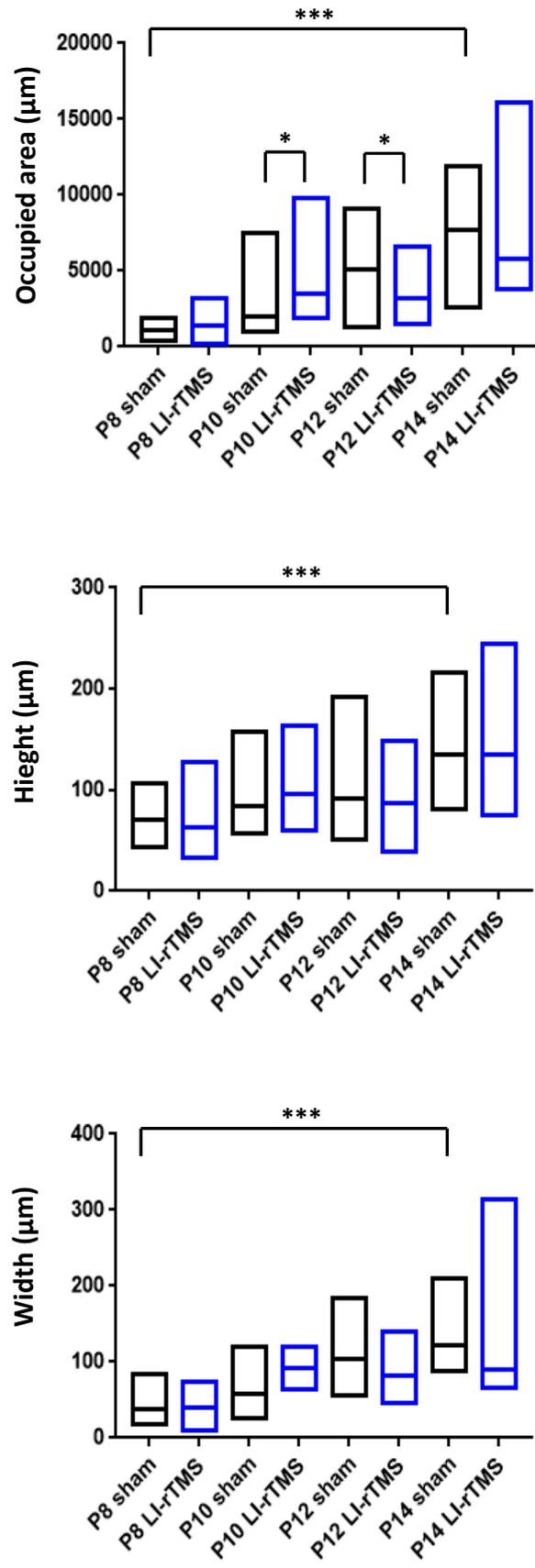
**A and B:** In the probe test of the MWM, LI-rTMS treatment increased the number of times the adult animals crossed the platform location (A), and entered the platform's quadrant (B) (1-way ANOVA and Tukey post-hoc pairwise comparisons  $p=0.0166$  and  $p=0.02$  respectively). This effect was not seen in the aged animals ( $p>0.05$ ).

**C:** In the probe test of the MWM, LI-rTMS treatment tend to increase the time that adult animals spend over the platform (1-way ANOVA and Tukey post-hoc pairwise comparisons  $p=0.0626$ ) although not significantly.

**D:** Heat map representing the merged localisation of mice during the probe tests of the Morris Water Maze. Adult animals treated with LI-rTMS spent more time over the platform location (white circle) than adult sham treated animals, as indicated by the red colour. This effect was not observed in aged mice.

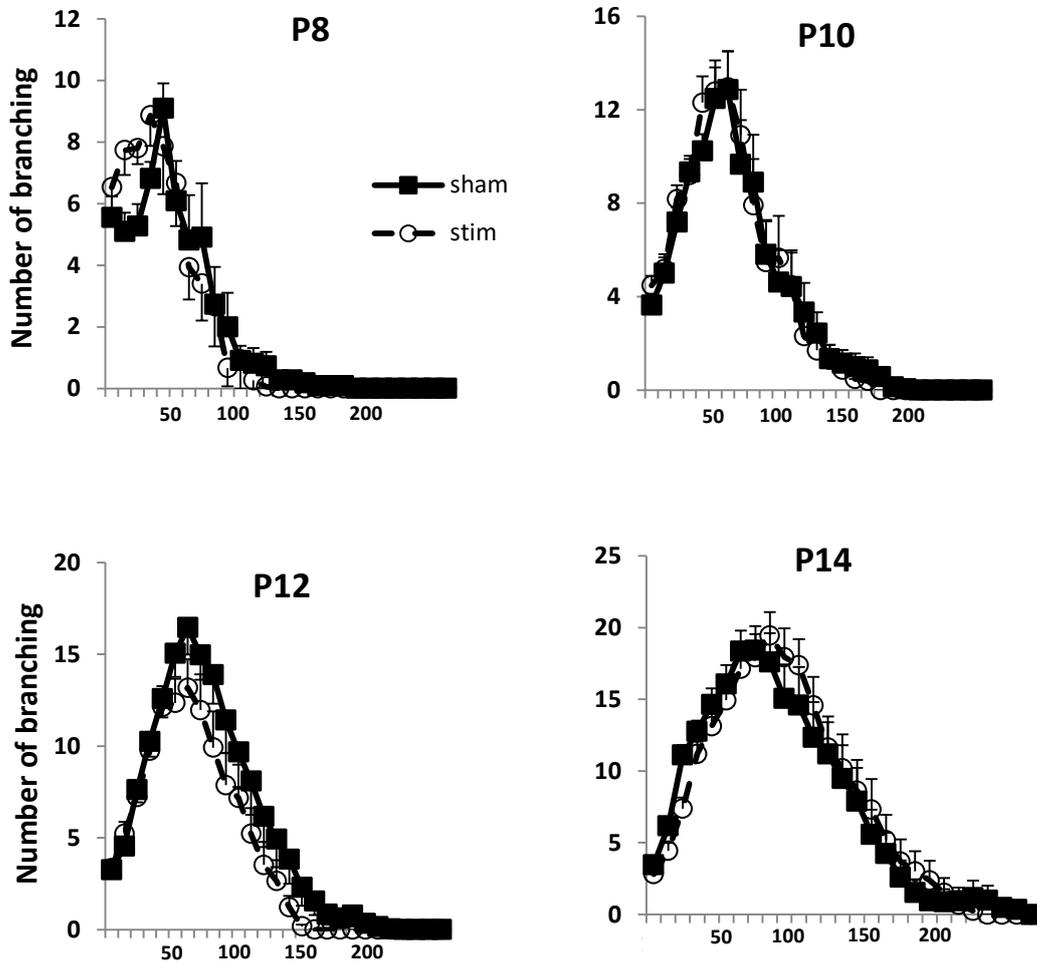
\* $p\leq 0.05$ ; \*\* $p\leq 0.01$

Figure 1

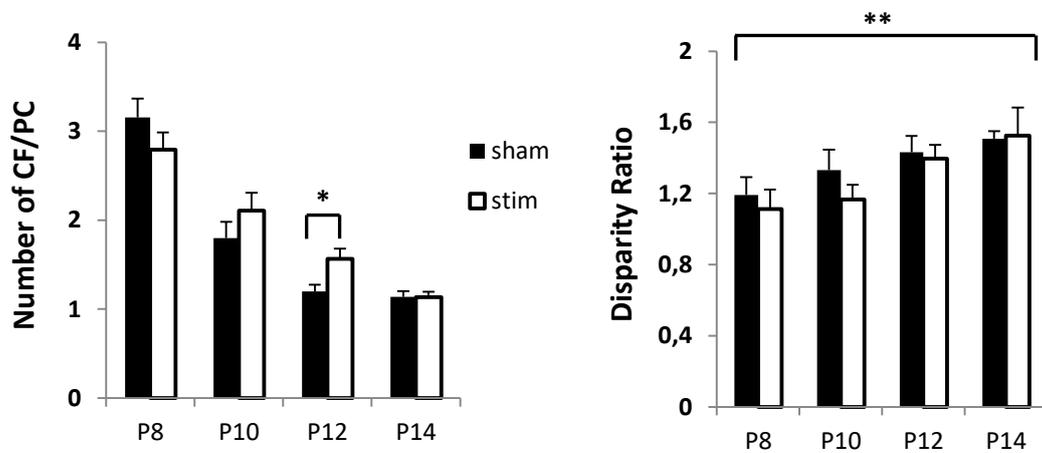


**Figure 2**

**A**



**B**



**Figure 3**

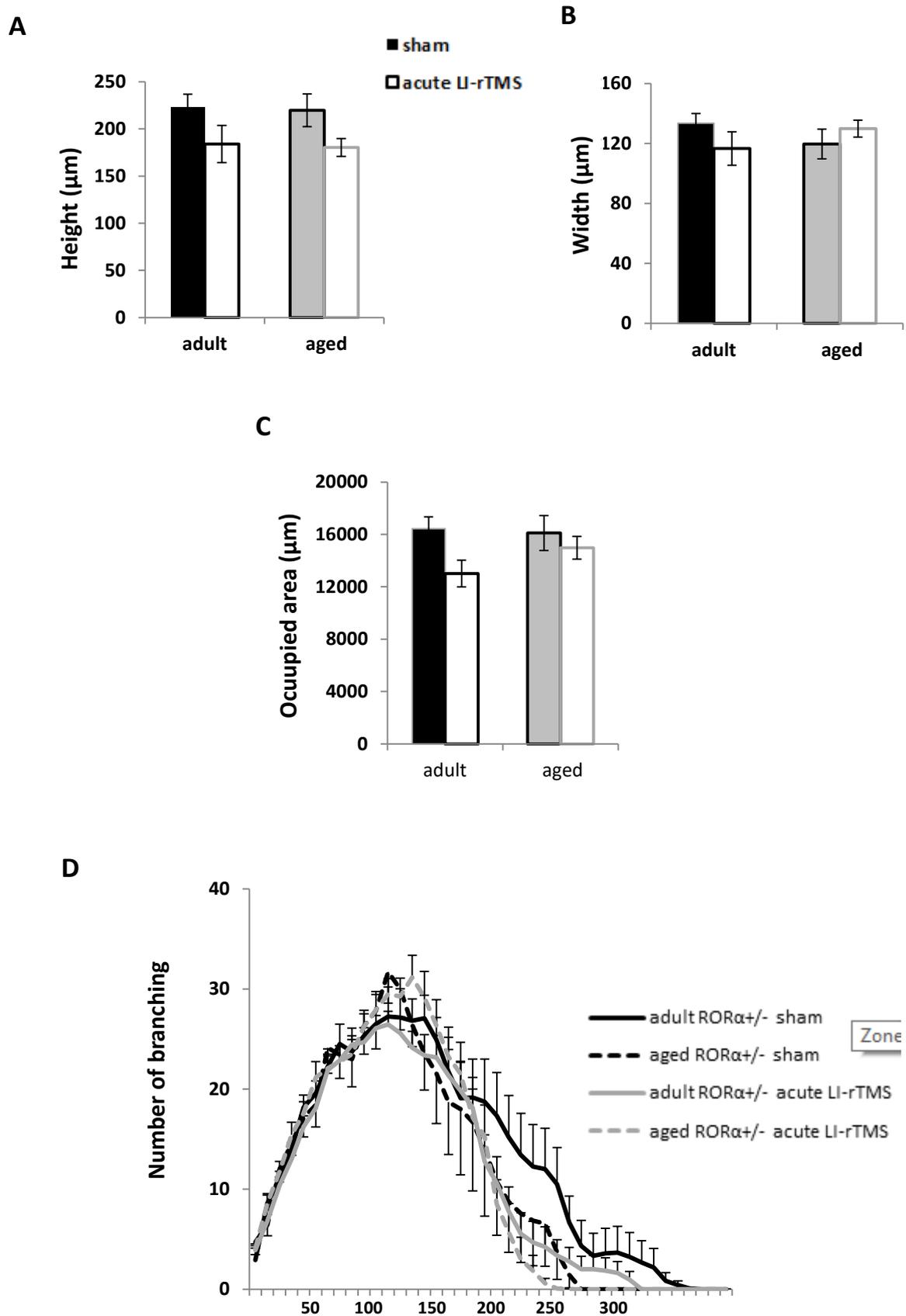


Figure 4

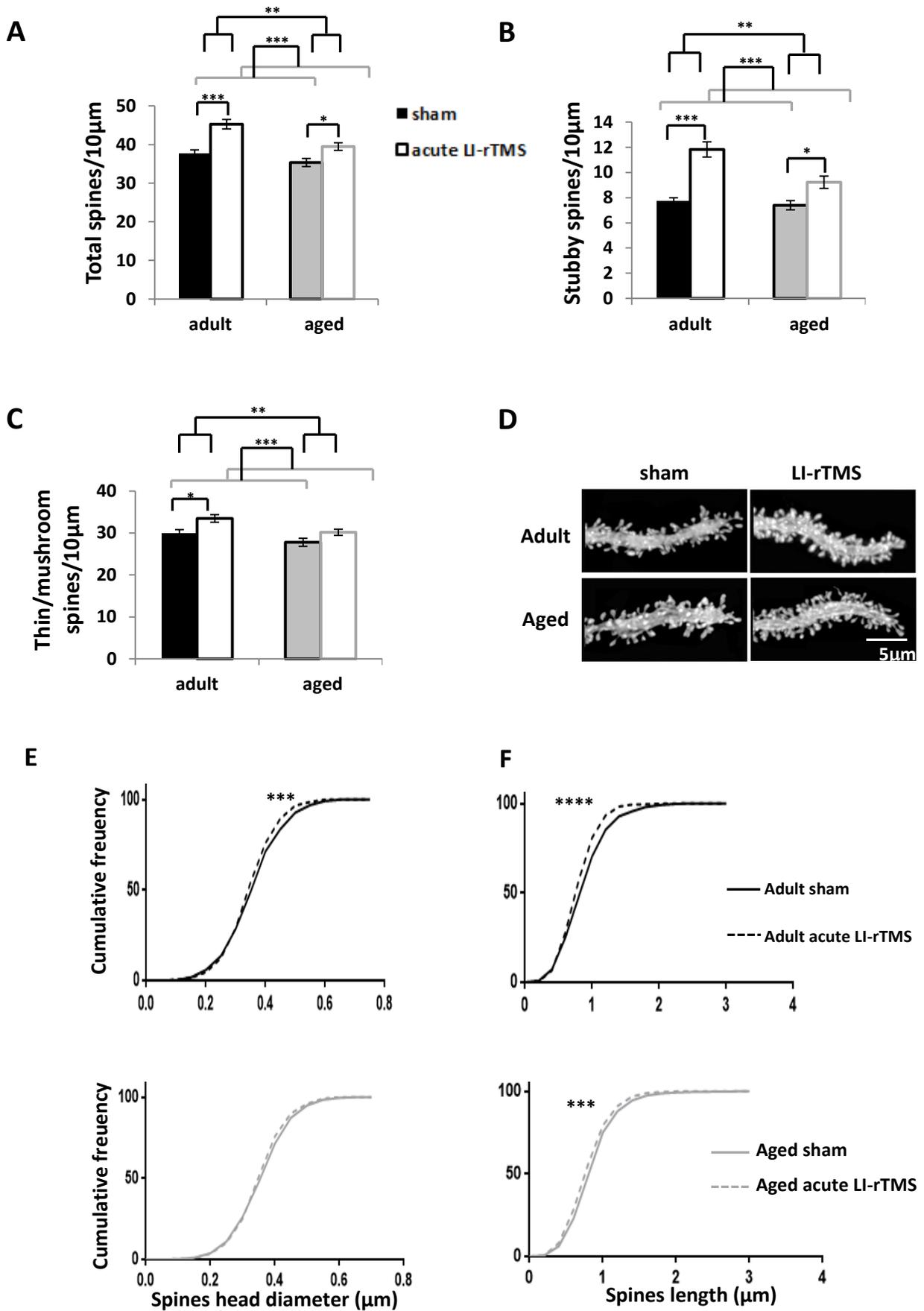


Figure 5

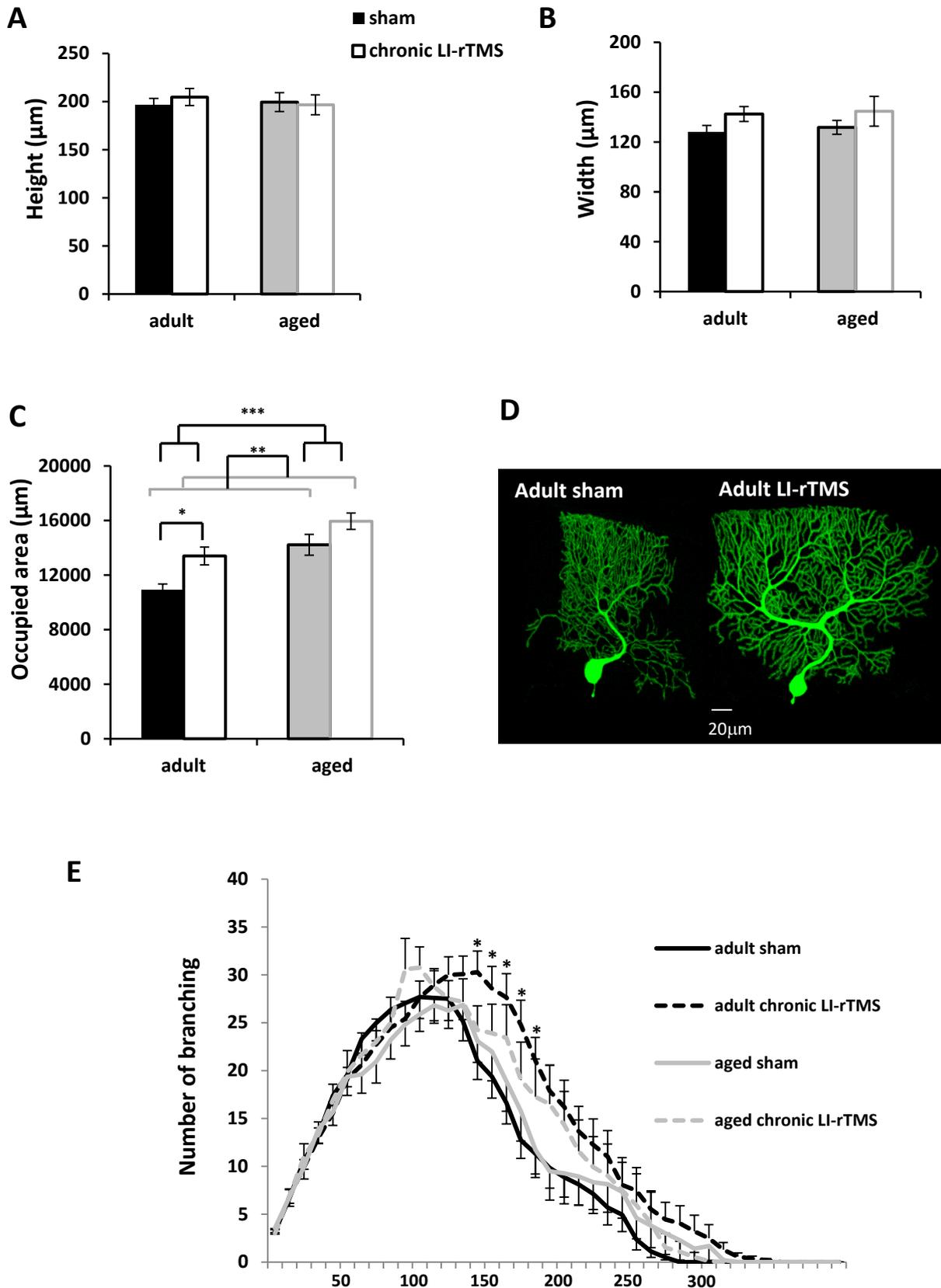


Figure 6

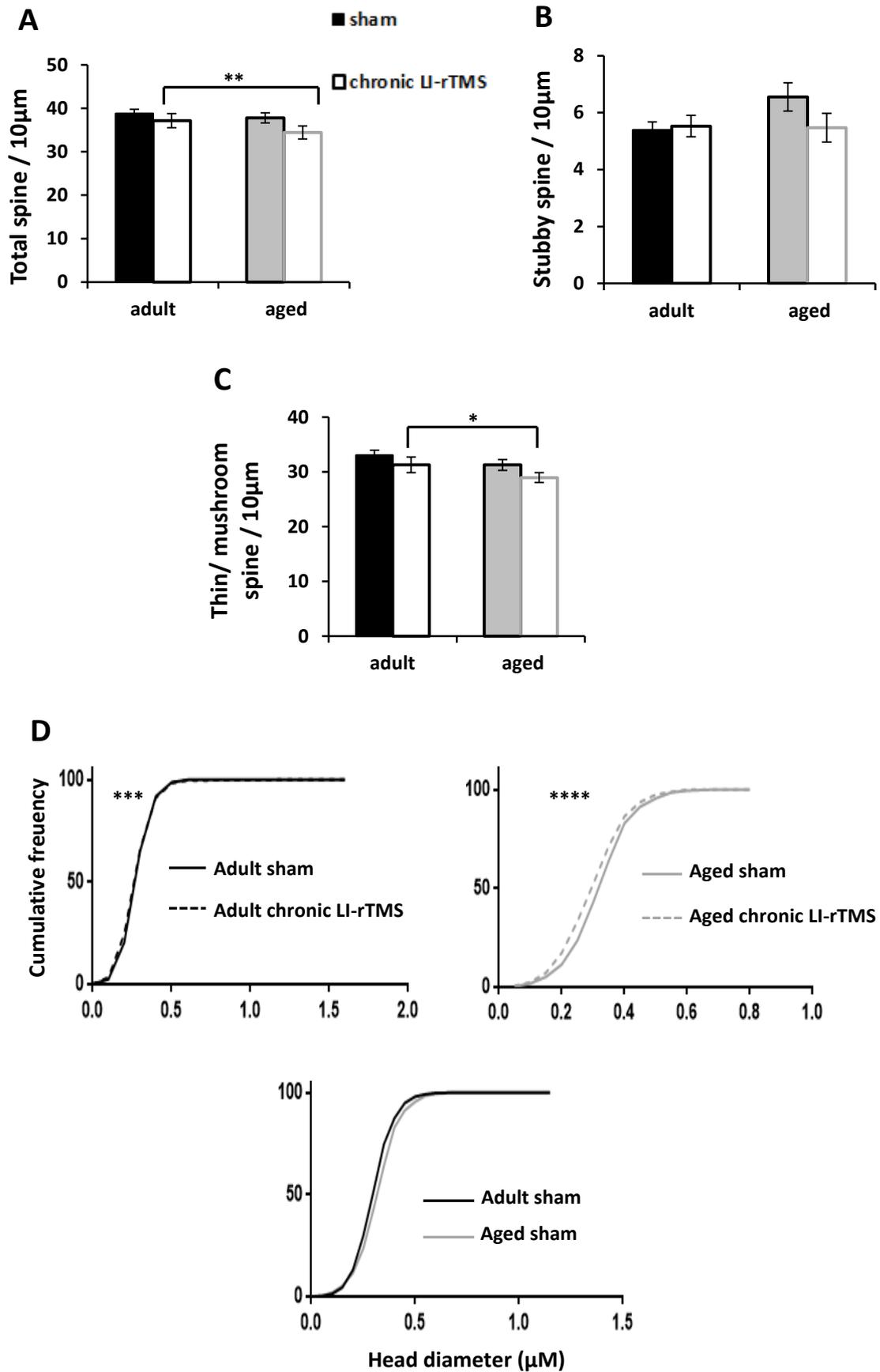


Figure 7

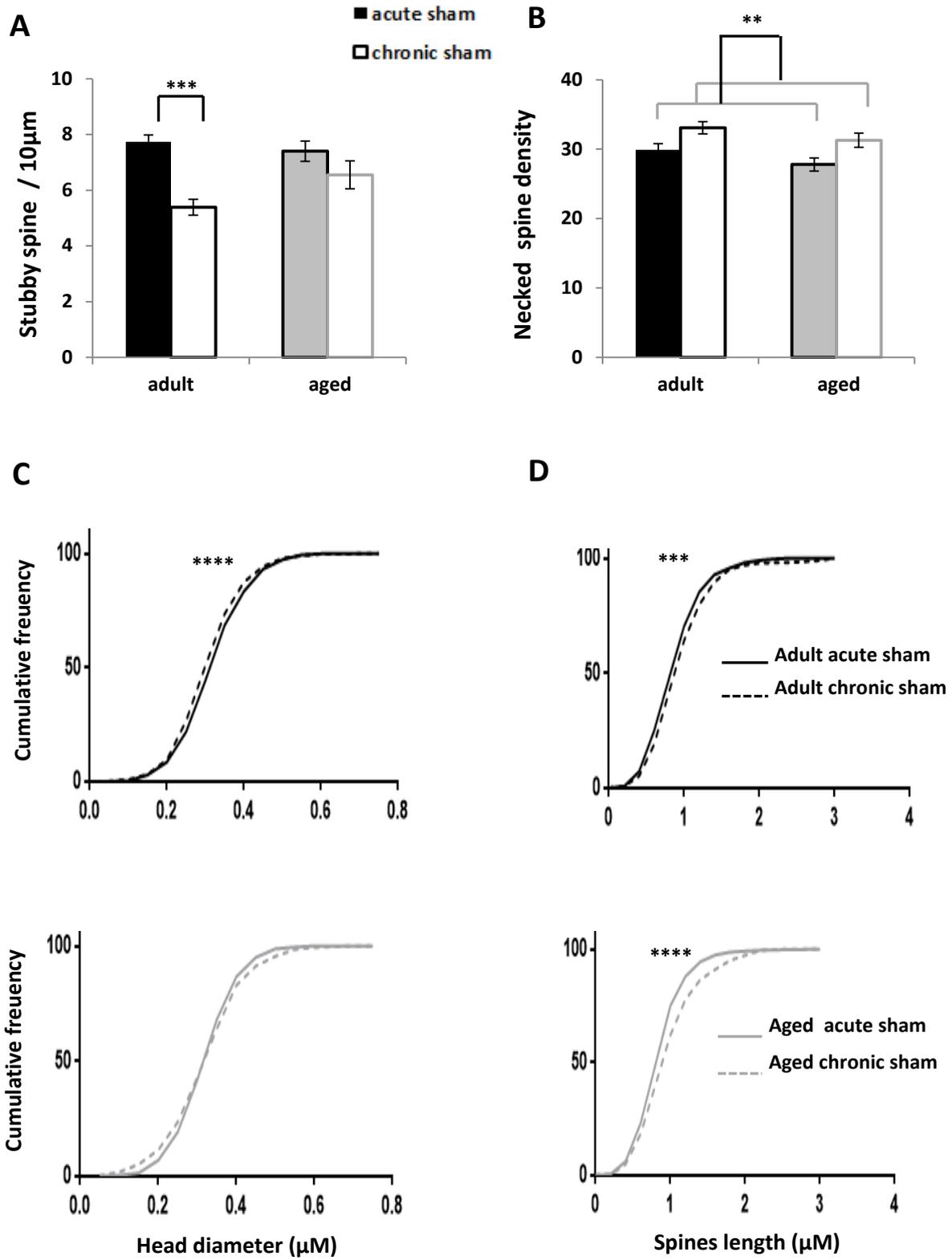
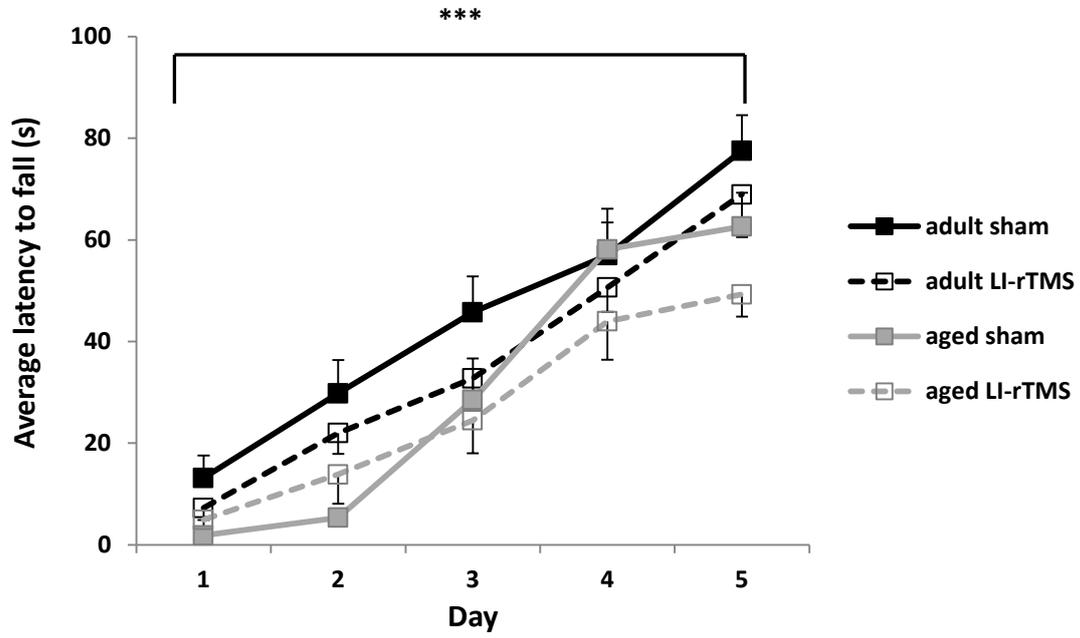


Figure 8

A



B

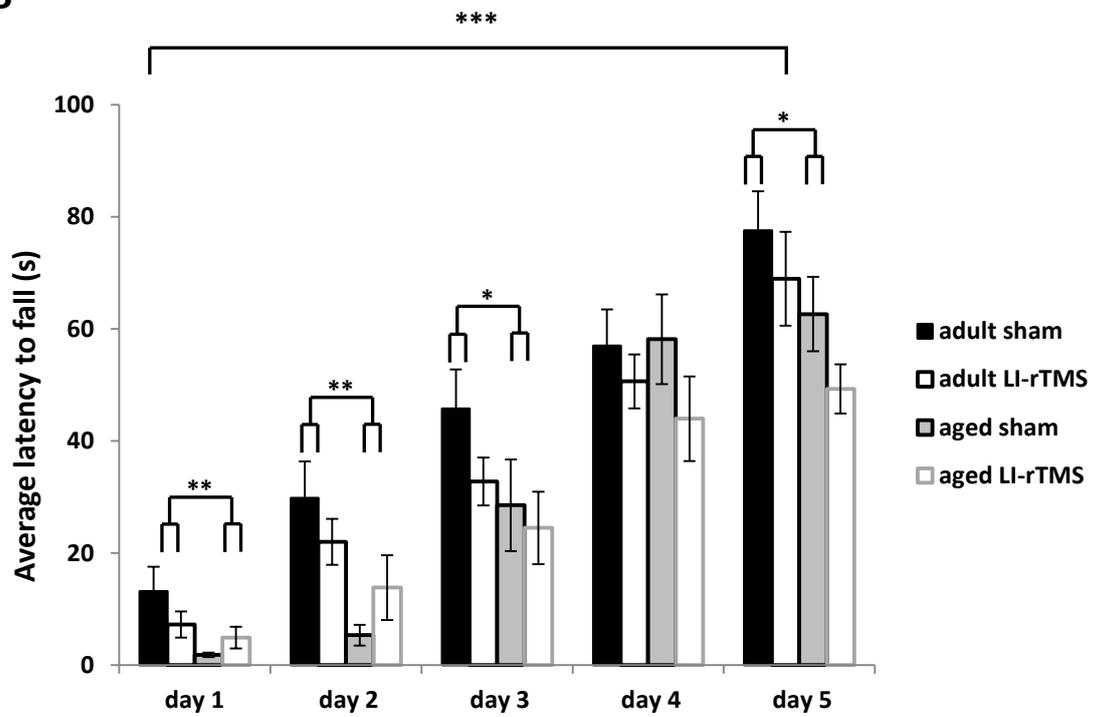
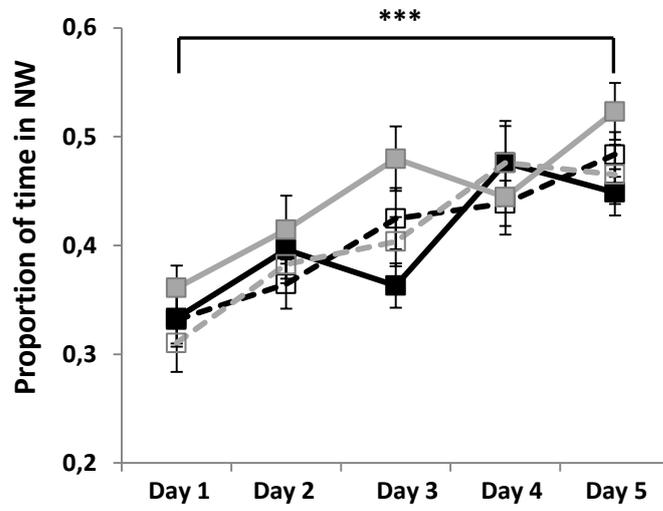
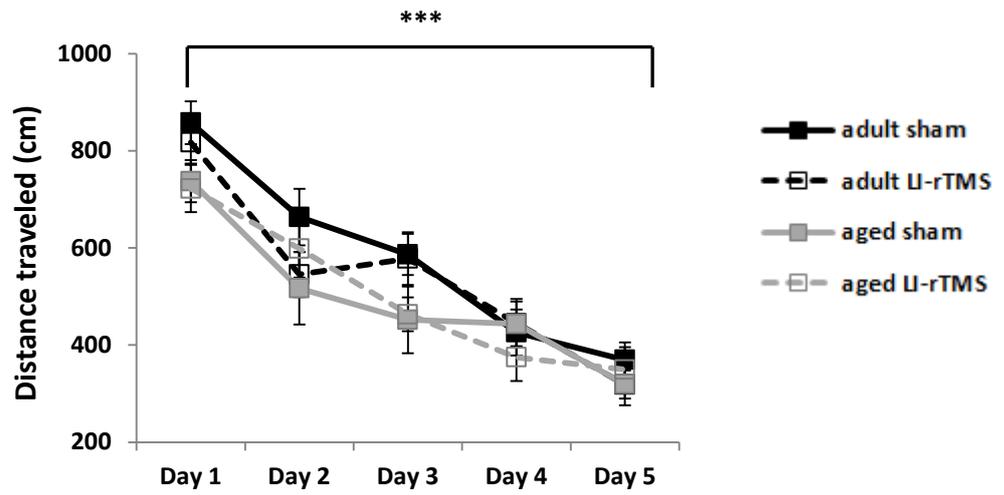


Figure 9

A



B



C

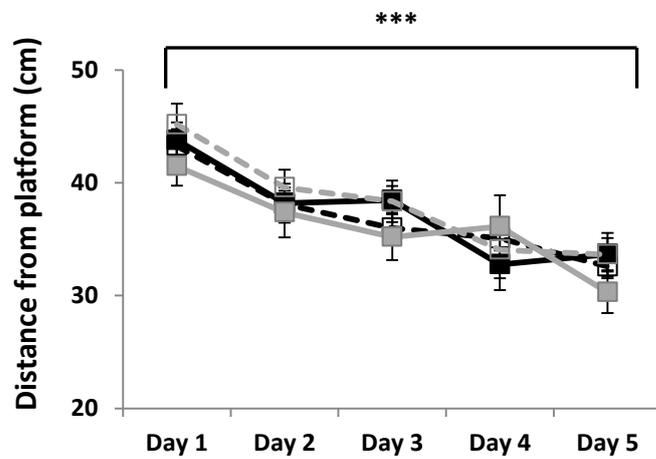
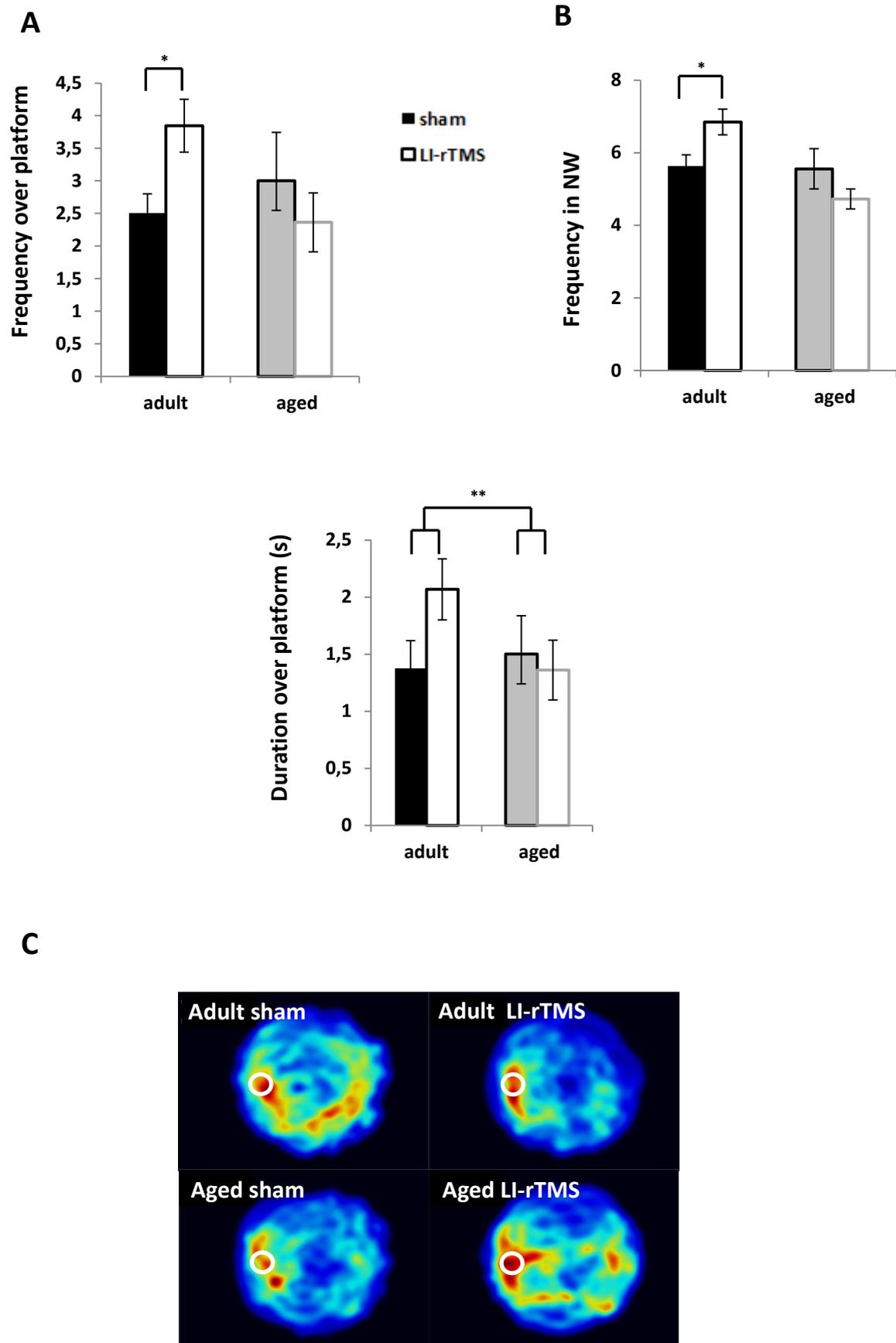


Figure 10



## **Chapter III – Article 2**

## Chapter III – Article 2

### **Patterns matter: low intensity rTMS induces neural circuit repair but depends critically on stimulation pattern and the presence of cryptochrome magnetoreceptors**

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## Abstract

The clinical outcomes of treatments for human disease using non-invasive brain stimulation by magnetic fields are variable. This is partly due to the lack of standardised stimulation protocols between studies and our limited knowledge of the cellular mechanisms induced by such stimulation. High intensity transcranial magnetic stimulation (rTMS) aims to depolarise underlying cortical neurons to modulate activity in specific circuits. Low intensity rTMS (LI-rTMS) does not directly stimulate action potentials but can modify neural function through mechanisms, which remain incompletely identified; in particular how such non-depolarising magnetic fields interact with their target tissue. In this study we tested a range of LI-rTMS stimulation parameters using the repair of a lesioned neural circuit as the readout, to try to clarify what stimulation parameters may have beneficial effects on neural circuits and what are the cellular processes they induce.

We used the olivocerebellar path, *in vivo* and *in vitro*, and systematically screened a range of LI-rTMS frequencies/rhythms to test whether any could induce axonal outgrowth and olivocerebellar reinnervation, and then searched for cellular processes that could explain the observed effects.

We show that 14 days of low-intensity magnetic field stimulation delivered in a high frequency pattern can induce olivocerebellar reinnervation *in vivo* and *in vitro*, with decreasing efficacy as the distance of axonal outgrowth increases. We also identified that this repair process is highly dependent on stimulation pattern, with biologically relevant rhythms being most effective, and that gene expression was modified in concert with biological effect: i.e. LI-rMS patterns that induced reinnervation also upregulated growth promoting genes, and vice versa. Finally, we demonstrated that these effects required detection of the magnetic (vs. induced electric) field, through the presence of the potential magnetoreceptor, cryptochrome. In the absence of cryptochrome, LI-rMS was unable to induce olivocerebellar reinnervation.

**Keywords:** LI-rTMS, stimulation patterns, gene expression, olivocerebellar pathway, reinnervation, cryptochromes.

## INTRODUCTION

The application of magnetic fields to the treatment of human disease is increasing, specifically in neurology and psychiatry (Lefaucheur et al., 2014; Pell et al., 2011). However, clinical outcomes of extrinsic brain stimulation are variable (Wassermann and Zimmermann, 2012), consistent with the lack of standardized stimulation protocols and our limited understanding of the cellular mechanisms induced by magnetic fields. In human neurological science, repetitive transcranial magnetic stimulation (rTMS) delivers focal high intensity stimulation to depolarize underlying neurons and modulate specific neural circuits; it is presumed through mechanisms of activity-dependent plasticity (Pell et al., 2011). Alternatively, low-, or pulsed-, field magnetic stimulation (LFMS, PMF), which delivers diffuse low-intensity stimuli that do not induce neuronal firing, is similarly effective (Di Lazzaro et al., 2013), raising the question of *how* these weak magnetic fields induce their diverse physiological effects (Capone et al., 2009; Cook et al., 2004; Legros et al., 2015; Martiny et al., 2010; Robertson et al., 2010; Rohan et al., 2014; Volkow et al., 2010) and whether a specific dose (number of pulses) or delivery (frequency/pattern) of LFMS/PMF are required. To address these questions and optimize magnetic field stimulation to treat human pathologies we systematically evaluate defined stimulation parameters and a potential receptor of weak magnetic fields, the chromophore cryptochrome (Maeda et al., 2012), in an appropriate experimental model.

We have developed a suitable experimental model delivering focal low-intensity transcranial magnetic stimulation (LI-rTMS) to the mouse brain, and demonstrated that 2 weeks LI-rTMS removes abnormal neural circuits to recover behavioural deficits (Makowiecki et al., 2014; Rodger et al., 2012) and modulates neuronal calcium concentration and gene expression (Grehl et al., 2015). The physiological effects of magnetic stimulation are critically influenced by stimulation parameters (frequency, pattern, duration) (Fitzgerald, et al., 2006; Hoogendam et al., 2010), which are limited in high intensity rTMS for technical and safety reasons (Rossi et al., 2009; Rossini et al., 2015). In contrast, LFMS/PMF devices generate a wide range of stimulation frequencies and patterns (Di Lazzaro et al., 2013) that modulate post-lesion neuronal function (Markov, 2007; De Pedro et al., 2005; Walker et al., 1994; Yang et al., 2012), neuroplasticity (Gunay and Mert, 2011; Sherafat et al., 2012), neuronal survival

(Boland et al., 2002; Di Loreto et al., 2009), and calcium signalling (Grassi et al., 2004; Grehl et al., 2015; Hogan and Wieraszko, 2004; Piacentini et al., 2008). The goal of this study was to test a range of LI-rTMS stimulation parameters using the repair of a lesioned neural circuit as the readout.

We used the mouse olivocerebellar path, which has well-defined topography and a one-to-one relationship between afferent climbing fibre axons (CF) from the inferior olivary nucleus and their target cerebellar Purkinje cell (PC) (Sugihara and Shinoda, 2004). After unilateral transection of the olivo-cerebellar pathway the axotomised ION degenerates, and injection of brain derived neurotrophic factor (BDNF) induces the remaining ION to develop axon collaterals that partially reinnervate the denervated hemocerebellum (Dixon and Sherrard, 2006; Willson et al., 2008). The *in vitro* olivocerebellar path model has also been optimized (explant; Letellier, et al., 2009), and closely resembles the *in vivo* system during development and post-lesion reinnervation, and thus facilitates the comparison of a large number of different LI-rMS parameters. We find that 14 days of low-intensity magnetic field stimulation can induce olivocerebellar reinnervation *in vivo* and *in vitro*, that this repair process is highly dependent on stimulation pattern, and that cryptochrome magneto-receptors must be present.

## **METHODS**

### **Animals**

Adult C57Bl/6j mice were purchased from the Animal Resources Centre (Murdoch, Western Australia) and timed pregnant Swiss mice from Janvier-Labs (Villejuif, France). Cryptochrome knockout mice ( $Cry1^{-/-}Cry2^{-/-}$ , DKO) were a generous gift from Dr Xiao-mei Li and Pr Francis Lévi.  $Cry1^{-/-}Cry2^{-/-}$  embryos were obtained from timed mating between  $Cry1^{-/-}Cry2^{-/-}$  male and  $Cry1^{-/-}Cry2^{+/-}$  females. The mouse phenotype and genotyping protocol have been described previously (van der Horst et al., 1999). Animal housing and all procedures followed guidelines established by le Comité National d’Ethique pour les Sciences de la Vie et de la Santé, in accordance with the European Communities Council Directive 2010/63/EU and regulations of the NH&MRC of Australia and the NIH.

### **In vivo olivocerebellar axonal transection (pedunculotomy)**

WT mice were anaesthetized with xylazine (10 mg/kg i.p.) and ketamine (70 mg/kg i.p.: Ilium, New South Wales Australia) and underwent unilateral transection of the left inferior cerebellar peduncle as previously described (Bower and Waddington, 1981; Dixon et al., 2005). Briefly, the skin over the neck was incised longitudinally and the muscles retracted to expose the atlanto-occipital membrane. A capsulotomy knife (MSP, 3mm blade) was inserted parallel to the brainstem into the fourth ventricle and rotated to the left to cut the left inferior cerebellar peduncle. After recovery from the anaesthetic, animals were returned to the cage. Food and water were provided ad libitum.

### **Organotypic Cultures and cerebellar denervation**

The olivocerebellar path and its lesion has also been optimized *in vitro* using hindbrain organotypic explants which contain the whole circuit, are highly reproducible and readily manipulated (Chédotal et al., 1997; Letellier et al., 2009). Hindbrain explants were cultured from embryonic Swiss or Cryptochrome knock-out ( $Cry1^{-/-}::Cry2^{-/-}$ ) mice at embryonic day 14 (E14) as previously described (Chédotal et al., 1997; Letellier et al., 2009). E0 was the mating day. Following anaesthesia and cervical dislocation of pregnant females, the E14 embryos were removed and their brains quickly dissected in ice-cold Gey's balanced salt solution (Eurobio) containing 5 mg/mL glucose. The hindbrain, including the cerebellar anlage and inferior olivary nucleus, was isolated and the meninges removed. The right and left cerebella plates were separated at the midline (Fig 2A) and the explants transferred onto 30mm Millicell membranes (pore size 0.4 $\mu$ m, Millipore) and cultured at 35°C with medium containing 50% basal medium with Earle's salts (Gibco), 2.5% Hank's Balance Salt Solution (Gibco), 25% horse serum (Gibco), 1mM L-glutamine (Gibco), and 5mg/mL glucose at 35°C in humidified air with 5 % CO<sub>2</sub>. The culture day was designated 0 day in vitro (DIV). The medium was replaced every 2–3 days.

To denervate (Dx) cerebellar tissue and induce olivocerebellar reinnervation, the cerebella plates were removed from their explant brainstem at *DIV 21* (equivalent to P15) and co-cultured (graft) adjacent to the cerebellar plate of an intact explant (host; Fig 2A). Twenty-four hours after co-culture some cerebella plates were treated (DxB) with 1 $\mu$ l (4 $\mu$ M) recombinant *human brain derived neurotrophic factor* (hBDNF; Alomone, 0.1% BSA in H<sub>2</sub>O),

which induces climbing fibre reinnervation in vivo (Dixon and Sherrard, 2006; Sherrard and Bower, 2001).

## **Magnetic stimulation**

### *In vivo*

To evaluate whether LI-rTMS can induce olivocerebellar reinnervation, pedunculotomised mice recovered for 3 days post-surgery before receiving stimulation or sham treatment for 10 minutes per day for 14 consecutive days, as previously described (Makowiecki et al., 2014; Rodger et al., 2012). We used a commercially available electromagnetic pulse generator (Global Energy Medicine Perth, Australia) modified for attachment of a custom designed copper wire coil with an outer diameter of 8mm, 300 windings and a 6mm diameter steel bolt in the centre to increase field penetration (Epstein and Davey, 2002; Makowiecki et al., 2014). The coil delivers a magnetic field of 12 mT at its surface, without any sound or vibration above background (Grehl et al., 2016; Makowiecki et al., 2014). The coil size was designed to ensure a similar coil-to brain ratio as used for focal magnetic stimulation in humans (Weissman et al., 1992).

### *In vitro*

To investigate which parameters were most effective for inducing reinnervation, pedunculotomised (Dx) explants were stimulated inside the incubator, 10 min per day for 14 days, using a custom built copper wire coil (10 mm inside diameter, 26 mm outside diameter, 199 turns) placed 4 mm below the well (Fig 4A) and driven by a 24 V magnetic pulse generator (Grehl et al., 2016). The non-sinusoidal monophasic 300 $\mu$ s pulse had a measured 100 $\mu$ s rise time and generated an intensity of 10 mT at the explant without sound or vibration above background (Grehl et al., 2016). Each culture plate was isolated using mu-metal to ensure no eddy current spill over. The induced electric field in the explant's regions of interest (cerebellar lobes and inferior olive nucleus) has been previously modelled at 0.05 Vm<sup>-1</sup> (Grehl et al., 2016). This electric field is at least two orders of magnitude below the electric field amplitude reported for activation of cerebellar neurons (Chan and Nicholson, 1986).

We chose stimulation patterns that are used in human clinical rTMS: simple frequencies at 1 Hz and 10 Hz; complex frequencies at theta-burst stimulation (TBS; 3-pulse bursts at 50 Hz

repeated at 5 Hz) delivered either continuously (c TBS), intermittently (iTBS) for 2 s repeated every 10 s, or randomly (riTBS: 3-pulse bursts at 50 Hz repeated randomly at 2-60 Hz, for 2 s, all repeated every 10 s). We also tested a complex biomimetic high-frequency stimulation (BHFS), which we have previously shown can modulate neural circuits (Makowiecki et al., 2014; Rodger et al., 2012). Sham treatment was delivered identically but without activation of the stimulation coil.

### **Immunohistochemistry**

The effects of LI-r(T)MS on olivocerebellar reinnervation and the explant cell populations it activated were evaluated by immunohistochemistry. Pedunculotomised mice were euthanized 10d post-Px with an overdose of sodium pentothal and perfused transcardially with 0.9% saline and 4% paraformaldehyde in 0.1M phosphate buffer and post-fixed in fresh 4% paraformaldehyde overnight at 4°C prior to cryoprotection in 30% sucrose and taking coronal 20 µm sections of the cerebellum and brainstem.

For *ex vivo* experiments, 24h after the last (14<sup>th</sup>) day of stimulation explants were fixed with 4% paraformaldehyde for 4h at 4°C. Sections and explants were labelled by immunohistochemistry. Fixed tissue was rinsed 3x5 min in phosphate buffered saline (PBS) containing 0.25% Triton X (PBS-T) and blocked in 20% donkey serum for 2h at RT prior to incubation overnight at 4°C in primary antibody diluted in PBS-TG (PBS-T containing 0.2% gelatine and 0.018g/ml L-Lysine). The next day sections or explants were washed 3x5 min in PBS-T and labelling was visualised with fluorescent-conjugated secondary antibodies in PBS-TG for 2h at RT. Finally, sections or explants were rinsed and mounted in Mowiol.

To identify **CF reinnervation**, Purkinje cells were labelled with rabbit anti-calbindin-28k (CB) antibody (1:3000; Swant; Celio, 1990) and CF terminals with polyclonal guinea pig anti-VGLUT2 antibody (1:2000, Millipore; Hioki et al., 2003; Letellier, et al., 2009). Primary antibodies were visualised using Cy3-conjugated donkey anti-guinea pig and Alexa Fluor (AF) 488-conjugated donkey anti-rabbit (1:200 and 1:400 respectively; Jackson Laboratories).

To identify which **cells were activated** by the magnetic stimulation *in vitro* we labelled c-fos 2.5h after a single 10 min stimulation session 72h after lesion and co-culture. Fixed explants were labelled with rabbit anti c-fos (Synaptic System, 1:3000) plus one of 4 different antibodies to label specific cell populations (Celio, 1990; Weyer & Schilling, 2003): PCs with

monoclonal mouse anti-CB-28k (1:2000; Swant), molecular GABAergic interneurons (basket and stellate cells) with goat anti-Parvalbumin (PV, 1:3000; Swant), granule cells with monoclonal mouse anti-NeuN (1:200; Millipore; Weyer & Schilling, 2003) or oligodendrocytes with monoclonal mouse anti-olig2 (1:400; Merck-Millipore). Primary antibody binding was visualised using fluorescently labelled secondary antibodies: Cy3-conjugated donkey anti-rabbit, AF488 conjugated donkey anti-goat and Cy5 conjugated donkey anti-mouse (all 1:200; Jackson Laboratories).

## **Histological Analysis**

### *Quantification of olivocerebellar reinnervation*

*In vivo* experiments: To quantify the extent of reinnervation, the cerebellum of pedunculotomised animals was divided into a series of parasagittal zones (500  $\mu\text{m}$  wide) extending from the midline to the left lateral hemiserebellum. Within each zone, the amount of VGLUT2 positive CF reinnervation was scored in each lobule using an arbitrary scale i.e. 1 = few strands, 2 = one-fourth CF-filled lobule, 3 = half lobule, 4 = three-fourth lobule and 5 = completely CF-filled lobule. Lobule scores in each 500  $\mu\text{m}$  zone were summed to generate a reinnervation value for each cerebellar cortical zone and functional region (Willson et al., 2008). The functional regions were designated medial (0–1500  $\mu\text{m}$ ), intermediate (1500–2500  $\mu\text{m}$ ) and lateral hemiserebellum (>2500  $\mu\text{m}$ ) according to the olivo–cortico–nuclear zones (Sugihara et al., 2003). To compare the density of CF reinnervation between experimental groups, we used the reinnervation values of all animals in each group for each zone to obtain an average score for each parasagittal zone.

*Ex vivo* experiments: Labelled explants were examined using epifluorescence microscopy (DM 6000F; Leica) and z-stack images taken for analysis. The amount of CF reinnervation was measured by the number of CB-positive PCs (soma and primary dendrites) co-localised with VGLUT2 per field of view (grid) and expressed as percentage PCs per field. This quantification was made systematically on z-stacks taken in rows through the cerebellar graft with increasing distance from the host-graft interface. Data from rows 1 and 2 were defined as the proximal zone, and those from rows 3-5 were defined as the distal zone.

### *Cellular activation: c-fos*

Explants were examined using epifluorescence microscopy and z-stack images were taken at 3 semi-randomly selected sites of  $0.073\text{mm}^2$  for each co-cultured cerebellar plate. Total c-fos

positive staining was counted per z-stack and double-labelled or triple-labelled profiles were quantified visually for co-labelling. C-fos, CB and PV positive cells were counted per image to identify the proportions that were activated by LI-rMS. Results are expressed as mean number of c-fos positive cells per mm<sup>2</sup>

### **qRT-PCR and gene analysis**

To investigate the molecular events triggered by different frequency LI-rMS that may be involved in reinnervation, changes in gene expression were examined in a separate series of explants following effective and ineffective reinnervation protocols. Explants were denervated and co-cultured at DIV21 (P15) and 72h after the lesion they received 3 daily sessions of LI-rMS. The cerebellar plate of Dx (LI-rMS/sham) explants was taken 6h after last stimulation (P18). RNA was extracted from pooled (6) cerebellar plates. Total RNA was extracted using Trizol (Life Technologies) according to manufacturer's instructions (Chomczynski & Sacchi, 1987) and stored at -80°C.

cDNA was transcribed from 400 ng of total RNA using the RT<sup>2</sup> Easy First Strand cDNA Synthesis Kit (Qiagen). For each sample, 400 ng of cDNA was applied to the RT<sup>2</sup> Profiler™ PCR Array Mouse Neurotrophins & Receptors (Qiagen) and amplified on a Lightcycler480. Results were analysed on the Qiagen RT<sup>2</sup> Profiler PCR array data analysis (v3.5) using the geometric mean of housekeeping genes (hsp90ab1). Normalized mean expression levels ( $\log_2(2 - \Delta Ct)$ ) were used to determine differentially expressed genes between each group and control.

To identify the functions of LI-rTMS regulated genes, GO (Gene Ontology) terms enrichment was assessed using the R topGo package, in which each list of genes was analysed by the topGo overrepresentation test in comparison to the mouse muscles reference list. GO terms for biological process, molecular function or cellular component (GO.db 3.3) were analysed. The Bonferroni correction for multiple testing was applied.

To identify genes which could potentially be modified by our transcription factors (TF) of interest (e.g. cryptochrome 1), we used R (3.3.0) and the Bioconductor suite (3.3). For the determination of TF binding sites (sequences), we applied Biostrings (2.40) and GenomicFeatures (1.24). TF binding matrices were obtained from the R package MotifDb (1.14). Binding of a TF to a target sequence was determined using R and Biostrings. In this study, we used the function matchPWM to match the PWMs (Position-Weight-Matrix)

against target sequences. For every gene, the sequences 2 kbp upstream were scanned with Position (90%) for the PWM.

### **Statistical Analysis**

All data was explored for normality, outliers and fulfilment of statistical test assumptions in SPSS 22 (IBM).

Reinnervation percentages were analysed with Repeated Measures ANOVA (F) (location x stimulation). When significant interaction between stimulation and location were present univariate ANOVA were performed to evaluate the effect of the treatment in proximal and distal rows independently. Tukey post-hoc comparisons were performed where appropriate. C-fos activation was analysed with univariate ANOVA. Tukey post-hoc comparisons were performed where appropriate. CB/c-fos Pv/c-fos were analysed with Fisher exact test. Gene expression levels were compared by two sample *t*-test. All values are expressed as mean  $\pm$  SEM and considered significant at  $p < 0.05$ .

## **RESULTS**

The aim of this study was to evaluate whether low-intensity magnetic stimulation (LI-rMS) could induce neural circuit repair in the olivocerebellar projection model of axonal injury. We assessed if LI-rMS induces olivary axonal outgrowth and reinnervation to denervated Purkinje cells in the maturing cerebellum. We tested different stimulation protocols to identify optimal parameters to induce repair, specifically aiming to identify whether frequency, pattern, or total number of pulses is the most important factor.

### **LI-rTMS induces CF reinnervation in vivo**

We have shown that BHFS LI-rTMS modifies abnormal connections in the visual system (Makowiecki et al., 2014; Rodger et al., 2012), increases intracellular calcium concentrations and modifies expression of genes related to neurite outgrowth (Grehl et al., 2015). Here we applied the same stimulation to adult mice following unilateral lesion of the olivocerebellar path (pedunculotomy). We found that after 2 weeks sham stimulation there was no reinnervation to the left hemicerebellum (Fig 1A). In contrast, BHFS LI-rTMS induced VGLUT2-positive terminals in the molecular layer of the left hemicerebellum. The terminal

morphology was consistent with CFs, being identical to reinnervating CF terminals previously shown in the rat (Dixon and Sherrard, 2006; Willson et al., 2008) (Fig 1 A and B). VGLUT2 labelling extended into the vermis and paravermis, with very little labelling in the hemisphere (Fig 1D).

### **LI-rMS induces PC reinnervation *in vitro* in a frequency dependent manner**

The post-lesion reinnervation we observed *in vivo* indicates that BHFS LI-rTMS can promote axonal outgrowth as well as retraction of aberrant axon terminals (Makowiecki et al., 2014; Rodger et al., 2012). However we wanted to know whether other stimulation patterns, particularly those already used in the clinic, could have similar or even more pronounced effects. Because systematic testing requires the analysis of a large number of cases in controlled conditions, we used our *ex vivo* model of the same neuronal pathway.

We repeated our *in vivo* study using organotypic hindbrain explants, to investigate the efficacy of different LI-rMS patterns to induce climbing fibre reinnervation of denervated PCs. BDNF treatment of denervated co-cultured explants (DxB) was used as a positive control (Dixon & Sherrard, 2006; Willson, et al., 2008). Reinnervation is observed as ingrowth of VGLUT2-positive terminals, which localise around the PC somata and primary dendrites (Letellier et al 2009; Fig 2C).

Since repeated-measure ANOVA showed an interaction between treatment and distance from the host-graft junction with the proximal region consistently presenting more reinnervated PCs than the distal region ( $F=7.043$ ,  $p=0.000$ ), we therefore performed one-way ANOVAs separately in proximal and distal region. First we tested BHFS stimulation to confirm its effect *ex vivo*. In comparison to sham Dx explants, BHFS significantly increased the amount of VGLUT2 labelling in grafted cerebellar plates in both proximal region, with  $26.23 \% \pm 1.72$  ( $p=0.000$ ) of PCs contacted by VGLUT2-positive terminals, and distal region with  $13 \% \pm 1.75$  ( $p=0.012$ ) (Fig 3A). We also tested frequencies used in human rTMS for inhibition (1Hz, continuous theta-burst = cTBS) or facilitation (10Hz, intermittent TBS = iTBS) of cortical excitability (Pell et al 2011). LI-rMS at 1Hz and cTBS showed a small percentage of VGLUT2-contacted PCs in both proximal and distal region (proximal:  $12.05 \% \pm 1.74$  and  $14.27 \% \pm 1.87$  respectively; distal  $6.52 \% \pm 1.91$  and  $6.89 \% \pm 1.87$  respectively (all  $p>0.05$ ; Fig 3A). In contrast, iTBS significantly increased the number of VGLUT2-contacted PCs in both proximal and distal region ( $27.19 \% \pm 1.1$ ;  $P=0.000$  and  $13.88 \% \pm 1.5$ ;  $P=0.005$ ; Fig 3A) with

similar effect to BHFS; while 10Hz significantly increased the number VGLUT2-contacted PCs only in the proximal region ( $15.02 \% \pm 2.7$ ;  $P=0.048$ ; Fig3A) and to a lesser extent than iTBS or BHFS ( $p=0.001$ ).

Our data reveal that excitatory high/frequency LI-rMS is effective for inducing olivocerebellar reinnervation in our model, specifically complex patterns iTBS and BHFS, in contrast to inhibitory low/frequency stimulation. However there does not appear to be a simple relation between stimulation frequency/pattern and reinnervation, since different stimulation protocols within a defined group have different effects to each other: e.g. complex frequencies (cTBS vs. iTBS), high frequencies (10Hz vs. BHFS). We thus tested the relation of pulse number to reinnervation density and found no correlation (Pearson coefficient,  $p=0.353$ ; Fig 3B). We confirmed this by creating a randomized pattern of intermittent TBS (riTBS) which delivers the same number of high-frequency bursts in the 2s stimulation, but repeated at variable frequencies rather than at the theta rhythm (5Hz). Two weeks stimulation with this pattern did not increase the amount of VGLUT2-PC reinnervation with respect to sham either in proximal or distal regions ( $9.89 \% \pm 1.05$  and  $7.7 \pm 2.43$  respectively;  $p>0.05$ ; Fig3A). This result confirms that pulse number is not fundamental to reinnervation (riTBS = iTBS), and adds that it is the theta rhythm of pulse-burst delivery which is essential. Moreover, this theta rhythm must be delivered intermittently (iTBS vs. cTBS) to induce reinnervation in the olivocerebellar explant (Fig 3A).

### **LI-rMS induced reinnervation requires simultaneous stimulation of both pre- and post-synaptic structures of the OCP**

In human neuropathology it is not necessarily possible to stimulate an entire dysfunctional neural system, e.g. both the motor cortex *and* the spinal cord. Therefore we tested whether stimulation of both pre and post-synaptic components of the OCP were essential to induce axon growth and PC reinnervation by CF; or if stimulation of either the denervated cerebellum or the reinnervating ION were sufficient. Therefore we isolated the explants cerebellum or brainstem from magnetic fields using mu-metal shielding and compared the reinnervation induced by BHFS LI-rMS of either cerebellum or brainstem compared to sham and BHFS stimulation of the whole explant. Two weeks daily BHFS LI-rMS targeted to either the cerebellum or the brainstem induces a similar small but not significant increase in the

percentage of VGLUT2-contacted PCs in proximal region ( $13.73\% \pm 1.92$ ;  $p=0.202$  and  $14.62\% \pm 4.05$ ;  $p=0.122$  respectively; Fig 3C) suggesting that simultaneous stimulation of both pre and post synaptic structures are required to observe significant reinnervation of denervated PCs.

### **LI-rMS activates Purkinje cells and interneurons**

As cerebellar stimulation is necessary for effective LI-rMS induced reinnervation, we used c-fos expression to identify what population(s) of cerebellar cells is activated by the low-intensity magnetic field. After a single 10min session of LI-rMS (sham; iTBS or BHFS), the number of c-fos positive cells were significantly increased by BHFS stimulation ( $821.32/\text{mm}^2 \pm 79.75$ ) compared to sham ( $439.2/\text{mm}^2 \pm 43.12$ ;  $p=0.001$  One way ANOVA), while iTBS resulted in an intermediary increase of c-fos labelled cells that was not significantly different from either sham or BHFS ( $599.6/\text{mm}^2 \pm 64.1$ ;  $p>0.05$ ; One way ANOVA; Fig 4B). Counterstaining with specific cell markers revealed c-fos labelling in neurons (PCs, GABAergic interneurons, granule cells and deep cerebellar nuclei) and oligodendroglia, but not astrocytes.

Since PCs are the major target for reinnervation, we examined the percentage of c-fos positive PCs. Both iTBS and BHFS increased the percentage of c-fos positive PCs compared to sham (Fisher exact test  $p<0.0001$  and  $p<0.0001$  respectively; Fig 4C). Also, in the cerebellar molecular layer parvalbumin expressing GABAergic interneurons (PVIN) also receive CFs, and their counterparts in the cerebral cortex are modulated by high-intensity iTBS (Benali et al., 2011; Hoppenrath and Funke, 2013; Hoppenrath et al., 2016; Mix et al., 2014) Thus we also counted the percentage of c-fos positive PVINs. As for PCs, both iTBS and BHFS increased the percentage of c-fos positive PVINs (Fisher exact test  $p=0.0002$  and  $p=0.0062$ ; Fig 4C). These data reinforce the importance of stimulation pattern to biological effect; although iTBS does not significantly increase the overall number of c-fos positive cells, it activates PCs and PVINs to a similar extent as BHFS, which globally induces greater activation of cerebellar cells.

### **LI-rMS modulates gene expression appropriately for PC reinnervation**

To identify mechanisms underlying LI-rMS-induced CF-PC reinnervation, we examined gene expression in grafted cerebellar tissue 6h after the last of 3 LI-rMS sessions (BHFS, iTBS, rTBS or sham), i.e. during the process of reinnervation. As BDNF induces OCP reinnervation

(Dixon and Sherrard, 2006; Willson et al., 2008) and is upregulated by LI-rTMS (Makowiecki et al., 2014; Rodger et al., 2012) we studied expression changes in an 84 gene array of molecules associated with neurotrophin signalling. We identified 11 genes (Table 1; Fig 5) for which expression was either significantly up or down regulated by at least one of the three LI-rMS frequencies compared to sham ( $p < 0.05$ ). LI-rMS frequencies that induced significant PC reinnervation (iTBS and BHFS) upregulated genes involved in different biological functions such as “axonogenesis, axonguidance, chemotaxis or response to axon injury” (Table 1; Figure 5 A and B). In contrast riTBS, that did not induce PC reinnervation, either did not change or tended to down-regulate gene expression (Table 1; Fig 5). Two genes, beta2-microglobulin (*b2m*) and neurotrophin-3 (*ntf3*), which were upregulated by iTBS and BHFS (*b2m*: x2.23  $p=0.048$  and x1.8  $p=0.021$  respectively; *ntf3*: x1.38  $p=0.026$  and x1.52  $p=0.094$  respectively; Fig 5), are both involved in post-lesion reinnervation (Duricki et al., 2016; Oliveira et al., 2004; Schnell et al., 1994; Sherrard and Bower, 2001). Moreover, nerve growth factor receptor (*Ngfr*), which was upregulated by iTBS (x2.25  $p=0.045$ ), is associated with neuronal survival and sprouting of autonomic axons (Glebova and Ginty, 2004; Mount et al., 1998; O’Keeffe et al., 2008)

Thus complex pattern LI-rMS (BHFS and iTBS) changes gene expression appropriately for inducing reinnervation of the olivocerebellar pathway.

### **Cryptochrome is required for LI-rMS-induced post-lesion repair**

Given the clear effects of LI-rMS on neuroplasticity and gene expression, combined with the weak induced electric field (Grehl et al., 2016) being sub-threshold for cerebellar neuron stimulation (Chan and Nicholson, 1986), we wanted to see how the magnetic field *per se* is detected by the neuronal tissue. Cryptochromes (CRYs) are potential magnetoreceptors, mediating magnetic field dependent behaviours (Yoshii et al., 2009) and neuronal excitation (Marley et al., 2014) in *drosophila*. Although direct evidence of CRYs involvement in mammals’ magnetoreception has not been showed, we examined their possible role in the LI-rMS-induced OCP reinnervation.

Explant cultures from Cry1/Cry2 double KO (DKO) embryos were prepared as described, lesioned, stimulated with BHFS LI-rMS and observed for reinnervation. In DKO explants, BHFS failed to induce significant reinnervation: only 8.56 %  $\pm$  1.46 PCs contacted by VGLUT2-

positive CFs in the proximal zones, compared to sham-treated WT explants ( $7.17 \% \pm 1.39$   $p > 0.05$ ; Fig 7B).

To determine whether DKO explants were unable to respond to the LI-rMS stimulation or whether they were unable to demonstrate post lesion repair at all, we treated DKO explants with BDNF, our positive control. In these cases, BDNF treatment resulted in significant CF-PC reinnervation ( $23.28 \% \pm 3.4$ ;  $p = 0.000$ ; Fig7B), as in WT  $20.13 \% \pm 1.57$ ;  $p > 0.05$  (Sebastian Jara, personal communication). Thus Cry DKO explants are able to show repair, but not to respond to the magnetic stimulation.

## DISCUSSION

Here we investigated whether LI-rMS can induce neural circuit repair, using the olivocerebellar projection model of axonal injury *in vivo* and *in vitro* (Willson et al 2008 (Letellier, et al., 2009). We also identified optimal stimulation frequencies and underlying mechanisms through systematic application of different LI-rMS protocols to the whole circuit organotypic model, and evaluating reinnervation of PCs after CF denervation, which cell populations were activated and gene expression changes.

### Post-lesion repair depends on stimulation pattern, not numbers of pulses

We investigated the amount of CF-PC reinnervation after different frequencies/patterns of LI-rMS. Here we show for the first time that repeated high-frequency LI-rMS induces olivocerebellar axonal outgrowth and target PC reinnervation after lesion of the normal neural circuit. Consistent with its capacity to modify abnormal neural circuits (Makowiecki et al., 2014; Rodger et al., 2012) we show that BHFS induced CF-PC reinnervation, both *in vivo* and *in vitro*, with decreasing efficacy as distance of axonal outgrowth increases: *in vivo* reinnervation decreases from vermis to hemisphere and *in vitro* from proximal to distal zones (Figs 1E and 3A).

There are few studies that evaluate the effects of magnetic stimulation on axonal growth and neosynaptogenesis. ELF-MF increases peripheral nerve regeneration and functional recovery in rats (Markov, 2007; Mohammadi et al., 2014; De Pedro et al., 2005) but not in

mice (Baptista et al., 2009). Results of high intensity rTMS also vary; enhancing hippocampal mossy fibre sprouting after chronic rTMS in mice (Lisanby and Belmaker, 2000) and dendritic and axonal sprouting to produce a greater number of synaptic contacts in mouse hippocampal cell cultures following rTMS (1.14T; 1Hz) (Ma et al., 2013). Surprisingly, in the same study, a slightly higher intensity rTMS (1.55T; 1Hz) reduced dendritic and axonal arborisation as well as the number of synaptic contacts (Ma et al., 2013), suggesting toxicity. These studies reinforce the importance of systematic investigations into the effects of different stimulation parameters, particularly intensity, on post lesion reinnervation of the central nervous system.

We also showed, using our in vitro system as a screening tool, that LI-rMS-induced reinnervation was dependent on some components of the stimulation pattern. Stimulation frequencies used in human high intensity rTMS to facilitate cortical excitability (10Hz and iTBS) induced significant PC reinnervation, although the complex pattern is significantly more efficient (10Hz vs iTBS: proximal, 15% vs. 27 %; distal, 6% vs. 14%) and similar to BHFS. In contrast, treatment with frequencies used in human rTMS to inhibit cortical excitability (1Hz and cTBS) did not increase overall reinnervation above sham controls. Moreover riTBS, which delivers the same number of pulses within 10min as iTBS, but in a random rather than repeated rhythm, did not induce PC reinnervation, revealing the importance of a “biological” theta rhythm (5 Hz). Thus, although the relation of stimulation frequency to effect has been shown functionally in terms of cortical excitability (Fitzgerald et al., 2006; Huang et al., 2005; Rossini et al., 2015), we show for the first time that, rather than the number of pulses delivered, the stimulation pattern is critical to produce structural changes to neuronal circuitry. This hypothesis is supported by the greater efficiency of LI-rMS delivering complex bursts of pulses (iTBS and BHFS; 1800 and 226000 pulses respectively) than LI-rTMS delivered at tonic 10Hz (6000 pulses). Similar observations were made in the electrophysiology literature, where induced neuronal burst firing produces greater cortical neuronal activation compared to tonic firing (Lisman, 1997; Sherman, 2001); as well as in the rTMS literature, where iTBS and cTBS, produce the most consistent and long-lasting effects on motor and visual cortex, despite delivering lower intensity and shorter duration rTMS than tonic 1Hz and 10Hz (Hoogendam et al., 2010; Huang et al., 2005; Di Lazzaro et al., 2005). Nonetheless it is very important to emphasize that we are using low intensity

stimulation that is not thought to *directly* elicit action potentials; so the mechanisms through which LI-rMS modulate neural plasticity, and more specifically CF outgrowth, are potentially different from those induced by high intensity TBS. Furthermore we show that the observed effects of the TBS are dependent on the rate and rhythm at which the burst are repeated, i.e. a regular physiological theta band 5Hz. This is consistent with burst rTMS applied at other frequencies of the EEG, such as alpha (10Hz) and beta (20Hz), successfully suppressing tinnitus (De Ridder et al., 2007). Thus it could be interesting to systematically investigate the potential of these other burst frequencies to induce neural plasticity.

### **LI-rMS modifies gene expression in a frequency-dependent manner**

The hypothesis that the effect of LI-rMS on post lesion neural circuit repair is determined by the stimulation pattern of frequency rather than the number of pulses was supported by our gene expression study. Indeed we showed that riTBS delivering the same number of pulses as iTBS results in lesser reinnervation and fewer gene-expression changes than iTBS and BHFS stimulation. Thus our data are coherent with previous studies showing quantitative transcriptional changes that are ELF-MF intensity and frequency dependent in other systems (Choi et al., 2003; Goodman et al., 1992; Wei et al., 1990).

Moreover direction of the modulation of gene expression induced by iTBS and BHFS seems to be appropriate for neural circuit repair since they systematically upregulated genes involved in neural plasticity mechanisms (Table1). B2m gene, is upregulated by both iTBS and BHFS; it is a central molecule of the major histocompatibility complex, and is involved in selective synaptic stabilization on motoneurons and their axonal regeneration after a peripheral nerve lesion (Oliveira et al., 2004). The neurotrophin gene family was also modulated. Specifically, neurotrophin 3 (*ntf3*) gene is upregulated by iTBS (significantly) and BHFS (trend). NT3, the protein synthesized by *ntf3*, promotes functional locomotor circuit repair (Chen et al., 2002, 2006; Zhou et al., 2003) through axon growth, sprouting and synaptic plasticity in several locomotor pathways including the corticospinal tract (Duricki et al., 2016; Fortun et al., 2009; Grill et al., 1997; Schnell et al., 1994). Furthermore NT3 is also involved in olivocerebellar plasticity in rats; exogenous injection of NT3 induces reinnervation of denervated PCs by CFs (Sherrard and Bower, 2001). Additionally, the nerve growth factor receptor (*ngfr*) gene is upregulated by iTBS. NGF is required for innervation and terminal sprouting of sympathetic axons within many distal targets (Glebova and Ginty,

2004; O’Keeffe et al., 2008) and, in our system, facilitates survival of PCs (Mount et al., 1998). In contrast rITBS did not upregulate any of our tested genes, but down-regulated pro-apoptotic *bax* and neuroprotective *gdnf*, consistent with its lack of induced CF-PC reinnervation. These results give us information on the power of LI-rMS showing that specific stimulation patterns activate appropriate signalling pathways to elicit axonal growth and neosynaptogenesis following a lesion. It would be interesting to see the use of higher intensity and/or longer duration of stimulation could potentiate the activation of these molecular to a higher degree. Additionally one would need to test these LI-rMS frequencies and their modulatory effects on gene expression in other model of neural circuit repair such as in the hippocampus or the corticospinal pathway to assess if they are generic or system specific.

### **LI-rMS potentially activates c-fos in PC and interneurons**

To identify which cellular populations are activated by BHFS and iTBS stimulation that could explain the molecular mechanisms underlying their effects on reinnervation, we measured cellular activation 2.5h after a single 10min session of these two LI-rMS frequencies. We show that specific low intensity magnetic stimulation (BHFS) increases the immediate early gene, c-fos (Bullitt, 1990; Kawashima et al., 2014; Schilling et al., 1991) in accordance with previous findings after high intensity rTMS to organotypic slice cultures (Hausmann et al., 2001). There, c-fos expression was dependent on sodium channel activity, suggesting membrane depolarization (Hausmann, et al., 2001). In contrast, computational modelling of the induced electric field (Chan and Nicholson, 1986; Grehl et al., 2016) is at least two orders of magnitude below the depolarization threshold for cerebellar neurons (Chan and Nicholson, 1986), however it increases calcium release from intracellular stores (Grehl et al., 2015) which can explain the c-fos expression.

Some of the increase in the number of cfos positive cells was due to greater PC c-fos labelling. Although it has been suggested from human studies that PCs are activated by cerebellar high intensity rTMS (Koch et al., 2008), we provide the first direct evidence for Purkinje cell activation by low intensity magnetic stimulation. Formation of CF-PC synapses is dependent on a variety of signalling interactions between CF and PCs during development (Watanabe and Kano, 2011), where the PC state is a central factor for CF-PC synapse formation (Letellier, et al., 2007 and 2009). Hence, the demonstration of PC activation is

consistent with induction of plasticity promoting mechanisms underlying reinnervation in the mature olivo-cerebellar circuit.

Parvalbumin-positive interneurons (PVIN) of the cerebellar molecular layer were also activated by our LI-rMS. PVINs finely tune Purkinje cell activity through powerful feed forward inhibition (Barmack and Yakhnitsa, 2008; Mittmann et al., 2005; Scelfo et al., 2008). These results are coherent with high intensity iTBS altering cortical inhibition through modulation of parvalbumin expression and activity of fast spiking interneurons (Benali et al., 2011; Hamada et al., 2013; Hoppenrath and Funke, 2013; Hoppenrath et al., 2016; Mix et al., 2014).

However, the total increase of PC and PVIN c-fos labelling is relatively slight, consistent with the absence of depolarization activating c-fos expression by the classically described flow of calcium ions through NMDA receptors or voltage gated calcium channels (Bito et al., 1997; Flavell and Greenberg, 2008; Kawashima et al., 2014). It is known that c-fos expression requires a strong, consistent increase of calcium within a short period and that a stimulus can activate a neuron without turning on c-fos expression (Appleyard, 2009). Thus, LI-rMS might selectively activate c-fos expression through an intracellular mechanism such as release of calcium by intracellular stores (Grehl et al., 2015), or in neurons with lower activation thresholds, as suggested by Koch and colleagues following iTBS delivered below motor threshold (Koch, 2010; Koch et al., 2008).

### **Cryptochromes magnetoreceptors are key elements for the transduction of the magnetic field into biological effects.**

A key issue for our LI-rMS, and other ELF-MF, studies aiming to identify their potential therapeutic application is *how* such small magnetic fields, which are sub-threshold for inducing neuronal firing, can have biological effects. However in addition to, or instead of, induced electric fields mediating tissue effects, direct interaction between magnetic fields and biological tissues, magnetoreception, is well described in plants, insects and birds and allows orientation to the ambient geomagnetic field (GMF) (Gegear et al., 2010; Kavaliers et al., 1984; Wiltschko and Wiltschko, 2012). The magnetoreceptor involved is a blue light photoreceptor, cryptochrome (CRY), that generates a radical-pair in response to light or magnetic stimulation (Dodson *et al.*, 2013; Liedvogel and Mouritsen, 2010). In turn the radical pair induces ROS (Arthaut et al., 2017; Jourdan et al., 2015; Usselman et al., 2014)

and alters Cry inhibitory action on the CLOCK/Bmal1 transcriptional activity (Zaporozhan and Ponomarenko, 2010). Similar magnetosensitivity of mammalian CRY is less clear, but transgenic insertion of human cry 2 (hCry2) into CRY-deficient insects rescues light-dependent static magnetic field-induced behaviours such as T-maze conditioning (Foley et al., 2011), negative geotaxis (Fedele et al., 2014a), directional magnetic responses (Bazalova et al., 2016) and hyperactivity in the presence of low frequency pulsed magnetic fields (3 and 50Hz; 50-500 $\mu$ T) (Fedele et al., 2014b). Moreover, mouse orientation is affected by a changing radiofrequency magnetic fields (0.9 to 5MHz; 25-50nT) (Malkemper et al., 2015). These data suggest that mammalian CRY can respond to magnetic fields. However, as mouse brain slices containing the suprachiasma nuclei did not change their circadian cycling under blue light and low frequency magnetic field exposure (Fedele et al., 2014b), the question remains open.

In the present study we showed that BHFS LI-rMS in Cry DKO explants was not associated with significant reinnervation while it did induce reinnervation in WT explants. We also demonstrated that this was not secondary to an abnormality of the CRY DKO system: BDNF application to the lesioned hemocerebellum did induce CF-PC reinnervation. Therefore we provide the first direct evidence that cryptochromes are required in mammals for the transduction of LI-rMS into biological effects, such as axon growth and post-lesion neosynaptogenesis. This is of crucial importance in the understanding of the biological, and potential clinically translatable, effects of low intensity magnetic fields that do not directly trigger action potential firing. From these results we can hypothesise that the magnetic field *per se* potentially activates biological cellular pathways through its action on cryptochromes.

## **Conclusions**

Our data show that LI-rTMS can induce olivocerebellar reinnervation *in vivo* and in an organotypic explant model. This reinnervation is dependent on stimulation frequency and pattern, and in particular iTBS and BHFS are effective. We show that this reinnervation requires the presence of cryptochromes as a magnetoreceptor and it also involves intracellular cascades such as appropriate change of gene expression.

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## Figure legends

**Figure 1: LI-rTMS induces transcommissural CF reinnervation *in vivo* after left pedunculotomy in adult mice.**

**A.** Photomicrographs of the cerebellar vermis region of a mouse that received sham treatment after left pedunculotomy. Vglut2-positive innervation (thin vertical white parallel lines) is present in the molecular layer of the right hemocerebellar lobules but absent in the denervated left hemocerebellum. The dotted-line represents the midline between left and right hemicerebella.

**B,C.** Photomicrographs of the left hemocerebellum of a mouse stimulated with LI-rTMS after left pedunculotomy. Vglut2-positive reinnervation (delicate white dotted lines) is present in the molecular layer of the lesioned left hemocerebellum.

**D.** Schematic diagram of the coil (in blue) in relation to the mouse head.

**E.** Diagram of an unfolded cerebellum (adapted from (Willson et al., 2008) showing the amount (score) of Vglut2- labelled CF reinnervation, in 0.5 mm wide parasagittal zones, induced by LI-rTMS.

**F.** Diagram of an unfolded cerebellum (adapted from (Willson et al., 2008) showing the intensity of the magnetic field delivered by LI-rTMS in different regions of the mouse cerebellum.

These experiments and analysis were done by Dr Alex Tang.

**Figure 2: Experimental protocol for LI-rMS to an *ex vivo* model of the olivocerebellar path.**

**A.** Diagram of the pedunculotomy and co-culture procedure. The two hemocerebellar plates are removed from an explant (dark dotted line) at P15 and placed next to the hemocerebellar plates of another intact explant (dark arrows). LI-rMS-induced collateral sprouting of climbing fibre axons (thin white arrow) into the denervated hemocerebellar plate (red dotted arrows) can then be studied.

**B.** Schematic representation of the spatial relation between the LI-rMS stimulation coil and the olivocerebellar explant. The lesioned (denervated) hemocerebellar plates and the ION (both in purple) are stimulated by a homogeneous magnetic field (Grehl et al., 2016).

**C.** Schematic diagram of climbing fibre reinnervation analysis. Hemocerebellar plates were divided into 5 rows (1-5) parallel from the host-graft delimitation. For further analysis, data

from rows 1 and 2 were pooled and called the proximal region, and data from rows 3, 4 and 5 were pooled and called the distal region. Moving along each row, PCs (CB staining in green) found within an ocular grid field were checked for CF reinnervation (small Vglut2-positive puncta, in red, indicated by the white arrows). Vglut2-positive Mossy fibres are larger red-labelled terminals not localised on the PC (white star). The white scale bar at the left bottom corner is 20 $\mu$ m.

**D.** Diagram of the different “complex” pattern of LI-rMS tested in this study. BHFS delivers bursts of 20 pulses at very high frequency (the first 10 at 200Hz and the last 10 at 1.2kHz) which lasts 62.6ms, at a 9Hz frequency (Makowiecki et al., 2014; Rodger et al., 2012). iTBS consist of bursts of three pulses at 50Hz repeated at 5Hz. Trains of bursts last 2s and are repeated every 10s. rITBS is similar to iTBS but the bursts are repeated at random frequencies ranging from 2-60Hz. cTBS is similar to iTBS but the trains of burst are delivered continuously for the 10min of stimulation.

**Figure 3: LI-rMS induced reinnervation is stimulation-pattern-dependant and requires the stimulation of both the ION and the Cerebellum**

**A.** Percentage of reinnervated PCs in the proximal and distal areas of the cerebella plate are determined as the % CB-positive PCs colocalised with VGLUT2-positive terminals. iTBS and BHFS induced significant reinnervation in both the proximal and distal regions compared to sham treatment (One way ANOVA with Tukey *post hoc* pairwise comparison; proximal,  $p=0.000$  for both iTBS and BHFS; and distal,  $p=0.005$  for iTBS and  $p=0.012$  for BHFS; iTBS  $n=8$  and BHFS  $n=11$ ). 10Hz induced significant PC reinnervation in the proximal area compared to sham treatment ( $p=0.048$ ;  $n=8$ ), but to a lesser extent than iTBS and BHFS ( $p=0.001$ ) and did not induced significant reinnervation in the distal area ( $p>0.05$ ). 1Hz, cTBS and rITBS did not induce significant reinnervation compared to sham treatment ( $p>0.05$ ; 1Hz  $n=6$ , cTBS  $n=8$ , rITBS  $n=7$ ). The amount of PCs colocalised with Vglut2 is greater in the proximal region compared to the distal region (Repeated Measure ANOVA;  $p=0.000$ ). Significant differences from sham treatment in denervated explants \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ .

**B.** Scatterplot representation of the % reinnervation as a function of the number of pulses delivered per 10 min session. There is no correlation between pulse number and reinnervation density (Pearson coefficient,  $p=0.353$ )

C. LI-rTMS stimulation of either the upper part of the explant with only the cerebellum (Cbm), or the lower part of the explant with only the brainstem (and thus the ION) does not induce significant reinnervation (One way ANOVA with Tukey *post hoc* pairwise comparison;  $p > 0.05$ ;  $n = 4$ ). Significant differences from sham treatment in denervated explants  $***p < 0.001$ .

**Figure 4: LI-rMS activates c-fos synthesis in Purkinje cells and molecular layer interneurons in the denervated hemicerebellar plate.**

A. An example of c-fos labelling after stimulation by iTBS. In this case c-fos (red) is co-labelled with calbindin (cyan) or parvalbumin (green) to reveal Purkinje cells and interneurons. C-fos labelling is located in some of the Purkinje cell and interneuron nuclei (\*).

B. Histograms show the number of c-fos positive cellular profiles per  $\text{mm}^2$  in sham (non-stimulated) controls, and explants stimulated with iTBS or BHFS ( $n = 8$  for each group; all three groups contain explants from the same 3 litters). Error bars are standard error of the mean. Cellular labelling in comparison to sham:  $***p < 0.0001$  (ANOVA followed by *post hoc* Tukey pairwise comparisons).

C. Histograms showing the percentage of double-stained c-fos/calbindin or c-fos/parvalbumin positive cellular profiles per  $\text{mm}^2$  of the total number of calbindin or parvalbumin positive cellular profiles in sham (non-stimulated) controls, and explants stimulated with iTBS or BHFS ( $n = 8$  for each group; all three groups contain explants from the same 3 litters). Error bars are standard error of the mean. Cellular labelling in comparison to sham treated denervated explant:  $*p < 0.05$   $***p < 0.0001$  (ANOVA followed by *post hoc* Tukey pairwise comparisons).

**Figure 5: Specific LI-rMS patterns modulate gene expression appropriately for PC reinnervation**

A,B,C. Volcano plots showing changes in gene expression induced by BHFS (A), iTBS (B) and riTBS (C) compared to sham treatment ( $n = 5$  for each group). Red dots represent genes that are significantly upregulated ( $p < 0.05$ ); orange dots represent genes that tend to be upregulated ( $0.05 < p < 0.1$ ); dark blue dots represent genes that were significantly down

regulated ( $p < 0.05$ ); light blue dots represent genes that tend to be down regulated ( $0.05 < p < 0.1$ ).

**D.** Heat map showing changes in expression of genes that were either significantly regulated ( $p < 0.05$ ) or tended to be regulated ( $0.05 < p < 0.1$ ) by BHFS, iTBS or riTBS compared to sham. The colour code is the same as in A, B and C.

**Figure 6: Relationships between genes regulated by different LI-rMS pattern.**

Pathway diagram obtained from STRING showing the relationships between genes regulated by different LI-rMS patterns in our study. The colour code of the big circles is the same as in Figure 5. Legends for the different type of protein-protein interactions.

**Figure 7: Cryptochromes are required for LI-rMS induced post-lesion repair.**

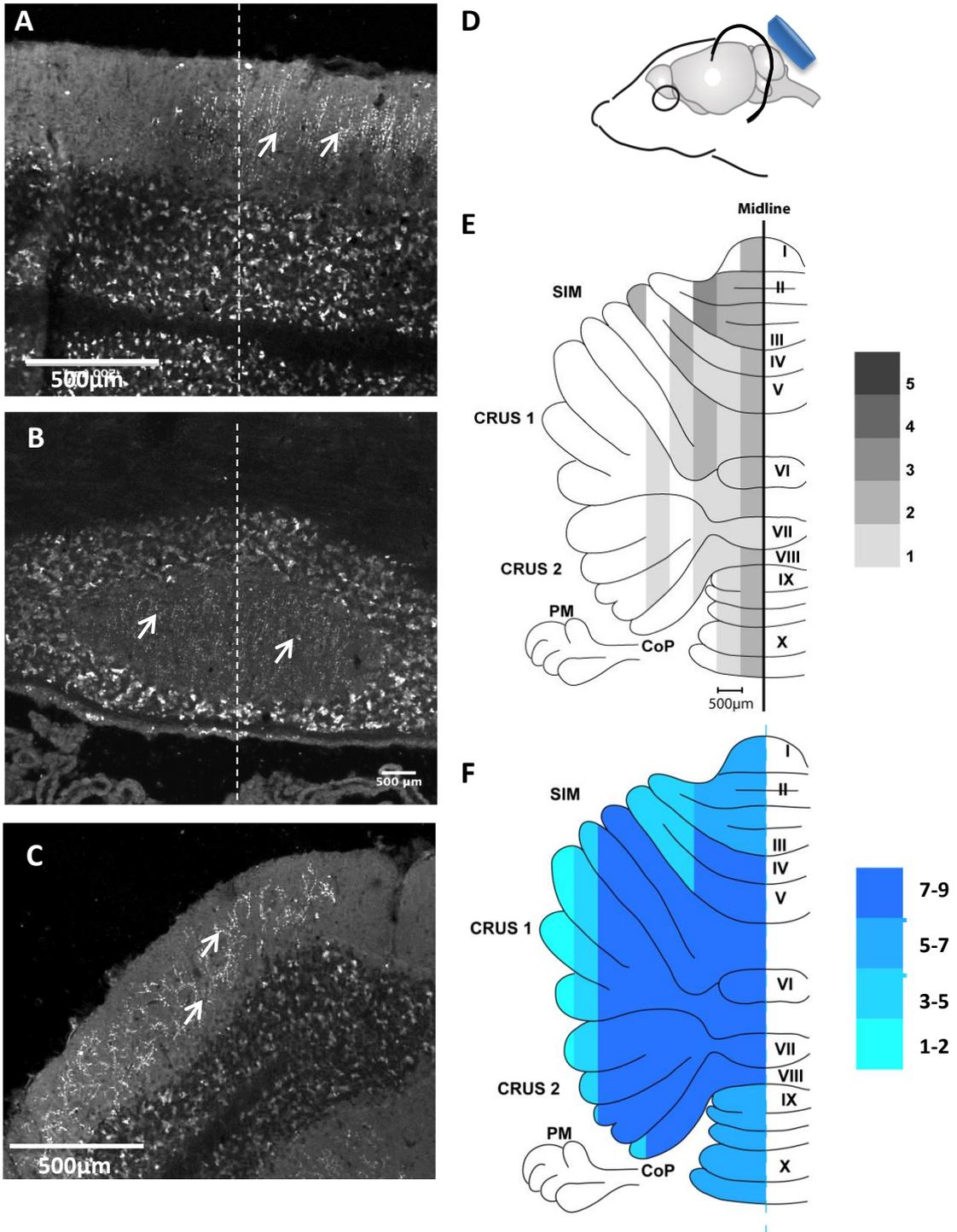
**A.** Immunofluorescent labelling for Foxp2, specific for the ION, in a frontal cryosection of a  $Cry1^{-/-}::Cry2^{-/-}$  fixed explant showing that ION neurons are present and normally-located in the central part of the brainstem.

**B.** In Cry DKO explants, BHFS did not induce significant reinnervation in the proximal zone of the lesioned hemicerebellum compared to sham treatment (One way ANOVA with Tukey *post hoc* pairwise comparison;  $p > 0.05$ ;  $n = 7$ ), whereas BDNF treatment resulted in significant reinnervation in the proximal zone of the lesioned hemicerebellum compared to sham ( $p = 0.000$ ;  $n = 7$ ), to a similar degree as BHFS-induced reinnervation in WT explants ( $p > 0.05$ ). Significant differences from sham treatment in denervated explants \*\*\* $p < 0.001$ .

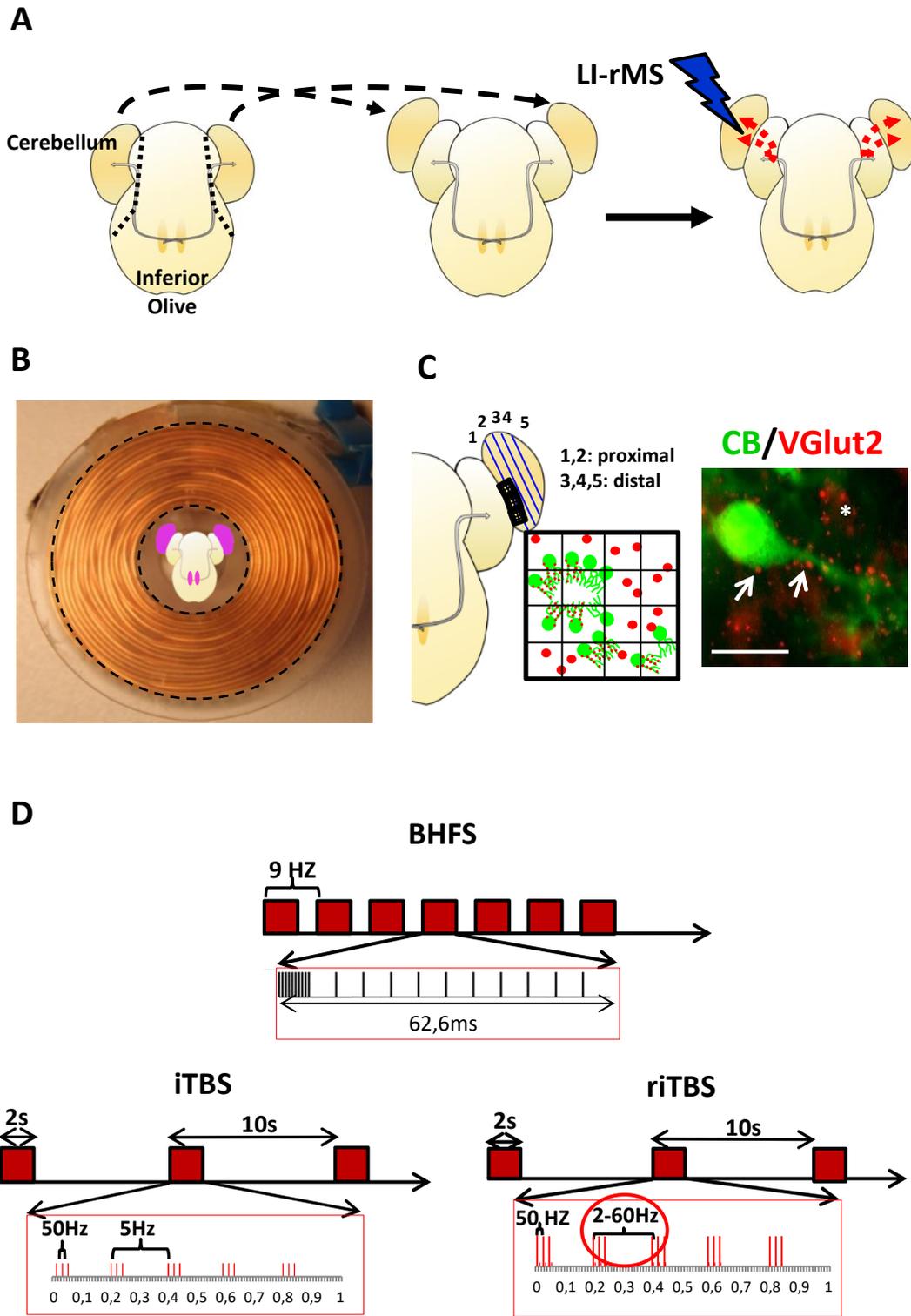
**Table 1: Biological pathways of genes regulated by LI-rMS**

Biological pathways in which LI-rMS regulated genes are implicated. The colour code is the same as in Figures 5 and 6. Gene ontology (GO) terms enrichment was assessed using an *in silico* approach against the mus musculus reference list, using GO biological process, molecular function or cellular component. The Bonferroni correction for multiple testing was applied.

Figure 1



**Figure 2**



**cTBS:** Like iTBS but continuously for 10mn

**Figure 3**

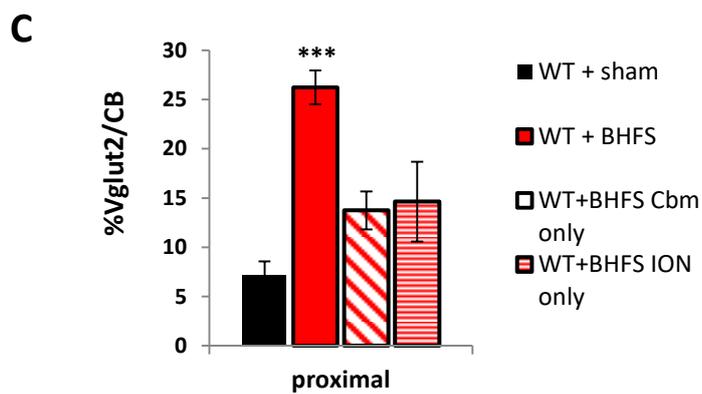
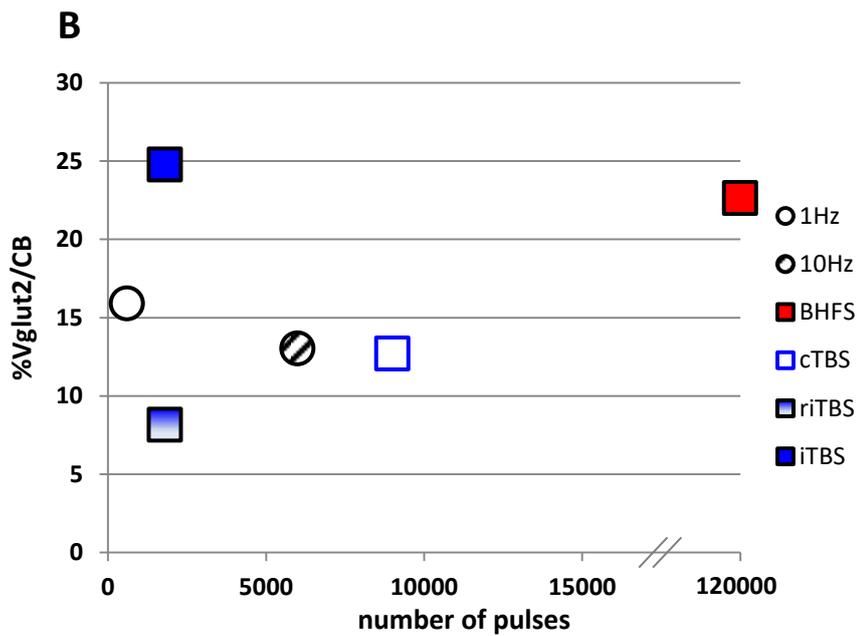
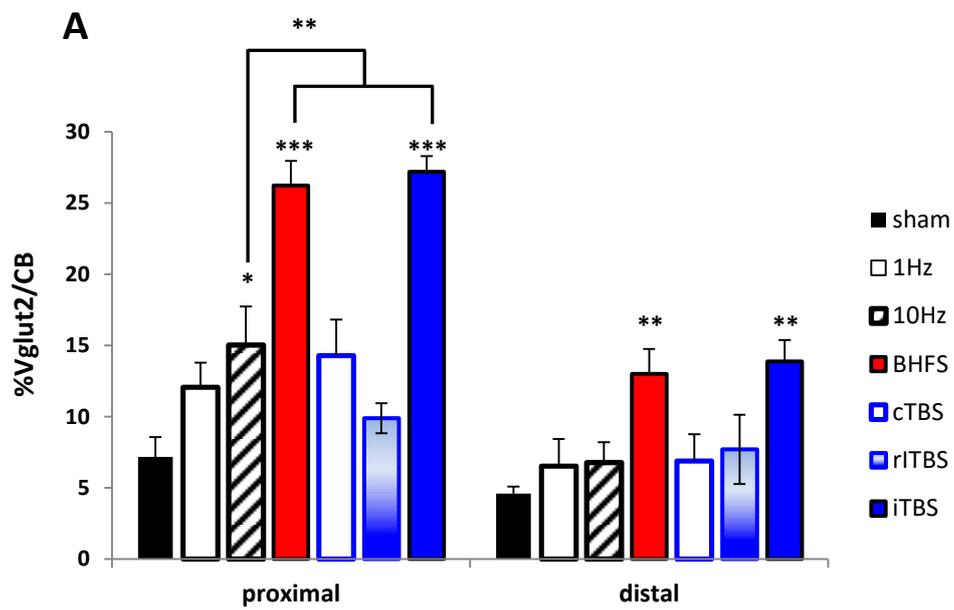
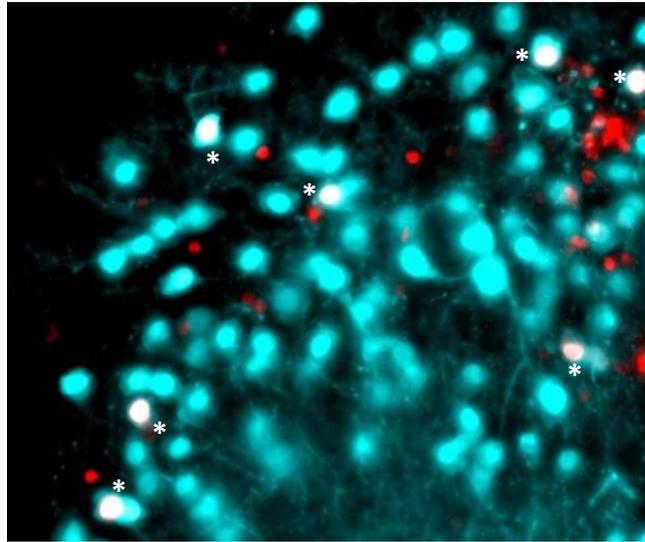


Figure 4

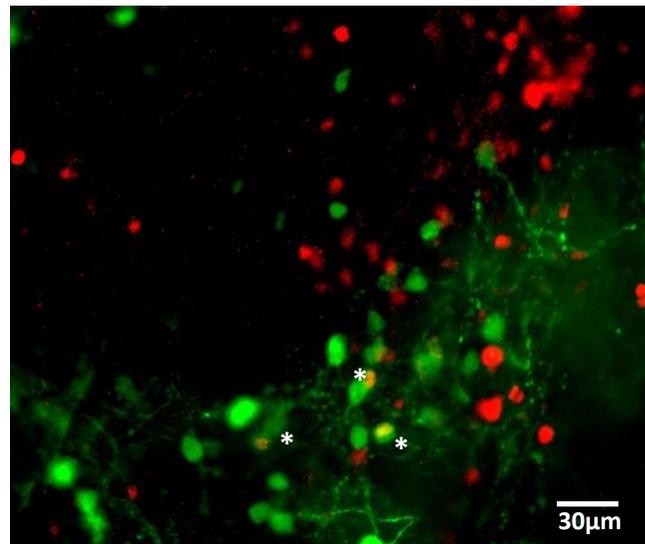
A

CB/cfos

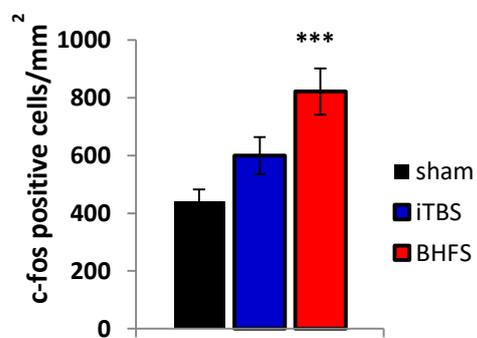


B

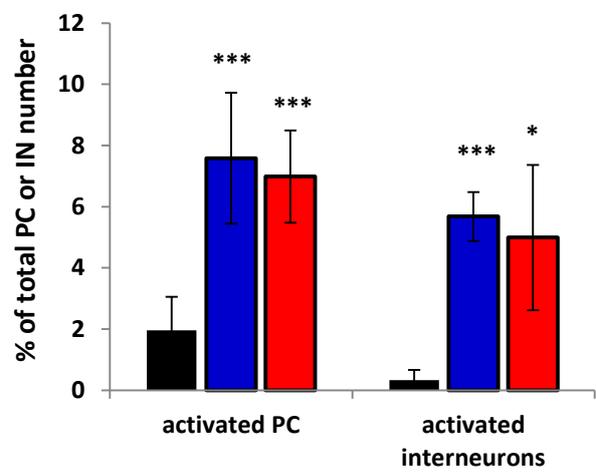
Pv/cfos



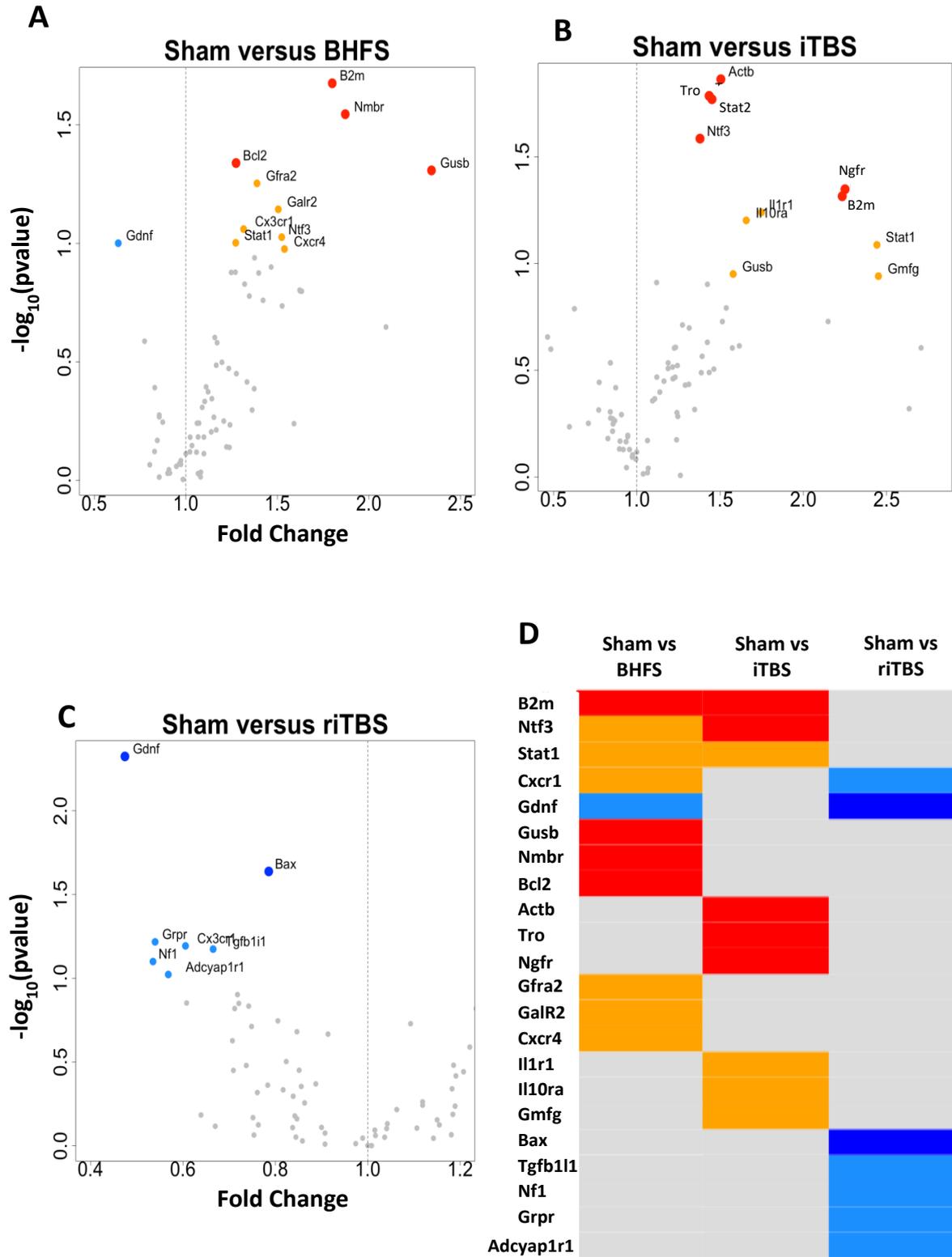
C



D

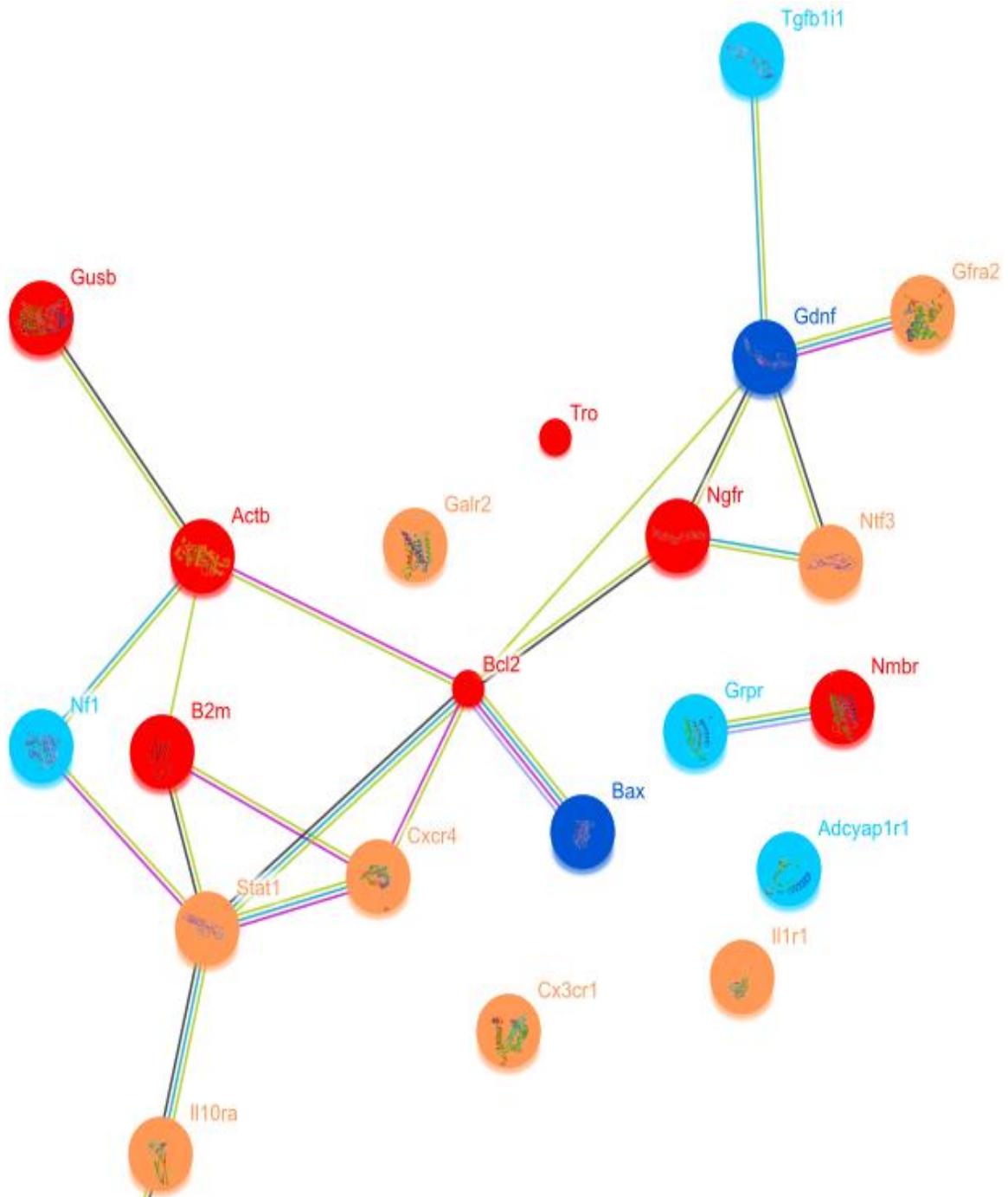


**Figure 5**



**Figure 6**

**A**



**B**

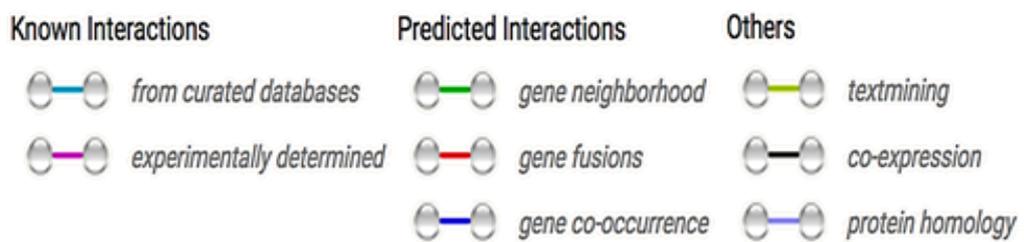
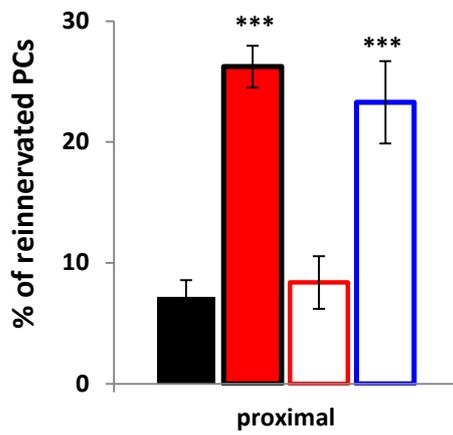
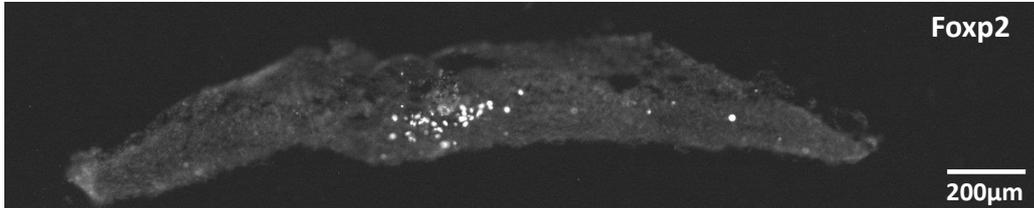


Figure 7

$Cry1^{-/-}::Cry2^{-/-}$  explants



- WT + sham
- WT + BHFS
- DKO Cry 1 and 2 + BHFS
- DKO Cry 1 and 2 + BDNF



**Table 1**

Genes modulated by LI-rMS	Pathway description
Bax,Bcl2,Gdnf,Nf1,Ngfr,Stat1,Tgfb1i1	Regulation of anatomical structure morphogenesis
Bax,Bcl2,Cx3cr1,Gdnf,Nf1,Ngfr,Ntf3,Stat1	Regulation of apoptotic process
Galr2,Nf1,Nmbr,Ntf3	Synaptic transmission
Bax,Bcl2,Cxcr4,Galr2,Gdnf,Gfra2,Nf1,Ntf3	Nervous system development
Bcl2,Cxcr4,Galr2,Gdnf, Ngfr,Ntf3	Neuron development
Bax,Bcl2,Cx3cr1,Cxcr4,Nf1,Ntf3	Brain development
Bax,Bcl2,Cx3cr1,Gdnf,Nf1, Ngfr,Ntf3,Stat1,Tgfb1i1	Regulation of signal transduction
Cx3cr1,Nf1	Regulation of long-term synaptic potentiation
Bcl2,Cxcr4, Ngfr,Ntf3	Axonogenesis
Bcl2,Cxcr4,Ngfr,Ntf3	Axon development
Bcl2,Cx3cr1,Cxcr4,Nf1,Ntf3	Regulation of neurogenesis
B2m,Bcl2,Cxcr4,Ill10ra,Ngfr,Ntf3,Stat1	Response to external stimulus
Bax,Bcl2,Cxcr4,Gdnf,Nf1,Ngfr,Ntf3,Tgfb1i1	Anatomical structure morphogenesis
Cx3cr1,Cxcr4,Ngfr,Ntf3	Chemotaxis
Cxcr4,Ngfr,Ntf3	Axon guidance
Bax,Bcl2,Galr2,Ill1r1,Ngfr,Stat1	Homeostatic process
Adcyap1r1,Cx3cr1,Galr2,Grpr,Nmbr	G-protein coupled receptor signaling pathway
Bax,Bcl2	Neuron apoptotic process
Bax,Bcl2	Response to axon injury
Cx3cr1,Cxcr4	Chemokine-mediated signaling pathway

## **Chapter IV – Article 3**

## Chapter IV – Article 3

### **ROR $\alpha$ haplodeficient mice have reduced responses to LI-rTMS**

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## Abstract

Mechanisms of action of LI-rTMS likely depend upon the structure stimulated, and we know that the age of the animal is important (Chapter II). In this study we wanted to test whether an abnormal neural structure would respond equally well to LI-rTMS. Previous studies have shown that abnormal neural circuits are specifically corrected in a mutant system without overtly perturbing normal axons. We wanted to examine effects of LI-rTMS in the context of a mutation that subtly alters neuronal development and ageing. ROR $\alpha$  is a transcription factor with important roles in the development, maintenance and survival of cerebellar Purkinje cells. In this study we investigated the short-term and long-term effects of LI-rTMS on dendritic morphology, behavior, and post-lesion repair in ROR $\alpha^{+/-}$  haplodeficient mice.

We observed that ROR $\alpha$  haplodeficiency prevents effective post-lesion repair in response to LI-rMS in our olivocerebellar explant model, in contrast to the robust repair observed in with the same stimulation in wild-type explants. Also inferior olivary expression of appropriate levels of ROR $\alpha$  seems particularly important for responding to LI-rMS to induce olivocerebellar axonal outgrowth.

We then examined the potential for LI-rTMS *in vivo* to induce morphological plasticity in Purkinje cell dendrites in ROR $\alpha^{+/-}$  haplodeficient mice, with associated behavioral improvement. This stimulation produced only minor modifications in dendritic spines and branch patterns of adult and aged ROR $\alpha^{+/-}$  mice, and behavior was not modified by the stimulation at either age. We identified some changes in gene expression in the ROR $\alpha^{+/-}$  cerebellum that could partly explain the reduced responses to LI-rTMS, notably in cryptochrome 1, which has a role in magnetoreception.

**Keywords:** ROR $\alpha$ , cryptochrome, LI-rTMS, magnetic fields, cerebellum, reinnervation, Purkinje cells

## Introduction

Low intensity repetitive transcranial magnetic stimulation (LI-rTMS) is gaining popularity as a treatment for neurological dysfunction, and we have shown that it can alter neuronal morphology and behaviour (see Chapter II). Mechanisms of action of LI-rTMS likely depend upon the structure stimulated and we know the age of the animal is important. In this study we wanted to test whether an abnormal neural structure would respond equally well to LI-rTMS. Previous studies have shown that abnormal neural circuits are specifically corrected in a mutant system without overtly perturbing normal axons (Rodger et al., 2012) i.e. that normal neural circuits appear less responsive to external brain stimulation, and this is possibly due to inherent metaplasticity of neural circuits that maintain their stability (Karabanov et al., 2015) We wanted to examine effects of LI-rTMS in the context of a mutation that subtly alters neuronal development and ageing (Boukhtouche et al., 2006a, 2006b; Chen et al., 2013)

We use the mouse cerebellum as a model because of its clearly defined structure and known behavioural functions, and the highly complex dendritic tree of the Purkinje cells (PCs), which are the centre of the cerebellar cortical circuit and integrate multiple afferents to transmit the combined signals to the deep cerebellar nuclei (Cerminara et al., 2015; Ruigrok et al., 2015). The cerebellum and particularly the PCs require the transcription factor  $ROR\alpha$  for normal development and synaptic maintenance.  $ROR\alpha$  is a transcription factor that binds to DNA response elements (Hamilton et al., 1996) and is implicated in ageing pathologies such as atherosclerosis (Mamontova et al., 1998), increased inflammation and associated immune dysfunction (Kopmels et al., 1992), as well as deficits in bone formation and mineralization (Meyer et al., 2000). In the cerebellum,  $ROR\alpha$  is expressed at high levels in Purkinje cells (Hamilton et al., 1996) and plays an important role in Purkinje cell development, maintenance and survival (Boukhtouche et al., 2006; Chen et al., 2013). In mice that lack  $ROR\alpha$ , at one month after birth, there is severe PC loss and degeneration of the cerebellum, which is associated with severe ataxia, tremor and hypotonia and deficits in spatial learning (Sidman et al., 1962; Lalonde et al., 1996; Steinmayr et al., 1998). In contrast,  $ROR\alpha^{+/-}$  haplo-insufficient mice display a normal phenotype at birth and young adulthood (Sidman et al., 1962). However, PC numbers are reduced as early as 1 month of age compared to WTs (Doulazmi et al. 2006) and there are deficits in motor coordination and

balance as young as 3 months (Caston et al. 2003). This gives us a well-defined model to evaluate whether LI-rTMS can ameliorate the abnormalities induced by  $ROR\alpha$  haploinsufficiency.

Animal studies of LI-rTMS are necessary to investigate cerebellar neuronal plasticity at the cellular and functional levels and allow some insight into molecular mechanisms underlying responses to stimulation. In this study we investigated the short-term and long-term effects of LI-rTMS on dendritic morphology, behaviour, and post-lesion repair in  $ROR\alpha^{+/-}$  haploinsufficient mice.

## **Materials and Methods**

### **Animals**

Heterozygous  $ROR\alpha^{+/-}$  mice were maintained on a C57Bl/6J genetic background, and controls were wild-type  $ROR\alpha^{+/+}$  littermates of the same age and gender. Animals were bred at Université Pierre et Marie Curie, Paris, France. They were housed in single-sex, mixed genotype cages on a 12 h light-dark cycle and with water and food *ad libitum*.

To determine the effects of short term LI-rTMS on PC morphology,  $ROR\alpha^{+/-}$  and wild-type mice aged 3-7 months (adult) and 14-17 months (aged) were stimulated for 10 minutes per day for 3 days. At 24 hours following the last stimulation, animals were euthanized for PC visualization.

To determine the effects of long term LI-rTMS on motor function and PC morphology,  $ROR\alpha^{+/-}$  and wild-type animals aged between 3-5 months (adult) and 14-16 months (aged), were transferred to the Centre de Recherche et Développement, Institut de la Longévité, Ivry, France, 2-4 weeks prior to the start of experimental procedures. Mice were treated with LI-rTMS for 4 weeks, followed by 2 weeks of behavioural testing. The same animals were then used for visualization of Purkinje cells.

All animal procedures were carried out under the guidelines established by 'le comité national d'éthique pour les sciences de la vie et de la santé' and approved by the "Comité d'éthique en expérimentation animale Charles Darwin, N° 5".

### **Organotypic Cultures and cerebellar denervation**

Hindbrain explants were cultured from embryonic ROR $\alpha$ <sup>+/-</sup> and ROR $\alpha$ <sup>+/+</sup> mice at embryonic day 14 (E14) as previously described (Letellier et al., 2009). E0 was the mating day. Following anaesthesia and cervical dislocation of pregnant females, the E14 embryos were removed and their brains quickly dissected in ice-cold Gey's balanced salt solution (Eurobio) containing 5 mg/mL glucose. The hindbrain, including the cerebellar anlage and inferior olivary nucleus, was isolated and the meninges removed. The right and left cerebella plates were separated at the midline and the explants transferred onto 30mm Millicell membranes (pore size 0.4 $\mu$ m, Millipore) and cultured at 35°C with medium containing 50% basal medium with Earle's salts (Gibco), 2.5% Hank's Balance Salt Solution (Gibco), 25% horse serum (Gibco), 1mM L-glutamine (Gibco), and 5mg/mL glucose at 35°C in humidified air with 5 % CO<sub>2</sub>. The culture day was designated 0 day in vitro (DIV). The medium was replaced every 2–3 days.

To denervate (Dx) cerebellar tissue and induce olivocerebellar reinnervation, the cerebella plates (grafts) were removed from their explant brainstem at DIV 21 (equivalent to P15) and co-cultured adjacent to the cerebellar plate of an intact explant (host).

### **Genotyping**

Mice were genotyped at weaning by PCR using genomic DNA prepared from mouse tails by incubating in proteinase K. Embryo brains were genotyped using genomic DNA obtained from the forebrain and spinal cord. Primer pairs used were:

5' TGT GAG CGA GTA ACA ACC CGT CGG ATT CT 3',

5' TTC AGG AGA AGT CAG CAG AGC 3',

5' TCA CCG GCT AGT TGG CTG ATT CC 3'.

### **Administration of LI-rTMS**

*In vitro*: Pedunculotomised (Dx) explants were stimulated inside the incubator, 10 min per day for 14 days, using a custom built copper wire coil (10 mm inside diameter, 26 mm outside diameter, 199 turns) placed 4 mm below the well and driven by a 24 V magnetic pulse generator (Grehl et al., 2016). The non-sinusoidal monophasic 300 $\mu$ s pulse had a measured 100 $\mu$ s rise time and generated an intensity of 10 mT at the explant without sound

or vibration above background (Grehl et al., 2016). Each culture plate was isolated using mu-metal to ensure no eddy current spillover.

*In vivo*: LI-rTMS was applied to the cerebellum, as previously described (Rodger et al., 2012; Makowiecki et al., 2014). Focal cerebellar stimulation was induced with a custom made magnetic coil designed for use on mice (copper wire, 300 windings, 16Ωm, inner diameter 5mm, outer diameter 8mm), and powered by an electromagnetic pulse generator (EC10701; Global Energy Medicine Pty Ltd, Perth, Western Australia). The intensity of the magnetic field was 9mT at the tissue and was measured using a Hall device magnetic field detector. For LI-rTMS treatment, animals were placed in a small box and the stimulation coil held directly above the scalp, without touching it, over the cerebellar region, for 10 minutes each day. To minimize stress, adult or aged mice were handled and habituated to the researcher and to the small box for 5 days prior to the start of stimulation. The mice showed no signs of discomfort or stress during or after the stimulation, and explored the box or groomed during the procedure. Control mice received sham stimulation: they were handled identically to those that received the LI-rTMS, but the stimulator was turned off. The sound emitted by the coil was tested previously (Rodger et al., 2012) and was below the audible threshold of mice. Previous tests have shown that this low-intensity stimulation does not change the coil temperature (Makowiecki et al., 2014).

In both types of experiment, the frequency used for stimulation was a high frequency, complex pattern, comprised of 59.9ms trains of 20 pulses at 3 different frequencies as follows; 1 min at 6.71Hz, 8 min treatment at 10.1Hz and 1 min at 6.26Hz (Rodger et al., 2012; Makowiecki et al., 2014), which is based on the Patent PCT/AU2007/000454 (Global Energy Medicine Pty Ltd., Western Australia).

## **Behavioural analysis**

### ***Open field- General motor activity***

To assess general motor activity and anxiety, animals were placed in an open field arena (square 500cm x 500cm) box and recorded for 5 minutes using Ethovision X10 software (Noldus Information Technology, France). Parameters measured were the total distance

moved and time spent moving, as well as time spent in the centre and the periphery of the box.

### ***Grip strength***

Grip strength of the two front paws was measured using a Grip strength meter (Ugo Basile). The animals were picked up by the tail and the 2 front paws placed on the bar of the grip meter and the tail pulled gently backwards until the animals lost their grip. The test was repeated three times and the average grip strength was used and divided by weight of the animals to correct for differences in weight.

### ***Catwalk- Analysis of gait***

The gait of each animal was analysed using the CatWalkXT (Noldus Information Technology, France). The catwalk consisted of a 7 cm x 2m corridor with a lit-up glass runway floor, through which mouse gait was filmed by a camera placed under the walkway. Two-three trials, where the animal walked in a straight trajectory, with constant speed between 15- 30 cm/s, were analysed. Parameters measured were stride length, stride width (distance between hind paws), paw angle (from the axis of the walking direction) of the hind paws and regularity index (% of regular step patterns). Results were averaged for each animal.

### ***Rotarod- motor coordination***

To test cerebellar motor function and coordination, we used an accelerating rotarod (TSE Systems, Bad Homburg, Germany) with a 3 cm diameter rod. The accelerating protocol consisted of an initial speed of 4 rpm and an increase of 4rpm every 30 seconds for a maximum of 5 minutes (40 rpm). Animals were placed on the rotating rod and the latency to fall was measured. Mice received 3 trials per day for 5 days and the average latency to fall was calculated for each day.

### ***Morris Water Maze- spatial learning and memory***

#### ***Learning phase***

To test spatial learning, mice were placed in a circular water tank (120 cm diameter) filled with water (23°C) and were trained to find an invisible clear Plexiglas platform (10cm diameter) hidden 1 cm under the surface of the water. External cues were placed on each wall in the room and were kept constant for all experiments. The animals were given 6 trials per day for 5 consecutive days, with the platform always in the same (northwest) quadrant. In each trial, the mice were released from one of 4 randomly-selected starting points (north, east, south or west). Mice were released facing the tank wall and were given 60 seconds to

locate and climb onto the platform. If the mouse did not find the platform in that period, it was retrieved by the experimenter, placed on the platform, and left there for 15 seconds. Mice were tracked using with EthoVision XT software (Noldus Information Technology, France). Parameters that were measured were latency to platform, path length, time spent in the northwest quadrant, swim velocity, mean distance from the platform during the swim and the percentage of direct swims. For each day, the average for the six trials for each animal was calculated.

#### *Probe test*

To assess spatial memory, two hours following the final trial on the final day, a probe trials was performed. The platform was removed and the animals were released from the south or east positions. Parameters measured were the latency to the platform, frequency of crossings and duration over the platform, time spent in the northwest quadrant, mean distance from the platform and number of direct swims.

#### *Visual Platform test*

To ensure that any navigation deficit was not caused by a decreased ability to orientate and swim to the platform, on the final day, we performed two visible platform tests. The platform was moved to the south east quadrant and was raised above the surface with a highly visible object marking its position and with a light shining directly onto it. Parameters measured were distance moved, latency to platform and swim velocity.

### **Electrophysiological recordings and biocytin filling**

Sagittal cerebellar slices were prepared from LirTMS-treated animals and sham-treated controls. Mice were sacrificed by rapid decapitation and the cerebellum removed, and sagittal vibratome slices (250  $\mu\text{m}$ ) were cut from the cerebellar vermis at 4°C in an oxygenated external solution (125 mM NaCl, 2.5 mM KCl, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 25 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM Glucose). The slices closest to the midline were used for patch clamping.

Whole-cell patch-clamp recordings were made from Purkinje cells, in the same external solution, at room temperature. PCs were identified in lobules VI and VII, with an attempt to fill an equal number of cells at the external surface of the lobule and deeper on the lobule bank. Patch pipettes were filled with an internal solution containing 120 mM Cs-D-gluconate,

13 mM biocytin, 10 mM HEPES, 10 mM BAPTA, 3 mM TEACl, 2 mM Na<sub>2</sub>ATP, 2 mM MgATP, 0.2 mM NaGTP, pH 7.3, 290–300 mOsm.

## **Immunohistochemistry and Histological Analysis**

### *Olivocerebellar Reinnervation*

The effects of LI-rMS on olivocerebellar reinnervation were evaluated by immunohistochemistry. 24h after the last (14th) day of stimulation explants were fixed with 4% paraformaldehyde for 4h at 4°C. Fixed tissue was rinsed 3x5 min in phosphate buffered saline (PBS) containing 0.25% Triton X (PBS-T) and blocked in 20% donkey serum for 2h at RT prior to incubation overnight at 4°C in primary antibody diluted in PBS-TG (PBS-T containing 0.2% gelatine and 0.018g/ml L-Lysine). The next day explants were washed 3x5 min in PBS-T and labelling was visualised with fluorescent-conjugated secondary antibodies in PBS-TG for 2h at RT. Finally, explants were rinsed and mounted in Mowiol.

To identify CF reinnervation, Purkinje cells were labelled with rabbit anti-calbindin-28k (CB) antibody (1:3000; Swant; Celio, 1990) and CF terminals with polyclonal guinea pig anti-VGLUT2 antibody (1:2000, Millipore; Letellier, et al., 2009). Primary antibodies were visualised using Cy3-conjugated donkey anti-guinea pig and Alexa Fluor (AF) 488-conjugated donkey anti-rabbit (1:200 and 1:400 respectively; Jackson Laboratories).

Labelled explants were examined using epifluorescence microscopy (DM 6000F; Leica) and z-stack images taken for analysis. The amount of CF reinnervation was measured by the number of CB-positive PCs (soma and primary dendrites) co-localised with VGLUT2 per field of view (grid) and expressed as percentage PCs per field. This quantification was made systematically on z-stacks taken in rows through the cerebellar graft with increasing distance from the host-graft interface. Data from rows 1 and 2 were defined as the proximal zone, and those from rows 3-5 were defined as the distal zone.

### *Purkinje cell morphological analysis*

*Biocytin revelation:* After filling multiple PCs, slices were fixed in 4% paraformaldehyde overnight at 4°C. To visualize the biocytin-filled cells, slices were washed in phosphate buffered saline (PBS) containing 0.25%Triton X-100 (PBS-T 0.25%). In order to reduce background staining, sections were incubated for 30 min in 70% methanol at room

temperature. Sections were then washed in PBS-T 0.25%, and incubated for 2 hours at room temperature with Streptavidin AF488 conjugate (1:400; Invitrogen, Molecular Probes) diluted in PBS- containing 0.25% Triton X, 0.2% gelatine, and 0.1M lysine (PBS-TG). Following washes with PBS-T 0.25%, slices were mounted on slides with Mowiol (Sigma) mounting medium.

*Confocal imaging:* Image stacks of Purkinje cells were acquired using a confocal laser-scanning microscope (Leica TCS SP5, Leica), at 40x (NA=1.25) or 63x (NA=1.4) objective under oil. Images were acquired with an argon 488 laser in 570-620nm emission range, with a step size of 0.5  $\mu\text{m}$ . For analysis of PC morphology, the images were flattened using the maximum intensity for each pixel, and the resulting image was used for analysis. The properties of the PCs measured were total area of the dendritic tree, height (measured from the soma along a straight line perpendicular to the edge of the dendritic tree closest to the edge of the molecular layer), apical width, basal width, and width halfway between the basal and apical ends. The average width of the dendritic tree was calculated. The length and width of the primary dendrite was also measured, as well as the soma area. The image was also thresholded, to eliminate any staining below a certain intensity, and the fraction of the area containing any labelling was measured, allowing calculation of the total occupied dendritic area. The level of thresholding was established for each cell, at a point where dendritic spines could still be seen but where as much background as possible was eliminated. Results were expressed as the average  $\pm$  the SEM for each group.

*Sholl analysis:* To measure dendritic branching complexity, we performed Sholl method on the z-stacks of biocytin labelled PCs in lobules VI and VII. First, the NIH ImageJ plugin tubeness ( $\sigma$  value of 0.8; <http://rsb.info.nih.gov/ij/index.html>) was applied to the image stack to reinforce tubular structures (i.e., dendrites). Images were then converted to 8-bit grayscale and flattened. For each neuron, the NIH Image J plugin Sholl analysis (Ferreira et al. 2014) was used to count the number of intersections of dendrites with a series of concentric spheres at 10 $\mu\text{m}$  intervals from the centre of the cell body, with a starting radius of 10 $\mu\text{m}$  and the ending radius the length of the dendritic arbour.

*Spine density and morphology:* To analyse spines, two separate distal dendrites of one PC located in lobule VI and VII were imaged (3-5 PCs per animal) using a confocal laser-scanning microscope (Leica TCS SP5, Leica). Images were taken with an objective of 63x (NA= 1.4) under oil, with an optical zoom of 4 (final optical magnification = x252), and a pinhole of 1 airy unit, at a step size of 0.13mm. Images were deconvoluted using Huygens 3.7 software (Scientific Volume Imaging). Theoretical PSF was used and the Maximum Likelihood Estimation algorithm was performed in classical mode with 150 iterations,. Background intensity was averaged from the voxels with lowest intensity, and signal to noise ratio values was set to 20. For each neuron, a tertiary distal dendritic segment of 20-40  $\mu\text{m}$  in length was measured. Dendritic spines were analysed with Neuronstudio software (Rodriguez et al. 2008); version 9.92; <http://research.mssm.edu/cnic/tools.html>). The dendrite was reconstructed and the spines were automatically detected and categorized as thin, stubby or mushroom spines. Manual correction was required for a minority of spines. The number of spines for each category and the total number for each cell was counted and expressed as the density of spines per 10 $\mu\text{m}$  of dendrite. The head diameter and spine length were automatically calculated by the software. Spine head diameter corresponds to the rayburst diameter (i.e. the minimal diameter of the ellipse describing the spine head, calculated in the xy axis). Data generated by Neuronstudio were saved in text file format, which was imported into graph pad prism to create frequency distribution of spine head diameter and length for each group. (Heck et al. 2012).

### **qRT-PCR**

To investigate the molecular events triggered by LI-rTMS that may be involved in neuronal plasticity of  $\text{ROR}_{\alpha}^{+/-}$  PCs, changes in gene expression were examined in the cerebellum of  $\text{ROR}_{\alpha}^{+/-}$  mice of 3-5months (adult) or 15-16 months (aged), 6h after the last of 3 daily LI-rTMS sessions. Total RNA was extracted from cerebellar tissue using Trizol (Life Technologies) according to manufacturer's instructions (Chomczynski and Sacchi, 1987) and RNA concentration was measured by a Nanodrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA,USA) before being stored at at  $-80^{\circ}\text{C}$ . 1 $\mu\text{g}$  of total RNA was reverse transcribed in 20 $\mu\text{l}$  using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). cDNA was amplified on a LightCycler<sup>®</sup> 480 (Roche Applid Biosystems, USA) in

10µl reaction volume using SYBR Green I Master Mix (annealing temperature 58°C, 50cycles)

House keeper primers were TUB5 and ARBP.

TUB5 : Forward GCTAAGTTCTGGGAGGTGATAAGG,

Reverse CCAGACTGACCGAAAACGAAGTTG;

ARBP: Forward TGCCAGCTCAGAACACTGGTCTA,

Reverse GGGAGATGTTTCAGCATGTTTCAGCA;

Cry1 Forward GTGGATCAGCTGGGAAGAAG,

Reverse CACAGGGCAGTAGCAGTGAA ;

Cry2 Forward TGACCTAGACAGAATCATCGAACTG,

Reverse GGCTGATGAGGGCCTGAA ;

RORα Forward ACCGTGTCCATGGCAGAAC,

Reverse TTTCCAGGTGGGATTTGGAT ;

MMP2 Forward CCACGTGACAAGCCCATGGGGCCCC,

Reverse GCCAGCTCAGCAGCCTAGCCAGTCG

All samples were amplified in triplicate and the mean was used to calculate gene expression in each tissue sample. Raw data were pre-processed with Lightcycler 480 software (Roche Applied Biosystems, USA) according to Pfaffl's equation. Target gene expression was normalised to the harmonic mean of 2 housekeeper genes.

### **Statistical analysis**

All data were tested for normal distribution and homogeneity of variance and squareroot or  $\log_e$  transformation were applied if needed. When appropriate, two-way ANOVA and Bonferroni post hoc tests with treatment and age as between-subject factors were used. If interactions between factors were observed one-way ANOVA and pairwise comparisons with Tukey post hoc tests were performed. When data did not fulfil the requirements for parametric tests, Kruskal-Wallis with Dunn-Bonferroni correction and Mann-Whitney U tests were used.

For the learning phase of Morris Water Maze two-way ANOVA repeated measures tests were done. For the rotarod task, Friedman non parametric RMANOVA followed by Wilcoxon ranked tests were performed. For spine morphology analysis Kolmogorov-Smirnoff distribution tests were employed. For gene expression analysis, t tests with Bonferroni

correction for multiple comparisons were performed. Statistical analyses were performed using GraphPad Prism version 7 and SPSS version 22 software.

## **RESULTS:**

In this study we used the  $ROR\alpha^{+/-}$  mouse cerebellum to evaluate the effects of LI-rTMS on a mildly perturbed central nervous system structure, whose development is essentially normal but which undergoes prematurely an 'age-related'-type of neuronal loss, in adult (4-6 months) and aged (15-17 months) mice. We examined the potential for LI-rTMS to induce plasticity in the system at cellular (PC dendritic tree morphology), behavioural, and circuit (CF-PC reinnervation) levels.

### **Post-lesion repair by LI-rMS is impaired in $ROR\alpha$ haplodeficient hindbrain explants**

Our team has previously shown that BHFS LI-rTMS can promote retraction of aberrant axon terminals to correct an aberrant neural circuit (Makowiecki et al., 2014; Rodger et al., 2012) but has a frequency dependent effect on neurite outgrowth (Grehl et al., 2015). However, in Chapter III we showed that high frequency patterns of LI-rMS induced reinnervation of denervated PCs in our olivocerebellar lesion model in vitro, as observed by VGLUT2-positive terminals, which localize around the PC somata and primary dendrites.

We thus treated lesioned explant cultures from  $ROR\alpha^{+/-}$  or WT embryos with the same pattern of BHFS stimulation that promoted significant reinnervation in WT explants, and quantified climbing fibre reinnervation of PCs in the grafted cerebellar plate (Fig 1A).

The proximal part of the graft consistently received more reinnervating fibres than distal zones (Repeated measures ANOVA,  $F_{1,33}=100.970$ ,  $P=0.000$ ), so we analysed proximal and distal compartments separately. BHFS-stimulated WT explants have significantly more calbindin-positive PCs co-localized with VGLUT2 than sham controls (1 way ANOVA, Tukey post-hoc: proximal compartment,  $p=0.000$ ; distal compartment,  $p=0.000$ ) as described in Chapter III. In contrast, in  $ROR\alpha^{+/-}$  explants there was very little reinnervation in the proximal compartment after LI-rMS, which was not different from sham (1 way ANOVA, Tukey post-hoc,  $p=0.962$ ), and no reinnervation observed in the distal zone (Fig 1B).

Given this unexpected reduction in reinnervation we checked whether this was  $ROR\alpha^{+/-}$  PCs that could not be reinnervated or the  $ROR\alpha^{+/-}$  ION that could not grow reinnervating axons. We thus co-cultured mixed-genotype explants, confronting WT brainstems with  $ROR\alpha^{+/-}$  grafted cerebellar plates, or vice versa. We found that BHFS-induced reinnervation is partially restored in the proximal region of the graft, if the reinnervating olivocerebellar axon comes from a WT inferior olivary nucleus ( $p=0.001$ ). However this LI-rTMS induced reinnervation was still significantly less than in normal WT explants ( $p=0.003$ ). Moreover in the distal zone, reinnervation was not significantly greater than sham treated explants ( $p=0.107$ ). In contrast, if only the cerebellar plate is wild-type, reinnervation by  $ROR\alpha^{+/-}$  ION axons is not different from sham controls in either the proximal or distal zones ( $p=0.224$ ) and is effectively the same as that observed when both components (reinnervating ION and denervated PCs) were  $ROR\alpha^{+/-}$  (Fig 1B).

These results indicate that the  $ROR\alpha^{+/-}$  nervous system is much less able to respond to LI-rTMS to grow reinnervating axons, in this case climbing fibres. This is supported by the greater disruption caused by  $ROR\alpha$  haploinsufficiency in the ION than its absence in the cerebellum.

#### **Four weeks LI-rTMS and psychomotor activity moderately alters PC dendritic morphology**

The induction of post-lesion axonal outgrowth and neosynaptogenesis comprise a major degree of plasticity in the mature mammalian central nervous system, which does not happen spontaneously and is extremely difficult to induce, even partially (Chen and Zheng, 2014). Therefore, we next carried out 4 weeks LI-rTMS followed by behavioural testing to adult and aged  $ROR\alpha^{+/-}$  mice, to see whether they were able to develop PC dendritic plasticity to improve deficits in motor and spatial learning and spatial memory (Caston et al., 2003, 2004).

#### *$ROR\alpha^{+/-}$ PC morphology changes little with age*

Given that it has been proposed that  $ROR\alpha$  haploinsufficient mice may be a model of precocious ageing, due to their premature PC loss (Doulazmi et al., 2006; Hadj-Sahraoui et al., 2001), we established baseline PC dendritic morphology in mice aged 3-4 months ("adult") and 15-17 months ("aged"). No differences were found between PC dendritic tree

height, width, or occupied area (1-way ANOVA, Bonferroni post-hoc tests: height  $F_{1,39}=2.2557$   $p=0.1118$ ; width  $F_{1,39}=0.809$   $p=0.374$ ; area  $F_{1,39}=0.408$   $p=0.527$ ; Fig 2A-C), nor in dendritic complexity following Sholl analysis (Mann Whitney U,  $p > 0.05$ ; Fig 2D). However, compared to adults, aged PC dendrites had signs of less robust synaptic input with lower spine density ( $F_{1,26}=5.100$ ,  $p=0.033$ ; Fig 2E-G), in particular of thin/mushroom spines ( $F_{1,26}=6.029$ ,  $p=0.021$ ), which were also thinner (Kolmogorov-Smirnoff test for cumulative distribution,  $p=0.0019$ ; Fig 2H). Thus the  $ROR\alpha^{+/-}$  PC dendritic arbor changes little during aging from 3-4 months to 15-17 months, showing only spine changes; these observations served as a point of comparison for the subsequent analysis of stimulated and sham-treated animals.

#### *Chronic LI-rTMS and behaviour testing moderately alters PC dendritic morphology*

Sham-stimulated, handled aged  $ROR\alpha^{+/-}$  animals had larger PC dendritic trees compared to equivalent adult  $ROR\alpha^{+/-}$  animals (Kruskal-Wallis tests: width,  $p=0.000$ ; height,  $p=0.000$ ; area,  $p=0.009$ ; Fig 3A-C). They also had more distal dendritic branching (Kruskal-Wallis,  $p < 0.05$ ; Fig 3D) compared to adults. But no effect of LI-rTMS was found for any of these measures.

As for the naive animals described above, spine density was lower in aged  $ROR\alpha^{+/-}$  PCs than in adults (Kruskal-Wallis with Dunn-Bonferroni correction; sham,  $p=0.018$ ; LI-rTMS,  $p=0.019$ ; Fig 4A,B) and did not change with LI-rTMS for either group (Kruskal-Wallis,  $p=0.576$ ). However, LI-rTMS did change spine morphology, reducing length (Fig 4D) in adult (Kolmogorov-Smirnoff test,  $p=0.0004$ ) and aged  $ROR\alpha^{+/-}$  PCs ( $p=0.0453$ ), but altering spine head diameter (Fig 4C) differently according to age: reducing it in aged  $ROR\alpha^{+/-}$  PCs (Kolmogorov-Smirnoff test,  $p=0.0336$ ), but increasing it in adult  $ROR\alpha^{+/-}$  PCs (Kolmogorov-Smirnoff test  $p=0.0182$ )

#### *Handling and behavioural testing in aged $ROR\alpha^{+/-}$ mice altered PC dendritic complexity and spine density*

Long-term treated animals undergo 7 weeks of daily handling during the stimulation period and the behavioural testing. We thus compared sham-treated  $ROR\alpha^{+/-}$  animals from the short-term (3-day) treatment group with the long-term (4 week) sham-treated animals, to identify whether handling and behaviour testing alone could alter PC dendritic morphology. Analysis of dendritic tree height, width, dendritic density, and branching complexity showed

that aged  $ROR\alpha^{+/-}$  PCs were more sensitive to handling stimulation than adult cells. In the aged long-term handling group, PC dendritic arbor width was increased (Kruskal-Wallis with Dunn-Bonferroni pairwise comparison correction, height and area,  $p>0.05$ , width,  $p=0.013$ ; Fig 5A-C), and there was greater branching in the distal tree (310-380  $\mu\text{m}$  from the soma; Kruskal-Wallis,  $p<0.05$ ; Fig 5D). There was no difference in spine density (Two way ANOVA:  $F_{1,68}=0.017$ ,  $p=0.896$ ; data not shown), although head diameter of thin/mushroom spines decreased (Fig 6A) and their length increased (Fig 6B) in both adult and aged groups as a result of handling (head diameter: adult and aged,  $p<0.001$ ; length: adult,  $p=0.0035$  and aged,  $p<0.0001$ ).

### **Chronic LI-rTMS treatment does not alter motor or spatial behaviour of $ROR\alpha^{+/-}$ animals**

We have shown that wild-type mice treated with LI-rTMS have improved spatial memory in association with increased PC dendritic tree size and branching complexity (See Chapter II). We did behavioural testing of the  $ROR\alpha^{+/-}$  mice to see whether LI-rTMS could ameliorate deficits in behaviour, despite only-minor morphological changes. We carried out a series of tests of activity, strength, and motor coordination, and then tested spatial learning and memory.

As for the WT animals, aged  $ROR\alpha^{+/-}$  mice were weaker and less active in the open field (open field: aged less active than adult, Kruskal-Wallis  $p=0.029$ ; lower grip strength for aged, Kruskal-Wallis  $p=0.000$ ; data not shown), and less coordinated on the rotarod than adult  $ROR\alpha^{+/-}$  mice (more foot-slips for aged, Two-way ANOVA,  $F_{1,26}=29.947$   $p=0.000$ ; aged fell from the rotarod sooner than adults, 2-way ANOVA  $F_{1,34}=19.510$ ,  $p=0.000$  for day 1;  $F_{1,34}=8.656$ ,  $p=0.006$  for day 2; Fig 7). But LI-rTMS had no effect on these deficits.

Also in the Morris Water Maze test of spatial learning and memory there were no treatment-dependent differences either during the learning phase (2-way ANOVA,  $p>0.05$ , swim velocity – not shown; distance travelled – Fig 8A; time in the platform quadrant – Fig 8B; escape latency – Fig 8C) or during the probe test of spatial memory (2-way ANOVA with Bonferroni post-hoc,  $p>0.05$ ). The only differences observed were that aged mice spent less time near the platform region compared to adults (2-way ANOVA,  $F_{1,45}=9.175$ ,  $p=0.004$ ; Fig 9).

We conclude that  $ROR\alpha^{+/-}$  mice fail to respond to long-term LI-rTMS with improved spatial memory, consistent with the minor PC morphological change in response to LI-rTMS. Moreover, the changes in PC dendrites and spines in aged  $ROR\alpha^{+/-}$  mice are induced by handling and behaviour testing rather than LI-rTMS, and do not convey a functional behavioural response.

### **$ROR\alpha^{+/-}$ PC responses to acute LI-rTMS treatment are small and differ with age**

Given the apparent lack of sensitivity of  $ROR\alpha^{+/-}$  neural tissue to demonstrate sufficient neuronal plasticity to change PC dendritic morphology, spatial behaviour or post-lesion axon collateral outgrowth, we decided to assess whether there was any measurable cellular response to LI-rTMS at all. We applied 3x10 min daily sessions of LI-rTMS to the cerebellum of adult and aged  $ROR\alpha^{+/-}$  mice then compared dendritic tree morphology, spine density and gene expression with sham-treated controls.

Although no differences were found in the global dendritic tree height, area, or complexity between sham and stimulated groups (Two way ANOVA: height  $F_{1,75}=1.102$ ,  $p=0.297$ ; area  $F_{1,75}=0.319$ ,  $p=0.574$ ; complexity Kruskal-Wallis,  $p>0.05$ ; Fig 10A,C,D), in contrast to the non stimulated PCs, LI-rTMS exposed aged PCs had narrower dendritic trees than LI-rTMS treated adult PCs (pairwise comparison with Tukey post-hoc  $p=0.02$ ; Fig 10B). This suggests that LI-rTMS tends to have a different effect on young adult PCs (increasing width) than aged PCs (reducing width). Stimulation also altered PC dendritic spines, most clearly in aged rather than adult groups: in contrast to adult PCs, in comparison to the sham-treated group LI-rTMS increased spine density (Kruskal-Wallis and Dunn-Bonferroni corrections,  $p=0.001$ ; Fig 11A-C), specifically stubby spines ( $p=0.003$ ), shortened spine length (Kolmogorov-Smirnoff test,  $p<0.0001$ ; Fig 11F lower graph) and increased head diameter ( $p<0.0001$ ; Kolmogorov-Smirnoff tests; Fig 11E lower graph) in aged animals.

To try to understand why the  $ROR\alpha^{+/-}$  neuronal tissue was less responsive to LI-rTMS, we looked at the expression of a gene involved in the regulation of PCs dendrites and spine morphogenesis, MMP2 (Verslegers et al., 2015), and genes involved in magnetoreception,

cryptochrome 1 and 2, and ROR $\alpha$  itself which is known to interact with Cry (Akashi and Takumi, 2005; Duez and Staels, 2009; Sato et al., 2004).

Cry1 was expressed at a lower level in sham-treated adult ROR $\alpha$ <sup>+/-</sup> cerebella than in corresponding WT cerebella (t-test with Bonferroni correction for multiple comparison;  $p < 0.026$ ); the LI-rTMS-treated group showed that stimulation tended to restore this expression ( $p = 0.096$ , n.s.). No other significant differences were found for adult animals (Figure 12).

Aged ROR $\alpha$ <sup>+/-</sup> animals showed clearer modifications of gene expression. Notably, MMP2 was significantly higher in sham-treated aged ROR $\alpha$ <sup>+/-</sup> mice (compared to WT adult sham; t-test,  $p = 0.000$ ) and ROR $\alpha$  expression was lower ( $p = 0.014$ ). LI-rTMS treatment tended to reduce the overexpression of MMP2 (t-test,  $p = 0.072$ ; n.s.).

Taken together, these results indicate that some elements of magnetoreception (CRY1) and spinogenesis (MMP2) are altered in ROR $\alpha$ <sup>+/-</sup> cerebella, which may contribute to the blunted plastic response to LI-rTMS by ROR $\alpha$ <sup>+/-</sup> PCs.

## **DISCUSSION :**

In a previous study (Chapter II) we showed that LI-rTMS can alter PC dendritic morphology and spine density, as well as spatial memory, and that these effects were dependant on the age of the animal. In this study we extended those experiments to a known model of abnormal cerebellar structure/maintenance, mutant mice haplodeficient for ROR $\alpha$ . Because normal levels of ROR $\alpha$  are necessary for normal development and synaptic maintenance (Chen et al., 2013; Gold et al., 2007; Jetten and Joo, 2006), and because ROR $\alpha$  interacts with magnetoreceptor Cry, it was likely that this system would respond differently to magnetic stimulation.

We wanted to investigate the capacity of LI-rTMS to induce neuronal plasticity and olivocerebellar reinnervation in a mildly disturbed neural circuit, the ROR $\alpha$ <sup>+/-</sup> mouse. We have already shown that post-lesion repair of the olivocerebellar path can be produced in vivo and in vitro (see Chapter III) in wild-type animals. We now show that haploinsufficiency

in ROR $\alpha$  prevents effective repair in response to LI-rMS, and that the inferior olivary expression of appropriate levels of ROR $\alpha$  seems particularly important for inducing olivocerebellar axonal outgrowth.

In view of the lack of LI-rTMS induced olivocerebellar reinnervation we also examined a less extensive form of plasticity, such as can be induced in dendrites and their spines. As a baseline we examined adult and aged PCs from non-treated (naive) animals in adult ROR $\alpha$  +/- compared to aged ROR $\alpha$ <sup>+/-</sup> mice to identify age-related changes. Aged animals had lower spine density and smaller spine head diameters, (which is consistent with the effect of age in WT PCs-- Chapter II). After 4 weeks LI-rTMS and behaviour tests, PCs in the aged group had increased dendritic tree size although spine density did not recover. Moreover, spine morphology also changed with this chronic stimulation: spine length decreased and head diameter was modified in both adult and aged. Importantly, the handling and activity of behaviour testing in these mice, accounted for most of the dendritic plastic changes, i.e. increasing the width of aged PC dendrites. Consistent with these minor changes, there were no improvements in any behavioural tests; the only differences were age-related decreases in basic motor behaviour and muscle strength. These results contrast with findings in WT mice and indicate that ROR $\alpha$  mice have reduced responsiveness to LI-rTMS.

The known roles of ROR $\alpha$  in PC development and maturation (Boukhtouche et al., 2006a; Chen et al., 2013) suggest that it regulates genes involved in neural plasticity. Indeed ROR $\alpha$ -deficient cerebellar are profoundly deficient in BDNF (Qiu et al., 2007) and ROR $\alpha$  is known to down-regulate calcium signalling genes (Gold et al., 2003), which could be involved in cytoskeletal reorganisation for spine and dendritic plasticity. Additionally, ROR $\alpha$  haplodeficient mice show characteristics of premature-onset ageing (Janmaat et al., 2011; Jarvis et al., 2002; Mamontova et al., 1998). Reduced neural plasticity is also characteristic of an ageing nervous system and similar mechanisms may be activated in the ROR $\alpha$  +/- nervous system.

An alternative hypothesis is that ROR $\alpha$ <sup>+/-</sup> mice show reduced plasticity because of faulty magnetoreception due to ROR $\alpha$ 's interaction with the Cryptochrome transcription factors. Circadian behaviours dependant on the periodic expression of Cry and other genes (Akashi

and Takumi, 2005; Crumbley et al., 2010) are abnormal in *Rora* deficient mice. *RORα* promotes the transcription of *BMAL1*, which forms heterodimers with the *CLOCK* protein and activates transcription of *Cry* and *Per* (Akashi and Takumi, 2005; DeBruyne et al., 2007; Duez and Staels, 2009; Sato et al., 2004). *Per* and *Cry* form heterodimers and once the *PER/CRY* heterodimer concentration reaches a critical level, they enter the nucleus and inhibit *BMAL1/CLOCK* heterodimer formation and consequently *Cry* transcription. The relationships between these different genes are thus complex and non-linear, but a lack of *RORα* would tend to reduce the activity of the *Cry/Per* regulation system. We did find reduced *Cry1* expression in the adult *RORα<sup>+/-</sup>* mice, which showed less response to LI-rTMS than the aged *RORα<sup>+/-</sup>* mice. In addition, *RORα* itself was reduced in aged *RORα* mice; and *MMP2* was expressed at higher levels in aged *RORα* mice. Similarly, *MMP2* overexpression is consistent with a reduced number of spines (Verslegers et al., 2015), as seen in aged *RORα<sup>+/-</sup>* mice; LIrTMS tended to correct this overexpression, which would allow new spines to be formed.

In conclusion, it is entirely possible that both a reduced magnetoreception and a general lack of plasticity contribute to the observed reduced responsiveness of the *RORα<sup>+/-</sup>* nervous system to LI-rTMS. Future experiments will examine other methods of inducing post-lesion plasticity (such as application of *BDNF*, overexpression of *PSA-NCAM*, or alteration of the extracellular environment) in *RORα<sup>+/-</sup>* cerebella to determine whether the reduced reinnervation response only occurs with magnetic stimulation.

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## Figure legends

### Figure 1: Post-lesion repair by LI-rMS is impaired in $ROR\alpha$ haplodeficient hindbrain explants

**A.** Diagram showing the different genotype combinations in the explants. We tested the efficiency of BHFS on post-lesion reinnervation in explants where both the ION and denervated hemicerebellar plates were  $ROR\alpha^{+/-}$  (left image), explants with  $ROR\alpha^{+/-}$  ION and WT denervated hemicerebellar plates (middle image), and explants with WT ION and  $ROR\alpha^{+/-}$  denervated hemicerebellar plates.

**B.** In proximal region, BHFS in WT explants induces significantly more reinnervation than sham treated WT explants (One way ANOVA with Tukey *post hoc*;  $p=0,000$ ;  $n=10$ ).

When both the ION and the denervated hemicerebellar plates are  $ROR\alpha^{+/-}$ , BHFS failed to induce significant reinnervation compared to sham treated explants ( $p>0.05$ ;  $n=5$ ). BHFS in explants with  $ROR\alpha^{+/-}$  ION and WT denervated hemicerebellar plates induces no significant reinnervation compared to sham treated WT explants or to BHFS in  $ROR\alpha^{+/-}$  explants (both ION and Cbm) ( $p>0.05$ ;  $n=6$ ).

BHFS in explants with a WT ION and  $ROR\alpha^{+/-}$  denervated hemicerebellar plates induces higher reinnervation than in WT sham treated explants and  $ROR\alpha^{+/-}$  explants (both ION and Cbm) treated with BHFS ( $p=0.001$ ;  $p=0.03$ ;  $n=6$ ) but to a lesser extent than BHFS stimulation in WT explants ( $p=0.003$ ).

In the distal region, only BHFS in WT explants induce significantly more reinnervation than sham treatment ( $p=0.000$ ). No significant reinnervation is induced by BHFS in any of the other explant configurations listed in (A) ( $p>0.05$ ).

Significant differences from WT sham treated explants ( $*p\leq 0.05$ ;  $***p\leq 0.001$ ). Difference between two other groups are indicated by appropriate bars ( $**p\leq 0.01$ ).  $n$ = number of explants.

**Figure 2: Ageing in ROR $\alpha$ <sup>+/-</sup> mice alters PC dendritic spines but not the general dendrite morphology.**

**A,B,C,D.** No differences in PC dendritic width (A), height (B) and density (C) between adult and aged ROR $\alpha$ <sup>+/-</sup> mice (One way ANOVA; all  $p>0.05$ ;  $n=5$ ; adult  $N=33$ ; aged  $N=21$ ) nor in dendritic complexity as shown by Sholl Analysis, which indicates the number of branch points as a function of distance from the soma (in  $\mu\text{m}$ ) (D) (Mann Whitney U; all  $p>0.05$ ).  $n$ = number of animals;  $N$ =number of cells analysed.

**E,F,G.** Density of total (E) and thin/mushroom (G) spines is lower in aged ROR $\alpha$ <sup>+/-</sup> mice compared to adult ROR $\alpha$ <sup>+/-</sup> mice (One way ANOVA;  $p=0.033$  and  $p=0.021$  respectively;  $n=5$ ; adult  $N=13$  and aged  $N=16$ ). Stubby spine density is not altered by age (F) (One way ANOVA;  $p>0.05$ ).  $n$ = number of animals;  $N$ =number of terminal dendrites analysed.

**H.** Head diameter of dendritic thin/mushroom spines in PCs from aged ROR $\alpha$ <sup>+/-</sup> mice is smaller than in adult ROR $\alpha$ <sup>+/-</sup> mice (Kolmogorov-Smirnoff test;  $p=0.0019$ ). ( $n=5$ ; adult  $N=1060$ , aged  $N=1384$ ).  $n$ = number of animals;  $N$ =number of dendritic spines analysed  
**\*\* $p\leq 0.01$ .**

**Figure 3: Chronic LI-rTMS (4 weeks) does not modify PC dendritic morphology in adult or aged ROR $\alpha$ <sup>+/-</sup> mice.**

**A,B,C.** Aged ROR $\alpha$ <sup>+/-</sup> animals have larger PC dendritic tree height (A), width (B) and area (C) compared to adult ROR $\alpha$ <sup>+/-</sup> animals (Kruskal-Wallis tests;  $p=0.009$ ,  $p=0.000$  and  $p=0.000$  respectively). LI-rTMS does not modify any of these PC dendritic measurements (all  $p>0.05$ ; adult chronic LI-rTMS  $n=13$ ;  $N=37$  and aged chronic LI-rTMS  $n=11$ ;  $N=26$ )

**D.** Aged ROR $\alpha$ <sup>+/-</sup> animals have a higher PC dendritic complexity in the distal part of the dendrites as shown by Sholl analysis (Kruskal-Wallis tests, all  $p\leq 0.05$  between 280-360  $\mu\text{m}$ ). Significant difference between adult ROR $\alpha$ <sup>+/-</sup> and aged ROR $\alpha$ <sup>+/-</sup> animals.

\* $p\leq 0.05$ . \*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$ .

**Figure 4: Chronic LI-rTMS does not significantly change PC dendritic spine density but alters their morphology.**

**A, B.** Total (A) and thin/mushroom (B) spine density are lower in aged sham animals compared to adult sham animals (Kruskal-Wallis with Dunn Bonferroni correction tests;  $p=0.018$  and  $p=0.01$  respectively).

**C.** PC dendritic thin/mushroom spine head diameter is larger in adult mice stimulated with LI-rTMS compared to sham-treated adult animals (Kolmogorov-Smirnoff test;  $p=0.0182$ ; adult chronic LI-rTMS  $N=1917$ ) and smaller in aged LI-rTMS treated animals compared to sham-treated aged animals ( $p=0.0336$ ; aged chronic LI-rTMS  $N=1053$ ).

**D.** PC dendritic thin/mushroom spine length is shorter in adult (top) and aged LI-rTMS treated animals compared to sham-treated animals (Kolmogorov-Smirnoff test;  $p=0.004$  and  $p=0.0182$ ).

\* $p\leq 0.05$ ; \*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$

**Figure 5: Handling and behavioural tests alter PC dendritic tree morphology in aged but not in adult  $ROR\alpha^{+/-}$  animals.**

**A,C.** PC dendritic height (A) and occupied area (C) are not modified by chronic handling and behavioural tests (Kruskal-Wallis with Dunn-Bonferroni corrections;  $p<0.05$ ; *adult  $n=11$  and aged  $n=14$ ; adult  $N=41$  and aged  $N=27$* ).  $n$ = number of animals;  $N$ =number of cells analysed.

**B.** PC dendritic width (B) is increased by chronic handling and behavioural tests in aged animals (Kruskal-Wallis with Dunn-Bonferroni corrections;  $p=0.013$ ) but not in adult mice ( $p>0.05$ ).

**D.** Chronic handling and behavioural tests increased distal PC dendritic complexity in the aged animals, as shown by Sholl analysis (Kruskal-Wallis tests, all  $p\leq 0.05$  between 280-360  $\mu\text{m}$ ) but not in adult mice ( $p>0.05$ ). Significant differences between aged  $ROR\alpha^{+/-}$  acute sham and aged  $ROR\alpha^{+/-}$  chronic sham (\* $p\leq 0.05$ ).

\* $p\leq 0.05$ ; \*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$ ;

**Figure 6: Handling and behavioural tests alters PC dendritic spine morphology in both adult and aged ROR $\alpha$ <sup>+/-</sup> animals.**

**A.** Handling and behavioural tests decreased PC dendritic thin/mushroom spine head diameter in adult (top) and aged mice (Kolmogorov-Smirnoff test; both  $p < 0.0001$ ; adult  $N=1896$  and aged  $N=854$ ).

**B.** Handling and behavioural tests increased PC dendritic thin/mushroom spine length in aged mice (Kolmogorov-Smirnoff test; both  $p < 0.0001$ ) and in adult mice ( $p < 0.05$ ).

\* $p \leq 0.05$ ; \*\*\*\* $p < 0.0001$

**Figure 7: Chronic LI-rTMS does not alter motor coordination or learning of adult and aged ROR $\alpha$ <sup>+/-</sup> mice.**

**A.** In the footslip test, aged animals have significantly more footslips per meter than adult mice (Two way ANOVA,  $p=0.000$ ; adult sham  $n=9$ , adult LI-rTMS  $n=10$ , aged sham  $n=6$ ; aged LI-rTMS  $n=5$ ), showing reduced motor coordination in these animals. Chronic LI-rTMS did not change the number of footslips per meter in either adult or aged animals ( $p > 0.05$ ).

**B.** In the accelerating rotarod test all groups learned the task during the 5-day test period (Friedman nonparametric RMANOVA and Wilcoxon ranked test,  $p=0.000$ ; adult sham  $n=14$ , adult LI-rTMS  $n=13$ , aged sham  $n=11$ , aged LI-rTMS  $n=11$ ).

**C.** In the accelerating rotarod test there were no differences between LI-rTMS and sham treated groups (2-way ANOVA; all  $p > 0.05$ ). The aged mice, whether treated or sham, generally fell from the rotarod sooner than adult mice (2-way ANOVA, on day 1  $p=0.001$ ; day 2  $p=0.006$ ).

\*\* $p \leq 0.01$ ; \*\*\* $p \leq 0.001$

**Figure 8: Chronic LI-rTMS does not alter spatial learning of adult or aged ROR $\alpha$ <sup>+/-</sup> mice.**

**A,B,C.** In the learning phase of the Morris Water Maze (MWM) all groups improved their performance, as measured by reduced distance travelled (A), greater time spent in the

correct quadrant (B), and reduced escape latency (C) (Repeated measures ANOVA; all  $p \leq 0.03$  between subsequent days). \*\*\* $p \leq 0.001$

**Figure 9: Chronic LI-rTMS does not improve spatial memory of adult or aged  $ROR\alpha^{+/-}$  mice.**

**A.** In the probe test of the MWM, aged animals crossed the platform zone location less frequently than adult mice (Two way ANOVA;  $p=0.004$ ) but chronic LI-rTMS did not modify the number of times the animals crossed the platform zone either in adult or aged mice ( $p > 0.05$ ). \*\* $p \leq 0.01$

**B,C.** No differences between groups were observed for the duration over the platform location (B) or the latency to reach the platform (C) (Two way ANOVA;  $p > 0.05$ ).

**D.** Heat map representing the merged localisation of mice during the probe tests of the Morris Water Maze. Adult animals treated with LI-rTMS did not spend a greater time over the platform (white circle) than adult sham treated animals as indicated by the red colour. In aged mice we observed a tendency of the LI-rTMS treated mice to spend more time over the platform than sham treated animals but this was not significant in any quantitative parameters measured during the MWM probe test (see A,B,C).

**Figure 10: Acute LI-rTMS (3 days) does not significantly alter PC dendritic size or complexity in adult or aged  $ROR\alpha^{+/-}$  mice.**

**A,C.** No differences in dendritic height (A) or occupied area (C) were observed in the different groups (Two-way ANOVA, all  $p > 0.05$ ; adult acute sham  $n=5$ ,  $N=33$ ; adult acute LI-rTMS,  $n=5$ ,  $N=18$ ; aged acute sham,  $n=5$ ,  $N=18$ ; aged acute LI-rTMS,  $n=5$ ,  $N=24$ ).

**B.** There was no difference in PC dendritic width of adult  $ROR\alpha^{+/-}$  sham and aged  $ROR\alpha^{+/-}$  sham animals. However since adult and aged animals respond differently to the stimulation, the aged  $ROR\alpha^{+/-}$  LI-rTMS mice have narrower PC dendritic trees than adult  $ROR\alpha^{+/-}$  LI-rTMS mice (One way ANOVA;  $p=0.013$ ). \*\* $p \leq 0.01$

**D.** No differences between groups were observed in Sholl analysis, quantifying the number of branch points as a function of the distance from the soma (in  $\mu\text{m}$ ) (Kruskal-Wallis tests, all  $p > 0.05$ ).

**Figure 11: PC dendritic spines density and morphology are more affected by acute LI-rTMS (3 days) in aged ROR $\alpha$ <sup>+/-</sup> animal than in adult ROR $\alpha$ <sup>+/-</sup> animal.**

**A,B,C.** Acute LI-rTMS (3 day) increases density of total (A) and stubby (B) spines in aged ROR $\alpha$ <sup>+/-</sup> animals (Kruskal-Wallis with Dunn-Bonferroni corrections;  $p=0.001$ ,  $p=0.003$  respectively; adult acute sham,  $N=13$ ; adult acute LI-rTMS,  $N=24$ ; aged acute sham,  $N=16$  ; aged acute LI-rTMS,  $N=24$ ) but not in adult mice ( $p>0.05$ ).

**D.** PC terminal dendrite showing the increased number of dendritic spines induced by LI-rTMS in aged ROR $\alpha$ <sup>+/-</sup> mice (right ) compared to sham stimulation (left ).

**E.** Acute LI-rTMS decreases PC dendritic spine head diameter in adult mice (Kolmogorov-Smirnoff test;  $p=0.0218$ ; adult acute sham  $N=1060$ , adult acute LI-rTMS  $N=2150$  ) but increases it in aged mice (Kolmogorov-Smirnoff test  $p<0.0001$ ; aged acute sham  $N=1384$ , aged acute LI-rTMS  $N=2197$ ).

**F.** Acute LI-rTMS decreases PC dendritic spine length in aged (Kolmogorov-Smirnoff test;  $p\leq 0.0001$ ) but not in adult (top graph)  $p>0.05$ .

\* $p\leq 0.05$ ; \*\* $p\leq 0.01$ ; \*\*\* $p\leq 0.001$ ; \*\*\*\* $p\leq 0.0001$

**Figure 12: LI-rTMS tends to normalize altered expression of some genes involved in magnetoreception and spinogenesis in ROR $\alpha$ <sup>+/-</sup> mice cerebellum.**

**A.** Cry1 expression is lower in the cerebellum of sham-treated adult ROR $\alpha$ <sup>+/-</sup> mice compared to sham-treated WT adult mice (t test with Bonferroni corrections for multiple comparisons;  $p=0.026$ ). Acute LI-rTMS tends to normalize this expression ( $p=0.096$ ) although not significantly. Cry1 expression is not altered in aged ROR $\alpha$ <sup>+/-</sup> animals and acute LI-rTMS does not modify the level of Cry1 expression. Significant differences from adult WT sham-treated mice ( $*p\leq 0.05$ ). Number of animals ( $n$ )=9-11 in adult groups and  $n=5$  in aged groups.

**B.** ROR $\alpha$  expression is lower in the cerebellum of sham-treated aged ROR $\alpha$ <sup>+/-</sup> mice compared to sham treated WT adult mice (t-test with Bonferroni corrections for multiple comparison;  $p=0.014$ ). ROR $\alpha$  expression is non-significantly lower in adult ROR $\alpha$ <sup>+/-</sup> animals compared to WT animals ( $p=0.1$ ). Acute LI-rTMS does not modify the level of ROR $\alpha$  expression in either

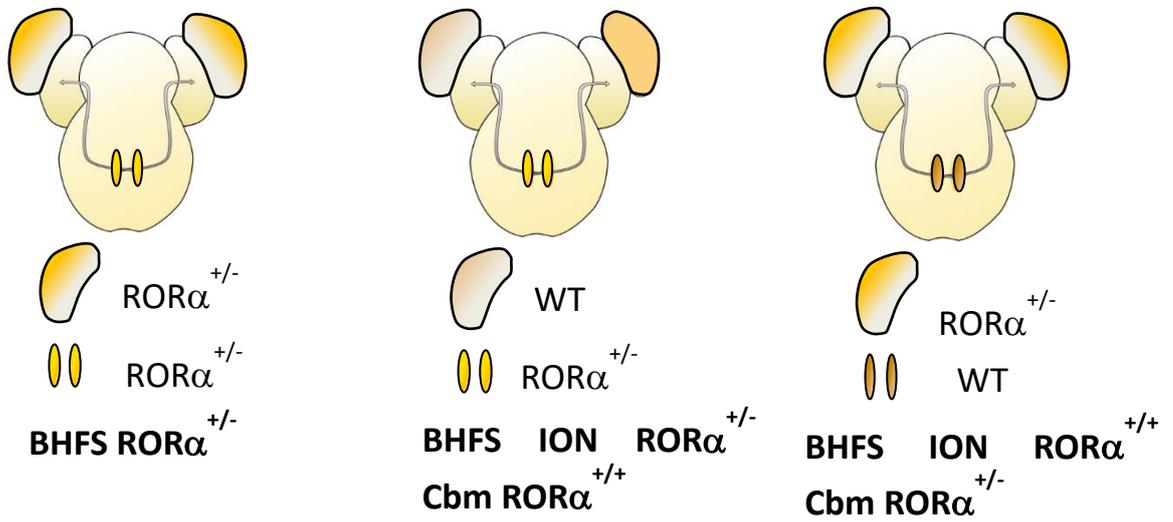
adult or aged  $\text{ROR}\alpha^{+/-}$  animals  $p>0.05$ . Significant differences with adult WT sham treated mice,  $*p\leq 0.05$ .

**C.** MMP2 expression is aberrantly high in the cerebellum of sham-treated aged  $\text{ROR}\alpha^{+/-}$  mice compared to sham-treated WT adult mice (t test with Bonferroni corrections for multiple comparison;  $p=0.000$ ). Acute LI-rTMS tends to normalize this aberrant level of expression ( $p=0.072$ ) although not significantly. MMP2 expression is non-significantly lower in adult  $\text{ROR}\alpha^{+/-}$  animals compared to adult WT sham treated mice ( $p=0.062$ ) and acute LI-rTMS does not modify MMP2 level of expression in these animals  $p>0.05$ . Significant differences with adult WT sham treated mice:  $*p\leq 0.05$

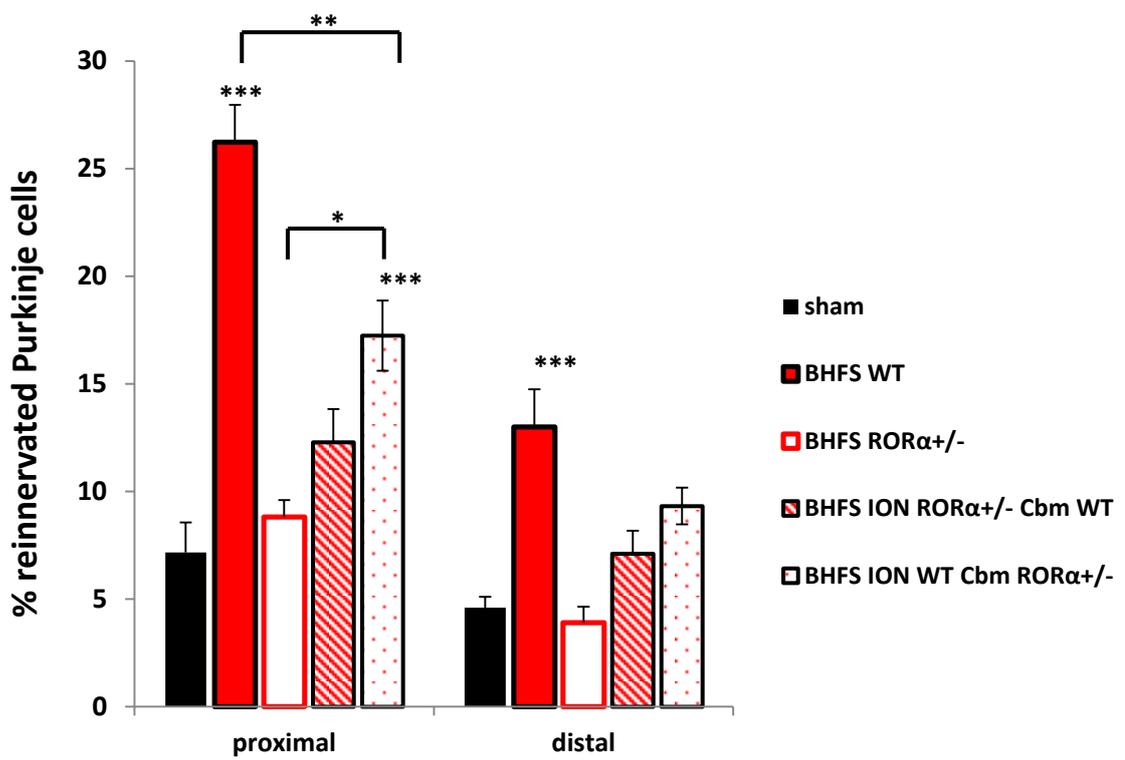
**D.** Cry2 expression is not altered by acute LI-rTMS nor by age in  $\text{ROR}\alpha^{+/-}$  mice (all  $p>0.05$ ).

**Figure 1**

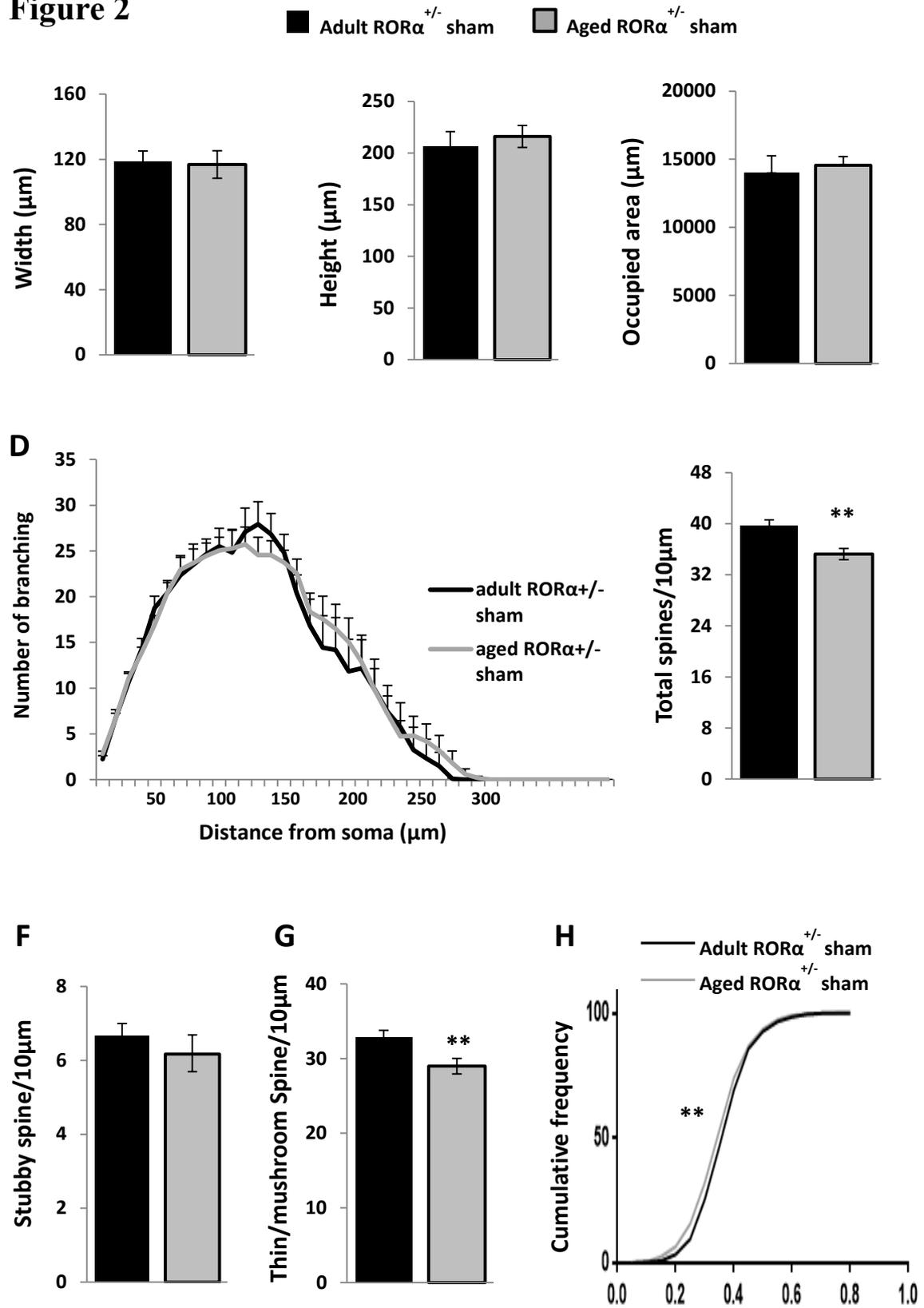
**A**



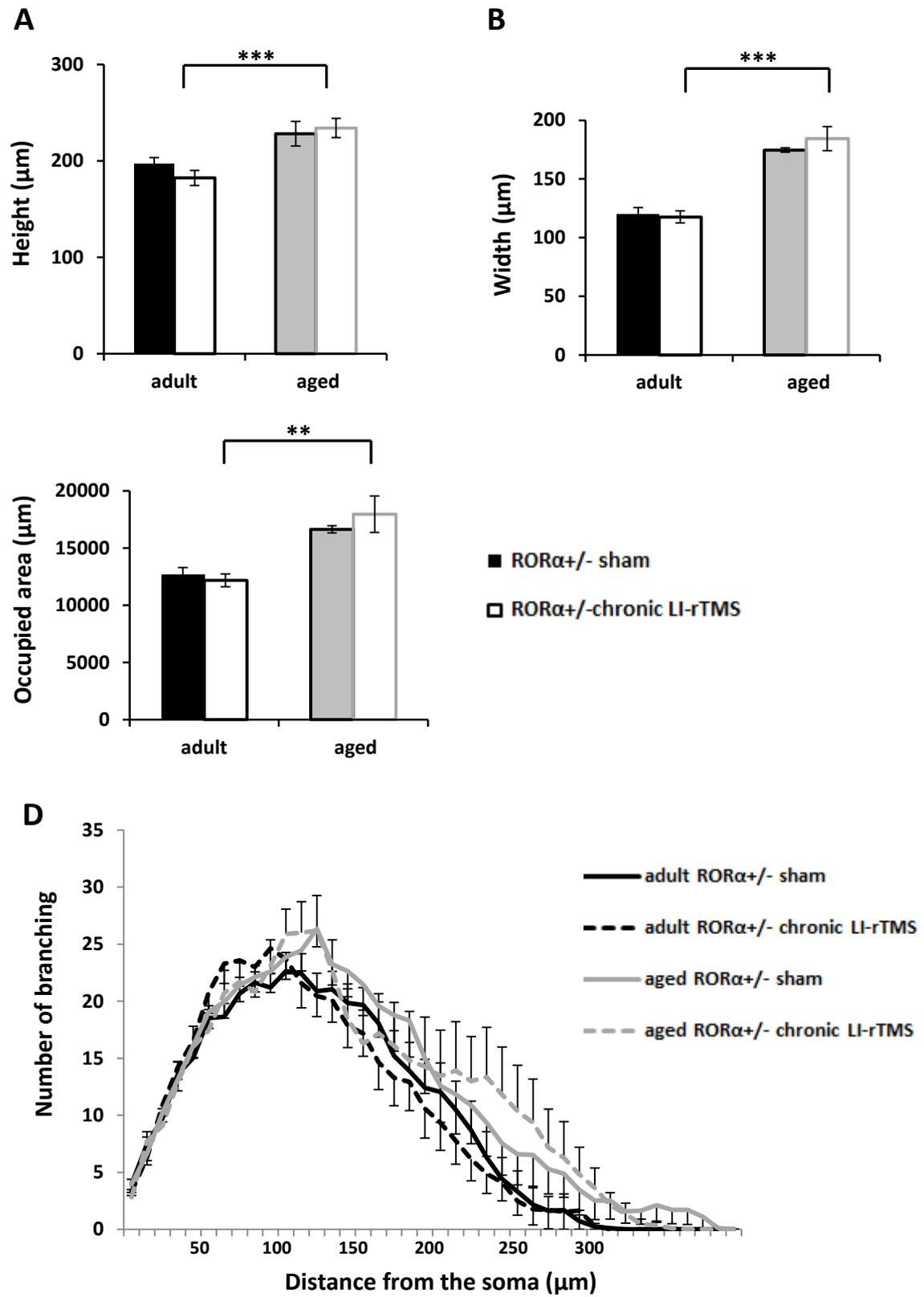
**B**



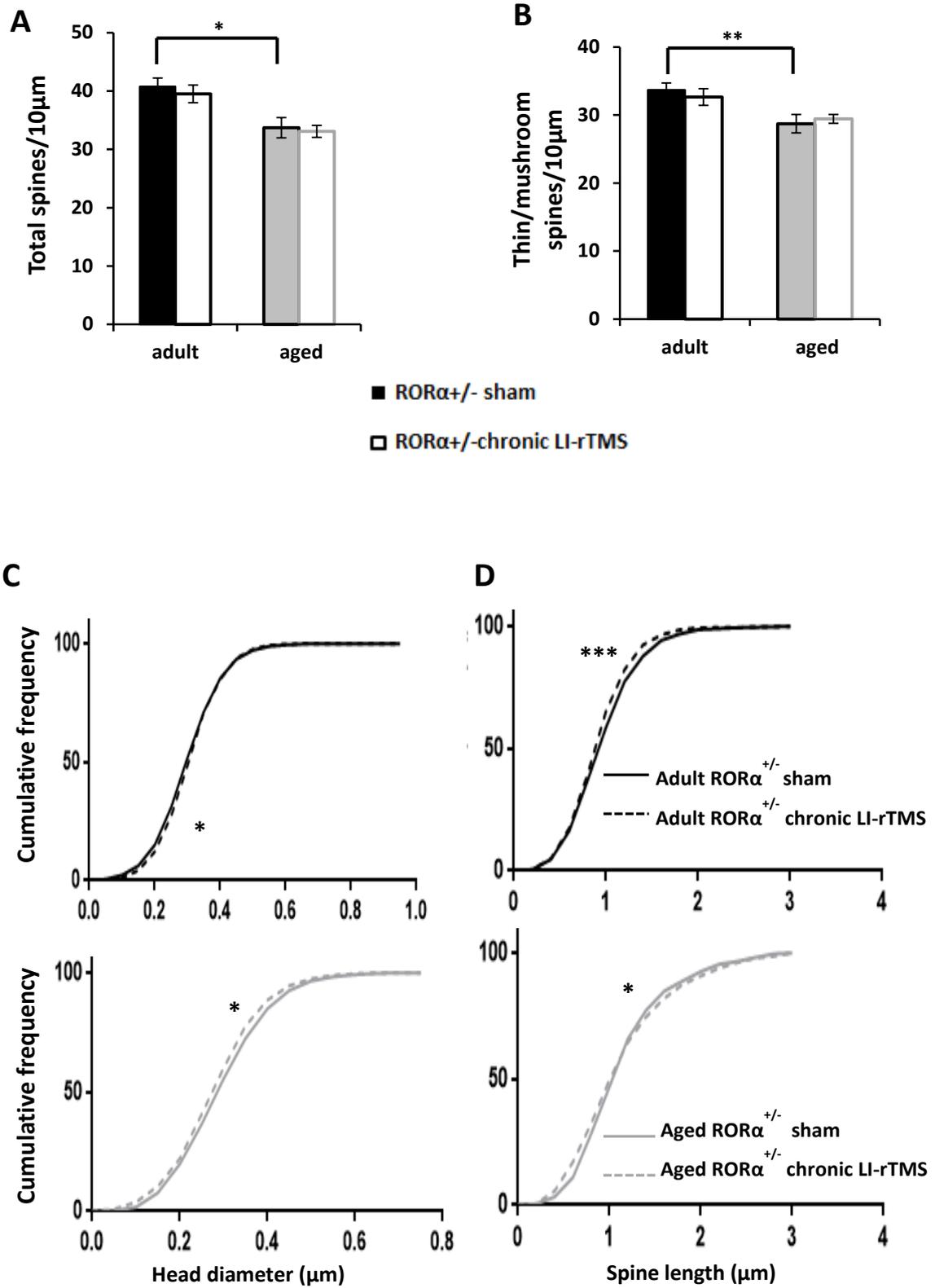
**Figure 2**



**Figure 3**



**Figure 4**



**Figure 5**

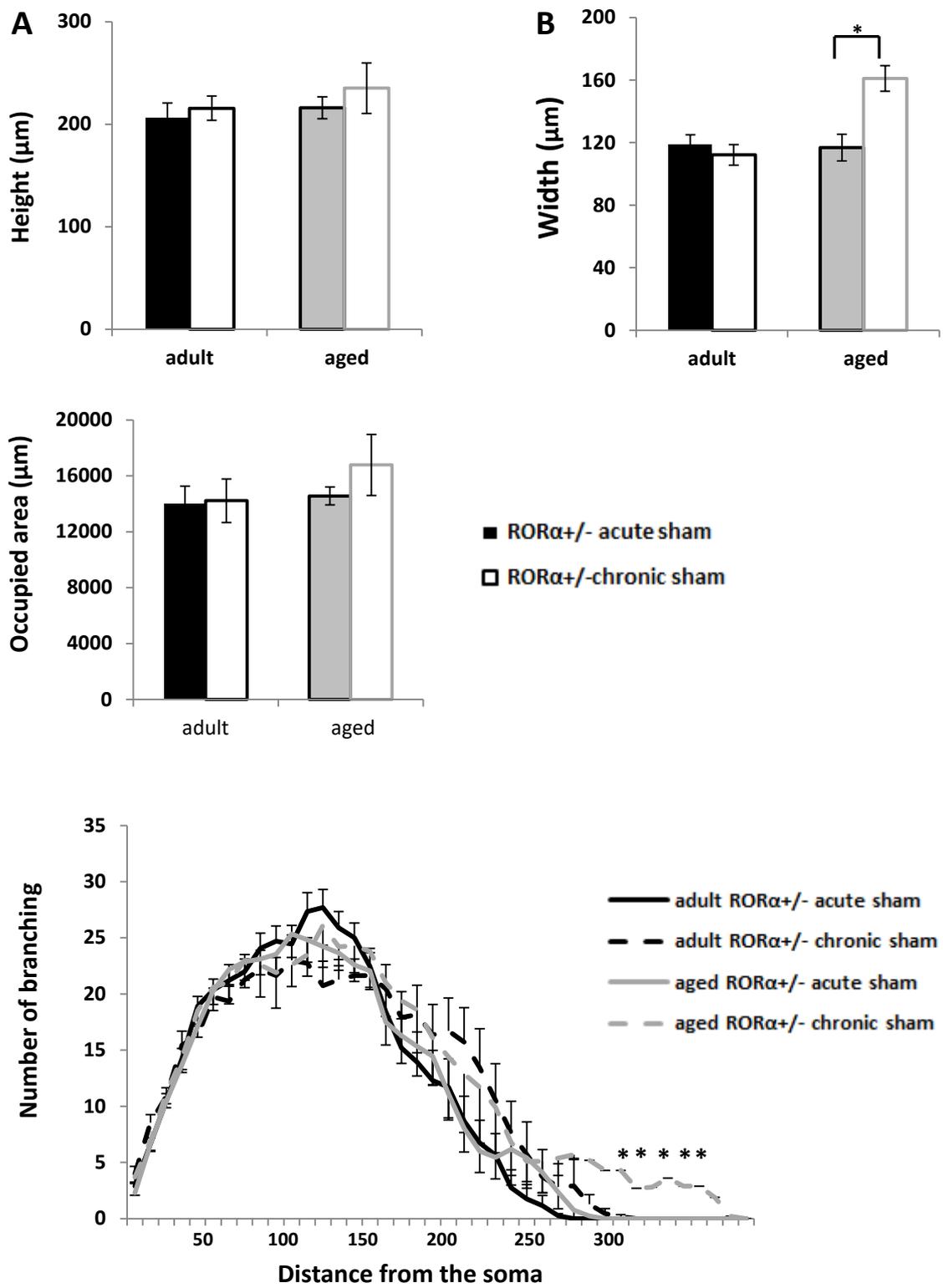
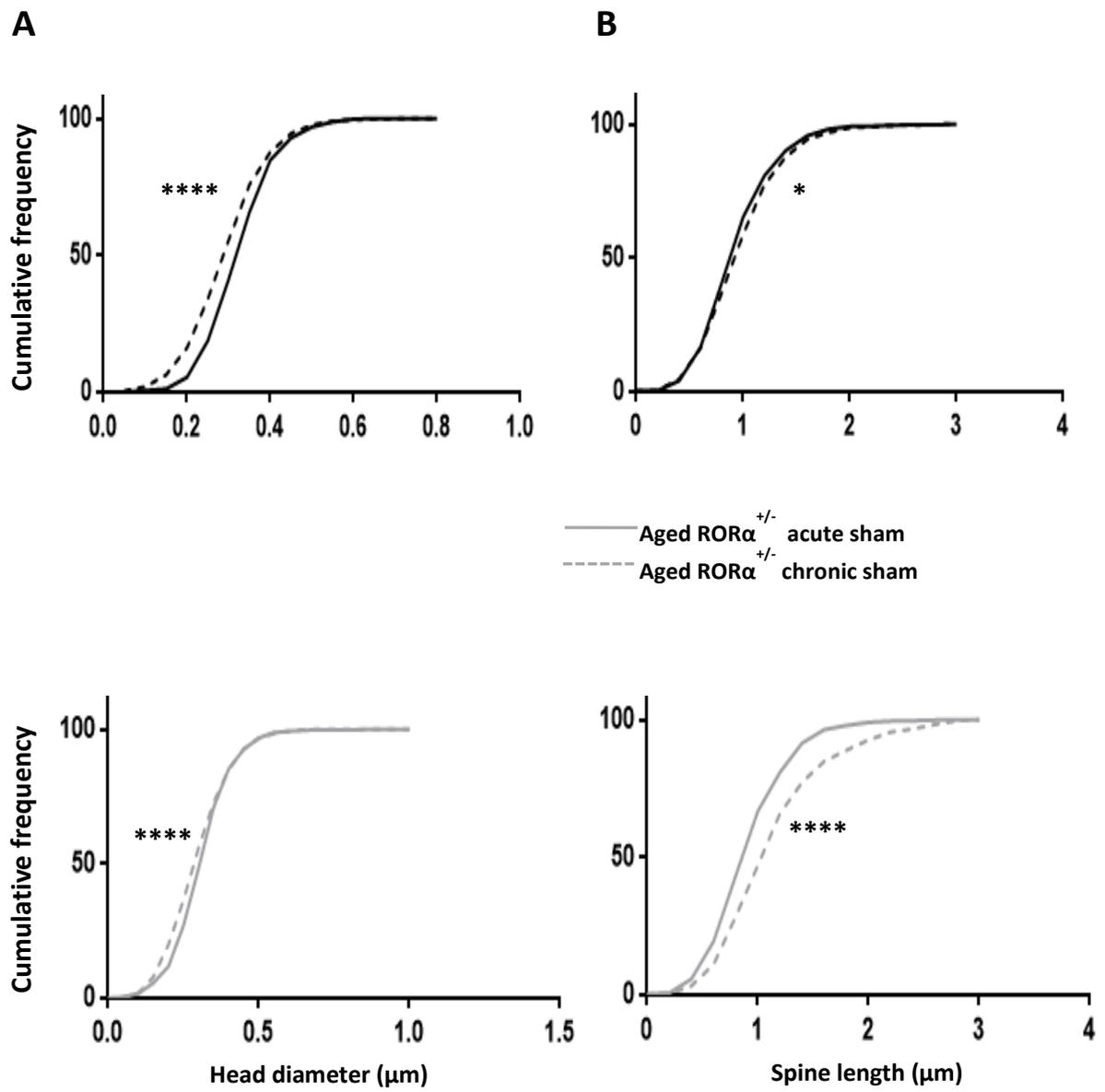


Figure 6



**Figure 7**

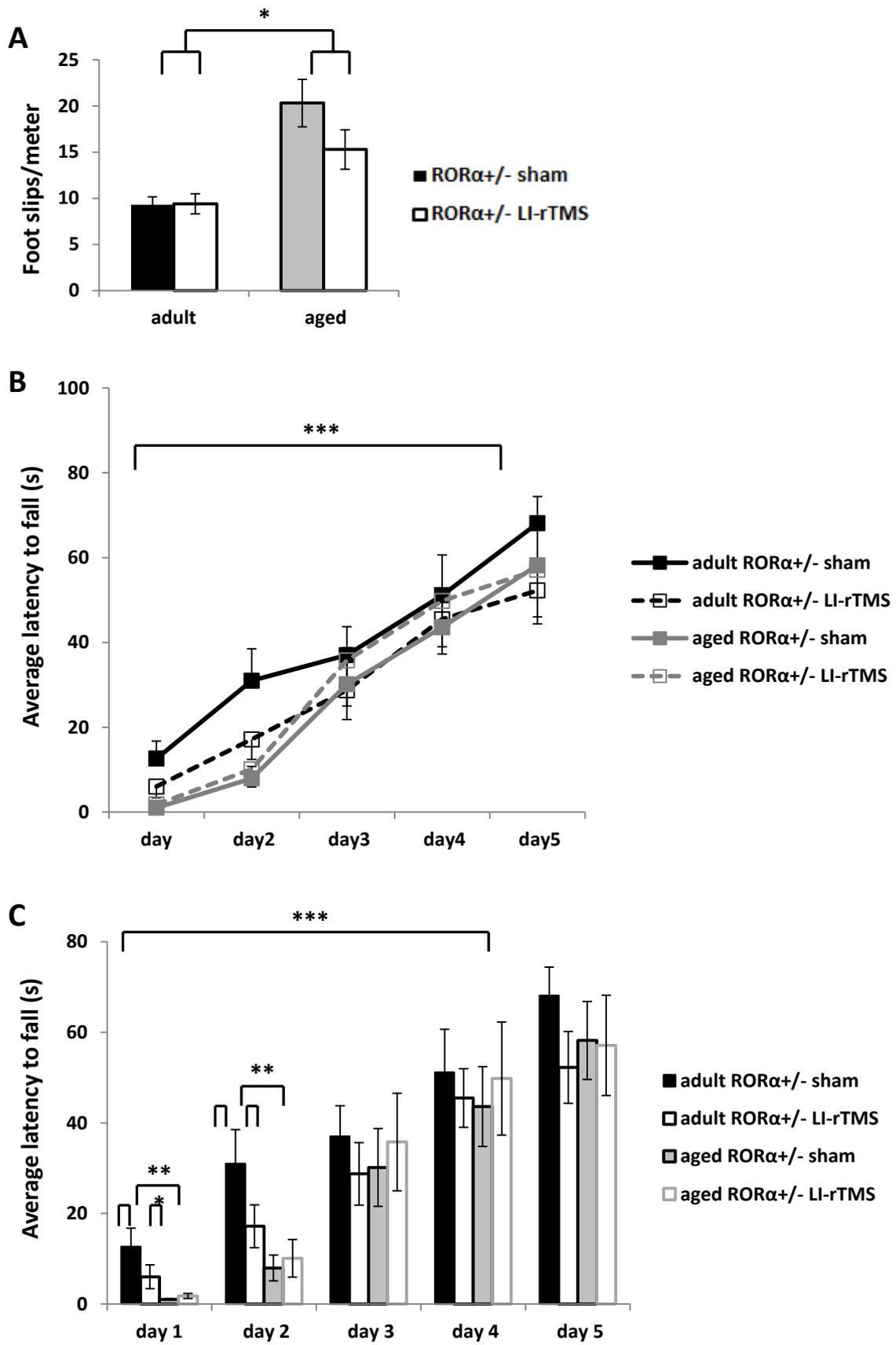
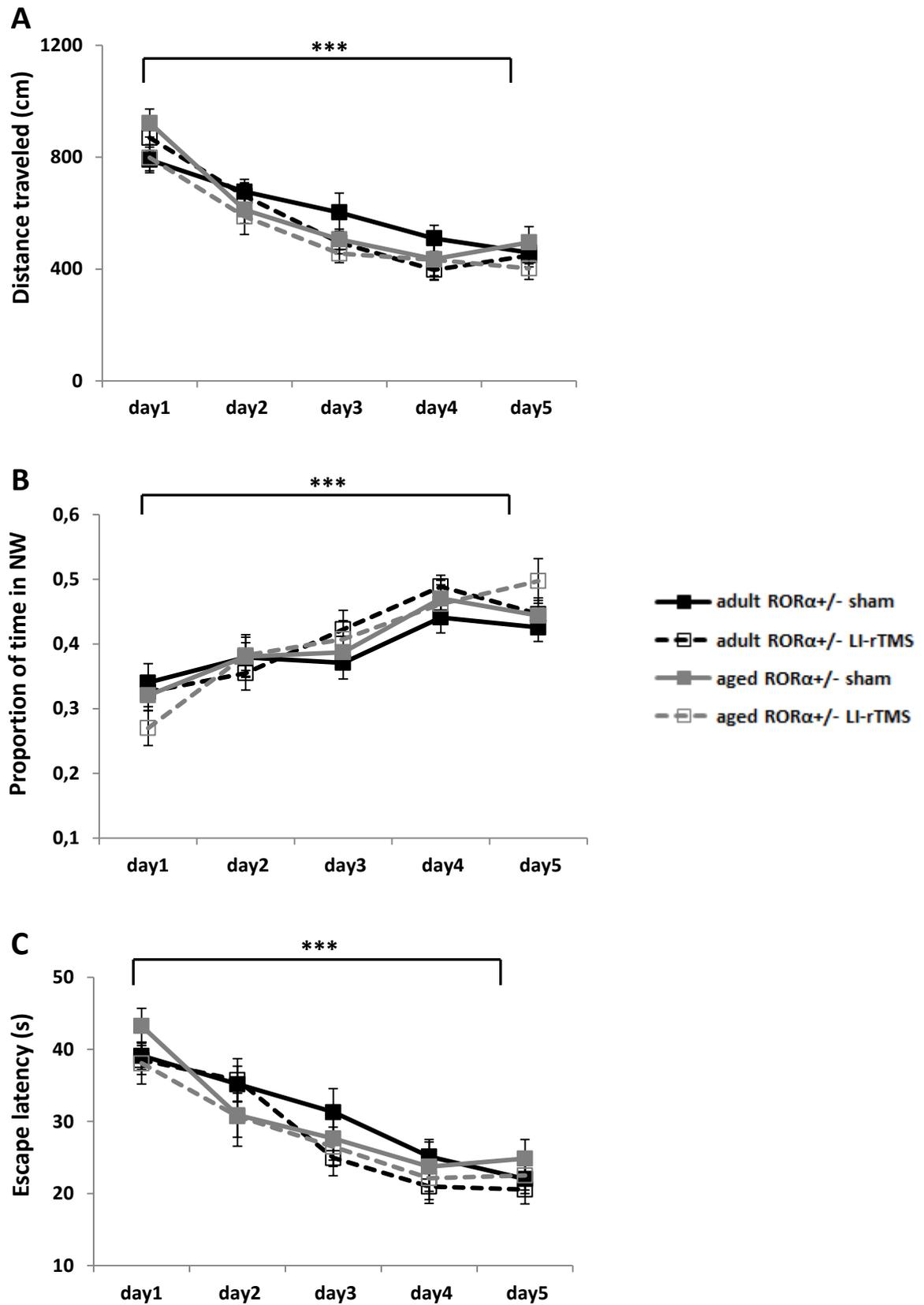
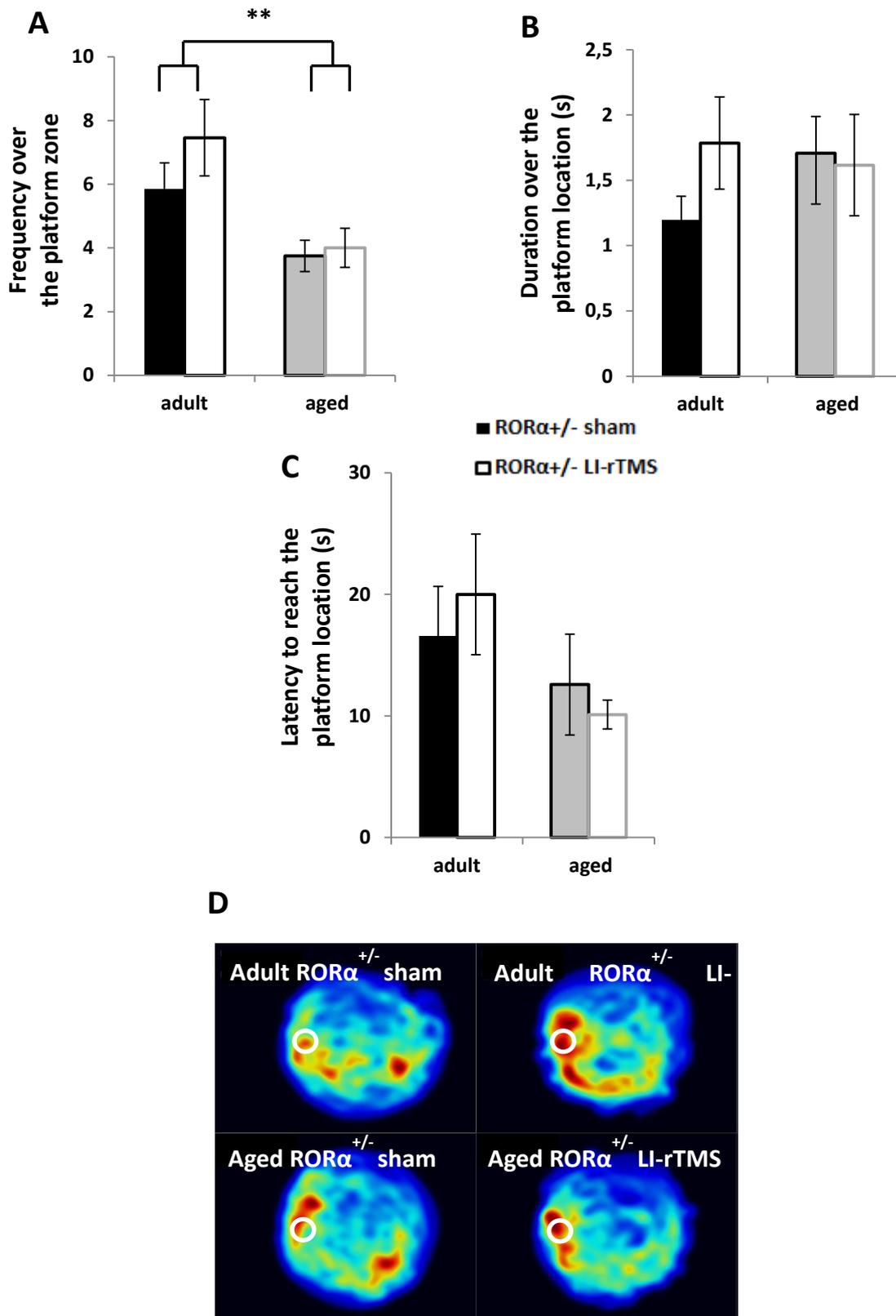


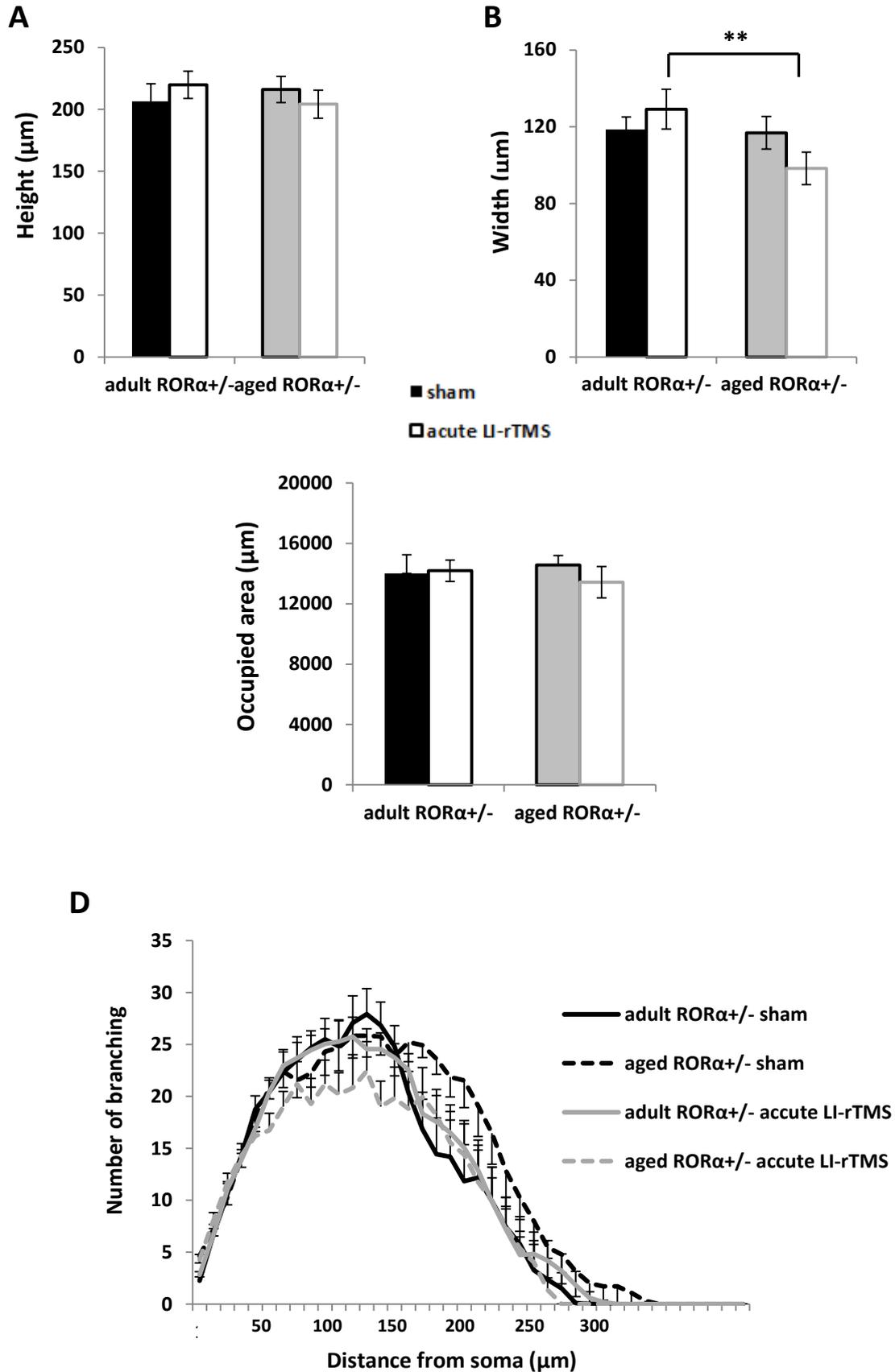
Figure 8



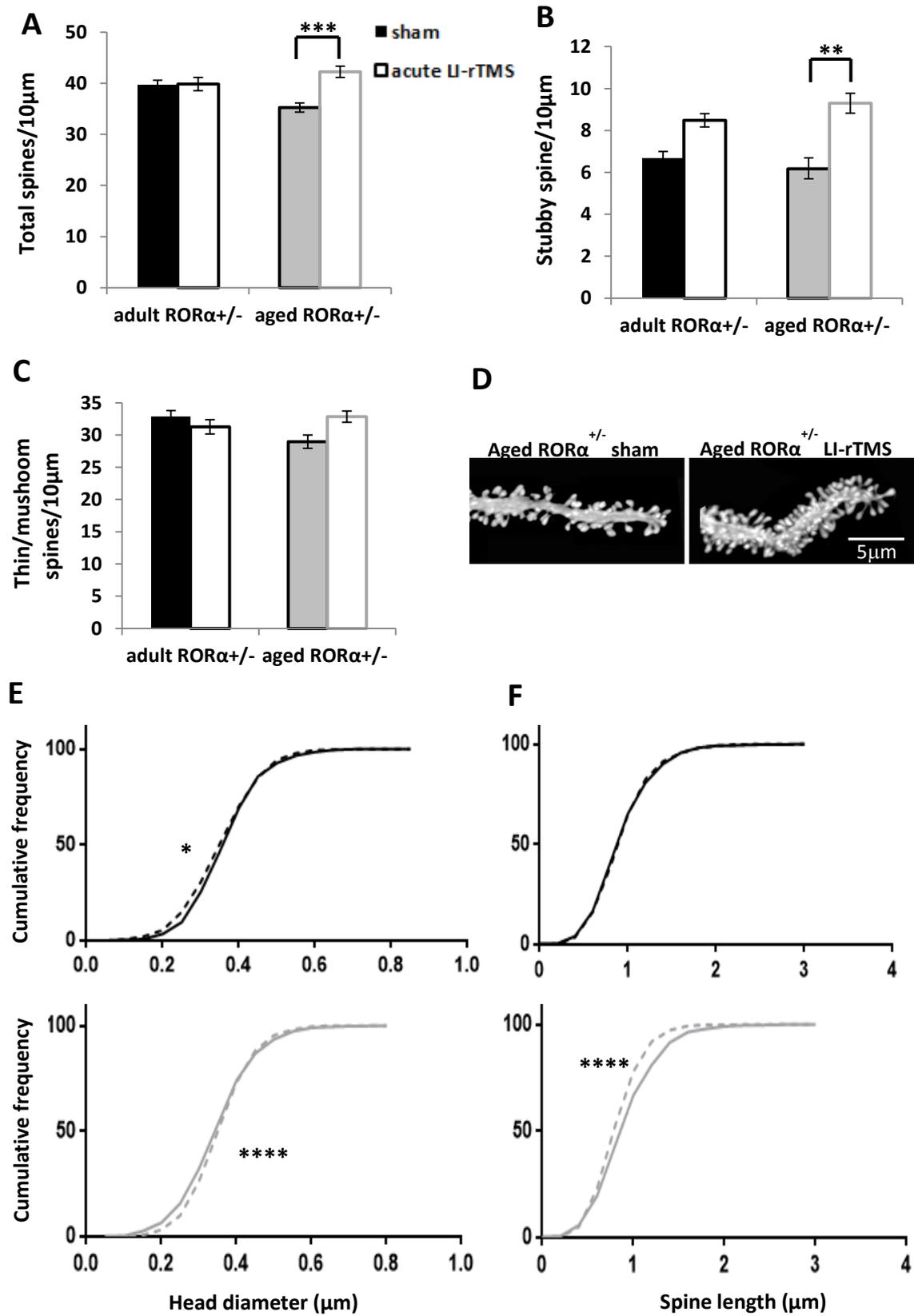
**Figure 9**



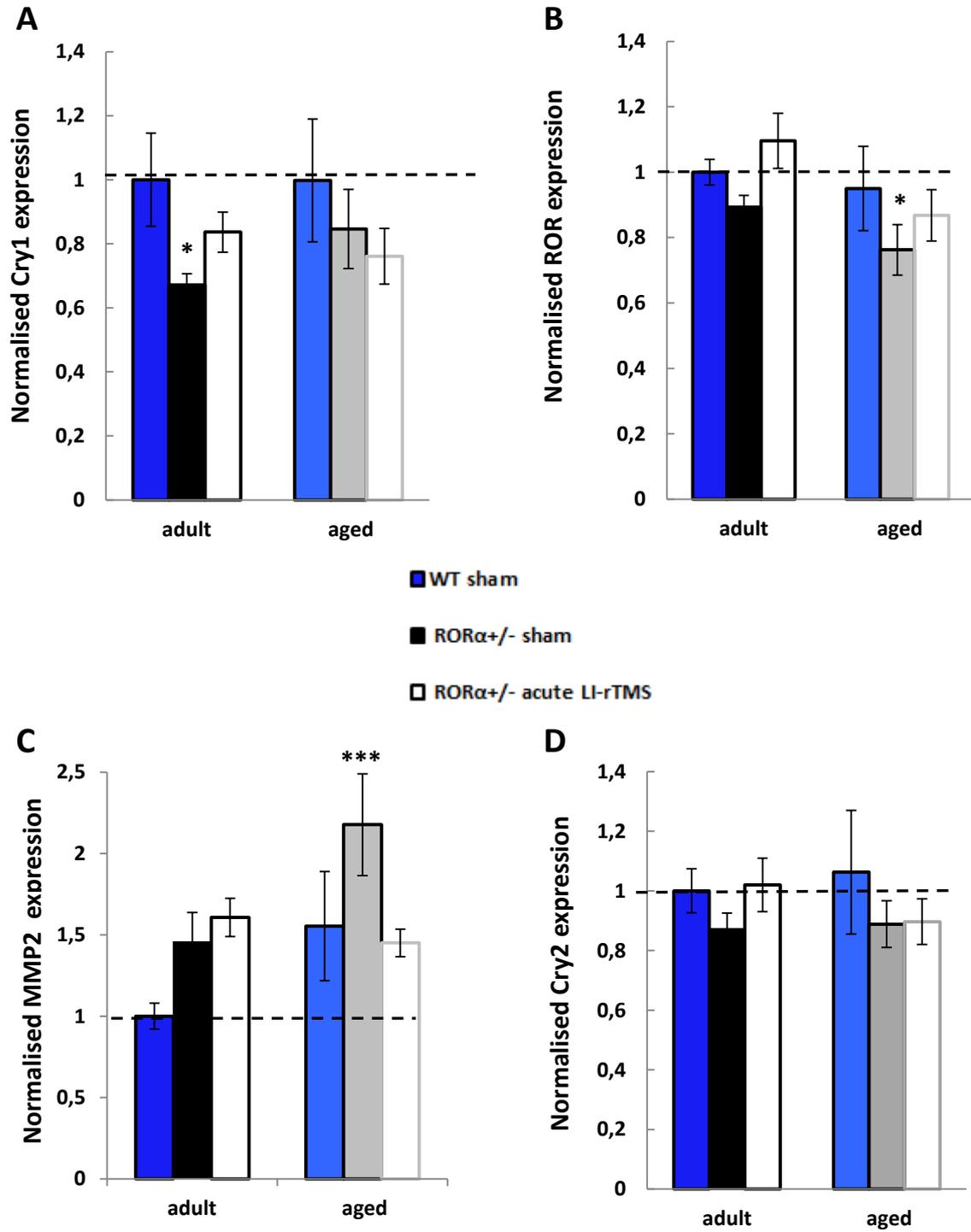
**Figure 10**



**Figure 11**



**Figure 12**





## **Chapter V – General Discussion**

## **V) Discussion**

### **V.1) Mechanism underlying the effects of LI-rTMS**

#### **V.1.1) LI-rTMS pattern of frequency are crucial for the induction of biological effects**

Using our well-described *in vivo* model of unilateral transection of the OCP (Dixon and Sherrard, 2006; Letellier et al., 2007; Willson et al., 2008), we have shown that sub-threshold LI-rTMS with a complex pattern of frequency (BHFS) is able to stimulate the growth of CF axons over a considerable distance, and to appropriately contact dendrites of denervated PCs with VGLUT2-positive terminals. The morphology of these terminals is consistent with previously-described spontaneous reinnervation in the developing rat cerebellum (Willson et al., 2008), as well as BDNF-induced reinnervation in the mature rat cerebellum (Dixon and Sherrard, 2006; Willson et al., 2008). This finding is important since neural rehabilitation using non-invasive treatments to promote post-lesion neural circuit repair is as yet extremely limited (Ellaway et al., 2014; Lefaucheur et al., 2014) and showing that synaptic contacts can be formed is a crucial step.

Fundamental research is required to optimize the parameters of existing treatments and to better define mechanisms by which they facilitate neurorehabilitation. To systematically investigate the actions of different LI-rTMS parameters on post-lesion repair and gene expression of the mature OCP, we have used our organotypic explant culture model (Letellier et al., 2009). This model allows us to easily screen a large number of treatment protocols, and thus we have shown for the first time that the frequency pattern of LI-rTMS pulses determines the biological effects, not simply the number of pulses delivered within a session or overall treatment plan. We have notably shown that patterns of stimulation that are described as having inhibitory effects on cortical excitability in clinical rTMS studies (ie 1Hz and cTBS; Fitzgerald et al., 2006; Huang et al., 2005; Rossini et al., 2015), do not promote post-lesion olivocerebellar reinnervation. As discussed in chapter III, few studies have evaluated the potential of rTMS to promote post-lesion axon growth but a study from our group showed that 1Hz LI-rTMS significantly inhibited neurite outgrowth in cultured cortical neurons (Grehl et al., 2015); this is coherent with our findings. In contrast, stimulation frequencies that are described as “excitatory” in the rTMS literature (10Hz and iTBS; (Fitzgerald et al., 2006; Huang et al., 2005; Rossini et al.,

2015) seem to be more appropriate to promote post lesion axon growth and neosynaptogenesis. Independently of the question of post-lesion repair, it would be interesting to see if different “inhibitory” and “excitatory” LI-rTMS patterns have similar effects on the activity of neuronal networks and therefore determine whether this terminology, derived from studies using high-intensity rTMS, remains accurate for low intensity magnetic fields.

Our findings show that complex burst high frequency LI-rTMS patterns (iTBS and BHFS) are more efficient than tonic high frequency stimulation (10Hz) for inducing axon collateral outgrowth to repair the OCP after unilateral transection. In other experimental models it has been shown that the information transmitted by bursts of action potentials provide more-precise information than tonic firing in brain structures with known computational roles (Lisman, 1997). In the hippocampal slice, for example, the application of an acetylcholine agonist induced a physiological theta rhythm network oscillation (5-8Hz), which is present in the hippocampus during normal brain function. Under these conditions a single brief (15ms) burst applied at the peak of theta oscillations is sufficient to produce LTP (Huerta and Lisman, 1995) . These results support the notion that during appropriate neuromodulatory conditions, synaptic modifications are particularly sensitive to the types of bursts that occur *in vivo* (in this case, theta burst). The greater efficiency of burst firing to modulate cortical activation has also been described in the rTMS literature, where iTBS and cTBS produce the most consistent and long-lasting effects on motor and visual cortex, even if delivered at lower intensity and shorter duration than tonic 1Hz and 10Hz stimulation (Hoogendam et al., 2010; Huang et al., 2005; Di Lazzaro et al., 2005). This is coherent with our *in vitro* results showing greater efficacy of LI-rMS delivering complex bursts of pulses (iTBS and BHFS) than tonic 10Hz LI-rMS for inducing axonal and synaptic structural plasticity.

A more complete picture of optimal stimulation parameters comes from our comparison between intermittent theta burst (iTBS) and our randomized theta burst stimulation (riTBS). We clearly show the importance of delivering each burst at a regular theta rhythm in order to observe the desired biological effects of iTBS LI-rMS; riTBS did not stimulate post-lesion repair, while the same number of bursts delivered in a regular pattern (iTBS) did. This observation is strengthened by our gene expression analyses that show the upregulation of

genes from signalling pathways related to the observed biological outcomes, such as synaptic plasticity axon growth and chemotaxis, only by the iTBS. In contrast the irregular rITBS does not have a profound effect on gene expression, although it did downregulate two genes with pro apoptotic and neuroprotective function. The theta rhythm at which pulse bursts are repeated is the only parameter that varies between iTBS and rITBS; this strongly suggests an important role for this biological rhythm.

In the context of the olivocerebellar pathway, it has been shown that action potentials elicited in the ION are translated into a burst of axonal spikes in CFs (Mathy et al., 2009). These bursts can propagate into the cerebellar cortex in vivo and have strong influence on PCs by increasing dendritic spikes and enhancing short-term and long-term plasticity at parallel fibre synapses (Mathy et al., 2009). Interestingly, synchronized sub-threshold membrane potential oscillations present in olivary neurons have a mean oscillation frequency of about 5Hz (i.e. within the theta band) that modulates afferent synapse-triggered CF axonal bursts (Mathy et al., 2009). The presence of electrical synapses between olivary neurons underlies their oscillatory synchronization and might render them more responsive to sub-threshold patterned stimulations (Connors and Long, 2004; Mathy et al., 2014).

Entrainment of EEG in specific frequency bands by corresponding rTMS (Romei *et al.*, 2016; Thut *et al.*, 2011) and ELF-MF (Bell et al., 1994) frequencies have been described. Thus in our case it may be interesting to design an LI-rTMS pattern that delivers bursts at frequencies mimicking the physiological rhythm of ION or PCs during reinnervation, to see if PC reinnervation can be further increased. In a general context it would be interesting to test the efficiency of rTMS or LI-rTMS delivering burst of pulses at different physiological EEG frequency bands depending on the targeted brain structure, its natural oscillatory rhythm, and the pathology of the patient, as suggested by (De Ridder et al., 2007). Indeed if LI-rTMS promotes neural plasticity through the entrainment of a physiological rhythm in stimulated neurons, then the random frequency at which bursts of pulses are delivered in the random iTBS would fail to entrain a physiological rhythm in neurons and thus might explain the absence of clear effects on olivocerebellar reinnervation or gene expression.

In this thesis we investigated the effects of different LI-rMS frequency patterns with a fixed duration of daily stimulation (10min), based on previous work from our group (Grehl et al., 2015; Makowiecki et al., 2014; Rodger et al., 2012) and on a study evaluating the optimal duration of mechanical vibrissae stimulation for appropriate functional outcome after peripheral nerve injury (Angelov et al., 2007). Thus in contrast to most human rTMS studies comparing different stimulation patterns with the same number of pulses but different durations, we were able to test the effect of pulse number and how it is delivered eg the same number of pulses at different rhythms (iTBS vs riTBS) or a different number of pulses within the same time frame (ie variable dose density) as opposed to total dose (i.e. number of pulses) on reinnervation density. Our results show that there is no simple linear correlation between the dose (number of pulses delivered in a given time) and the effects (neural circuit plasticity), in accordance with high intensity TBS studies in humans (Gamboa et al., 2010; Nettekoven et al., 2014) and animals (Volz et al., 2013). Moreover, our low intensity stimulation does not heat the stimulation coil as in high-intensity protocols, which allowed us to stimulate continuously for 10min without stopping to cool the apparatus. This allowed us to show that induced biological outcomes of LI-rMS are critically dependent on pattern rather than dose of stimulation (frequency vs number of pulses). iTBS induced reinnervation of the OCP but cTBS did not; since the break between episodes of TBS is the only parameter that varies among these two patterns, this result demonstrates that the interval between theta bursts critically influences reinnervation of the OCP induced by LI-rTMS as it does with its effect on activity for high-intensity TBS ((Huang et al., 2005; Di Lazzaro et al., 2005). In contrast, due to technical limitations, to date in human rTMS studies, the high intensity electrical field produces heating of the coil and thus often requires coil cooling intervals within a session of stimulation (Rossi et al., 2009; Rossini et al., 2015). Therefore it would be worth considering the contribution of these coil "cooling breaks" on induced cortical plasticity.

We have shown that patterns of LI-rMS are crucial to observe biological effects both *in vivo* and *in vitro*, but we also wanted to understand how biological tissues can detect a time-variable magnetic field, and how this interaction could produce structural changes in neuronal circuits.

### **V.1.2) LI-rTMS requires cryptochromes to interact with biological tissue and potentially activate the transcription of signalling pathways.**

We have found that cryptochromes are required in mice for the transduction of LI-rMS into biological effects, such as axon growth and post-lesion neosynaptogenesis. This observation introduces a novel role of the magnetic field *per se* in the human magnetic stimulation. We show for the first time that the magnetic field can potentially activate biological cellular pathways through its action on cryptochromes. The activated signalling pathway that results in neural circuit repair of the OCP are unknown, but some hypotheses can be developed based on the literature and our findings.

Mammalian cryptochromes (CRY) are ubiquitously expressed nuclear proteins (Lin and Todo, 2005) and inhibit the transcriptional activity of the CLOCK/BMAL1 circadian complex which controls the expression of a large number of genes (Chaves et al., 2006; Kondratov et al., 2006; Zhu et al., 2003) and thus accounts for the circadian control of many physiological events (Panda and Hogenesch, 2004). Magnetic field effects on CRY signalling activity is thought to involve conformational changes at the active site of the CRY protein, promoting interaction with its downstream signaling partners (Lin and Todo, 2005). Thus exogenous magnetic fields might modify Cry functional activity and thus remove its inhibitory action on the CLOCK/Bmal1 transcriptional activity (Zaporozhan and Ponomarenko, 2010). This potential mechanism could account for the otherwise surprising altered circadian rhythm and increased gene transcription in animals and cells exposed to ELF-MF (Choi et al., 2003; Goodman et al., 1992; Wei et al., 1990). Importantly, the quantitative transcriptional changes depend on ELF-MF intensity and frequency (Litovitz et al., 1990; Wei et al., 1990), which is coherent with the crucial importance of frequency patterns described previously in this discussion. Therefore the low intensity magnetic fields generated by LI-rMS could be detected by cryptochromes and induce biological changes by indirectly modulating the transcription of a wide range of genes in a stimulation pattern-specific manner.

It is known that Bmal1/CLOCK heterodimers have transcription factor functions, and *in silico* searches of transcription factor binding sequences (TFBSs) for Bmal1 and CLOCK showed that they have target sequences present on several genes that were significantly up- or down-regulated (or showed tendencies) by different LI-rMS patterns in our study (see chapter III). Our findings, indicate that LI-rMS modulated the expression of genes implicated in the induction of neural circuit plasticity in a pattern-dependent manner. This is coherent with

cryptochromes regulating gene expression and therefore mediating the transduction of pattern-specific magnetic fields into long-term functional and structural modifications of neurons (Jones et al., 2001).

Whether cryptochrome activation by time-varying magnetic fields directly modulates gene transcription or if it does so through the activation of other second messengers is still not known.

### **V.1.3) Intracellular calcium may be a second messenger mediating the biological effects of cryptochrome activation by LI-rMS**

#### **V.1.3.1) Intracellular calcium levels and downstream signalling cascades**

Modulation of intracellular calcium and other downstream signalling pathways by ELF-MF has been thoroughly demonstrated in various models (Kavet *et al.*, 2001; Morabito *et al.*, 2010; Saunders and Jefferys, 2007; Zhang *et al.*, 2017). These intracellular calcium concentration changes have been described to arise from activation of voltage-gated calcium channels (VGCC) (Barbier *et al.*, 1996; Grassi *et al.*, 2004; Piacentini *et al.*, 2008);, including L-type calcium channels (Morgado-Valle *et al.*, 1998), or G protein signalling (Zhang *et al.*, 2017). Other studies have demonstrated calcium release from intracellular stores (Aldinucci et al., 2000; Grehl et al., 2015; Pessina et al., 2001) in response to ELF-MF. Results from our group showed increased intracellular calcium concentration, due to release from intracellular stores, induced by LI-rMS with parameters similar to those used in our study (BHFS; 10mT) (Grehl et al., 2015). Although we don't have direct evidence of the modulation of intracellular calcium concentration through activation of mammalian CRY, calcium homeostasis is suggested as a second messenger for cryptochrome signal transduction in *Arabidopsis thaliana* (Folta and Spalding, 2001; Lin and Shalitin, 2003; Long and Jenkins, 1998). Therefore our results together with the results from Grehl et al (2015) suggest that intracellular calcium may be a central, action potential independent, cellular mechanism underlying the effects of LI-rMS on neural circuits, and a potential second messenger candidate for cryptochrome signal transduction.

It is known that an increase in intracellular calcium concentration within a short period can activate calcium-dependent kinase cascades; some of these lead to the phosphorylation of

CREB and to the transcription of immediate early genes (IEGs) including c-fos (Appleyard, 2009; Bito et al., 1997; Flavell and Greenberg, 2008; Kawashima et al., 2014). In turn c-fos can regulate the expression of later-response genes leading to long-term functional and structural plasticity in the neuron (Jones et al., 2001). We showed that BHFS and iTBS LI-rMS increased the number of c-fos-positive cells 2.5h after a single stimulation, including the proportion of Purkinje cells and interneurons that were c-fos positive. Thus our findings are consistent with the possibility that the c-fos-dependent signalling pathway described above contributes to the gene expression changes and long term structural plasticity induced by LI-rMS in the OCP. Further systematic investigation of the effects of several LI-rMS frequency patterns on c-fos activation in each cell type of the OCP would be required to make a direct link between frequency pattern, c-fos activation, gene expression changes, and structural plasticity.

Ultimately it would be interesting to find direct evidence of cryptochrome activation by specific LI-rMS patterns and to show a possible correlation with modulation of intracellular calcium concentration and gene expression.

#### **V.1.3.2) ROS production**

Results from this thesis strongly suggest that cryptochrome activation is necessary to transduce LI-rMS signal into biological effects. Transient formation and accumulation of reactive oxygen species (ROS) such as of  $O_2^\bullet$  and  $H_2O_2^-$  are byproducts of cryptochrome activation and may contribute to its signaling role (Arthaut et al., 2017; Consentino et al., 2015; Jourdan et al., 2015; Usselman et al., 2014). Additionally almost all studies that tested the effects of EMFs above 1mT found small “sub-toxic” (30-60%) modifications of ROS, compatible with both the protective effects of ELF-MF exposure (Mattsson and Simkó, 2014) and an inhibitory effect of high levels of ROS on the AKT pathway and Foxp3 expression which enhances ELF-MF induced tumor lysis in metastatic melanoma (Tang et al., 2016). Thus ROS production at “moderate” levels are proposed to be an early response of ELF-MF exposure (Di Loreto *et al.*, 2009; Mattsson and Simkó, 2014; Simkó, 2007).

As described above (see chapter V.1.3.1), intracellular calcium homeostasis is also a possible cellular mechanism underlying the effects of LI-rMS and a potential second messenger candidate for cryptochrome signal transduction. It is interesting to note that ROS and  $Ca^{2+}$  both act as cellular signalling molecules and mutually interact: ROS regulates calcium

cellular signalling and increased intracellular calcium from intracellular stores (mitochondria and endoplasmic reticulum) activates ROS production (Gordeeva et al., 2003). These interactions are important for fine tuning of cellular signalling networks, and altering them can lead to neurodegenerative diseases, such as amyotrophic lateral sclerosis, Parkinson's disease and Alzheimer's disease (Cali et al., 2013; Görlach et al., 2015; Tadic et al., 2014).. Although we did not quantify ROS production in our explant, further research into potential common pathways between LI-rMS-induced cryptochrome activation, ROS production, and calcium signalling might help to optimize novel therapeutic strategies.

#### **V.1.4) ROR $\alpha$ seems necessary to observe neural circuit plasticity and behavioural improvements induced by LI-rTMS**

Previous results from our group have suggested that LI-rTMS was more likely to affect an altered neuronal system, than a normal one (Makowiecki et al., 2014; Rodger et al., 2012). Thus we studied the short-term and long-term effects of LI-rTMS on PC dendritic morphology, gene expression, cerebellar-related behaviour and post-lesion repair in mutant mice haploinsufficient for ROR $\alpha$  gene. This mouse is a model of abnormal cerebellar development, structure/maintenance and ageing (Boukhtouche et al., 2006a; Chen et al., 2013; Gold et al., 2007; Jetten and Joo, 2006). We found that LI-rTMS, in these ROR $\alpha$ <sup>+/-</sup> animals and olivocerebellar explants, show only small short term effects on dendritic spine density and gene expression but no sustained long term effects on PC morphology, cerebellar behavior or neural circuit plasticity. In comparison with results obtain in WT mice and explants, these observations suggest that ROR $\alpha$ <sup>+/-</sup> mice are less responsive to LI-rTMS. ROR $\alpha$  interacts indirectly with cryptochrome by promoting the transcription of BMAL1, which activates transcription of Cry and Per (Akashi and Takumi, 2005; DeBruyne et al., 2007; Duez and Staels, 2009; Sato et al., 2004). The complex cyclic feedback loops between Cry and ROR $\alpha$  could partially explain the decreased ROR $\alpha$ , but normal cry1 expression in the cerebellum of aged ROR $\alpha$ <sup>+/-</sup> mice, in association with PC plasticity. Whereas, Cry1 expression was reduced in the cerebellum of adult ROR $\alpha$ <sup>+/-</sup> and these animals were the less responsive to the stimulation; this is potentially coherent with the central role of cryptochrome in the transduction of LI-rTMS signals into biological effects (as previously discussed).

Overexpression of ROR $\alpha$  in cortical neurons has been shown to reduce ROS production (Boukhtouche et al., 2006b) through increased expression of *Gpx1* and *Prx6*, which are involved in ROS degradation; this ROR $\alpha$  overexpression led to reduced sensitivity to apoptotic stimuli compared to controls (Boukhtouche et al., 2006b; Hoehn et al., 2003; Salmon et al., 2004). It is also known that several genes involved in calcium signalling and buffering are overexpressed in the developing cerebellum of ROR $\alpha$  mutant mice (Gold et al., 2003). Therefore it is possible that in ROR $\alpha$ <sup>+/-</sup> mice, the reduced expression of ROR $\alpha$ , affects the normal regulation of intracellular calcium and ROS production to a “moderate” level, similar to that induced by LI-rMS cryptochrome activation, and therefore obscures the effect induced by LI-rTMS with its downstream signalling modifications to neural and behavioural plasticity. However further studies would be required to find direct interactions between ROR $\alpha$  expression, LI-rTMS induced cryptochrome activation and subsequent intracellular calcium and ROS signalling.

Our results also show that the presence of ROR $\alpha$  in the ION seems to be more important than in the cerebellum for the development of LI-rMS induced post-lesion reinnervation of PCs by CFs. Although it is difficult to interpret this observation with certainty, it suggests that ROR $\alpha$ 's actions are preferentially on plasticity of the presynaptic neuron (e.g. axon growth mechanisms of the ION neurons) rather than plasticity of the post-synaptic neurons (e.g. molecular cues secreted by PC).

ROR $\alpha$ 's roles in PC development and maturation have been extensively described (Boukhtouche et al., 2006c; Chen et al., 2013; Jetten and Joo, 2006; Shirley and Messer, 2004) and suggest that it regulates the expression of genes involved in neural plasticity. ROR $\alpha$  haploinsufficient mice present premature-onset ageing characteristics (Janmaat et al., 2011; Jarvis et al., 2002; Mamontova et al., 1998), where ageing is characterized by a decreasing level of neural plasticity (Freitas et al., 2011a, 2011b). Thus it is possible that ROR $\alpha$  haplodeficient mice have generally reduced levels of neural plasticity; this might explain the lower level of LI-rTMS induced neural changes compared to those observed in WT animals, quite independently of a role in magnetoreception.

Whether due to altered cryptochrome transcription, calcium and ROS signalization, or a general lack of plasticity the ROR $\alpha$ <sup>+/-</sup> nervous system is clearly much less responsive to LI-

rTMS. Future experiments will examine other methods of inducing postlesion plasticity (such as application of BDNF or overexpression of PSA-NCAM,) in  $ROR\alpha^{+/-}$  explants to determine whether the reduced reinnervation response only occurs with magnetic stimulation. Moreover, since we showed crucial importance of the pattern of stimulation to observe neural circuit plasticity it will be interesting to test the effect of other LI-rTMS pattern on reinnervation in  $ROR\alpha^{+/-}$  explants to see whether other patterns of LI-rTMS are more effective.

## **V.2) Biological effects of LI-rTMS**

### **V.2.1) LI-rTMS targeting the cerebellum induces neuronal structural plasticity and improvements in cerebellar related behaviour in adult mice.**

Recent studies have shown that high-intensity rTMS of the cerebellum in humans modulates neuronal circuits between the cerebellum and the primary motor cortex, changing cerebellar excitability to alter motor and cognitive function (Casula et al., 2016; Grimaldi et al., 2014; Popa et al., 2013; Schutter and van Honk, 2009). Thus cerebellar rTMS is thought to have potential wide clinical applications. However since cerebellar plasticity and long term neural sequelae induced by cerebellar rTMS are not yet established, experts in the field estimate that further research is necessary before possible clinical use (Grimaldi et al., 2014). In our *in vivo* study of the LI-rTMS effects on cerebellar neuronal development, morphology and associated behaviour in WT mice, we provide the first evidence that sub-threshold magnetic stimulation is able to induce dendritic spinogenesis after only 3x10min LI-rTMS sessions, and long-term increase of dendritic density and complexity with longer treatment (4weeks). Importantly, this long-term PC dendritic size increase was accompanied by improved spatial learning and memory, behaviors that have been shown to require effective parallel fibre-Purkinje cell LTD (Lefort et al., 2015; Rochefort et al., 2011, 2013). These results are of particular interest since high-intensity cerebellar rTMS is technically challenging, due to discomfort associated with induced neck muscle contractions (Demirtas-Tatlidede et al., 2011; Hardwick et al., 2014); this problem could be avoided with the use of LI-rTMS.

In this thesis we described in detail the LI-rTMS-induced structural plasticity in the central neuron and sole output of the cerebellar cortex, the Purkinje cell. The rapid increase in PC dendritic spine density, which is primarily due to a sharp increase in stubby spines, is coherent with the activation of a plasticity mechanism (Knott et al., 2006; Lai and Ip, 2013). Acute cerebellar LI-rTMS thus seems able to increase the number of synaptic inputs onto PC (Arellano et al., 2007; Hering and Sheng, 2001; Lai and Ip, 2013) and could reshape connectivity within cerebellar neuronal circuits. Spinogenesis in adult animals has been shown to underlie memory formation (Fu et al., 2012; Segal, 2005) and other adaptive changes of the brain (Hofer et al., 2009; Holtmaat et al., 2006). Further investigation on the molecular and cellular mechanisms underlying LI-rTMS-induced new spine formation could identify the stimulation parameters which optimally activate those mechanisms. This would be of particular interest since the impairment of activity-dependent spine morphogenesis underlies many neurological disorders including age related cognitive decline (Lai and Ip, 2013; Penzes et al., 2011).

Our results showing the long-term increase in PC dendritic tree size and branching complexity induced by LI-rTMS in adult mice are consistent with morphological PC changes observed following periods of motor learning or environmental enrichment in rats (De Bartolo et al., 2015; González-Burgos et al., 2011; Lee et al., 2007, 2013) and which also suggest a modulation of synaptic efficacy (Lee et al., 2005). It is known that dendritic branching complexity influences action potential propagation efficacy along the dendrites (Vetter et al., 2001) which will have a key role in the integration of synaptic inputs and in the induction of synaptic plasticity mechanism such as LTP and LTD (Johnston et al., 1996; Linden, 1999). Interestingly, the spatial memory of adult mice was improved by LI-rTMS, providing a correlation between morphological and behavioural effects. However we cannot make a direct link between the PC morphological changes and spatial memory improvement, since this behaviour involves other structures such as the hippocampus. Nonetheless it would be interesting to evaluate the effects of LI-rTMS on the plasticity of PF-PC synapses (LTD) *in vivo* in mice (Lefort et al., 2015; Rochefort et al., 2011, 2013).

### **V.2.2) Reduced neural plasticity in aged mice may explain reduced LI-rTMS effects on cerebellar structural plasticity and behaviour**

Although we found evidence of acute dendritic spine formation with 3 days of stimulation in the PCs of aged mice, this spinogenesis was reduced compared to that in adult mice. Moreover these short term plastic changes were not maintained, and we saw no increased PC dendritic complexity or behavioral improvement in aged mice after 4 weeks of stimulation. These results suggest that ageing PCs are less responsive to plastic changes induced by external stimuli (in this case LI-rTMS), a phenomenon already described in the electrophysiology literature. Indeed it has been shown that aged mouse neurons are characterized by slower firing rates, shorter spike duration, higher variability in spike intervals than in younger adult neurons, and importantly decreased L-type calcium currents (Branch et al., 2014). Additionally, it has been shown that expression of GluA2 and PKM $\zeta$  in dendritic spines is altered in aged monkeys and that this is associated with memory deficits (Hara et al., 2012). Thus even if short-term LI-rTMS slightly increases dendritic spine density in aged mice, this altered synaptic plasticity and neuronal function due to age might explain the absence of sustained morphological changes in the PC dendritic complexity, and could prevent LI-rTMS-dependent behavioral improvement.

However it is also essential to consider the changes in the ageing brain at a more complex level, such as functional connectivity and the oscillatory activity of brain areas. Indeed evidence for reduced hemispheric lateralization (Cabeza et al., 1997; Grady, 2008; Rossi et al., 2001) as well increased recruitment of frontal areas together with reduced occipital activity (Cabeza et al., 2004; Davis et al., 2008; Madden et al., 2002) have been thoroughly described in the ageing brain. These phenomena have been proposed to be potential compensatory mechanisms to overcome age-related functional decline (Cabeza et al., 1997; Nielson et al., 2002; Stebbins et al., 2002), but more recently an alternative hypothesis has been proposed suggesting that this engagement is more likely to be an inefficient allocation of brain resources rather than a functional compensatory mechanism (Grady, 2008; Tatti et al., 2016). However this recruitment of additional brain areas for a specific task might partially explain the absence of functional improvement induced by LI-rTMS in aged mice in our study, which targets the cerebellum and not the hypothetical additional brain areas. Simultaneous LI-rTMS stimulation of both the cerebellum and the hippocampus, for

example, to see if this leads to greater functional improvement in the aged mice would partly answer this question.

EEG alterations have also been observed during physiological aging (Tatti et al., 2016), however the direction of the changes in specific brain oscillatory patterns is still not clear. Some studies show increased delta (1–3 Hz) and theta (4–7 Hz) power (Dujardin et al., 1994, 1995; Klass and Brenner, 1995), but others show a decrease in these same frequency bands (Klimesch, 1999; Stomrud et al., 2010). Considering this altered oscillatory activity in aged neurons and our previous results in chapter III showing that the biological outcomes of LI-rTMS seem to be stimulation-pattern dependent, further experiments systematically testing other stimulation frequencies and patterns in aged mice could identify better stimulation parameters to induce long term structural and functional change in these animals.

Reduced neuroplasticity has been well described in elderly population (Freitas et al., 2011a; Pascual-Leone et al., 2011), and recent rTMS studies in animals suggest that multiple sessions of stimulation within the same day separated by close intervals can produce more durable plasticity (Goldsworthy et al., 2015). In the aged population it would therefore be useful to test different numbers of stimulations and time intervals. LI-rTMS is subthreshold, in that it does not directly trigger action potential in the stimulated neurons; we suggest that it acts on intracellular mechanisms to induce a form of metaplasticity. Aged neurons may be less susceptible to be activated by such subthreshold mechanisms and therefore require either higher intensity of stimulation or multiple consecutive stimulation sessions to be achieve the same level of activation.

### **V.2.3) Further investigation into the effects of magnetic stimulation are required prior to eventual clinical use in young patients**

Because our results in adult and aged mice showed that LI-rTMS modifies the structure of Purkinje cells and cerebellar-related behavior in an age dependent manner, it was important to evaluate if this stimulation could alter PC morphological and synaptic development.

Our results on young animals show that LI-rTMS did not induced gross perturbations of the PC morphological and synaptic development; only slight morphological and synaptic

variations were detected around P12 but these changes had disappeared by P14. This is important for potential clinical applications because it suggests that the normal developing cerebellum, and perhaps the whole nervous system, may not undergo gross perturbations as a result of LI-rTMS treatment and that more subtle therapeutic changes, if they occur, may not be disruptive. Developmental critical periods are potentially interesting time windows for modulation of neural plasticity by non-invasive brain stimulation, but interventions during these periods may have unintended results as well. Indeed during this period, high levels of plasticity are necessary for the physiological establishment of neural circuitry and function (Ganguly and Poo, 2013) and have been shown to be experience and activity dependent in both the visual system (Anderson et al., 2011; Chapman and Stryker, 1993; Espinosa and Stryker, 2012) and the corticospinal tract (Anderson et al., 2011; Martin, 2005). As both rTMS and LI-rTMS modulate cortical network activity (Di Lazzaro et al., 2013; Pell et al., 2011), their application to modulate brain activity during critical periods of development in young patients with neurodevelopmental disorders such as autism spectrum disorder (ASD) might be a promising tool, since ASD is thought to result from a disrupted balance between excitatory and inhibitory neurotransmission (LeBlanc and Fagiolini, 2011). However it is important to note that maladaptive plasticity can also occur, notably leading to devastating effects following some early brain injuries (Kolb et al., 2000).

Considering the serious harmful effects that any (LI-)rTMS-induced maladaptive plasticity would represent to young patients, caution is required during the interpretation of our results. In this initial study we looked for gross perturbations to PC morphology and synaptic development, but further animal research on more subtle changes such as dendritic spine and receptor plasticity, *in vivo* electrophysiological properties of stimulated neurons, and behavioral consequences would need to be undertaken. Moreover systematic investigations of different stimulation parameters (intensity, frequency pattern and duration) should be done in different brain structures before concluding on the safety of (LI-)rTMS in the immature brain. However temporary, the alterations to PC dendritic morphology reinforce the need for caution, especially since low intensity stimulation, such as used in this study, could occur brain regions adjacent to the structures targeted by high-intensity rTMS. In contrast with the ageing brain where lower levels of plasticity are described (Freitas et al., 2011a, 2013; Pascual-Leone et al., 2011), the developing brain is highly plastic. Thus LI-rTMS

seems better suited than high intensity rTMS for application in young subjects since subthreshold stimulation may be more susceptible to induce neural plastic changes in these subjects compared to elderly population, with the advantage of minimising the risk of undesired side effects (Davis and van Koningsbruggen, 2013).

In chapter III we showed that the pattern of stimulation is critical to induce post-lesion plasticity of the olivocerebellar pathway; it is possible that the BHFS pattern we used in the development study in vivo was not appropriate to induce PC morphological and synaptic plasticity in the developing mouse cerebellum. In section V.1 of this discussion we hypothesised that electrical synapses between olivary neurons underlying their oscillatory synchronization might render them more responsive to sub-threshold patterned stimulations (Connors and Long, 2004; Mathy et al., 2014). Interestingly, membrane potential oscillations in olivary neurons, recorded in brainstem slices from immature rats, were absent before P9 but were present in 41% of neurons between P10-15 and in 95% of neurons after P16 (Bleasel and Pettigrew, 1992); this could also partly explain the absence of gross developmental perturbation at the ages we stimulated (P2-P14).

Previous work, using the same parameter of stimulation as in our study has shown that abnormal neural circuits can be induced to undergo considerable remodelling while normal ones appear unchanged (Makowiecki et al., 2014; Rodger et al., 2012; Sykes et al., 2013). Further experiments in animal models of neurodevelopmental disorders and/or abnormal developmental circuits would allow us to see if under these conditions LI-rTMS can correct structural abnormalities and promote associated functional improvement. The highly consistent cerebellar abnormalities present in ASD suggest that cerebellar functional alteration might play a crucial role in its aetiology (Buckner, 2013; D'Mello and Stoodley, 2015; Hampson and Blatt, 2015). Moreover ROR $\alpha$  has been recently identified as a novel candidate gene for autism spectrum disorder (ASD) (Nguyen et al., 2010; Sarachana and Hu, 2013) and we know that ROR $\alpha$  deficient mice have cerebellar developmental abnormalities (Boukhtouche et al., 2006c; Gold et al., 2007; Shirley and Messer, 2004). Further research on the effects of LI-rTMS on the cerebellar development of these, and other potential ASD candidate gene mutant/transgenic, animals might give us key information on the potential of LI-rTMS to correct neurodevelopmental abnormalities.

## VI ) Conclusions

Repetitive transcranial magnetic stimulation (rTMS) is commonly used to modulate cortical excitability and shows promise in the treatment of some neurological disorders. It is assumed that the effects observed are mainly due to the induction of activity dependent mechanisms.

In this thesis we have shown that focal low intensity magnetic stimulation (LI-rTMS), which does not directly elicit action potentials in the stimulated neurons, can also induce neural plasticity within the cerebellar cortex and olivocerebellar pathway, specifically: (1) changes at the levels of gene expression and cerebellar dendritic spines after short-term (3x10 min) stimulation; and (2) increased neuronal connectivity at the levels of dendritic branching, or axonal outgrowth and reinnervation after long-term (14-28x10min daily sessions) stimulation. It is probable that some effects of short-term LI-rTMS contribute to the longer-term effects. Indeed we have shown that 3 days of stimulation with a BHFS pattern were able to alter PC dendritic spine density and morphology and thus modify PC connectivity and function, which is then increased with further LI-rTMS (chapter II and IV). Indeed the functional sequelae of modifying PC connectivity are illustrated, in part, by the improved spatial memory that accompanied the LI-rTMS-induced neuronal structural plasticity.

We also identified that age had considerable effect on the response to LI-rTMS. Remarkably, the long term induced plasticity was only present in adult mice but not in aged mice nor in  $ROR\alpha^{+/-}$  mice that have altered neural development and ageing (chapter II and IV). Although in  $ROR\alpha^{+/-}$  mice 3 days of stimulation tended to normalize aberrant expression of genes involved in magnetoreception and spinogenesis (Chapter IV), the acute changes in dendritic spines associated with this gene regulation did not result in long-term alteration of PC dendritic morphology and cerebellar-related behavioural improvement. To understand why, requires more extensive studies involving different stimulation patterns and/or intracellular signalling and gene expression analyses.

In chapter III we showed that 2 weeks BHFS stimulation to the mouse cerebellum *in vivo* induced olivocerebellar reinnervation after unilateral transection of the inferior cerebellar peduncle. Previous experience shows that once this CF-PC reinnervation has developed, it is

permanent and does not subsequently regress (Sugihara et al., 2003). We then deepened our investigation *in vitro* and demonstrated the critical importance of the *pattern of stimulation*, rather than number of pulses, for promoting reinnervation. Specifically we show that magnetic stimulation delivering regular complex bursts of pulses are more efficient than straight tonic frequencies, with BHFS and iTBS being the most efficient stimulation patterns to induce post-lesion PC reinnervation in the mature olivocerebellar pathway. The relevance of these different LI-rTMS patterns/frequencies is supported by our gene expression data, in which, after only 3 daily sessions, reinnervation-inducing stimulation patterns/frequencies upregulated genes involved in axon growth and guidance, appropriately for reinnervation to occur, whereas stimulation that did not induce reinnervation either did not change, or down-regulated, growth-promoting genes.

Very importantly we have shown for the first time that cryptochrome, which has magnetoreceptor properties in flies and birds, must be present for the response to magnetic stimulation to be transduced into biological effects (Chapter III). This result suggests that the magnetic field *per se* has effects, which gives new insight for the interpretation of studies about the effects of low intensity magnetic stimulation. Further experiments trying to find direct evidence of cryptochrome activation by specific magnetic stimulation patterns, and identifying the consequent second messenger systems would give important information about the mechanisms underlying low intensity magnetic stimulation. For future therapeutic strategies this would ultimately help to adapt the stimulation parameter to the neuronal context in each individual person, which seems to critically influence the outcomes of a given stimulation.

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