What Does Low-Intensity rTMS Do to the Cerebellum?

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Conflict of Interest

There are no current or potential conflicts of interest for any of the authors.

Abstract:

Non-invasive stimulation of the human cerebellum, such as by transcranial magnetic stimulation (TMS), is increasingly used to investigate cerebellar function and identify potential treatment for cerebellar dysfunction. However, the effects of TMS on cerebellar neurons remain poorly defined. We applied low intensity repetitive TMS (LI-rTMS) to the mouse cerebellum in vivo and in vitro and examined the cellular and molecular sequelae. In normal C57/Bl6 mice, 4 weeks of LI-rTMS using a complex biomimetic high-frequency stimulation (BHFS) alters Purkinje cell (PC) dendritic and spine morphology; the effects persist 4 weeks after the end of stimulation. We then evaluated whether LI-rTMS could induce climbing fibre (CF) reinnervation to denervated PCs. After unilateral pedunculotomy in adult mice and 2 weeks sham or BHFS stimulation, VGLUT2 immunohistochemistry was used to quantify CF reinnervation. In contrast to sham, LI-rTMS induced CF reinnervation to the denervated hemicerebellum. To examine potential mechanisms underlying the LI-rTMS effect, we verified that BHFS could induce CF reinnervation using our in vitro olivocerebellar explants in which denervated cerebellar tissue is co-cultured adjacent to intact cerebella and treated with BDNF (as a positive control), sham or LI-rTMS for 2 weeks. Compared with sham, BDNF and BHFS LI-rTMS significantly increased CF reinnervation, without additive effect. To identify potential underlying mechanisms, we examined intracellular calcium flux during the 10 min stimulation. Complex high frequency stimulation increased intracellular calcium by release from intracellular stores. Thus even at low intensity, rTMS modifies PC structure and induces CF reinnervation.

Introduction:

Non-invasive stimulation to the cerebellum is increasingly being investigated not only to clarify cerebellar function and its influence on other regions of the brain, but also as a potential treatment for neurological dysfunction. Such stimulation can be induced through repetitive transcranial magnetic stimulation (rTMS) or transcranial direct current stimulation (tDCS) and their effects vary according to the method of stimulation delivery (rTMS frequency, tDCS anode/cathode) [1,2]. While both rTMS and tDCS have been shown to modulate function of cerebello-thalamo-cortical loops [see review 2], the long-term consequences of cerebellar stimulation remain unknown including the effects of such stimulation on the cerebellum itself and its intrinsic circuits. In order to better understand the behavioural or psychiatric outcomes of cerebellar stimulation, animal studies are necessary to identify underlying cellular or network mechanisms of non-invasive cerebellar stimulation.

Cerebellar Purkinje cells (PCs) are large macro-neurons at the centre of the cerebellar cortical circuit, which modulate activity in the deep cerebellar nuclear outflow. Synchronous activity in groups of PCs selects appropriate muscle groups to perform skilled movements [4,5]. Purkinje cell synchronous firing is strongly regulated by their climbing fibre afferents from the inferior olive (ION) [4]. Olivocerebellar axons cross the midline in the medulla and ascend the contralateral inferior cerebellar peduncle to terminate on PCs organised in narrow parasagittal zones. This precise parasagittal organisation is refined during the developmental process of transient PC multiple innervation by CFs arriving from both contralateral and ipsilateral inferior olives [6] and the subsequent selective synapse stabilisation and elimination of supernumerary synapses [7] and ipsilateral projections [6].

Thus any modulation of Purkinje neurons and/or their CF afferents by magnetic stimulation is likely to affect cerebellar-mediated behaviours.

Since it has been proposed that homeostatic mechanisms render the brains of healthy volunteers less responsive to rTMS than those of patients with neurological conditions [8] we also lesioned the olivocerebellar path to induce a condition in which we could more easily identify rTMS-induced cerebellar plasticity. Plasticity within the olivocerebellar pathway can be induced by cutting it unilaterally (pedunculotomy: Px) in rodents and injecting neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) [9], into the denervated hemi-cerebellum. This stimulates the remaining (ipsilateral) ION to grow a new pathway, which reinnervates the denervated hemicerebellum [9,10]. The induced reinnervation is incomplete, but respects the parasagittal organisation [9] and supports recovery of motor and spatial function [10]. Given that intracranial injection of BDNF is not readily transferrable to the clinic and that rTMS can upregulate BDNF synthesis [11], we wanted to investigate whether non-invasive cerebellar stimulation could induce similar olivocerebellar reinnervation.

Low intensity rTMS alters Purkinje cell morphology

To investigate the effect of magnetic stimulation on cerebellar PCs, we applied a low intensity (10mT) high frequency complex pattern of stimulation (LI-rTMS), which we have previously shown modulates axonal projections in the visual system [3], to the cerebellum of healthy mice aged 3 or 7 months, 10 min per day for 4 weeks. Sham treated animals underwent the same handling but the stimulator was not turned on. We then evaluated gait

on a CatWalk XT (Noldus Information Technology, Wageningen, The Netherlands), motor coordination on an accelerating rotarod (TSE Systems, Bad Homburg Germany) and spatial learning in a Morris Water maze according to our established protocols [10, 12]. All our experiments 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.

General motor activity and gait were normal in all groups with no differences between LI-rTMS treated mice and sham controls. However when we evaluated spatial learning and memory in the Morris water maze, we found that while LI-rTMS did not alter parameters of spatial learning, in the probe test there was a positive effect of LI-rTMS on spatial memory in comparison to sham-treated controls ($p<0.05$; Fig 1).

We then wanted to see if these modifications in spatial behaviour were accompanied by morphological changes. After behavioural testing, mice were euthanized and the cerebellum prepared for electrophysiology [10]. We carried out whole cell patch clamp recordings of Purkinje cells in cerebellar slices, with biocytin in the internal pipette solution to allow visualisation of the cell morphology after fixation and streptavidin revelation. Confocal imaging of fluorescent PCs was followed by simple measurements of dendritic arbor dimensions. In the LI-rTMS treated mice, PC dendritic arbors were taller and had a greater surface area ($p< 0.05$; Fig 1). Higher power images were used to quantify spine density on these same PCs; we found that spine density was increased in LI-rTMS treated mice ($p<0.01$). Whether these spine changes are directly due to the LI-rTMS, or indirectly through the improved spatial learning and memory, cannot be identified from this study. However, since these morphological studies were undertaken after the 3 weeks of

behavioural testing, these data reveal that one month of daily LI-rTMS can induce stable changes to PC morphology in conjunction with modified spatial learning and memory.

Low intensity rTMS induces CF reinnervation

In a second series of experiments we also tested whether magnetic stimulation parameters can promote PC reinnervation post-lesion. These experiments were undertaken using our explant model of the olivocerebellar system *in vitro* [13,14], in which the cerebellum and brainstem are cultured "en bloc" to retain all cell populations and circuits. These explants develop as *in vivo* and can be manipulated to replicate the pedunculotomy lesion and PC reinnervation [14]. They are highly reproducible and thus form an easily controlled experimental paradigm for examining the effects of magnetic stimulation on olivocerebellar neosynaptogenesis. Following deafferentation of the hemicerebellum and co-culture adjacent to an intact explant, we treated them with our complex pattern low intensity repetitive magnetic stimulation (LI-rMS) [3] for two weeks. VGLUT2-positive CF reinnervation was induced by our LI-rMS. To evaluate potential mechanisms underlying this process, we used qPCR to identify that BDNF was upregulated in the explant. Moreover, the density of reinnervation induced by LI-rMS is not additive with BDNF-induced repair, suggesting that the same pathways mediate these two responses. We are currently investigating whether this effect of LI-rTMS is reproducible *in vivo.* Our preliminary data indicate that our complex frequency pattern LI-rTMS can induce post pedunculotomy olivocerebellar reinnervation.

Finally, to try to understand the cellular signalling processes likely to underlie these LI-rTMS effects we have undertaken experiments in cell culture [15]. In primary cultures of cortical neurons, we performed calcium imaging using Fura2AM ratiometric fluorescence changes from 5 minutes pre- to 5 minutes post- repetitive magnetic stimulation. We found rMS increased cytoplasmic calcium and pharmacological analysis showed that this was due to release from intracellular stores rather than influx from the extracellular milieu (Fig 1). This increase in calcium was associated with altered expression of genes of interest for neuronal survival (JunD, hexokinase) and cytoskeleton modification (Cyr61) [15].

Conclusion

In conclusion, our data show that repeated low-intensity magnetic stimulation to the cerebellum modifies Purkinje cell dendrites and can facilitate reorganisation of the olivocerebellar circuit; effects which need to be considered during the clinical application of cerebellar stimulation for ameliorating cerebellar dysfunction or modulating extra-cerebellar motor circuits.

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Figure Legend

Fig 1

A: A schematic diagram showing the relation of the stimulation coil (blue) to the mouse cerebellum (modified from [3]).

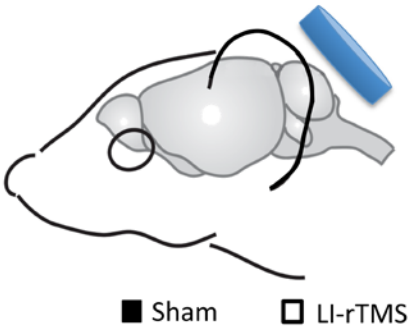
B: Calcium imaging of cultured cortical neurons during 10min LI-rMS reveals that in comparison to sham (black bar), LI-rMS increased Fura-2 fluorescence intensity (open bar) even in calcium-free media (hatched bar). However treatment with thapsigargin, to deplete intracellular calcium stores, abolished this effect (Th bar) indicating that the rise in calcium was due to release from intracellular stores. (Graphic based on data from [15]; Mann Whitney U, ** = $p<0.01$; *** = $p<0.001$).

C: Four weeks LI-rTMS improves spatial memory as revealed in the probe test by treated mice crossing the previous location of the escape platform more often that sham mice (2-way ANOVA, $F_{1,49} = 4.66$, * = $p<0.05$).

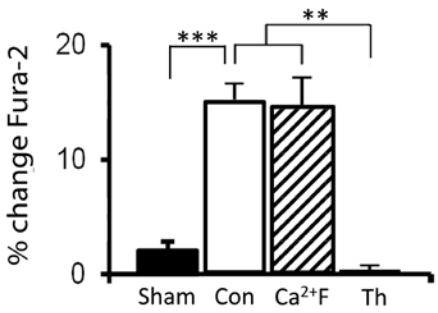
D: LI-rTMS increased the size of the Purkinje cell dendritic tree (student t-test; * = $p<0.05$).

3m = 3 month mice; 7m = 7 month mice; Con = LI-rMS in control media; $Ca^{2+}F$ = LI-rMS in calcium-free media; Th = LI-rMS after thapsigargin treatment.

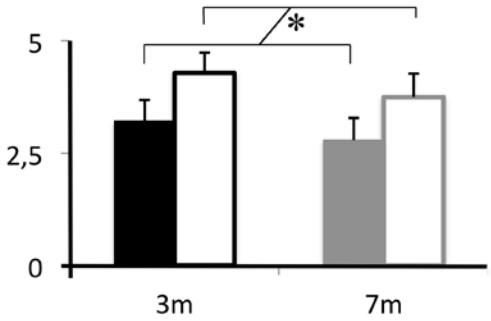
A: Cerebellar LI-rTMS



B: LI-rMS increases intracellular Ca^{2+}



C: Spatial Memory - Platform crossings



D: PC Dendritic Tree Area (μm^2)