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RESEARCH ARTICLE

Opposite impact on ^{14}C-2-deoxyglucose brain metabolism following patterns of high and low frequency repetitive transcranial magnetic stimulation in the posterior parietal cortex

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Abstract Repetitive transcranial magnetic stimulation (rTMS) appears capable of modulating human cortical excitability beyond the duration of the stimulation train. However, the basis and extent of this "off-line" modulation remains unknown. In a group of anesthetized cats, we applied patterns of real or sham focal rTMS to the visuo-parietal cortex (VP) at high (HF) or low (LF) frequency and recorded brain glucose uptake during (on-line), immediately after (off-line), or 1 h after (late) stimulation. During the on-line period LF and HF rTMS induced a significant relative reduction of ^{14}C-2DG uptake in the stimulated VP cortex and tightly linked cortical and subcortical structures (e.g. the superficial superior colliculus, the pulvinar, and the LPl nucleus) with respect to homologue areas in the unstimulated hemisphere. During the off-line period HF rTMS induced a significant relative increase in ^{14}C-2DG uptake in the targeted VP cortex, whereas LF rTMS generated the opposite effect, with only mild network impact. Moderate distributed effects were only recorded after LF rTMS in the posterior thalamic structures. No long lasting cortical or subcortical effects were detected during the late period. Our findings demonstrate opposite modulation of rTMS on local and distant effects along a specific network, depending on the pattern of stimulation. Such effects are demonstrated in the anesthetized animal, ruling out behavioral and non-specific reasons for the differential impact of the stimulation. The findings are consistent with previous differential electrophysiological and behavioral effects of low and high frequency rTMS patterns and provide support to uses of rTMS in neuromodulation.

Keywords Posterior parietal cortex · Plasticity · Neuromodulation · Spatial attention · Neglect · Rehabilitation

[†]Prof. Payne passed away in May 2004. This article is submitted in his memory.

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Introduction

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive neurophysiologic technique capable of temporarily modifying activity in cortical circuits (Kobayashi and Pascual-Leone 2003; Walsh and Pascual-Leone 2003). Such modulation of brain activity and its associated behavioral consequences can outlast the duration of the rTMS trains ["off-line" effects (Robertson et al. 2003) or "after effects" (Siebner and Rothwell 2003)], and may have therapeutic utility in a variety of psychiatric and neurological pathologies in which brain function might be altered (Pascual-Leone et al. 2001; Wassermann and Lisanby 2001). Depending on

stimulation parameters, particularly frequency, the modulatory effects of rTMS appear to be different (Pascual-Leone et al. 1998; Siebner and Rothwell 2003), and recent experiments imply that with appropriate choice of rTMS frequencies or intertrain intervals it might be possible to induce effects similar to those observed in long-term depression (Iyer et al. 2003) and long-term facilitation (Huang et al. 2005).

Most of these insights are derived from studies in humans, particularly of rTMS to the motor cortex, where the outcome measure is the amplitude of motor evoked potentials and the confounding effect of motor activation or sensory feedback even a subthreshold levels are not easy to control for (Valero-Cabré et al. 2001; Gerschlager et al. 2002; Valero-Cabré and Pascual-Leone 2005). Human and animal studies combining rTMS with various brain mapping methods have been used to explore this question and frequency related changes on regional cerebral blood flow signal have been described in motor regions (Fox et al. 1997; Paus et al. 1997; Siebner et al. 1998, 2001; Civardi et al. 2001; Bestmann et al. 2003; Chouinard et al. 2003; Lee et al. 2003; Okabe et al. 2003; Bestmann et al. 2004; Takano et al. 2004; Rounis et al. 2005; Valero-Cabré et al. 2005). The off-line behavioral impacts of high and low frequency rTMS on prefrontal (Robertson et al. 2001; Mottaghy et al. 2003), occipital (Ganis et al. 2000), or parietal cortex (Hilgetag et al. 2001; Thut et al. 2005) have been also reported, but the difficulty obtaining an independent, physiologic output measurement of rTMS effects makes it challenging to establish their neural bases. As a result, relatively little is still known about such off-line and frequency dependent effects of rTMS when applied to non-motor cortical regions.

To specifically address this question, we applied patterns of high (20 Hz) or low (1 Hz) frequency rTMS to the posterior and inferior parietal cortex (visual parietal VP cortex) in anesthetized cats (Fig. 1a) and studied with high spatial resolution their impact on brain metabolic activity, as measured with ^{14}C-2DG (Kennedy et al. 1975; Sokoloff et al. 1977; Payne and Lomber 1999; Valero-Cabré et al. 2005) during (on-line), immediately after (off-line), or 1 h after (late) rTMS (Fig. 1b). The use of anesthetized animals has the advantage of allowing to control for the different scalp sensations, neck, face and scalp muscle contractions, and patterns of auditory stimulation induced by rTMS at various frequencies. The choice of VP cortex as target of stimulation has the benefit that the nodes and pathways of the involved cortico-subcortical network have been well characterized in the cat in previous anatomical, electrophysiological and metabolic studies combined with cooling or rTMS reversible deactivation (Palmer et al.

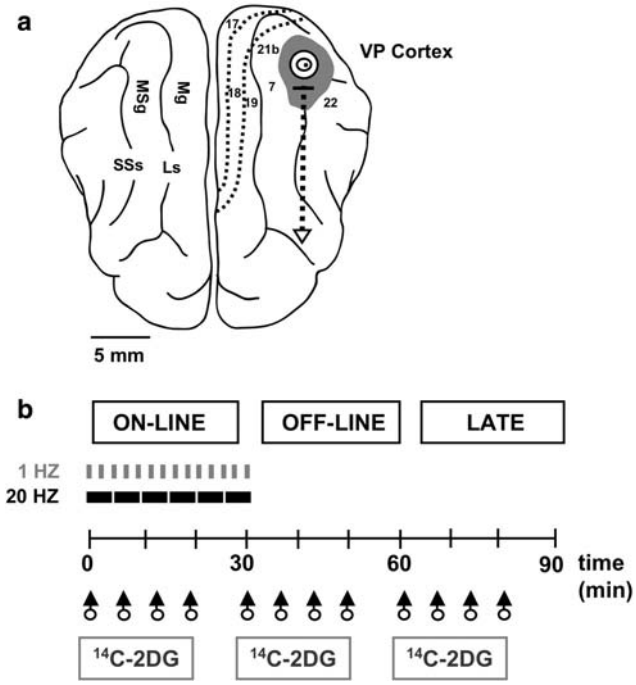

Fig. 1 **a** Dorsal view of the cat's brain. Location of visuo-parietal (*VP*) cortex is identified (*shading*) with the target on the area of rTMS stimulation (◎). Visual areas 17, 18 and 19 are identified with the marginal gyrus (*Mg*), middle suprasylvian gyrus (*Msg*), lateral sulcus (*Ls*) and suprasylvian sulcus (*SSs*). Areas 21b and 7 are both located between the lateral and the suprasylvian sulci, medial from VP, whereas area 22 is localized lateral to VP. **b** Time scale of the application of rTMS and administration of ^{14}C-2DG. Patterns of rTMS were delivered at 20 Hz (2 s trains, 28 s inter train interval, 2,240 pulses) or 1 Hz (1,800 pulses of continuous stimulation). Four boluses (25 μCi/kg each) of ^{14}C-2DG were injected intravenously, during (on-line), immediately after (off-line) or 30 min after the end of rTMS trains (late). Perfusion fixation of brain took place 10 min after the end of the last ^{14}C-2DG injection in each condition

1978; Updyke 1981; Payne 1994; Payne et al. 1996; Vanduffel et al. 1997a; Galuske et al. 2002; Payne and Lomber 2003; Valero-Cabré et al. 2005). Behavioral experiments demonstrate that this region and its rivalrous interactions with its homologue in the contralateral hemisphere might be critical—in both felines and humans—for the ability to redirect attention, and orient head and eyes to look at novel stimuli (Payne et al. 1996; Hilgetag et al. 2001; Thut et al. 2005; Valero-Cabré et al. 2006). Only balanced levels of activity in both VP cortices, ensure an equal degree of inhibition between hemispheres and midbrain structures, so that the probability to respond to targets is the same at both visual hemifields (Payne and Rushmore 2004). Unilateral local disruptions caused by parietal damage (Payne and Lomber 2003) or reversible cooling deactivation (Payne et al. 1996) or TMS (Valero-Cabré et al. 2006) and resulting in a relative interhemispheric imbalance have shown to induce a behaviorally relevant neglect-like

syndrome. Those deficits can be paradoxically cancelled if interhemispheric balance is achieved through the deactivation of the intact homologue parietal region (Payne et al. 2003; Payne and Rushmore 2004).

Materials and methods

Animals

Ten adult female cats (2.4–2.7 kg) underwent real or sham rTMS applied to the VP cortex (Fig. 1a). Under deep halothane anesthesia (4%), each cat was intubated with an endotracheal tube and then had its head secured in a non-metallic stereotaxic frame (Kopf, Tujunga, CA). The skin and temporal muscles on both sides of the head were detached from the bone to expose the skull and cortical landmarks that permitted identification of VP on the posterior area of the middle suprasylvian gyrus (pMS). Thereafter, and throughout the experiment, including rTMS and ^{14}C-2DG injections, inhaled halothane was kept constant at 0.7–0.8% to minimize neural depression but maintain complete anesthesia. Inhaled O_2 (28–32%), nitrous oxide (68–72%), exhaled CO_2 (3.8–4.2%), heart rate (185–200 beats/min), respiratory rate (35–38 strokes/min), respiratory volume (30–35 ml), blood O_2 saturation (96–98%) and rectal temperature (38–38.5°C) were closely monitored and maintained within a tight range for the duration of the experiment. All procedures were approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine and follow the Policies on the Use of Animals and Humans in Neuroscience Research, revised and approved by the Society for Neuroscience in January 1995.

Transcranial magnetic stimulation

A tightly wound, 50 mm outer-diameter circular coil (Magstim Inc., UK) was held tilted at a 45° angle to the surface of the skull by a mechanical arm fixed to the stereotaxic frame and lowered until the anterior edge of the coil was in direct contact with the parietal bone over the left VP cortex (see Fig. 3 in Valero-Cabré et al. 2006, for details). This method of stimulation with an appropriately tilted, small circular coil held on edge to the scalp provides the most focal form of TMS (Amassian et al. 1990; Roth et al. 1991), inducing a metabolic effect that is limited to a cortical area of about 10–15 mm^2 (Valero-Cabré et al. 2005), and resulting into a significant and selective visuo-spatial disruption (Valero-Cabré et al. 2006). The thinness of the skull allowed precise identification of the cortical macroanatomical structures, such that the VP cortex could be localized and clearly marked to allow precise positioning of the TMS coil over the targeted structures. Repetitive TMS was delivered in patterns of 20 Hz (high frequency, HF group; $n = 4$) or 1 Hz (low frequency, LF group; $n = 4$) at 55% of maximal stimulator output intensity (~135% of resting motor threshold) using a Magstim Super Rapid magnetic stimulator. The motor threshold was determined prior to the experiment as the minimal level of TMS intensity applied over the left primary motor cortex inducing a 50 μV motor evoked potential in the contralateral *flexor carpi radialis* muscle in at least 5 out 10 attempts. Surface EMG electrodes located in the above-mentioned muscle were used for recordings as specified elsewhere (Valero-Cabré et al. 2005). HF animals received a total of 56 trains of 2 s duration with 28 s intertrain intervals (2,240 pulses; Fig. 1b), whereas LF animals received 1,800 pulses of continuing stimulation. The number of pulses in both paradigms was adjusted to account for the same total stimulation time. In two additional animals, sham rTMS was applied over the VP cortex to control for additional, non-rTMS-related experimental effects, such as the clicking sounds generated by the pulses, or the impact of positioning and holding the animal in the stereotaxic apparatus. This was done by positioning the flat surface of the coil at a 90° angle with respect to the scalp surface, such that the generated magnetic field was directed away from the brain (Valero-Cabré et al. 2005, 2006). The duration of rTMS application was adapted in all paradigms to fit the ^{14}C-2DG protocol in which at least 25–30 min were needed to ensure equal and relatively stable levels of tracer in the vascular compartment and reliable ^{14}C-2DG tissue accumulation (Valero-Cabré et al. 2005). The rTMS intertrain intervals and stimulation intensity were set to prevent the coil from overheating while delivering as many pulses of high intensity rTMS as possible. Similar stimulation parameters and durations have been successfully used in humans to induce behavioral impairment in a visual attention task (Pascual-Leone et al. 1994; Hilgetag et al. 2001; Valero-Cabré et al. 2006) or produce opposite lasting modulation of cortico-spinal excitability (Gangitano et al. 2002). Furthermore, we have previously demonstrated that this HF rTMS parameters, when applied on-line (i.e., when the tracer is injected during the rTMS application) resulted in significant effects on ^{14}C-2DG uptake at the site of stimulation and in distant areas along a specific neural in anesthetized cats (Valero-Cabré et al. 2005).

C^{14}-2-deoxyglucose metabolic labeling

A total of 100 μCi/kg ^{14}C-2DG (specific activity 310 mCi/mmol; Amersham, Piscataway, NJ, USA) was infused intravenously in four equal boluses of 25 μCi/kg ^{14}C-2DG at 5 min intervals during (on-line condition), immediately after (off-line condition), or starting 30 min after the end of the rTMS trains (late condition) (Fig. 1b). This protocol is an adaptation of Sokoloff's original method, which normally relies on a single bolus injection (Sokoloff et al. 1977). It has proved to be effective in consistently labeling metabolic changes across long periods of time (~30 min) during which cooling or TMS have been applied onto the VP or the primary visual cortex (Payne and Lomber 1999; Lomber et al. 2002; Valero-Cabré et al. 2005). We studied two cats in the on-line and late conditions, one exposed to HF and the other to LF rTMS. Only one animal per rTMS frequency was studied in the on-line condition as we simply sought to confirm our previously published results (Valero-Cabré et al. 2005). In the off-line condition, the main focus of the present study, we studied two animals receiving LF and two undergoing HF rTMS. Sham rTMS cats were injected with ^{14}C-2DG during the on-line and off-line periods, respectively to control for the potential side effects, associated with the delivery of TMS. After the 30 min of rTMS animals were given an overdose of sodium pentobarbital (50 mg/kg), and their vascular system was perfused with a flush solution (15% sucrose in 0.1 M phosphate buffer, pH = 7.4 during 1 min) followed by an aldehyde fixative solution (2% paraformaldehyde in 15% solution of 0.1 M phosphate buffer, pH = 7.4 during 5 min). Brains were dissected "in situ" and the exact position of application of rTMS to the cortex was identified before removing the parietal and frontal bones in their entirety. Brains were photographed, blocked, coated in albumin, and frozen as previously described (Payne and Lomber 1999). The parietal block of the brain was cut in 25 μm-thick coronal sections using a cryostat (−23°C). Every fourth section (each 100 μm) was collected on 0.5% gelatin–chrome–alum-subbed coverslips and warmed to 60°C on a copper plate. The coverslips were serially mounted onto cards in sets of 25, and exposed to photographic Agfa-Structurix D7 X-ray film (Ridgefield Park, NJ, USA) for 18 days at −80°C. Each card was accompanied with radioactive C^{14} calibration standards (Amersham). Additional sections were collected on glass slides and processed for Nissl substance or cytochrome oxidase and used to delineate areas, nuclei, layers, and other subdivisions. The spatial resolution of the technique has been estimated to be in the range of 100 μm (Lowel 2002).

Image analysis

Images were digitized using full spectrum illumination and a CCD camera (Payne and Lomber 1999) with a final resolution 0.047 mm/pixel. ^{14}C-2DG uptake was quantified by measuring optical densities in the ^{14}C-2DG radiograms using a computer controlled image analyzer (AIS^{TM}, Imaging Research Inc., St. Catherines, ON, Canada) and related to ^{14}C standards via calibration curves (Gonzalez-Lima 1992). Software was calibrated for each autoradiograph by quantifying the optical density of ^{14}C-2DG activity levels in the calibration standards, and linear calibration curves were constructed and used to quantify ^{14}C -2DG uptake in the sections. A circular sampling area of 100/250 μm radius was used to measure optical density in a variety of cortical locations, and in a number of subcortical structures. Sampling included sulcal and limited gyral areas of the VP cortex corresponding to middle suprasylvian (MS) cortex. The medial (VPm) and lateral banks (VPl) of the VP cortex were both sampled along their entire trajectory from the crown to the depth of the sulcus. These samples were pooled together and averaged. In the cortex, measurements were made in a strictly sequential order from lateral to medial but avoiding transition zones from one area to another. These were identified on brain sections stained for Nissl substance or reacted for the presence of cytochrome oxidase, and were related to the maps of the visual areas (Tusa et al. 1981). Between 55 and 70 sections per animal covering a 20 mm distance were analyzed in the cortex. Measurements taken in thalamic and midbrain structures also respected architectonic divisions (Kanaseki and Sprague 1974; Updyke 1981; Harting et al. 1992). The sample area had a diameter of 100 μm in the superior colliculus (SC), 250 μm in cortical regions, and 350 μm in the white matter. Between 25 and 32 sections per animal were sampled for the SC and the thalamic nuclei. General results are based on an average of 40,236 sample sites in each animal, amounting to a total of 402, 360 measurements.

Statistical analyses

Absolute levels of ^{14}C-2DG activity in each section (nCi/g) were measured and compared across homologous structures of the right (non-stimulated) and left (stimulated) hemispheres by means of paired *t*-tests, with Bonferroni corrections for multiple comparisons. Therefore, all reported changes refer to relative decrease or increase of the differential left–right interhemispheric ^{14}C-2DG uptakes across homologues brain structures and areas. These values were

calculated as [(left/right) × 100]−100, so that 0% indicates equal uptake in the stimulated (left) and non-stimulated (right) hemispheres. Values < 0% indicate either rTMS-induced decreases in ^{14}C-2DG uptake in the stimulated hemisphere, alone or combined with potential increases in uptake in the non-stimulated hemisphere. Similarly, values > 0% could be due to rTMS-induced increases in ^{14}C-2DG uptake in the stimulated hemisphere alone or combined with decreases in the non-stimulated hemisphere. This approach has been successfully used previously to label deactivation or activation effects on cortical and subcortical visual structures. It is optimal to minimize inter-subject variability in ^{14}C-2DG uptake, thus reducing to reasonable levels the amount of animals and analysis effort needed to initially explore a large number of conditions (Vanduffel et al. 1995, 1997a, b; Payne and Lomber 1999; Lomber et al. 2002). Inter-hemispheric differences in ^{14}C-2DG uptake levels were also compared across animals and conditions by means of non paired *t*-test. Significance level was set at $P < 0.05$ or $P < 0.01$ (after correction for multiple comparisons).

Results

"On-line rTMS": local impact

On-line rTMS at both 1 Hz (animal on-line-LF) and 20 Hz (animal on-line-HF) induced a significant decrease in the relative levels of ^{14}C-2DG uptake across the whole left VP region ($P < 0.01$). This effect is the result of an induced imbalance of metabolic activity between hemispheres. Twenty Hertz rTMS proved to locally reduce average metabolic activity down to significantly lower levels of uptake (−14.7 ± 0.9%) than 1 Hz (−3.8 ± 0.3%) ($P < 0.0001$) (Figs. 2, 3; Table 1). As described elsewhere, superficially located sulcal regions and layers within VP were more significantly impacted than deep regions and layers (Valero-Cabré et al. 2005).

The patterns of high frequency rTMS achieved its maximal impact right in the targeted cortical area (−20 ± 1.7%; $P < 0.001$). This effect wore-off rapidly with distance, reaching non-significant values for the inter-hemispheric comparison 11.8 mm rostrally to the targeted site. In addition, there was slight spread of the impact of HF rTMS medially and laterally into brain areas immediately adjacent to the targeted VP cortex. This included medially, area A21b/7 in which uptake was decreased by 6.5 ± 1.7% ($P < 0.05$), and laterally, A22, where activity was suppressed by 5.1 ± 1.9%; ($P < 0.05$). These findings confirm our previously reported results (Valero-Cabré et al. 2005) (see Fig. 1a for details on the anatomy of cortical gyri and sulci; Fig. 4; Table 1).

The impact of LF rTMS was less focal and less strong in the targeted area than that of HF rTMS. However, both, HF and LF rTMS resulted in a decrease in uptake in the targeted hemisphere as compared with the unstimulated hemisphere. LF rTMS decreased uptake in the targeted region by −5.9 ± 1% as compared with the homologous site in the non-stimulated hemisphere ($P < 0.02$). Unexpectedly, the interhemispheric imbalance was maximal in a region rostral from the target site by 11.3 mm, rather than right at the site of stimulation. This local effect decayed to non-significant values at around 13.3 mm

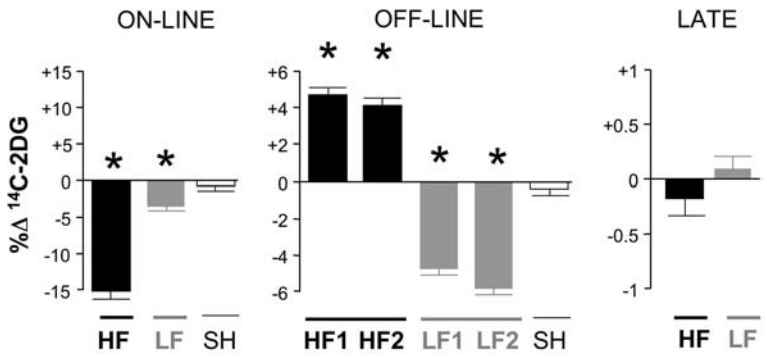

Fig. 2 Average impact of rTMS on primary sites in the cerebral cortex. *Histograms* represent the relative impact of real rTMS on the eight cats studied across the three rTMS conditions (on-line, off-line, late). Values in each cat are all relative to contralateral homologue areas [(left/right) × 100]−100 (0% = equal uptake on stimulated (*left*) and non stimulated (*right*) sides, < 0% = rTMS-induced decrease, > 0% induced increase in ^{14}C-2DG uptake). Impact is shown for the visuo-parietal cortex (*VP*) cortex. *HF*, *HF1* and *HF2* (*in black*) high frequency stimulated cat, *LF, LF1* and *LF2* (*in grey*) low frequency stimulated cats at each period, and *SH* (*in white*) sham stimulated cats at each period. *Asterisks* identify statistically significant decreases in ^{14}C-2DG uptake between hemispheres (*$P < 0.05$) of absolute (nCi/kg) values of 2DG comparing stimulated (*left*) with non stimulated (*right*) areas. Two additional cats were stimulated with sham rTMS during the on-line and off-line conditions, resulting in no significant impact

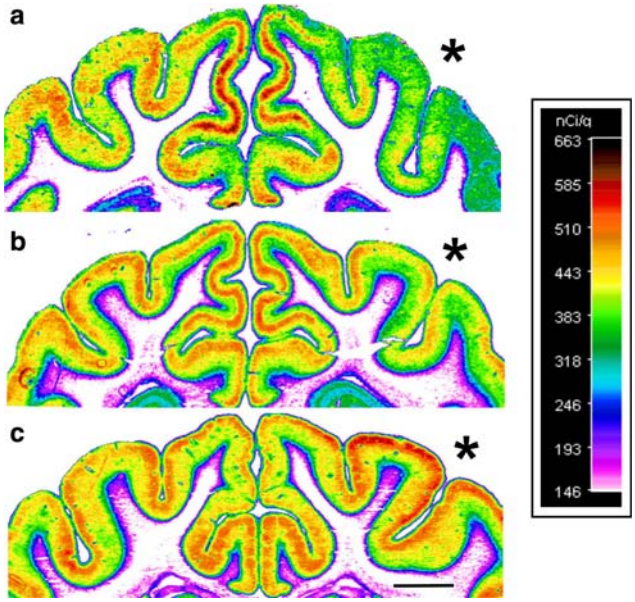

Fig. 3 Color coded ^{14}C-2DG uptake densities following stimulation of the visuo-parietal (*VP*) cortex in the following conditions (**a**) 20 Hz on-line, (**b**) 1 Hz off-line and (**c**) 20 Hz off-line. Notice the relative decrease of ^{14}C-2DG at the stimulated VP (*) respect to the contralateral VP area in **a** and **b** and the increase of ^{14}C-2DG in **c**. (*) rTMS stimulated VP area

from the stimulation center. Adjacent gyri medial (A21b/7) or lateral (A22) from the site of stimulation did not reveal any interhemispheric asymmetries in 2DG uptake (Table 1, Fig. 4). A sham stimulated cat analyzed during this on-line period did not yield as expected any significant difference between VP cortices ($-0.7 \pm 0.4\%$; $P > 0.05$). The white matter underlying the rTMS stimulated VP area of all those animals did not show any significant change with respect to its contralateral counterpart at either rTMS frequency (Table 1, Fig. 4).

"Off-line" rTMS: local impact

HF and LF rTMS resulted in opposite "off-line" effects. Following LF rTMS, we found decreased relative levels of metabolic activity in the targeted VP area as compared with the opposite hemisphere in both off-line-LF animals (-4.7 ± 0.3 and -5.8 ± 0.4, $P < 0.01$). However, following HF rTMS we measured an increase in 2DG uptake in the targeted VP cortex as compared with the non-stimulated side in both animals ($+5.0 \pm 0.4\%$ and $+4.0 \pm 0.4\%$; $P < 0.01$, respectively). These findings reveal a differential impact of LF and HF stimulation and a significant difference between on-line and off-line effects of HF rTMS (Fig. 2, 3, 4; Table 1). For both stimulation paradigms, effects proved to be more significant for superficial sulcal regions and layers, than those located deep within VP. A sham stimulated cat injected with 2DG during this off-line period did not show any significant difference between VP cortices ($-0.4 \pm 0.6\%$; $P > 0.05$).

As in the case of 1 Hz on-line rTMS, the local off-line impact of 1 Hz rTMS appeared to be displaced from the site directly under the coil. The interhemispheric comparison in 2DG uptake became first significant at 0.8 mm (LF1) and 1.6 mm (LF2) from the coil's center, and reached maximal interhemispheric asymmetry at 3.8 mm (LF1) and 6.3 mm (LF2) rostral from the targeted region. In both animals, the stimulated hemisphere showed significantly less 2DG uptake than the homologous areas in the non-stimulated hemisphere (maximal difference $-6.05 \pm 0.3\%$; $P < 0.001$ in LF1 and $-7.48 \pm 0.4\%$; $P < 0.005$ in LF2). This interhemispheric asymmetry, with a decrease in 2DG uptake in the stimulated hemisphere, became non significant at 12.8 mm (LF1) and 14.4 mm (LF2) from the site of stimulation (Fig. 4). In both LF animals, there was a significant spread of the rTMS effects towards areas medial from the stimulation site, such as A7/21b (-5.4 ± 2 and -3.7 ± 0.4; $P < 0.05$). There was no significant spread of the rTMS effects towards lateral portions A22 of the brain (Table 1).

The 20 Hz off-line effect on the targeted brain region also appeared slightly displaced from the precise site of stimulation in both HF animals. The 2DG uptake across the hemispheres became significantly different at 1.6 mm (HF1) and 2.8 mm (HF2) from the targeted area, and reached maximally significant asymmetry at 7.3 mm (HF1) and 10.9 mm (HF2) rostral from VP (Fig. 4). In both cases, and contrary to the effects of HF and LF on-line rTMS, and of LF off-line rTMS, 2DG uptake was greater in the stimulated than the non-stimulated hemisphere ($+7.2 \pm 1.05$; $P < 0.01$ in HF1, and $+8.3 \pm 1.0\%$ $P < 0.01$ in HF2). The interhemispheric asymmetry in 2DG uptake became non-significant at 13.3 mm (HF1) and 15.7 mm (HF2) from the coil's center. Impact did not significantly spread laterally from the targeted region, but it did spread medially to areas 7 and 21b in both animals ($+5.0 \pm 0.5$ and $+6.3 \pm 0.5\%$; $P < 0.05$) (Table 1).

Distant impact of "on-line" and "off-line" rTMS

On-line rTMS at 20 Hz patterns induced clear distant effects in areas holding rich connections with the VP cortex, thus confirming our previously reported results (see Payne and Rushmore 2004; Valero-Cabré et al. 2005) for details on cat's visuo-parietal network). For example, the superior colliculus (SC) ipsilateral to the stimulated VP cortex showed lower levels of ^{14}C-2DG

Table 1 Impact on ^{14}C-2DG uptake of rTMS on-line, off-line and late at different cortical and subcortical areas in stimulated animals

% Δ	On-line		Off-line		Off-line		Late	
	HF	LF	HF1	HF2	LF1	LF2	HF	LF
VP	**−14.7***	**−3.8#**	**+5.0***	**+4.0***	**−4.7***	**−5.8***	−0.2	+0.1
VPl	**−13.1***	**−3.5#**	**+4.7***	**+4.0***	**−4.5***	**−5.7***	−0.3	+0.1
VPm	**−16.1***	**−3.9***	**+5.2***	**+4.2***	**−5.0***	**−5.8***	−0.05	+0.2
A19	**−6.4***	+0.2	+1.2	−1.2	−2.1	−3.1	+0.1	+0.1
A18	**−9.2***	−0.9	+0.1	−0.7	−0.7	−1.8	−0.1	−0.2
A17	−2.0	−0.5	−0.3	−1.7	−0.03	−0.1	−0.8	+0.1
SVA	**−5.8***	−0.6	+2.7	−0.6	−0.4	−2.4	−0.1	−0.4
CVA	−0.4	−0.2	+1.8	−0.1	−0.5	−1.8	+0.1	−0.4
PCG	−1.7	−0.8	+0.6	+0.1	+0.7	−0.4	+0.6	−0.5
A7/21b	**−6.5***	−0.3	**+5.0***	**+6.3***	**−5.4***	**−3.7***	+0.5	+0.4
A22	**−5.1***	+0.1	+0.4	+0.4	−0.2	−0.1	−0.03	+0.1
SC	**−5.8***	**−2.8#**	−0.1	−0.1	−0.9	−1.3	+0.3	+0.1
SGS	**−9.6***	**−3.3#**	−0.6	−0.4	−1.1	−1.5	+0.1	−0.6
I	**−9.2***	**−3.0#**	−0.2	−1.6	−0.7	−0.5	+0.9	−0.1
II	**−9.6***	**−3.6#**	−1.3	+0.1	−1.0	−1.7	+0.5	+0.1
III	**−9.6***	**−3.0#**	−0.3	−0.1	−2.1	−2.2	−1.0	−0.3
SO	**−6.7***	**−2.6#**	+0.4	+0.4	−0.4	−2.4	+0.5	−0.4
SGI	−0.4	−0.7	+1.8	+0.9	−0.5	−0.5	+1.2	−0.2
SGP	+0.5	+0.2	+0.3	+0.6	−0.7	+0.3	+0.2	+0.4
IC	−2.3	−1.9	+0.9	+1.3	−0.9	+0.1	−0.2	−0.1
PUL	**−8.6***	**−3.1#**	+0.7	+0.1	**−4.5***	**−6.0***	+0.5	−0.5
LP	−2.9	−2.1	+1.8	+0.1	−3.8	−2.2	+0.2	−0.4
LPl	**−5.7***	**−3.0#**	+1.5	+0.4	**−4.1***	**−3.0#**	+0.5	−0.6
LPi	−0.2	−0.9	+1.2	−0.3	−3.4	−2.0	+0.3	−0.1
LPm	−0.2	−0.9	+0.8	+0.9	−0.9	−1.2	−0.8	−0.2
LGN	−1.0	+0.4	+0.2	+0.8	−1.3	−0.7	+0.1	−0.1
MGN	−2.5	−0.5	−0.3	−0.3	−0.4	−1.3	+1.0	−0.6
WM	−0.9	+0.01	+0.03	+0.06	−0.01	−0.07	−0.01	+0.03

Values are all relative to contralateral homologoue area [(left/right) × 100]−100 (0% = equal uptake on stimulated (left) and non stimulated (right) sides, < 0% = rTMS-induced decrease or > 0% increase in ^{14} C-2DG uptake. Two additional cats stimulated with sham rTMS during the on-line and off-line conditions resulted in no significant impact at any cortical or subcortical location (data not shown in the table, see Results and Fig. 2). Cortical regions: *VPm* medial bank of VP cortex, *VPl* lateral bank of VP cortex, *A17*, *A18*, *A19* primary visual areas 17, 18 and 19, *SVA* splenial visual area, *CVA* cingulate visual area, *pCG* posterior cingulate gyrus; tectal structures: *SC* superior colliculus; and its different layers: *SGS* stratum griseum superficiale; *SO* stratum opticum; *SGI* stratum griseum intermediale; *SGP* stratum griseum profundus; and *IC* inferior colliculus. Thalamic structures: *PUL* pulvinar, *LP* lateral posterior complex and its different divisions, *LPl*, *LPi* and *LPm* lateral, interadjacent and medial divisions of the lateral posterior complex (Updyke et al. 1981); *LGN* lateral geniculate nucleus; *MGN* medial geniculate nucleus; and *WM* white matter underlying VP. See details on the visuo-parietal anatomy and circuitry in Fig. 1a; Payne and Rushmore 2004 and Valero-Cabré et al. 2005. Asterisks identify statistically significant decreases in ^{14} C-2DG uptake between hemispheres (*$P < 0.01$, #$P < 0.05$) of absolute (nCi/kg) values of 2DG comparing stimulated (left) with non stimulated (right) areas; Significant effects in rTMS 1 or 20 Hz animals ($P < 0.05$ or $P < 0.01$) are presented in bold

uptake, as compared with the SC in the non-stimulated hemisphere ($-6.5 \pm 0.6\%$, $P < 0.05$). Consistent with the connectivity of VP cortex with SC, this impact was particularly prominent in the superficial layers of SC, stratum griseum superficiale (SGS -9.6 ± 1; $P < 0.05$) and stratum opticum (SO -6.7 ± 1.3; $P < 0.0001$), whereas no impact occurred in the stratum griseum intermediale (SGI) or deeper layers, such as the stratum griseum profundus (SGP) located only 200–400 μm deeper (Harting et al. 1992). A significant distant relative decrease in ^{14}C-2DG was also demonstrated in the ipsilateral pulvinar (-8.6 ± 0.8) and the lateral division of LP nucleus (LPl) ($-5.7 \pm 0.8\%$; $P < 0.01$) in the thalamus, as compared with homologous areas of the non-stimulated hemisphere. In addition, there was distant cortical impact of on-line 20 Hz rTMS in visual areas 18 ($-9.2 \pm 0.6\%$; $P < 0.01$) and 19 ($-6.4 \pm 1\%$; $P < 0.05$). Furthermore, the splenial visual area showed significant interhemispheric differences, with decreased uptake in the stimulated side (SVA -5.8 ± 0.8; $P < 0.01$). The site and system specific impact of rTMS was further verified by the absence of interhemispheric differences on other cortical regions such as the visual area 17 (A17), the cingulate visual area (CVA) and the posterior cingulate gyrus (pCG) and subcortical structures (LGN, MGN, IC) holding poor or no connections with VP (Vanduffel et al. 1997a; Valero-Cabré et al. 2005) (Table 1).

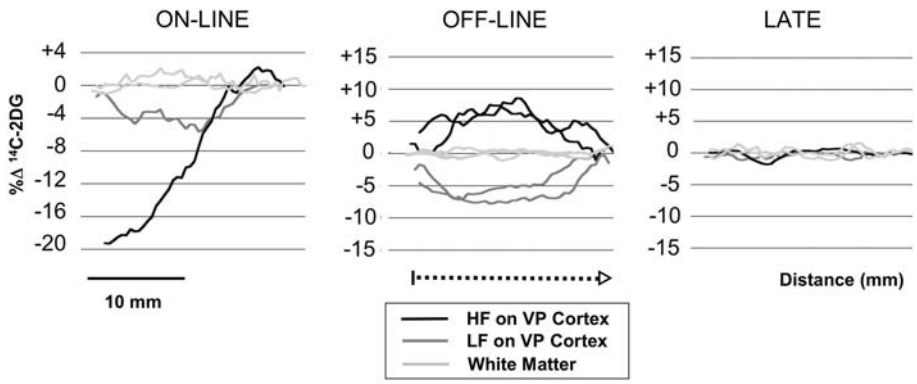

Fig. 4 Spatial distribution of ^{14}C-2DG uptake relative values (moving average with $n = 5$) across 20 mm in the caudal–rostral axis from beginning of VP cortex. Values correspond to (**a**) on-line ($n = 2$ animals) condition at high (*black*, $n = 1$) and low frequency (*dark grey*, $n = 1$) rTMS, (**b**) off-line ($n = 4$ animals) stimulated at high (*black*, $n = 2$) or low (*dark grey*, $n = 2$). For each condition values are compared to white matter ^{14}C-2DG left–right uptake differences in the set of same animals (*light grey*, undifferentiated). See statistically significant values and standard errors for the crucial data points of this graph in the results section. Uptake values are all relative to contralateral homologous area (100-[(left/right) × 100])−100 (0% = equal uptake on stimulated (*left*) and non-stimulated (*right*) sides, < 0% = rTMS-induced decrease, > 0% rTMS-induced increase in ^{14}C-2DG uptake)

During the on-line 1 Hz condition, only non-significant trends of similar distant impacts were detected in superior colliculus SGS ($-3.3 \pm 0.4\%$), the pulvinar ($-3.1 \pm 0.5\%$) and LPl nucleus ($-3.0 \pm 0.7\%$). During the off-line rTMS condition only the LF animals showed significant distant impact of the stimulation, while no interhemispheric differences in 2DG uptake in structures distant from the directly targeted VP cortex were noted in either off-line HF rTMS cats. In both off-line LF animals, the distant impact was strictly restricted to the Pulvinar and LPl thalamic nuclei, both of which showed decreases in 2DG uptake in the stimulated as compared with the non-stimulated hemisphere (pulvinar $-4.5 \pm 0.$ and $-6.0 \pm 0.7\%$, $P < 0.05$; LPl -4.1 ± 0.4, $-3.0 \pm 0.6\%$; $P < 0.05$). No distant effect was seen in the any of the layers of the SC, nor in any of the primary, splenial and cingulate visual areas (Table 1). Furthermore, in agreement with previous reports (Valero-Cabré et al. 2005) no significant effects were seen in any of those distant locations after sham rTMS (values between -0.8 ± 0.6 and $+1.0 \pm 0.8\%$; $P > 0.05$ for all regions).

Delayed impact of rTMS stimulation during the late period

When we allowed 30 min to pass between the rTMS and the first 2DG injection, no significant difference was found in VP cortex ^{14}C-2DG uptake across the hemispheres for HF or LF rTMS. Similarly, no significant inter-hemispheric differences were noted in these late animals for any of the other cortical or subcortical regions and structures that showed significant impact during on- or off-line rTMS (-0.3 ± 0.25; $+0.2 \pm 0.15\%$; $P > 0.05$) (Figs. 2, 4; Table 1).

Discussion

Our study in anesthetized animals reveals fundamental stimulation-frequency-dependent differences between the "on-line" and "off-line" effects of rTMS. During rTMS (on-line), ^{14}C-2DG uptake, a close correlate of neuronal presynaptic activity (Kennedy et al. 1975; Schoppmann and Stryker 1981), is decreased in the targeted brain region as compared with the homologous areas of the non-stimulated hemisphere. This effect is greater during our 20 Hz paradigm than during 1 Hz stimulation. The implication is that during rTMS neuronal firing might be locally suppressed and that these suppressive effects are greater with higher rTMS frequencies. On the other hand, immediately following rTMS (off-line), local ^{14}C-2DG uptake in the stimulated area depends on stimulation frequency pattern. If rTMS is applied at 1 Hz, uptake is decreased in the stimulated cortex as compared with the homologous area of the non-stimulated hemisphere. On the contrary, uptake is increased if rTMS is applied at 20 Hz. The implication is that the sign of off-line effects—relative suppression versus enhancement—is frequency dependent; and that following exposure to LF rTMS neuronal firing in the targeted brain region might remain suppressed for some time, while after exposure to HF rTMS, neuronal firing in the targeted brain area rebounds and is greater than in homologous areas of the non-stimulated hemisphere. Sham rTMS stimulated animals studied during the "on-line" and "off-line" periods and subjects stimulated and analyzed 30–60 min after the end of real rTMS trains did not show significant changes, thus ruling out potential spurious interhemispheric differences. The metabolic

modulation with opposite sign found in this study could be due to specific modulating properties of the frequencies themselves, or alternatively, secondary to differences between a continuous (1 Hz) versus an interrupted (20 Hz) pattern of rTMS stimulation (Huang et al. 2005) or the total number of pulses delivered in each paradigm.

Local "on-line" versus "off-line" impact

During rTMS (on-line) significant pools of the targeted cortical neurons are repetitively depolarized according to a specific stimulation frequency, which interferes with their normal firing rhythms. Supporting this notion, Funke and cols. showed that single or paired TMS pulses at high intensity were able to suppress firing in primary visual cortex of halothane anesthetized cats (Moliadze et al. 2003, 2005). It is reasonable to assume, that higher stimulation frequencies—thus interfering more frequently with intrinsic firing rhythms—will result in a greater suppression, as suggested by our on-line 2DG findings. On the other hand, "off-line" behavioral effects are likely due to long lasting alterations in intracortical excitability and neuronal firing (Walsh and Pascual-Leone 2003). Recent electrophysiological studies in primates, demonstrate that following 1 Hz rTMS (off-line), firing activity in the primary motor cortex of neurons encoding for a given movement direction is suppressed for a several minutes (J. Ashe et al. unpublished data). The effects of high frequency patterns on similar paradigms have not been studied yet, in part because it is difficult to train awake animals to tolerate the bursts of rapid tapping sensation, muscle twitching, and loud clicking noises. Nevertheless, several human studies have consistently shown "off-line" increases in cortico-spinal excitability (Pascual-Leone et al. 1994; Maeda et al. 2000; Gangitano et al. 2002; Peinemann et al. 2004; Takano et al. 2004; Huang et al. 2005) and regional blood flow changes (Okabe et al. 2003; Takano et al. 2004; Rounis et al. 2005) after rapid rTMS on motor regions. Therefore, long lasting increases in individual neuronal firing responses in the targeted cortical region should be expected as the main after-effect of high frequency patterns.

The spatial distribution of "on-line" and "off-line" impacts—specially at high frequencies—also suggests different mechanisms of action. On-line 20 Hz rTMS patterns induced a strong deactivation right under the coil, which decayed sharply with distance, likely to be the result of rhythmically persistent depolarization of neuronal clusters. Off-line rTMS, however, evoked not only a lower but also much less focused impact, which might be the result of a "mixed" effect combining a progressively decaying "off-line" impact with the counterbalancing efforts along the network targeted by rTMS. We hypothesize that compensatory phenomena could be primarily mediated locally so that altered neuronal firing can be corrected, but also affected by compensatory changes in cortical and subcortical sites in both, the ipsi- and contralateral hemispheres, with the aim of maximizing the likelihood of correct behavioral output. This phenomenon has been already reported in awake humans for example in the lack of behavioral consequences during effective primary motor cortex rTMS stimulation (Lee et al. 2003; Rounis et al. 2005). In other words, differences between "on-line" and "off-line" impacts might be due to the ability of the brain to functionally compensate the effects of such an "abnormal" physiological input with rapid plastic reorganization of the local and neighboring regions.

Duration of the "off-line" effects

Electrophysiological and behavioral experiments in different regions of the human cortex have consistently shown that the effect of a single rTMS session decays quickly with time (Munchau et al. 2002; Romero et al. 2002; Lee et al. 2003). However, a recent PET study in anesthetized monkeys reported long lasting effects (> 24 h) on the primary motor cortex and associated areas after a single session of rapid rTMS (Hayashi et al. 2004). In our experiment, the inter-hemispheric differences generated during the "on-line" and "off-line" periods, wore-off completely 30–60 min after the end of the rTMS application. This suggests complete restitution of the metabolic balance between bilateral regions and subcortical nodes within the network. Nevertheless, it is important to note that interhemispheric balance could have been achieved by both, the recovery of the system's original excitability trough the "wash out" of non-physiological firing rhythms from the targeted neurons, or by changes occurring at other network sites, for example, by an activity decrease at the contralateral homotopic VP region (Payne 1994; Payne et al. 2003). In the latter case, interhemispheric asymmetry of rTMS, and even behavioral effects of rTMS, might normalize without the neural impact of the stimulation having resolved (Payne et al. 1996).

Repetitive TMS frequency dependent effects

The most striking and novel result from the present study is the demonstration of the opposite impact of off-line rTMS on VP cortical metabolism depending on stimulation frequency pattern. These results are in close agreement with the observation that slow rTMS

on the human primary motor cortex leads to a decrease of intracortical and corticospinal excitability (Chen et al. 1997; Romero et al. 2002), whereas rapid rTMS tends to induce the opposite effect (Pascual-Leone et al. 1994; Maeda et al. 2000; Gangitano et al. 2002; Peinemann et al. 2004; Takano et al. 2004). Recently, similar frequency dependent changes (1–3 Hz mediated supression and 10 Hz mediated enhancements) have been elegantly reported in the primary visual cortex of anesthetized cats by measuring visual evoked potentials (Aydin-Abidin et al. 2006). In awake human experiments, however, differential but not opposite local and network regional blood flow (rCBF) changes were shown to occur after stimulation paradigms known to yield opposite excitability modulation, according to motor electrophysiological studies (Strafella and Paus 2001; Rounis et al. 2005). Those differences may be explained by the fact that cortical modulation mechanisms might not only be dependent on frequency patterns, but also on the specific microarchitecture of the targeted region. Thus reaction to identical stimulation patterns might greatly differ across brain areas, such as the motor or parietal brain regions. Finally experiments in awake human subjects are subject, even at subthreshold levels, to the confounding effects of auditory, scalp and peripheral sensory feedback due to the TMS.

Circumstantial analogies have been drawn between the depressant effect of "off-line" low frequency rTMS and long term depression (LTD), in which a long lasting decrease of excitatory synaptic transmission can be induced by 1 Hz electrical stimulation to the rat motor cortex (Castro-Alamancos et al. 1995; Iyer et al. 2003). Long term potentiation-like mechanisms (LTP-like) have been associated to particular patterns of "off-line" high frequency rTMS cortical facilitation (Huang et al. 2005). Our data prove that cortical presynaptic metabolic activity—which is the main source for ^{14}C-2DG cellular signal—can be long lastingly depressed or potentiated by rTMS in a frequency dependent manner. Nevertheless, our experimental methodology does not provide evidence for specific synaptic modulation, thus LTP and LTD as basis for the rTMS-induced effects remains tenuous and purely speculative. In fact, distributed network effects, rather than local synaptic changes, could well account for the same findings.

Impact of "on-line" and "off-line" rTMS on associated systems

In agreement with a rapidly growing body of literature, we found that "on-line" rTMS has significant repercussions in an extended neural network of cortical and subcortical nodes (Fox et al. 1997; Paus et al. 1997, 1998; Siebner et al. 1998, 2001; Civardi et al. 2001; Chouinard et al. 2003; Lee et al. 2003; Okabe et al. 2003; Takano et al. 2004; Rounis et al. 2005; Valero-Cabré et al. 2005). Consistent with anatomical studies, this distant impact was present only in cortical areas receiving rich projections from the targeted region (see Payne and Rushmore 2004 for a detailed review of the visuo-parietal circuitry; Vanduffel et al. 1997a; Payne and Lomber 2003; Valero-Cabré et al. 2005). Interestingly, the distant effect consisted in a relative deactivation at all significantly impacted sites (Valero-Cabré et al. 2005), as observed previously during cooling VP deactivation (Vanduffel et al. 1997a). The strictly local impact achieved by "on-line" 1 Hz as compared to 20 Hz rTMS might be explained by an insufficient supression of VP cortex to induce distant, transynaptic effects. Similarly, during the "off-line" period no consistent distant effects were detected, perhaps due to dissipating local impact. These observations are consistent with the notion that the efficiency in the deactivation of a population of neurons is progressively lost from the source of the perturbation along connectional chains (Payne and Lomber 1999; Valero-Cabré et al. 2005). The thresholds to achieve transynaptic effects in similarly distant targets under conditions of poor cortical VP impact—see "off-line" period—might differ as a function of connectivity richness. Accordingly, pulvinar and LP thalamic nuclei, which receive extremely rich projections from VP (Vanduffel et al. 1997a; Payne and Lomber 2003; Valero-Cabré et al. 2005) were the only transynaptic targets that remained impacted during the off-line period. We cannot resolve, however, whether distant effects during "off-line" high frequency stimulation were absent due to insufficient amount of net cortical change or the inability of postsynaptic stations to react accordingly to increases of local activity in their presynaptic nodes.

Methodological considerations

The combination of halothane, O_2 and N_2O used in our study is a standard inhalation anesthesia for recording cortical activity, showing moderate suppressive effects on visual responses in cats or monkeys (Ikeda and Wright 1974) and has been successfully used before in similar experiments as ours (Moliadze et al. 2003, 2005; Aydin-Abidin et al. 2006). Halothane is likely to have reduced rTMS cortical impact, since the potential for further suppression in an already inhibited system might be lower, whereas significant excitatory effects might be difficult to elicit in

this inhibitory environment. Due to this methodological constraint, for the time being, any direct inference to analogous potential effects of TMS in awake individuals in terms of sign and extent needs to be done cautiously. Furthermore, similar experiments in awake cat preparations need to be carried out and compared with the present data in the future. However, in our experiment anesthesia might have also clearly attenuated spurious changes due to rTMS side-effects present in awake individuals such as the tapping sensation on the scalp, the clicking noise of the discharging rTMS, or the repetitive contraction of facial and scalp muscles, along with startle reactions and restlessness. Those are frequent sources of biases that can be hardly corrected through the use of sham TMS coils, inducing similar clicking noise, but no scalp tapping sensation, neither cochlear activation through direct bone conduction.

It is also critical to remember that the measured effects of rTMS on ^{14}C-2DG uptake are relative differences between homologous sites in the stimulated and the non-stimulated hemispheres. This approach provides the only method that helps minimizing the impact of large interindividual and intrasubject variability of glucose metabolism rates (Vanduffel et al. 1995, 1997a, b; Payne and Lomber 1999; Lomber et al. 2002). This is crucial in a study such as ours, in which we aimed at exploring using a reasonable number of animals, a large array of TMS frequency and time-related conditions. Unfortunately, it means that only conclusions about shifts in interhemispheric activity can be reached. In other words, we cannot rule out if the significant differences our results account for are solely due to local changes in the targeted area alone or as it is most likely, arise from a combination of local effects with also frequency dependent changes in contralateral homologue regions. Therefore, as mentioned in the text, all our findings should be interpreted primarily as modulation of metabolism across a distributed bi-hemispheric network. Future work will specifically focus in a given TMS and time conditions, by using normalized methods in large population of animals and controls. In spite of this, the demonstrated changes in interhemispheric balance of activity are clearly due to the impact of TMS, since activity in both hemispheres remained balanced following sham rTMS, or as shown elsewhere after real rTMS in non-parietal regions (Valero-Cabré et al. 2005). Furthermore, the effects show a tight temporal correlation with the rTMS application, as no interhemispheric differences in glucose uptake were found in the two late animals, in which 2DG labeling was performed 30–60 min after rTMS. In the cat, a mixed population of callosal fibers, both inhibitory and excitatory, link homologous regions of the visual and parietal cortices (Buhl and Singer 1989) so that local cortical deactivations generated by *cooling* or parietal ablations yield extremely moderate contralateral decreases (Payne et al. 1991; Payne 1994), or null effects (R. Rushmore personal communication). In the light of previous observations and models in the cat (Payne et al. 1996; Payne and Rushmore 2004), we hypothesize that any intervention resulting in a disbalance of interhemispheric and/or intercollicular activity, by means of generating relative increases or decreases of local VP activity will result into a neglect-like syndrome. A recent TMS study in the awake moving cat has proved that hypothesis to be true when locally using 20 min of 1 Hz stimulation, but its comparison with the effects of unilateral high frequency stimulation remains to be tested (Valero-Cabré et al. 2006). Finally, it is important to remember that due to the low temporal resolution of ^{14}C-2DG labeling, our data represent the cumulative side-to-side rTMS effect achieved during a 30 min long period. Recent work combining TMS and PET in humans clearly demonstrates extremely rapid changes and adaptability of a distributed, bi-hemispheric network following application of rTMS to a focal cortical target (Lee et al. 2003; Rounis et al. 2005). Therefore, further animal experiments, using invasive techniques such as local field potential recordings and optical imaging, will be needed to study with better temporal resolution the detailed timing and dynamics of the complex local and network effects induced by rTMS.

Conclusions

Our findings demonstrate a differential metabolic modulation of rTMS on local and distant effects along a specific parietal network, which are dependent on two different patterns based on high and low frequency stimulation. Furthermore, we show that the same pattern might result into different or opposite metabolic modulation during "on-line" and "off-line" periods. The details of the neuronal mechanisms underlying such differential effects remain to be elucidated. However, the findings provide support to uses of rTMS to modulate cortical activity and modify behavior in normal and impaired neural systems.

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