L-Type Voltage-Gated Ca^{2+} Channels: A Single Molecular Switch for Long-Term Potentiation/Long-Term Depression-Like Plasticity and Activity-Dependent Metaplasticity in Humans

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The ability of synapses to undergo persistent activity-dependent potentiation or depression [long-term potentiation (LTP)/long-term depression (LTD)] may be profoundly altered by previous neuronal activity. Although natural neuronal activity can be experimentally manipulated *in vivo*, very little is known about the *in vivo* physiological mechanisms involved in regulating this metaplasticity in models of LTP/LTD. To examine whether Ca^{2+} signaling may influence metaplasticity *in vivo* in humans, we used continuous theta burst stimulation (cTBS) (Huang et al., 2005), a noninvasive novel repetitive magnetic stimulation protocol known to induce persistent alterations of corticospinal excitability whose polarity is changed by previous voluntary motor activity. When directed to the naive motor cortex, cTBS induced long-lasting potentiation of corticospinal excitability, but depression under the influence of nimodipine (NDP), an L-type voltage-gated Ca^{2+} channel (L-VGCC) antagonist. Both aftereffects were blocked by dextromethorphan, an NMDA receptor antagonist, supporting the notion that these bidirectional cTBS-induced alterations of corticospinal excitability map onto LTP and LTD as observed in animal studies. A short period of voluntary contraction and a small dose of NDP were each ineffective in blocking the cTBS-induced potentiation. However, when both interventions were combined, a depression was induced, and the magnitude of this depression increased with the dose of NDP. These findings suggest that Ca^{2+} dynamics determine the polarity of LTP/LTD-like changes *in vivo*. L-VGCCs may act as molecular switches mediating metaplasticity induced by endogenous neuronal activation.

Introduction

Neurons and neuronal ensembles respond to new experiences, or newly retrieved old memories, by changing the efficacy of synaptic connections. The term “metaplasticity” has been coined to refer to the phenomenon that neural activity at one point in time can change the ability of synapses to exhibit long-term potentiation (LTP) or long-term depression (LTD) after a later bout of activity (Abraham, 2008). The functional significance of metaplasticity may include, but is not limited to, controlling homeostasis of neural network excitability, thus maintaining synaptic efficacy in an operational range. Metaplasticity also may guard against potentially noxious excitability increases

(Abraham, 2008). However, the fact that, by virtue of metaplasticity, even the sign of synaptic efficacy changes may be changed (Seol et al., 2007) indicates that its significance extends beyond these “defensive” roles and may indicate higher complexity of information coding.

Metaplasticity implies the existence of mechanisms that monitor nerve activity and integrate this information over time (Davis, 2006). The molecular identity of an “activity monitor” remains unknown, although postsynaptic Ca^{2+} and CaMKII (Ca^{2+} /calmodulin-dependent protein kinase II) signaling are thought to be involved (Turrigiano et al., 1994; Thiagarajan et al., 2002). Neurons are equipped with numerous powerful mechanisms directly regulating increase of postsynaptic Ca^{2+} concentration (Augustine et al., 2003; Bloodgood and Sabatini, 2007; Sjöström et al., 2008). Among these, voltage-gated Ca^{2+} channels have properties suggesting a role in metaplasticity. For example, extended neuronal activation has been shown to shut down Ca^{2+} channels (Yasuda et al., 2003) and this leads to blockade of LTP (Yasuda et al., 2003). Interestingly, Ca^{2+} channels have also been implicated in controlling the polarity of efficacy changes of naive synapses *in vitro* (Bloodgood and Sabatini, 2007; Shor et al., 2009). This may indicate that Ca^{2+} channels have a dual and central role in regulating synaptic plasticity in response to both

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present and future experience. Although neuronal activity can be studied most meaningfully *in vivo*, very little is known about the *in vivo* physiological mechanisms involved in regulating metaplasticity (Abraham, 2008).

Here, we investigate the role of Ca^{2+} dynamics in human metaplasticity. We used magnetic continuous theta burst stimulation (cTBS), a novel protocol inducing enduring excitability changes in the human corticospinal system (Huang et al., 2005) that have been linked with synaptic plasticity. In animal preparations, TBS is a stimulation protocol that robustly and efficiently induces LTP (Larson et al., 1986; Otto et al., 1991). We previously showed that cTBS results in enhancement of corticospinal excitability [bursts containing three biphasic transcranial magnetic stimulation (TMS) pulses of 50 Hz repeated at 200 ms for a duration of 20 s (cTBS300)] (Gentner et al., 2008). Voluntary muscle activation of sufficient duration before the cTBS intervention reverses the direction of plasticity induced by subsequent cTBS, suggesting a metaplasticity phenomenon (Gentner et al., 2008). In the present study, we first established that nimodipine (NDP), a prototypical blocker of L-type voltage-gated Ca^{2+} channels (L-VGCCs) (Hess et al., 1984), is a powerful modulator of cTBS-induced plasticity. We then add critical evidence for the synaptic nature of cTBS bidirectional effects. This set of findings allowed the direct testing of the hypothesis that Ca^{2+} dynamics are involved in regulating activity-dependent metaplasticity.

Materials and Methods

Subjects

The study was approved by the Ethics Committee of the University of Würzburg, and written informed consent was obtained from all participants. Experiments were performed on 57 healthy volunteers (13 men; 44 women), aged 19–38 years (mean, 25.1 ± 4.2 years). None of them had a history of physical or neurological illness, and none of them was under any medication acting on the CNS. Fifty-six volunteers were right-handed. Handedness was assessed by self-report.

Stimulation and recording

TMS was performed using a figure-eight-shaped magnetic coil (MC-B70; Medtronic) connected to a MagPro X100 magnetic stimulator (Medtronic). The pulse shape was either monophasic (direction of current induced in the brain: posterior to anterior) or biphasic (direction of current induced in the brain: initially anterior to posterior), as detailed below.

Surface electromyographic (EMG) activity was recorded from the right abductor pollicis brevis muscle (APB) using Ag–AgCl surface electrodes (Fischer Medizintechnik) in a belly tendon montage. Raw signals were amplified using a 1902 signal conditioner (Cambridge Electronic Design) and bandpass filtered between 1 Hz and 2 kHz. EMG signals were digitized at 5 kHz by an A/D converter (model 1401 Plus; Cambridge Electronic Design).

Measures of corticospinal excitability

The resting motor threshold (RMT) was determined as the minimum stimulator output needed to produce a response of at least $50 \mu\text{V}$ in the relaxed APB in at least 5 of 10 consecutive trials. This was done using a monophasic (RMT_{Mono}) and a biphasic (RMT_{Bi}) pulse configuration. RMT_{Mono} may reflect membrane excitability of a more homogenous population of cortical neurons than RMT_{Bi} (Kammer et al., 2001). RMT_{Bi} was needed, because a biphasic pulse configuration was used for cTBS intervention. Corticospinal excitability was assessed by measuring the peak-to-peak amplitude of motor evoked potentials (MEPs) elicited in the APB muscle using single-pulse TMS (using the monophasic pulse configuration).

Input–output curves. Ten MEPs each were recorded with 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140% of RMT_{Mono} . The different stimulation intensities were applied in a pseudorandomized order.

For each subject, the peak-to-peak amplitudes were measured on each single trial to calculate the mean amplitude for each stimulation intensity.

Assessment of cTBS intervention-induced change of corticospinal excitability. The stimulation intensity producing MEP amplitudes of $\sim 1 \text{ mV}$ at rest ($\text{SI}_{1 \text{ mV}}$) was assessed using a monophasic pulse configuration. Pre-cTBS corticospinal excitability of APB muscle representation was established by collecting 30 MEP responses at $\text{SI}_{1 \text{ mV}}$ using a stimulation rate of $0.2 \pm 10\%$ Hz with the target muscle at rest. After the interventions (see below), subjects paused for 2 min. Post-cTBS corticospinal excitability was then probed by collecting 12 MEP responses at $\text{SI}_{1 \text{ mV}}$ using a stimulation rate of $0.2 \pm 10\%$ Hz with the target muscle at rest, beginning after completion of the second minute after intervention, every 2 min until completion of the 33rd minute after intervention. Identical stimulus intensities were used before and after intervention. Complete muscle relaxation during the resting periods and during TMS application was monitored by audiovisual feedback. Subjects were instructed to maintain attention to the task throughout the entire session.

Experimental procedures

Subjects were seated comfortably in an armchair. The optimal position (“hot spot”) of the magnetic coil for eliciting MEPs in the resting right APB was assessed over the left primary motor cortex at a moderately suprathreshold stimulation intensity using the monophasic pulse configuration. The hot spot was registered by a neuro-navigation system (Brainsight; Rogue Research) to ensure stability of the stimulation position over the course of an experiment.

Pharmacological interventions

We tested the effects of NDP, a blocker of L-type voltage-gated Ca^{2+} channels (Hess et al., 1984) and of dextromethorphan (DXM), an NMDA receptor antagonist (Wong et al., 1988), on corticospinal excitability and on cTBS-induced changes of corticospinal excitability in different situations. Subjects received either 30 or 15 mg of NDP (Nimodipin Hexal; Hexal). NDP at 30 mg results in cerebral concentrations in humans (Allen et al., 1983) similar to those inducing blockade of LTP (Bi and Poo, 1998; Fourcaudot et al., 2009). Subjects received a single dose of 120 mg of DXM (Hustenstiller-Ratiopharm; Ratiopharm). DXM at 120 mg results in brain concentrations (Hollander et al., 1994; Steinberg et al., 1996) known to block NMDA receptors (Wong et al., 1988; Aplan and Braitman, 1990) *in vitro*.

Timed isometric contraction

In some experimental sessions, cTBS was preceded by voluntary muscle activation. Subjects were instructed to perform isometric abductions with their right thumb against a force transducer (Grass CP122A; Grass Instruments) for a duration of 1.5 min ($\text{ACT}_{1.5}$) at $\sim 25\%$ of the maximal individual force. The force signal was fed back to the subject on a computer screen.

Continuous theta burst stimulation (cTBS300)

This consisted of bursts containing three magnetic pulses (biphasic pulse configuration) of 50 Hz repeated at 200 ms intervals (i.e., at 5 Hz) for a duration of 20 s (Huang et al., 2005). The stimulation intensity was subthreshold for eliciting a response in the target muscle ($70\% \text{RMT}_{\text{Bi}}$) as assessed beforehand.

Experiment 1 (effect of DXM and NDP on corticospinal excitability and cTBS-induced plasticity)

Experiment 1a was performed on 22 healthy volunteers (5 men; 17 women), aged 22–38 years (mean, 24.7 ± 4.0 years), and experiment 1b on 15 healthy subjects (6 men; 9 women), aged 23–38 years (mean, 26.5 ± 3.7 years). A schematic overview of the experimental timeline is provided in Figure 1a.

Experiment 1a. Subjects were tested in two sessions separated by at least 48 h. Sessions were performed in a double-blind counterbalanced design. At the beginning of each experimental session, RMT_{Mono} (before medication) and input–output curve (IO curve) (before medication)

were determined as described above. Subjects received either placebo (PBO) (PBO plus cTBS300) or a single dose of 30 mg of NDP (NDP_{30 mg} plus cTBS300).

Experiment 1b. Subjects were tested in three sessions separated by at least 48 h and blinded to the medication they received. A fully blinded design would not have been feasible with a single timing of placebo tablets because of the different pharmacokinetics of NDP and DXM. Subjects were either untreated (nil plus cTBS300) or treated with either a single dose of 120 mg of DXM (DXM_{120 mg} plus cTBS300) or with single doses of 120 mg of DXM and 30 mg of NDP (DXM_{120 mg} plus NDP_{30 mg} plus cTBS300). After a delay of ~25 min (NDP) or 2 h 10 min (DXM), RMT_{Mono}, IO curve, RMT_{Bi}, and SI_{1 mV} were determined. Thereafter, cTBS300 was applied. To synchronize the cTBS300 intervention with the timing of peak drug concentrations, cTBS300 was timed to be performed 45 min after NDP application (He et al., 2004) and 2 h 30 min after DXM application (Hollander et al., 1994; Steinberg et al., 1996).

Experiment 2 (effect of NDP on activation-induced metaplasticity)

Twenty healthy volunteers (3 men, 17 women), aged 19–38 years (mean, 24.4 ± 4.8 years), participated each in five different sessions separated by at least 48 h. A schematic overview of the experimental design is provided in Figure 2a. The order of the experimental sessions was pseudorandomized and balanced between subjects.

Except in one intervention (NDP_{0 mg} plus ACT_{1.5} plus cTBS300), subjects were premedicated with nimodipine at 15 mg (NDP_{15 mg} plus ACT₀ plus cTBS300 and NDP_{15 mg} plus ACT_{1.5} plus cTBS300) or nimodipine at 30 mg (NDP_{30 mg} plus ACT₀ plus cTBS300 and NDP_{30 mg} plus ACT_{1.5} plus cTBS300), respectively. RMT_{Mono}, RMT_{Bi}, and SI_{1 mV} were assessed in all interventions. Thereafter, subjects either performed the isometric thumb abduction for 1.5 min (ACT_{1.5}) and paused for 2 min before cTBS300, or no isometric voluntary activation (ACT₀) was done and subjects paused for 3.5 min. cTBS300 was timed 45 min after NDP application to be approximately synchronous with the peak drug concentration (He et al., 2004).

Subjects were repeatedly verbally reminded to maintain attention throughout experiments 1 and 2.

Statistical analysis

MEP amplitudes were measured peak-to-peak in each individual trial. For MEP recruitment (IO curves, experiment 1), for each subject and each stimulation intensity, MEP amplitudes were averaged. The IO curves were compared using an ANOVA model with repeated measurements to assess the interaction of “time” (before, after) and “stimulation intensity.”

For each subject, MEP amplitudes were averaged before cTBS300 intervention (30 responses, T0). For statistical analyses, post-cTBS300 values were binned at four successive time points (T1: 3, 5, 7, 9 min; T2: 11, 13, 15, 17 min; T3: 19, 21, 23, 25 min; T4: 27, 29, 31, 33 min; 48 responses each) to reduce variability of results. To compare the effect of interventions on corticospinal excitability, all averaged MEP amplitudes were normalized to “pre-cTBS300” assessed at T0 before cTBS intervention. The effect of cTBS300 on corticospinal excitability in experiment 1 was evaluated by a two-way repeated-measures ANOVA (ANOVA_{RM}) with “medication” as between-subjects factor with two levels (experiment 1a, PBO plus cTBS300, NDP_{30 mg} plus cTBS300) and three levels (experiment 1b, cTBS300, DXM_{120 mg} plus cTBS300, DXM_{120 mg} plus NDP_{30 mg} plus cTBS300), respectively, and time as within-subject factor with five levels (T0, T1, T2, T3, T4). The effect of cTBS300 on corticospinal excitability in experiment 2 was evaluated by a two-way ANOVA_{RM} with “intervention” as between-subjects factor with five levels (NDP_{15 mg} plus ACT₀ plus cTBS300, NDP_{30 mg} plus ACT₀ plus cTBS300, NDP_{0 mg} plus ACT_{1.5} plus cTBS300, NDP_{15 mg} plus ACT_{1.5} plus cTBS300, NDP_{30 mg} plus ACT_{1.5} plus cTBS300) and time as within-subject factor with five levels (T0, T1, T2, T3, T4). Additionally for each protocol, a one-way ANOVA_{RM} was performed with time as within-subject factor with five levels (T0, T1, T2, T3, T4), conditional on significant interaction terms time by intervention. Data were tested for nonsphericity using Mauchly’s test. In case of lack of sphericity, *p* values were corrected using the Green-

house–Geisser correction. Conditional on significant *F* test results, two-tailed Student’s *t* tests were performed for *post hoc* testing. The false discovery rate correction procedure was used for correction for multiple comparisons. Effects were considered significant if *p* < 0.05. All data are presented as means $\pm$ SD.

Results

Blockade of L-VGCC reverses the polarity of cTBS300-induced aftereffects (experiment 1a)

We tested the effects of NDP on parameters of corticospinal excitability (motor threshold at rest, RMT, and IO curve) and on cTBS-induced changes of corticospinal excitability. RMT is a global measure of corticospinal excitability and is sensitive to the excitability of axons activated by the TMS pulse, whereas IO curve, the function describing the increase of MEP amplitude with increasing intensity of magnetic stimulation, is additionally sensitive to glutamatergic synaptic transmission in elements pre-synaptic to the pyramidal output cell (Paulus et al., 2008). Thus, RMT and IO curve probe complementary aspects of cortical excitability (Paulus et al., 2008). Subjects received either PBO or a single dose of 30 mg of NDP in two different sessions (PBO plus cTBS300, NDP_{30 mg} plus cTBS300) (Fig. 1a). Given at this dose, NDP results in CSF concentrations (Allen et al., 1983) similar to those inducing blockade of LTP *in vitro* (Bi and Poo, 1998; Fourcaudot et al., 2009). RMT as determined by using a stimulation with monophasic stimulus configuration (RMT_{Mono}) was similar before and after NDP_{30 mg} application (before, $47 \pm 8\%$ of maximal stimulator output; after, $47 \pm 9\%$ of maximal stimulator output; *p* = 1.000) (Table 1) and NDP_{30 mg} did not change the recruitment curve (ANOVA_{RM}, medication by time by intensity interaction, $F_{(10,210)} = 1.642$; *p* = 0.189, corrected) (Fig. 1b). cTBS modulated the magnitude of MEP amplitudes recorded from the right APB muscle depending on medication with PBO or NDP. Consistent with previous findings (Gentner et al., 2008), cTBS300 applied after medication with PBO resulted in MEP facilitation, which was present for >30 min after its application (Fig. 1c, left panel). However, cTBS300 under the influence of 30 mg of NDP did not induce enhancement of corticospinal excitability. Rather, excitability was depressed (Fig. 1c, left panel). A two-way ANOVA_{RM} revealed a significant medication by time interaction ($F_{(4,84)} = 6.968$; *p* < 0.001), suggesting that the effect of time (by which the efficacy of cTBS300 is implicated) was dependent on the type of medication. To test for the presence of an order effect, experiment 1a was additionally analyzed by a three-way ANOVA_{RM} with medication with two levels (PBO plus cTBS300, NDP_{30 mg} plus cTBS300) and “order” with two levels (first PBO or NDP) as between-subjects factors and time as within-subject factor with five levels (T0, T1, T2, T3, T4). The interaction medication by time by order was not significant (*F* = 0.912; *p* = 0.461), suggesting that the interval of 48 h between any two sessions was sufficiently long to avoid carryover effects. For both groups, one-way ANOVA_{RM} revealed a significant effect of time (PBO plus cTBS300: $F_{(4,84)} = 4.523$, *p* = 0.002; NDP_{30 mg} plus cTBS300: $F_{(4,84)} = 3.919$, *p* = 0.006). *Post hoc t* testing revealed that a significant MEP facilitation was present at all time epochs of post-cTBS300 measurement (T1–T4) in PBO plus cTBS300, whereas suppression was present with NDP_{30 mg} plus cTBS300. Because RMT and recruitment curves were unchanged by NDP_{30 mg}, it is unlikely that excitatory neurotransmission was reduced, a result consistent with the finding that L-VGCCs are not substantially involved in the control of the release of glutamate from presynaptic nerve terminals (Miller, 1987; Kullmann et al., 1992; Reuter, 1996; Fourcaudot et al., 2009). This suggests

that the effect of NDP on cTBS300-induced effects may be directly related to its action on postsynaptic L-VGCCs. Animal studies have shown that limiting the increase of postsynaptic Ca^{2+} concentration may lead to induction of LTD, instead of LTP, after application of a stimulation protocol that induces LTP in the naive preparation (Hansel et al., 1997). Reversal of cTBS300-induced aftereffects from facilitation to depression may, therefore, be explained by induction of LTD, instead of LTP, in excitatory pathways connected to the pyramidal output neurons. According to results obtained in animal studies, NMDA receptors are involved in most forms of LTP and LTD (Sjöström et al., 2008). Therefore, demonstration of NMDA receptor dependency would provide considerable support for the notion that cTBS300-induced effects may be mapped onto these synaptic mechanisms. This question was addressed in the following experiment.

Bidirectional cTBS300-induced changes of corticospinal excitability are NMDA receptor dependent (experiment 1b)

Intermittent TBS, a protocol in which a train of bursts is applied over 2 s, and then repeated every 10 s for 190 s, may induce long-lasting enhancing aftereffects (Huang et al., 2005), which may be blocked by pharmacological blockade of NMDA receptors (Huang et al., 2007). However, it is unclear whether similar mechanisms are involved in cTBS300-induced enhancement of corticospinal excitability and in depression induced by cTBS applied after ingestion of NDP_{30 mg}. To examine these questions, we used DXM, a common antitussive and an NMDA receptor antagonist (Wong et al., 1988) and investigated its influence on corticospinal excitability and on cTBS-induced changes of corticospinal excitability. Subjects received a single dose of 120 mg of DXM, a dose leading to brain concentrations (Steinberg et al., 1996), similar to those that induce NMDA receptor blockade *in vitro* (Wong et al., 1988; Aplan and Braitman, 1990). A schematic overview of the experimental timeline is provided in Figure 1a. In agreement with a previous study (Ziemann et al., 1998), RMT_{mono} was similar before and after ingestion of DXM_{120 mg} (before, $47 \pm 9\%$ of maximal stimulator output; after, $47 \pm 9\%$ of maximal stimulator output; $p = 0.617$) (Table 1) and DXM did not decrease recruitment curve (ANOVA, medication by time by intensity interaction, $F_{(20,678)} = 0.028$; $p = 1.000$) (Fig. 1b), suggesting that excitatory transmission was not compromised. Figure 1c, right panel, illustrates that cTBS induced enhancement of corticospinal excitability, confirming results obtained in experiment 1a. However, DXM_{120 mg} blocked both the facilitation induced by cTBS300 and the depres-

Figure 1. *a*, Schematic overview of experiment 1 (expt. 1). Before and after ingestion of the medication (see inset), RMT_{mono} and IO curves were determined. After determining biphasic RMT (RMT_{BI}) and SI_{1 mV}, subjects paused for 2 min. Pre-cTBS corticospinal excitability (T0) was established and followed by the application of cTBS (timed 45 min after PBO, NDP application, and 2 h 30 min after DXM application, respectively). After a delay of 2 min, post-cTBS MEP responses were obtained (16 blocks consisting of 12 MEP responses each, followed each by 1 min of rest), up to 33 min after intervention. *b*, Effect of NDP_{30 mg} or DXM_{120 mg} on input–output properties of the corticospinal pathway. MEP responses were obtained at different magnetic stimulation intensities before and after medication with PBO, NDP_{30 mg}, and DXM_{120 mg}. *c*, Effect of PBO or NDP_{30 mg} (left panel) or DXM_{120 mg} or NDP_{30 mg} plus DXM_{120 mg} (right panel) on cTBS-induced plasticity. Time course of averaged changes in MEP responses normalized to pre-cTBS. Each data point represents the average of MEP amplitudes from 22 (experiment 1a) or 15 (experiment 1b) subjects. Vertical arrows represent time of cTBS intervention. Gaps in the x-axis indicate interruptions for each 1 min break. Examples of averaged MEP responses of one subject (for each of experiments 1a and 1b) obtained before (T0), 3–9 min (T1), 11–17 min (T2), 19–25 min (T3), and 27–33 min (T4) after intervention. Calibration: vertical, 0.5 mV; horizontal, 25 ms. The bar graphs indicate MEP amplitudes averaged after intervention (T1–T4). The asterisks denote significant differences after false discovery rate correction. Error bars indicate SD.

sion when cTBS300 was applied under the influence of NDP_{30 mg} (Fig. 1c, right panel). Two-way ANOVA_{RM} revealed a significant medication by time interaction ($F_{(8,112)} = 3.170$; $p = 0.018$, corrected). This was entirely attributable to the enhancement of corticospinal excitability induced by cTBS300, as shown by the results of one-way ANOVA_{RM} (time, cTBS300: $F_{(4,56)} = 4.935$; $p = 0.002$; DXM_{120 mg} plus cTBS300: $F_{(4,56)} = 1.340$; $p = 0.278$, corrected; DXM_{120 mg} plus NDP_{30 mg} plus cTBS300: $F_{(4,56)} = 0.257$; $p = 0.800$, corrected). The fact that bidirectional TBS-induced effects are blocked by antagonism at NMDA receptors supports the view that they are related to changes in synaptic efficacy.

Table 1. Pre-cTBS physiological data

Expt.	Intervention	RMT _{Mono} (% of MSO)	RMT _{Bi} (% of MSO)	SI _{cTBS} (% of MSO)	SI _{1 mV} (% of MSO)	MEP _{pre-cTBS} (in mV at T0)
1a	PBO + cTBS	48 ± 8	35 ± 6	25 ± 5	60 ± 11	0.9 ± 0.3
	NDP _{30 mg} + cTBS	47 ± 8	34 ± 6	24 ± 5	59 ± 11	1.1 ± 0.3
1b	Nil + cTBS	45 ± 9	34 ± 5	24 ± 4	57 ± 12	0.9 ± 0.4
	DXM _{120 mg} + cTBS	47 ± 9	34 ± 6	24 ± 4	59 ± 13	1.1 ± 0.3
	DXM _{120 mg} + NDP _{30 mg} + cTBS	45 ± 8	33 ± 6	23 ± 4	56 ± 13	0.9 ± 0.3
2	NDP _{15 mg} + ACT ₀ + cTBS	47 ± 8	33 ± 6	23 ± 4	62 ± 12	0.9 ± 0.3
	NDP _{30 mg} + ACT ₀ + cTBS	47 ± 8	33 ± 6	23 ± 4	61 ± 12	1.1 ± 0.3
	NDP _{0 mg} + ACT _{1.5} + cTBS	46 ± 8	33 ± 6	23 ± 4	62 ± 11	0.9 ± 0.2
	NDP _{15 mg} + ACT _{1.5} + cTBS	46 ± 8	33 ± 6	23 ± 4	61 ± 12	1.1 ± 0.3
	NDP _{30 mg} + ACT _{1.5} + cTBS	46 ± 9	33 ± 6	23 ± 4	61 ± 12	1.1 ± 0.4

RMT_{Mono}, Resting motor threshold, established with a monophasic pulse configuration; RMT_{Bi}, resting motor threshold, established with a biphasic pulse configuration; % of MSO, percentage of maximal stimulator output; SI_{cTBS}, stimulation intensity during cTBS (70% of RMT_{Bi}); SI_{1 mV}, stimulation intensity producing MEP amplitudes of ~1 mV at rest; MEP_{pre-cTBS}, mean MEP amplitude before cTBS.

Blockade of L-VGCC and voluntary activation act synergistically to reverse polarity of cTBS300-induced effects (experiment 2)

The results presented so far may suggest that Ca^{2+} entry into postsynaptic neurons is a major factor determining the sign of activity-dependent synaptic changes *in vivo*. Previously, we showed that the polarity of cTBS300-induced changes of corticospinal excitability may be reverted to depression if cTBS300 is preceded by voluntary activation (ACT) of long (5 min) duration, whereas previous voluntary activation of short (1.5 min) duration did not induce a similar effect (Gentner et al., 2008). If Ca^{2+} dynamics are involved in the regulation of metaplasticity, then it may be hypothesized that voluntary activation of a duration, which by itself is insufficient to lead to polarity reversal of cTBS300-induced effects, will be effective if combined with NDP, even if the dose of NDP alone is also insufficient to reverse polarity. To test this hypothesis, we examined the effects of cTBS300 under the influence of different combinations of NDP and voluntary activation (Fig. 2a). Baseline physiological parameters were similar between conditions (Table 1). Time courses of corticospinal excitability as indexed by MEPs are displayed in Figure 2b–f for each intervention. cTBS300 under the influence of 30 mg of NDP (NDP_{30 mg} plus ACT₀ plus cTBS300) did not induce enhancement of corticospinal excitability but induced depression of corticospinal excitability (Fig. 2c), confirming findings obtained in experiment 1a. Of note, a smaller dose of NDP (NDP_{15 mg} plus ACT₀ plus cTBS300) was ineffective in reversing the polarity of cTBS300-induced changes (Fig. 2b). cTBS300 performed without NDP after voluntary activation of 1.5 min duration (NDP_{0 mg} plus ACT_{1.5} plus cTBS300) induced enhancement of corticospinal excitability (Fig. 2d), in agreement with previous observations (Gentner et al., 2008). However, when medication with NDP_{15 mg} accompanied 1.5 min of voluntary contraction (NDP_{15 mg} plus ACT_{1.5} plus cTBS300), a depression of corticospinal excitability was induced (Fig. 2e). MEP suppression was present at all time epochs of post-cTBS300 measurement. Depression was more pronounced when voluntary contraction was performed under the influence of NDP at 30 mg (NDP_{30 mg} plus ACT_{1.5} plus cTBS300), suggesting a dose-dependent effect (Fig. 2f). Two-way ANOVA_{RM} revealed a significant intervention by time interaction ($F_{(16,304)} = 6.627$; $p < 0.001$, corrected), suggesting that the effect of time was dependent on the type of intervention. For each intervention, one-way ANOVA_{RM} revealed a significant effect of time (NDP_{15 mg} plus ACT₀ plus cTBS300: $F_{(4,76)} = 3.834$; $p = 0.019$, corrected; NDP_{30 mg} plus ACT₀ plus cTBS300: $F_{(4,76)} = 4.872$; $p = 0.009$, corrected; NDP_{0 mg} plus ACT_{1.5} plus cTBS300: $F_{(4,76)} = 3.297$; $p = 0.048$, corrected;

NDP_{15 mg} plus ACT_{1.5} plus cTBS300: $F_{(4,76)} = 4.024$; $p = 0.005$; NDP_{30 mg} plus ACT_{1.5} plus cTBS300: $F_{(4,76)} = 18.274$; $p < 0.001$). Figure 2g displays the effects of each intervention on MEP amplitudes averaged across all postinterventional time periods (T1–T4).

Discussion

Our findings suggest a prominent role, in humans, for Ca^{2+} dynamics in the native control of the polarity of LTP/LTD-like changes, as well as an important mechanism regulating metaplasticity induced by endogenous neuronal activation.

Ca^{2+} dynamics determine the direction of motor cortical LTP/LTD-like plasticity *in vivo*

In naive subjects, cTBS300 induced facilitation, whereas in subjects medicated with 30 mg of NDP, cTBS300 induced depression of excitability instead. This pattern of responses is best explained by a mechanism resembling LTP/LTD formation of excitatory synapses in motor cortex. The duration of both facilitation and depression was sufficiently long to be consistent with LTP/LTD of excitatory synapses (Sjöström et al., 2008). DXM, a blocker of NMDA receptors (Wong et al., 1988) and LTP formation in animal preparations (Krug et al., 1993), blocked cTBS300-induced facilitation. Since NDP relatively specifically affects L-type voltage-gated Ca^{2+} channels (Randall and Tsien, 1997), blockade of Ca^{2+} entry into the postsynaptic neuron via L-VGCCs is a likely explanation for polarity reversal of cTBS300-induced effects. The fact that NDP did not affect either motor threshold or recruitment curve provides support in favor of a postsynaptic mechanism, although recent findings suggest that blockade of presynaptic L-VGCC may interfere with expression of presynaptic LTP, despite the fact that L-VGCC barely contribute to baseline synaptic release (Fourcaudot et al., 2009). According to one influential theory (Lisman, 1989; Artola and Singer, 1993), whether activity-dependent LTP or LTD is induced, depends on the magnitude and dynamics of the different postsynaptic levels of Ca^{2+} induced by the stimulation (Artola et al., 1990; Kemp and Bashir, 2001; Massey and Bashir, 2007). This theory is supported by observations that a train of stimuli capable of inducing LTP can induce LTD during pharmacologically reduced activation of NMDA receptors (Hirsch and Crepel, 1991; Cummings et al., 1996; Mizuno et al., 2001) or blockade of Ca^{2+} entry via NMDA receptors by voltage clamping of the postsynaptic neuron at negative membrane potentials (Cummings et al., 1996). Similarly, trains of stimuli that normally induce LTP could result in LTD in the presence of intracellular Ca^{2+} chelators (Hansel et al., 1997). Of particular relevance for the present study, TBS, which ro-

bustly induces LTP in animal preparations (Larson et al., 1986), led to LTD in the presence of Ca^{2+} chelators (Kimura et al., 1990; Yoshimura et al., 1991). Moreover, in a rare study in which LTD was induced by weak TBS, this observation was associated with a lesser rise in postsynaptic Ca^{2+} concentration compared with LTP induced by strong TBS (Yasuda and Tsumoto, 1996). We propose that, under a sufficient dose of NDP, the remaining Ca^{2+} entry in the postsynaptic neuron via NMDA receptors (or release from intracellular Ca^{2+} stores) was just sufficient to induce LTD-like changes. This hypothesis gains support by our observation that the combination of NDP and DXM, which likely has affected both major pathways of Ca^{2+} entry into the postsynaptic neuron, via NMDA receptors and via L-VGCC, has completely blocked the efficacy of cTBS300 to induce excitability changes. Under this circumstance, any remaining Ca^{2+} influx was presumably insufficient to induce any lasting changes on synaptic efficacy. Our findings appear to provide indirect evidence supporting the hypothesis that the magnitude of postsynaptic Ca^{2+} concentration determines the outcome of LTP/LTD-inducing stimulation protocols (Lisman, 1989; Artola et al., 1990) *in vivo*. They also may indicate that L-VGCCs are elements powerfully regulating the polarity of synaptic changes. However, since NDP at 30 mg is unlikely to lead to complete blockade of L-VGCC, blockade of efficacy of cTBS300 under NDP plus DXM does not exclude the possibility that Ca^{2+} influx via NMDA receptors alone might be sufficient to induce LTD-like changes.

Regulation of activation-induced motor cortical metaplasticity by L-type voltage-gated Ca^{2+} channels

The polarity of excitability changes induced by cTBS300 could be changed by a combination of isometric contraction and NDP, even when the duration of isometric contraction was too short, or the dose of NDP was too small, to modulate the effects of cTBS300, if either of these interventions was applied on its own. The fact that a subliminal duration of isometric contraction was sufficient to reverse the polarity of cTBS300-induced effects under the influence of a subliminal dose of NDP suggests that Ca^{2+} dynamics are involved not only in regulating the polarity changes of naive synapses exposed to a plasticity-inducing stimulation protocol (as indicated above) but also are involved in the physiological events induced by isometric contractions. Ca^{2+} dynamics are regulated by several mechanisms, which include—apart from L-VGCC—ligand-gated channels and release from intracellular Ca^{2+} stores (Cavazzini et al., 2005). It is not a priori clear which of these mechanisms predominates. If isometric contraction changed Ca^{2+} dynamics by virtue of an effect on ligand-gated channels or on release from intracellular Ca^{2+} stores, then

Figure 2. *a*, Schematic overview of experiment 2. After application of medication and determination of RMT_{Bi} and SI₁ mV, subjects either performed isometric voluntary contraction for 1.5 min (ACT_{1.5}) and then paused for 2 min, or paused for 3.5 min (ACT₀). Pre-cTBS corticospinal excitability was assessed and followed by the application of cTBS. Post-cTBS corticospinal excitability was then established (16 blocks consisting of 12 MEP responses each, followed each by 1 min of rest, up to 33 min after intervention). Different combinations of NDP and short-lasting voluntary activation were then used to modulate cTBS-induced plasticity. *b–f*, Interaction of voluntary preactivation and NDP on cTBS-induced plasticity. Time course of changes in MEP responses normalized to pre-cTBS after different interventions. Each data point represents the average of MEP amplitudes from 20 subjects. Gaps in the x-axis indicate interruptions for each 1 min break. Examples of averaged MEP responses of one subject obtained before (T0), 3–9 min (T1), 11–17 min (T2), 19–25 min (T3), and 27–33 min (T4) after intervention. Calibration: vertical, 0.5 mV; horizontal, 25 ms. *g*, Synopsis of results. MEP amplitudes decreased after medication with 30 mg of NDP and combinations of medication with NDP and voluntary preactivation. The bar graphs indicate MEP amplitudes averaged after intervention (T1–T4). The asterisks denote significant differences after false discovery rate correction. Error bars indicate SD.

additional pharmacological blockade of L-VGCC might have constrained the cTBS300-induced increase of intracellular Ca^{2+} concentration to remain below a critical threshold necessary for LTP induction. Although we cannot exclude this possibility, it is important to note that prolonged ion channel modulation by neuronal activity has been found in pyramidal neurons (Beck and Yaari, 2008). In hippocampal area CA1 neurons, the activation of kinase signaling pathways by LTP-inducing synaptic input patterns not only leads to the expression of long-term synaptic plasticity but also to long-term modulation of specific dendritic ion channels (Frick et al., 2004). Long-term changes in active dendritic properties can also follow periods of increased postsynaptic action potential firing (Yasuda et al., 2003) such as that which likely accompanies prolonged voluntary contraction. Additional indirect support for a genuinely physiological role of L-VGCC is provided by considering their subcellular distribution. Different species of Ca^{2+} channels are inhomogeneously distributed with the neuron, suggesting that they may have different roles in synaptic neuronal plasticity, and metaplasticity (Magee, 1999).

Immunohistological experiments have localized L-VGCC predominantly in the soma and proximal dendrites of cortical neurons (Ahlijanian et al., 1990) where they are strategically positioned to control the integrative properties of the neuron. L-VGCC located at proximal dendrites may allow action potentials to propagate back into the dendritic tree, where they can then sum with incoming synaptic activity (Magee, 2000). Against this background obtained in *in vitro* experiments, the present findings provide support for the idea that activity-dependent modification of L-VGCCs may be a strong candidate mechanism mediating metaplasticity. The profound polarity-changing influence on LTP/LTD-like plasticity by pharmacological blockade of L-VGCCs suggests that manipulation of L-VGCC may have therapeutic potential in neuropsychiatric disorders in which manipulation of memory formation is desired, such as in post-traumatic stress disorder.

Conclusions

Our observations in the naive and activation-conditioned motor system suggest a link between an elementary mechanism governing the polarity of activity-induced persistent changes of synaptic efficacy and mechanisms underlying metaplasticity. In both circumstances, Ca^{2+} dynamics are implicated. Even though there may be no singular activity monitor at the molecular level, L-VGCCs may be an important final pathway in mediating (meta-)plasticity *in vivo*.

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