PHARMACOLOGICAL ALTERATIONS OF NEUROPLASTICITY IN THE HUMAN MOTOR CORTEX INDUCED BY DOPAMINERGIC AND CHOLINERGIC AGENTS

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Statement of Originality

I hereby declare that this thesis is my own work and has been written independently with no other sources and aids than quoted in the text, references and acknowledgements.

Göttingen, 13th December 2010

Nivethida Thirugnanasambandam

This doctoral thesis is dedicated with love and gratitude to my father, K.P.Thirugnanasambandam, to my mother, Parvady Thirugnanasambandam and to my sisters, Manasadevi and Mirunalini for their incredible support which lies behind every success in my life…

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Chapter 1 Introduction

Neuroplasticity is a marveling feature exhibited by neuronal networks of the simplest to the most complex brains in nature. By dynamically re-organizing the structure of neuronal networks or function or both, the brain is constantly responding to environmental stimuli and injuries (Boller, 2004). Long term- potentiation (LTP) and depression (LTD) are the most well-studied forms of neuroplasticity and are proposed to underlie the complex processes of learning and memory formation. In recent decades, non-invasive brain stimulation techniques have gained importance in the exploration of neuroplasticity in humans. Repetitive transcranial magnetic stimulation (rTMS), transcranial direct current stimulation (tDCS) and paired associative stimulation (PAS) are the most commonly used techniques (Huang et al., 2005; Nitsche and Paulus 2000; 2001; Stefan et al., 2000). These techniques are able to induce long-term plastic changes in the intact human brain thereby enabling us to study this phenomenon in humans (Cooke and Bliss, 2006).

Neuromodulators are substances that, depending on concentration, activation of subreceptors, and other factors, affect plasticity in neuronal networks. Acetylcholine, dopamine, serotonin and noradrenaline are the most important neuromodulators in the human central nervous system. Several studies have looked into their impact on neuroplasticity mainly in animals (Hasselmo and Barkai, 1995; Kung et al., 2007; Otani et al., 1998; 2003). Very few studies so far have studied the impact of neuromodulators on plasticity in humans (Kuo et al., 2007; 2008; Nitsche et al., 2009; Monte-Silva et al., 2009; 2010). In the present projects, we were interested to explore certain aspects of the impact of cholinergic and dopaminergic activation on neuroplasticity in humans. In the cholinergic system, we explored the specific impact of the nicotinic sub-receptor on neuroplasticity. In the dopaminergic system, we explored the dose-dependent effect of dopaminergic activation on plasticity.

The first chapter introduces basic concepts necessary for the further understanding of the studies included in the thesis. The second chapter consists of the papers presenting the research results. The concluding chapter summarizes the results of the studies and offers an outlook to future research in the field.

1.1 Plasticity in the central nervous system

Neuroplasticity is the phenomenon by which functional or structural reorganization of neuronal connectivity takes place depending on the amount and pattern of neuronal activity (Citri and Malenka, 2008). Long term potentiation (LTP) and long term depression (LTD) are the most extensively studied neuroplastic mechanisms considered to be the physiological basis of learning and memory formation. These processes are most detailed studied at glutamatergic synapses, especially in the region of the hippocampus, but also in other cortical and subcortical areas (Malenka and Bear, 2004). However, plasticity is not limited to the glutamatergic system, also the most important inhibitory cortical network, the GABAergic system, has neuroplastic properties (Kano, 1995). Both LTP and LTD at glutamatergic synapses are mediated by NMDA (N-methyl D-aspartate) receptors, which have calcium channel properties (Bliss and Collingridge, 1993; Malenka and Bear, 2004). Consequently, one of the major determinants of plasticity at the synapse is the postsynaptic intracellular calcium concentration (Lisman, 2003). It is known that moderate increase in intracellular calcium triggers signal transduction molecules like protein phosphatases that inactivate or remove AMPA receptors from the subsynaptic membrane, resulting in LTD (Cummings et al., 1996). Conversely, a large increase in intracellular calcium concentration beyond a critical threshold triggers other signaling molecules like the calcium-calmodulin kinase and other protein kinases, which enhance the insertion of AMPA receptors onto the postsynaptic membrane inducing LTP (Malenka and Nicoll, 1993; Malenka and Bear, 2004) (see figure 1).

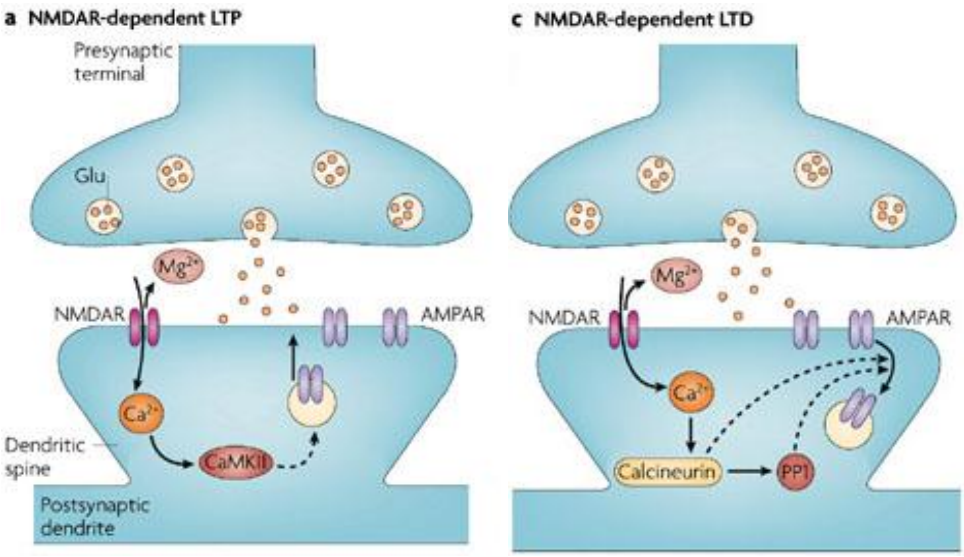

Figure 1: Illustration of the phenomena of LTP (left) and LTD (right) at a glutamatergic synapse. Depending on the level of intracellular calcium concentration, different signal transduction molecules are activated that result in either LTP or LTD via insertion or removal of excitatory AMPA receptors to and from the subsynaptic membrane and related processes, thus reducing or enhancing the efficacy of synaptic transmission (adapted from Kauer and Malenka, Nat Rev Neurosci, 2007).

Beyond glutamatergic plasticity, LTP and LTD are also known to occur at inhibitory GABAergic synapses. Also here, plasticity depends on calcium signaling however involving different cascades depending on the brain region under study (Kano, 1995; Gaiarsa et al., 2002). Since the majority of the neurons in the cerebral cortex are inhibitory interneurons that are GABAergic, their influence on global network plasticity should not be ignored (Evans and Viola-McCabe, 1996). However, since our studies deal primarily with plasticity of the glutamatergic system, a more detailed description of the mechanisms of GABAergic plasticity is avoided in this context.

1.2 Neuroplasticity in humans

Apart from animal experiments, studies conducted in recent years on healthy humans and patients have shown that neuroplastic alterations play a prominent role in adaptive processes of the human cerebral cortex. Functional MRI and other imaging studies in stroke patients have revealed changes in the motor/sensory maps following injury that recover to different extents depending on rehabilitation therapy (Hodics et al., 2006; Levy et al., 2001) and such changes correlate to functional recovery (Johansen-Berg et al., 2010; Seitz, 2010). Functionally beneficial plasticity also has been observed in blind individuals who show higher degrees of tactile discrimination abilities than normal-sighted individuals (Van Boven et al., 2000). This is accompanied by larger representations of the finger tips in the somatosensory maps of these subjects and is more pronounced in Braille readers (Pascual-Leone and Torres, 1993). Not only under pathological states, but also in physiological conditions, such kinds of plasticity have been observed, for example in musicians (Pantev et al., 2003) and following motor practice (Ziemann et al., 2001). In addition to these behaviorally induced neuroplastic processes in humans, it has been shown in recent years that cortical plasticity can be induced by non-invasive brain stimulation protocols, namely transcranial magnetic stimulation, transcranial direct current stimulation and paired associative stimulation (rTMS, Huang et al., 2005; tDCS, Nitsche and Paulus, 2000; 2001; PAS, Stefan et al., 2000). These techniques induce LTP- or LTD- like plasticity that can be monitored by changes of cortical excitability using TMS. Although they all increase or decrease cortical excitability, the underlying physiological mechanisms of plasticity induction and the foci differ to some extent. This will be discussed later in detail.

1.2.1 Motor system as a model for neuroplasticity in humans

Most of the studies on system level plasticity in humans, especially those using non-invasive brain stimulation techniques for plasticity induction, use the motor cortex as model system. The main reasons for this are that the physiology of the human motor cortex is relatively well explored, it is situated at the cerebral surface and thus is accessible for non-invasive brain stimulation, and an objective output parameter for probing cortical excitability is available, namely the motor evoked potential (MEP) amplitude, which can be measured non-invasively by transcranial magnetic stimulation (TMS). For obtaining MEPs, small hand muscles are most often used because of the superficial position of their motor cortex representation, low thresholds for stimulation and relatively large representations. In all studies mentioned in the thesis, MEPs have been obtained from the first dorsal interosseous or the abductor digiti minimi muscles. The MEP amplitude obtained by single pulse TMS is a measure of corticospinal excitability (Rothwell, 1993) that reflects the synaptic strength and the balance of excitatory and inhibitory inputs at the synapses of corticospinal neurons (Ziemann et al., 2003).

1.3 Non-invasive brain stimulation techniques

Since the 1980's several techniques have been introduced to stimulate or modulate cortical neurons non-invasively. The first attempt of non-invasively stimulating neurons of the intact brain was made by Merton and Morton (1980) by transcranial electrical stimulation (TES). This technique uses high voltage currents that penetrate the skull and induce action potentials in cortical neurons. Since this high intensity electrical stimulation also activates pain receptors on the scalp and induces contraction of head muscles, which is inconvenient and painful to the subjects, this technique went less popular and is nowadays seldom used to

stimulate awake human subjects. In 1985, Barker and colleagues developed an effective alternative to this technique – transcranial magnetic stimulation (TMS, Barker et al., 1985). This technique involves application of brief magnetic pulses to the brain through the scalp non-invasively. The magnetic field penetrates the skull painlessly unlike the high voltage electric current used in TES. Through electromagnetic induction, a secondary electric field is induced in the brain tissue that is sufficiently strong to induce neuronal action potentials. Single pulse TMS applied over the motor cortex induces motor evoked potentials, which are a convenient measure of corticospinal excitability (Rothwell, 1993), while TMS applied over the visual cortex is able to elicit phosphenes, which are subjectively perceived light flashes caused by activation of visual cortical neurones (Hallett, 2007). In recent years, sophisticated TMS protocols have been developed which are able to probe the functions of cortical subsystems. For example, it is possible to monitor intracortical inhibition and facilitation via paired pulse TMS protocols (Kujirai et al., 1993).

Apart from monitoring cortical excitability, specific TMS protocols have been shown to be suited to modify the same, and thus to induce neuroplasticity. When TMS is applied repetitively (rTMS), it induces enduring excitability changes, the direction depending on the frequency of stimulation (Fitzgerald et al., 2006; Huang et al., 2005; Pascual-Leone et al., 1994). Beyond high and low frequency rTMS, other non-invasive plasticity induction protocols have been developed in the last years. Stefan et al. in 2000 introduced a variant of TMS combined with peripheral nerve stimulation which induces an associative kind of plasticity called paired associative stimulation (PAS). Nitsche and Paulus (2000) introduced transcranial direct current stimulation (tDCS) which induces changes in cortical excitability using subthreshold electrical stimulation.

1.3.1 Transcranial Magnetic Stimulation

The basic principle of TMS is electromagnetic induction of current in brain tissue. A rapidly changing magnetic field in the TMS coil painlessly penetrates the skull and induces an electric field in the brain tissue underlying the coil oriented in the opposite direction. The intensity of the current – if sufficiently large - depolarizes the underlying neurons (Barker et al., 1980). The focality of stimulation depends on the coil geometry, orientation and pulse configuration. The figure of eight coil is most commonly used for stimulation of small hand muscles (Ueno et al., 1988). Such a coil is capable of stimulating superficial brain regions with adequate focality. TMS is thought to activate corticospinal neurons transsynaptically (Di Lazzaro et al., 1998). Considering the orientation of the pyramidal neurons in the primary motor cortex, TMS applied in the postero-anterior direction results in excitation of the output neurons that can be recorded as MEPs from the peripheral muscles using surface electromyography (EMG) (Hallett, 2007). In the present thesis, single pulse TMS is used as a technique to elicit MEPs and not as an intervention. However, as stated earlier, repetitive TMS (rTMS) can induce plastic changes in the cortical neurons depending mainly on the frequency of stimulation. This is analogous to high or low frequency stimulation induced-plasticity in animal slices preparations (Huang et al., 2005).

1.3.2 Transcranial direct current stimulation

This technique differs from rTMS in that it induces changes in cortical excitability by application of subthreshold currents, which do not elicit action potentials themselves. The application of a weak electrical field is able to modulate the resting membrane potential of the affected cortical neurons depending on electrode polarity. Anodal stimulation induces subthreshold depolarization and cathodal stimulation hyperpolarization of the neurons. Consequently, the depolarized neurons exhibit higher excitability whereas those that are

hyperpolarized show lower excitability. The concept of neuronal excitability alteration induced by weak electric field was first demonstrated by Bindman et al. (1964), and Purpura and McMurtry (1965) in animal slice preparations and in vivo animal experiments. In these experiments, anodal polarization resulted in higher frequency of neuronal spiking compared to the baseline whereas cathodal polarization reduced neuronal firing. Interestingly, the excitabilility and activity changes persisted for hours even after the electric fields were switched off, if stimulation duration exceeded some minutes (Bindman et al. 1964), and these neuroplastic excitability alterations were protein synthesis-dependent (Gartside 1968). Nitsche and Paulus (2000) could show that non-invasive transcranial application of weak direct currents to the human motor cortex, termed transcranial direct current stimulation (tDCS), induces similar excitability alterations in the motor cortex of awake humans. Whereas application for some seconds induces excitability modifications restricted to the time during stimulation, tDCS for some minutes induces neuroplastic after-effects lasting for up to one hour or longer (Nitsche and Paulus, 2000, 2001; Nitsche et al., 2003b). Similar to the animal experiments, anodal stimulation results in an excitability enhancement of the human motor cortex and cathodal stimulation diminishes neuronal excitability. Following sufficiently long stimulation duration, the respective excitability changes outlast the duration of stimulation. The neuroplastic excitability changes are both NMDA- and calcium channel-dependent (Nitsche et al., 2003a), similar to glutamatergic LTP and LTD elicited in animal experiments. On the contrary, the after-effects of tDCS are not prominently affected by GABAergic drugs (Nitsche et al., 2004). Since tDCS targets the neurons under the electrode area non-selectively (Purpura and McMurtry 1965), it is considered to induce relatively non-focal plasticity.

tDCS is applied to the human cortex via constant current stimulators. The anode and cathode, both of which are saline-soaked sponge electrodes, are placed on the scalp of the subject with rubber bands and are connected to the stimulator. For all the experiments where

tDCS was used in the current thesis, one electrode was placed over the motor cortex and the other electrode was placed over the contralateral supra-orbital region. The current intensity used was 1mA. Stimulation with such intensity for a duration of 9-13 minutes results in motor cortical excitability changes that outlast the stimulation duration for about one hour (Nitsche and Paulus 2001; Nitsche et al., 2003b).

1.3.3 Paired Associative Stimulation

PAS is a variant of TMS that induces an associative kind of plasticity. The technique is similar to the spike-timing dependent plasticity phenomenon at the cellular level (Dan and Poo, 2006) which is considered to be the underlying mechanism for several forms of learning and memory processes (Letzkus et al., 2007). Pairs of stimuli are applied – one stimulus is given to a peripheral nerve of the upper extremity and the other to the motor cortex. The stimulus applied to the peripheral nerve (usually at the wrist) travels via the spinal cord to the somatosensory cortex and reaches the motor cortex approximately 25 milliseconds after application via somatosensory-motor cortical connections. When the second stimulus is applied to the motor cortex at approximately the time when the peripheral nerve stimulus reaches the motor cortex, due to the synchronous activation of the somatosensory-motor cortical connections, facilitatory plasticity is induced (Stefan et al., 2000), which most likely reflects the kind of associative plasticity first described by Hebb (1949). When the two stimuli are asynchronous, inhibitory plasticity is induced (Wolters et al., 2003). Therefore, the interstimulus interval is the critical factor that determines the direction of plasticity. PAS induces long-lasting after-effects (approximately upto 60 minutes following the stimulation) which are, similar to tDCS, dependent on NMDA receptors and calcium channel activity (Stefan et al., 2002; Wolters et al., 2003). Therefore PAS is thought to induce LTP-like or LTD-like plasticity at glutamatergic synapses. Unlike tDCS, it is proposed that PAS induces

plasticity only in those neurons of the motor cortex that receive the priming stimuli from the somatosensory cortex (Stefan et al., 2000). Therefore, the technique induces input-specific, timing-dependent, associative plasticity (Stefan et al., 2000), which might mimic the phenomenon of learning and memory formation relatively closely. See Figure 2.

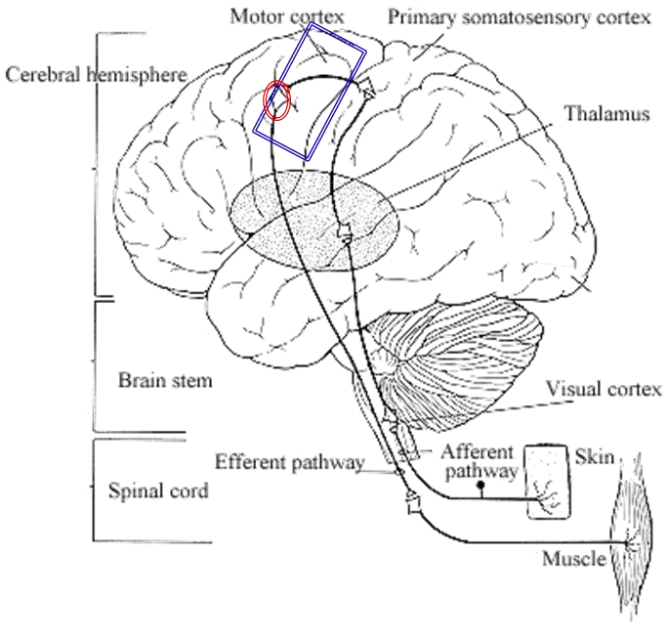

Figure 2: Shows the focality of stimulation by tDCS (blue) and PAS (red). tDCS targets non-selectively all the neurons beneath the electrodes. PAS affects the excitability of only those motor cortex neurons that were primed by the input from the somatosensory afferents. Hence PAS induces focal, synapse-specific plasticity whereas tDCS induces non-focal plasticity.

In our experiments, the first peripheral electrical nerve stimulus was given to the ulnar nerve at the level of the wrist. The intensity of the stimulus was three fold of the sensory threshold intensity. The second stimulus of the pair, which was a suprathreshold magnetic pulse, was applied to the corresponding region of the motor cortex. The two stimuli were delivered at interstimulus intervals of either 10 or 25 milliseconds at a frequency of 0.05 Hz for 30 minutes. When the interstimulus interval was 25 ms, which is the approximate time the peripheral stimulus takes to travel to the motor cortex, the two stimuli reached the motor

cortex approximately synchronously, which induces facilitatory plasticity (Stefan et al., 2000). When the interstimulus interval was 10 ms, the motor cortex received the second pulse much before the arrival of the first stimulus. Such asynchronous activation of the somatosensory-motor connections results in inhibitory plasticity (Wolters et al., 2003).

1.4 Neuromodulators

Neuromodulators are substances that influence plasticity but are not essential for the occurrence of plasticity (Malenka and Bear, 2004). Such substances act primarily on the drivers of plasticity, namely the NMDA receptors in case of plasticity of the glutamatergic system, usually in a non-linear fashion (Gu, 2002). Some of the major neuromodulators in the human central nervous system are dopamine, acetylcholine, serotonin and noradrenaline. The effects of these neuromodulators may be exerted either directly or indirectly on NMDA activity. These substances are known to alter plasticity by either altering the excitability levels of neurons, by enhancing the signal-to-noise ratio of neuronal responses or by altering the threshold of activity-dependent changes at the synapses (Gu, 2002). In any case, their effects depend on several factors of which the most important are (i) the type of subreceptors activated (ii) the dosage of the substance (iii) background cortical activity in the specific brain region. Because neuromodulators influence plasticity in neural networks considerably and neuroplasticity is assumed to be an important physiological basis of learning and memory formation, it might be speculated that neuromodulators exert their effects on cognition via their impact on neuroplastic processes. Indeed it has been shown that altered neuromodulator levels in healthy individuals influence cognitive performance, learning and memory formation. For example, dopamine improves motor and verbal memory in healthy subjects (Floel et al., 2005; Knecht et al., 2004). Altered neuromodulator levels are also observed in pathological conditions accompanied by learning and memory deficits, such as in patients

suffering from Lewy body dementia (dopamine), Alzheimer's disease (acetylcholine), and depression (serotonin), amongst others.

Alterations of neuromodulator levels can be induced in healthy humans by pharmacological interventions. This enables us to study the physiological effects of neuromodulators on plasticity and thus explore their impact on cortical functions. In our studies, we explored two important aspects of the effects of neuromodulatory activity on plasticity, namely (a) activity of subreceptors (nicotinic receptor activation in the cholinergic system), and (b) dose-dependency of the effects on plasticity (dopaminergic system).

1.4.1 Cholinergic system

The cholinergic system comprises two types of receptors, namely the muscarinic and nicotinic receptors. The muscarinic receptors are G-protein coupled (Ishii and Kurachi, 2006), whereas the nicotinic receptors are ligand-gated cation channels (Gotti et al., 2009). Some subtypes of the nicotinic receptors are calcium channels that play an important role in glutamatergic plasticity (Shen and Yakel, 2009). Acetylcholine is a non-specific agonist of both receptor types. Muscarine and nicotine are agonists specific to the respective receptor types. Cholinergic activation is known to improve attention, arousal, learning and working memory from many animal and human studies (Gold, 2003; Sarter et al., 2003). Such an impact of cholinergic activation could be secondary to its effects on neuroplasticity. Effects of acetylcholine on plasticity have been explored in several animal studies (Blitzer et al., 1990; Hasselmo and Barkai, 1995). However, the role of cholinergic activation on neuroplasticity at the system level in humans has been sparsely studied. Kuo et al. (2007) explored the impact of rivastigmine, a cholinesterase inhibitor that is therapeutically used in patients suffering from Alzheimer´s disease (Onor et al., 2007), on neuroplasticity induced by non-invasive brain stimulation protocols. The drug increases the availability of acetylcholine at the

synapses, thereby activating non-specifically both muscarinic and nicotinic receptors. The participants were administered rivastigmine along with excitability - enhancing or excitability – diminishing, focal (PAS) or non-focal (tDCS) brain stimulation. Rivastigmine enhanced focal facilitatory plasticity induced by PAS-25 and suppressed non-focal facilitatory plasticity induced by anodal tDCS. Non-focal inhibitory plasticity following cathodal tDCS and focal inhibitory plasticity were preserved or prolonged. Therefore it was concluded that cholinergic activation resulted in a focusing effect on facilitatory plasticity probably due to enhanced signal-to-noise ratio in the system. This might at least partially account for the improvement of learning and memory following cholinergic activation (Hasselmo and Barkai, 1995). In this thesis, we aimed to study the nicotinergic impact on neuroplasticity to explore further the contribution of these receptors to the plasticity alteration induced by cholinergic activity modulation. So far, nicotine has been shown to affect plasticity primarily in animal experiments (Radcliffe and Dani, 1998). Studies exploring nicotinic impact on human neuroplasticity are very sparse. The only available study by Swayne and colleagues (2009) revealed that nicotine exposure enhances and prolongs the facilitatory after-effects of intermittent theta burst stimulation in healthy subjects (Huang et al., 2005). Indirect hints for plasticity-improving effects of nicotine stem from animal and human experiments demonstrating a beneficial effect of nicotine on cognition, including learning and memory formation (Froeliger et al. 2009; Hahn and Stolerman 2002; Heishman et al., 2010; Kastner et al., 2010; Kumari et al. 2003). However, so far a systematic exploration of the nicotinic impact on plasticity, especially considering the likely focusing effect of the drug on facilitatory plasticity, is missing.

1.4.2 Dopaminergic system

The dopamine receptors are classified into D1-like (D1 and D5) and D2-like (D2,D3 and D4) receptor families. They are located at post-synaptic and also as autoreceptors at the

pre-synaptic sites of neuronal connections (Missale et al., 1998; Vallone et al., 2000). Depending on the subtype and location of these receptors, the functional effects of dopaminergic activation differs largely. The dopaminergic impact on neuroplasticity in addition to the receptor subtype and location also depends on several other factors, the major ones being the level of cortical activity, concentration level and the specific brain region involved (Seamans and Yang, 2004).

Several studies have explored the impact of dopamine on cortical activity and plasticity, both in animals and humans. With regard to animal experiments, the dopaminergic impact on neuroplasticity has been studied in several brain regions – striatum (Kung et al., 2007), hippocampus (O'Çarroll et al., 2006) and prefrontal cortex (Otani et al., 1998; 2003). It has been shown that dopamine exerts heterogeneous effects on plasticity depending on subreceptor specificity and dopamine concentration. D1 activation results in enhancement of both LTP and LTD mediated by higher NMDA activity (Bailey et al., 2000; Huang et al., 2004) whereas D2 activation has variable effects due to suppressed NMDA and GABA activities (Chen et al., 1996; Otani et al., 1998; Tseng and O'Donnel, 2004). The balance of NMDA and GABA activity, which is influenced by dopamine, determines the resultant plasticity at the synapse. Considering the effect of concentration of dopamine on plasticity, low and high levels impair neuroplasticity whereas moderate concentrations enhance it (Seamans and Yang, 2004). In the cognitive domain, presumably at least in part caused by the effect of dopamine on plasticity, it has been shown to improve working memory performance, acquisition, stabilization and retrieval of long term memory in animal experiments (Brozoski et al., 1979; Seamans et al., 1998). The heterogeneity of the effects of dopamine on neuroplasticity is thought to be reflected by its heterogeneous effects on cognition (Goldman-Rakic et al., 2000; Seamans and Yang, 2004; Zahrt et al., 1997).

The effects of dopamine on plasticity were also explored in a couple of studies in humans. Kuo et al. (2008) demonstrated that 100 mg l-dopa enhances focal (PAS-induced) facilitatory plasticity and suppresses non-focal (tDCS-induced) facilitatory plasticity in healthy human subjects, that is, dopamine had a focusing effect on facilitatory plasticity. Moreover, Monte-Silva et al. (2010) demonstrated an inverted-U shaped dose-response curve of l-dopa on tDCS-induced non-focal plasticity in healthy human subjects. Ueki and colleagues (2006) showed that in Parkinson's disease patients, who were off-medication and thus in a hypo-dopaminergic state, PAS-induced plasticity could not be induced, but was restored by administration of l-dopa. Functionally, these effects of dopamine on plasticity might at least partly explain the positive effects of dopaminergic activation on learning and memory formation in humans. Here 100mg l-dopa has been shown to be effective in improving verbal memory in healthy human subjects (Floel et al., 2005a; Knecht et al., 2004) and in stroke patients (Floel et al., 2005b). Since PAS induces a kind of plasticity which might reflect the neurophysiological basis of learning and memory processes relatively closely, it would be interesting to explore the dosage-dependent effect of dopaminergic activation on this kind of plasticity. Such studies however have so far not been performed. Moreover, since in some studies dopamine also failed to induce positive effects on cognitive performance (Ghilardi et al., 2007; Gotham et al., 1988; Shohamy et al., 2006), non-linear dose-dependent effect on PAS-induced plasticity, if observed, might partly account for the heterogeneous dopaminergic effects on cognition.

1.5 Aim of the thesis

In this dissertation, we aimed to explore specific aspects of the impact of two main neuromodulatory systems of the human central nervous system on plasticity, namely the cholinergic and dopaminergic systems. The first study included in the dissertation deals with the cholinergic system and aims at defining the specific contribution of the nicotinic receptors to the global cholinergic effects on plasticity. Considering the calcium channel properties of some of the nicotinic subreceptors, and the importance of calcium-dependent mechanisms for neuroplasticity, we expected a prominent contribution of nicotinic receptors to the global cholinergic effects on plasticity explored in a preceding study (Kuo et al., 2007). Moreover, since global cholinergic activation resulted in a focusing effect on facilitatory plasticity, we aimed to determine if this effect is caused by the nicotinic subreceptor.

In the second study we aimed to improve our understanding of the dopaminergic impact on plasticity. Specifically we were interested to learn if associative plasticity induced by PAS, which is thought to resemble Hebbian plasticity to a certain degree, is affected by modulation of dopaminergic activity in a non-linear dosage-dependent way. The results of previous studies using other plasticity-probing protocols (Monte-Silva et al. 2010) are in favor for such an effect, which however has not been described for associative plasticity before. If such an effect exists, it might help to understand the partly heterogeneous effects of dopaminergic activation on learning and memory formation, which are thought to be closely related to Hebbian plasticity.

Hereby, in the present thesis we explore two important factors that determine the impact of neuromodulators on plasticity in the human motor cortex, namely subreceptor specificity and dosage-dependency.

Chapter 2 Original articles and manuscripts

In this chapter, the manuscripts of two studies incorporated in the thesis are included. The first study focuses on the nicotinergic impact on focal and non-focal plasticity in healthy non-smoking human subjects. The second study explores the dose-dependent effect of l-dopa on focal, associative plasticity in healthy human subjects.

- **Thirugnanasambandam N**, Grundey J, Adam K, Drees A, Skwirba AC, Lang N, Paulus W, Nitsche MA. Nicotinergic impact on focal and non-focal neuroplasticity induced by non-invasive brain stimulation in non-smoking humans. *Accepted, Neuropsychopharmacology.*

- **Thirugnanasambandam N**, Grundey J, Paulus W, Nitsche MA. Dose-dependent non-linear effect of L-DOPA on paired associative stimulation-induced neuroplasticity in humans. *Submitted*

2.1 Nicotinergic impact on focal and non-focal neuroplasticity induced by non-invasive brain stimulation in non-smoking humans

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ABSTRACT

Nicotine improves cognitive performance and modulates neuroplasticity in brain networks. The neurophysiological mechanisms underlying nicotine-induced behavioral changes have been sparsely studied, especially in humans. Global cholinergic activation focuses plasticity in humans. However, the specific contribution of nicotinic receptors to these effects is unclear. Henceforth, we explored the impact of nicotine on non-focal neuroplasticity induced by transcranial direct current stimulation (tDCS) and focal, synapse-specific plasticity induced by paired associative stimulation (PAS) in healthy non-smoking individuals. Forty eight subjects participated in the study. Each subject received placebo and nicotine patches combined with one of the stimulation protocols to the primary motor cortex in different sessions. Transcranial magnetic stimulation (TMS) - elicited motor evoked potential (MEP) amplitudes were recorded as a measure of corticospinal excitability until the evening of the second day following the stimulation. Nicotine abolished or reduced both PAS- and tDCS-induced inhibitory neuroplasticity. Non-focal facilitatory plasticity was also abolished, whereas focal facilitatory plasticity was slightly prolonged by nicotine. Thus, nicotinergic influence on facilitatory, but not inhibitory plasticity mimics that of global cholinergic enhancement.

Therefore, activating nicotinic receptors has clearly discernable effects from global cholinergic activation. These nicotine-generated plasticity alterations might be important for the effects of the drug on cognitive function.

INTRODUCTION

Smoking tobacco is the single largest preventable cause of mortality and morbidity (Peto et al. 1992). Nicotine is the primary constituent of tobacco that is responsible for its addictive properties. Nicotine is the classical agonist at nicotinic acetylcholine receptors (nAchRs) which are ligand-gated cation channels. Studies in animals and humans have shown that nicotine improves attention as well as working and long-term memory (Froeliger et al. 2009; Hahn and Stolerman 2002; Kumari et al. 2003). While many studies focused on the behavioral effects of nicotine in healthy humans and patients (Jacobsen et al. 2004; Sacco et al. 2005), very few have investigated the nicotinergic impact on cortical excitability and plasticity, which are the likely neurophysiological basis for the cognitive effects of the substance. For global cholinergic enhancement, it was shown that cholinesterase-inhibitors reduce intracortical inhibition, increase facilitation, and enhance focal, but diminish non-focal facilitatory plasticity in healthy humans (Korchounov et al. 2005; Kuo et al. 2007). A study on tobacco smokers, who are under chronic nicotine exposure, revealed enhanced motor cortex inhibition and reduced facilitation (Lang et al. 2008). Nicotine also enhances and prolongs the facilitatory after-effects of intermittent theta burst stimulation in human motor cortex (Swayne et al. 2009). Thus, global cholinergic and nicotinergic activation might have at least partially dissimilar effects on cortical excitability.

Transcranial direct current stimulation (tDCS) and paired associative stimulation (PAS) are non-invasive brain stimulation techniques that induce neuroplastic cortical excitability alterations (Nitsche and Paulus 2000, 2001; Nitsche et al. 2003a; Stefan et al. 2000; Wolters et al. 2003). Both techniques induce NMDA- and calcium-dependent changes of cortical

excitability (Nitsche et al. 2003b; Stefan et al. 2002; Wolters et al. 2003). tDCS modulates spontaneous neuronal activity and excitability by either depolarizing or hyperpolarizing neurons. Anodal tDCS induces depolarization that enhances neuronal excitability whereas cathodal tDCS hyperpolarizes neurons, decreasing their excitability levels (Nitsche and Paulus 2000, 2001; Nitsche et al. 2003a). Since tDCS affects all neurons beneath the electrodes, it is thought to induce relatively non-focal plasticity. PAS, on the other hand, induces focal, synapse-specific, timing-dependent, associative neuroplasticity in the targeted neurons. Here an electrical pulse to a mixed peripheral nerve at an intensity which activated somatosensory fibres is followed by a suprathreshold magnetic pulse applied to the corresponding area of the primary motor cortex. Depending on the interstimulus interval, there occurs synchronous or asynchronous activation of somatosensory-motor cortical connections that enhance or reduce excitability respectively (Stefan et al. 2000).

In the current study we aimed to identify the specific contribution of nicotinic receptors to the cholinergic effect on focal and non-focal neuroplasticity by exploring the effects of nicotine on tDCS- and PAS-generated plasticity in healthy non-smoking humans to improve our comprehension of the cognition-enhancing and addictive properties of this substance. As in the foregoing studies, the motor cortex was taken as a model system in this single blinded, placebo-controlled, partial crossover study because it allows a convenient monitoring of excitability alterations by measuring motor evoked potential (MEP) amplitudes via transcranial magnetic stimulation (TMS).

MATERIALS AND METHODS

Subjects

Forty eight healthy human volunteers participated in the study. All of them were complete non-smokers, that is, none of them had smoked tobacco for at least 3 years prior to the study. They did not suffer from any chronic or acute medical illness or any history of neurological/psychiatric diseases, and did not take any chronic or acute medication. This information was obtained by a detailed free personal interview with the subjects. Pregnancy, family history of epilepsy, presence of any metallic implant or cardiac pacemaker was ruled out. All of them were right-handed according to the Edinburgh handedness inventory (Oldfield, 1971). The selection of subjects was not based on their results from previous plasticity experiments in our laboratory; most of them were naïve to the experimental procedure. Table 1 shows the demographic characteristics of the different groups. All subjects gave written informed consent before participating in the study. The experiments were approved by the local Ethics Committee and conformed to the Declaration of Helsinki. Allocation of the subjects to the respective experimental conditions as well as order of sessions was randomized (See table 1).

Assessing motor cortex excitability

Single transcranial magnetic (TMS) pulses were delivered from a Magstim 200 stimulator (Magstim Company, Whitland, Dyfed, UK) at a frequency of 0.25 Hz with a figure-of-eight shaped coil (diameter of one winding, 70mm; peak magnetic field, 2.2 T). The coil was held tangentially to the scalp at an angle of 45º to the sagittal plane with the coil handle pointing laterally and posterior. This induced a postero-anterior current flow in the brain at an angle that optimally activates the corticospinal system monosynaptically (Di Lazzaro et al., 1998). Motor evoked potentials (MEPs) were recorded using a surface electromyogram (EMG) set-up. Surface EMG electrodes (Ag-AgCl) were placed over the abductor digiti minimi muscle

(ADM) in a belly-tendon montage. Signals were amplified, band-pass filtered (2Hz - 2KHz), digitized (5KHz) and stored in a laboratory computer for offline analysis using Signal software and CED 1401 (Cambridge Electronic Design, Cambridge, UK). MEPs were elicited using single pulse TMS over the motor cortex representation of the ADM. The position of the coil on the scalp, where the stimulus elicited consistently the largest MEP amplitudes for slightly suprathreshold intensity was marked as the motor 'hotspot'. Stimulus intensity was then adjusted in order to obtain peak to peak MEP amplitudes of approximately 1mV (SI1mV). This TMS intensity was kept constant throughout the experiment. The mean MEP amplitude was calculated from at least 20 pulses for baseline, and post-intervention excitability monitoring. The change of the mean MEP amplitude over time reflects alterations of motor cortex excitability.

Transcranial direct current stimulation

Twenty four subjects participated in the tDCS experiments. tDCS was administered by a battery-driven constant current stimulator (Schneider Electronic, Gleichen, Germany) through rubber electrodes covered by saline soaked sponges (35 square cm). One electrode was placed over the motor cortex representation of the right ADM as determined by single pulse TMS and the other electrode over the contralateral supra-orbital region. All subjects received 1mA of either anodal (for 13 min) or cathodal stimulation (for 9 min), combined with nicotine or placebo medication in different experimental sessions. Therefore, twelve subjects received anodal tDCS with nicotine or placebo patches and the remaining twelve received cathodal tDCS with nicotine or placebo patches. This stimulation intensity and duration (13 min anodal tDCS and 9 min cathodal tDCS) generates after-effects on cortical excitability lasting for approximately 60 min after stimulation (Nitsche and Paulus 2001; Nitsche et al. 2003a). The two consecutive experimental sessions per subject were separated by at least one week interval.

Paired associative stimulation

Twenty four subjects participated in the PAS experiment. Here a peripheral electrical pulse over the right ulnar nerve at wrist level was followed by a TMS pulse over the motor cortex representation of the ADM at inter-stimulus intervals (ISI) of either 10 (PAS 10) or 25 milliseconds (PAS 25). The peripheral pulse was delivered from a Digitimer D185 multipulse stimulator (Digitimer, Welwyn Garden City, UK) at an intensity of 300% of the sensory perceptual threshold. The suprathreshold magnetic pulse was delivered from a Magstim 200 stimulator with an intensity which elicited MEP amplitudes of approximately SI1mV. The paired pulses were repeated 90 times at a frequency of 0.05 Hz. This protocol induces long-lasting excitability changes in the motor cortex depending on the ISI duration. An ISI of 10 ms induces excitability diminution whereas an ISI of 25 ms induces facilitation (Stefan et al. 2000; Wolters et al. 2003). The subjects were instructed to count the number of pulses they received at their wrist throughout the whole stimulation duration in order to guarantee sufficient attention to the procedure, which has been shown to be crucial to obtain the intended effects (Stefan et al., 2004).

Pharmacological intervention

Each subject participated in two sessions in randomized order. 30cm^2 nicotine transdermal patches, each containing nicotine 0.83mg/cm^2 releasing 15mg over 16 hours or placebo patches were administered to all subjects in combination with one of the stimulation protocols - anodal tDCS, cathodal tDCS, PAS-10 or PAS-25. By this dosage of nicotine, physiologically and behaviorally relevant plasma levels are accomplished (Tønnesen et al. 1991). Subjects received the patch 6 hours before the start of the stimulation. This was the approximate time for the plasma level of nicotine to reach its maximum following application of the patch (Nørregaard et al. 1992). The patch was retained until the end of the last after-

measurement of the experiment on the evening of the second day. In order to counteract possible systemic side effects of nicotine, the subjects were instructed to take 20mg domperidone, a peripheral acting dopamine D2-receptor antagonist with antiemetic effects, in case of need.

Course of the experiment

The subjects received either a placebo or nicotine patch, which was adhered to the left upper arm and remained there until the end of the last after-measurement on the following evening. They were given 20mg domperidone and asked to take it orally in case of any side effects. Unpublished results from our group show that domperidone alone does not have any significant effect on motor cortical excitability. Six hours later, subjects were seated comfortably in a reclined position on a dentist´s chair with proper arm and head rests and asked to relax completely. The EMG electrodes were placed at the right ADM as described above. The motor 'hotspot' was determined over the left motor cortex and marked with a water-proof skin marker, and the TMS intensity needed to induce MEP amplitudes of 1mV (SI1mV) size was determined. Twenty MEPs were recorded at this stimulus intensity and the mean MEP amplitude was calculated as the baseline. One of the stimulation protocols, either tDCS or PAS, was administered. At least 20 MEPs were recorded immediately following the stimulation (0min) and at time points of 5, 10, 15, 20, 25, 30, 60, 90, and 120 min. For the nicotine patch sessions, the after-measurements were also conducted in the evening of the stimulation day and in the morning and evening of the day following the plasticity induction procedure (See figure 1). We used a waterproof pen to mark the ADM electrodes and TMS coil positions and ensured that these were positioned over the same spot during the whole course of the experiment.

Data analysis and statistics

The individual means of the 20 MEP amplitudes recorded at each time point were calculated for all subjects. The post-intervention mean MEP amplitudes from each subject were then normalized to the respective individual mean baseline MEP amplitude. The normalized mean MEP amplitudes from all subjects were pooled together and the grand average across subjects for each time bin was calculated.

A repeated measures ANOVA was performed on the normalized data using MEP amplitude as the dependent factor including all time points up to 120 min after stimulation. Drug (Nicotine versus Placebo) and time points were included as within-subjects factors. Stimulation (anodal tDCS/ cathodal tDCS/ PAS-10/ PAS-25) served as between-subjects factors. The Mauchly test was performed to test for sphericity, and the Greenhouse-Geisser correction applied when necessary. Conditional on significant results of the ANOVA, we performed post-hoc comparisons using Student's t-tests (paired, two-tailed, $p<0.05$, not adjusted for multiple comparisons) where we compared (i) the mean MEP amplitudes at the time points after plasticity induction versus baseline and (ii) the mean MEP amplitudes following nicotine versus placebo at one time point within a stimulation condition. Moreover, we compared absolute baseline MEP values between the stimulation and drug conditions via Student´s t-tests to exclude a priori differences. Chi square test was performed to look for significant differences in gender distribution between the groups. For comparison of the age of subjects between the groups, Student's t-tests (two - tailed, unpaired, $p<0.05$) were performed.

RESULTS

All subjects tolerated the experimental procedure well. None of them complained of any side effects of either nicotine or the stimulation. Especially the participants did not complain of any sedative effects of the patch. During the experiment, they were completely alert and

relaxed. Since no systemic side effects of nicotine were perceived and none of the participants needed to take domperidone, the subjects were blinded effectively. Gender distribution did not differ significantly between the various groups tested by chi square test ($p = 0.083$). There were significant differences in the mean age between some of the groups as tested by Student's unpaired t-tests. However, the maximum difference of mean age between groups was 2.65 years. Absolute baseline MEP amplitudes did not differ significantly between groups (Student's t-test, two-tailed, paired, $p>0.05$ for all cases) or medication conditions (Student's t-test, unpaired, two-tailed, $p = 0.66$).

The ANOVA revealed a significant main effect of the between-subjects factor stimulation ($F(3,44) = 18.137$; $p < 0.001$), in accordance with different effects of inhibitory and facilitatory tDCS and PAS on MEP amplitudes. The main effects of either nicotine ($F(1,44) = 0.093$; $p = 0.762$) or time ($F(10,440) = 1.654$; $p = 0.089$) were not significant. However, the interactions between nicotine X stimulation ($F(3,44) = 5.498$; $p = 0.003$); time X stimulation ($F(30,440) = 3.070$; $p < 0.001$) were significant, showing that nicotine had different effects on the above-mentioned stimulation protocols, and that the time course of the effects of these stimulation protocols was not identical. The three-way interaction nicotine X time X stimulation ($F(30,440) = 1.848$; $p = 0.005$) was also significant. Thus, application of nicotine patch significantly influenced the after-effects of the different stimulation protocols differently over time.

Nicotinergic impact on tDCS-induced plasticity

As shown by the post hoc t-tests, in the control condition without nicotine, MEPs were significantly enhanced following anodal tDCS for up to 90 minutes. Cathodal tDCS diminished excitability levels significantly also for up to 90 minutes after stimulation. Under nicotine, both anodal and cathodal tDCS-induced after-effects were abolished. The post-hoc

test revealed that for anodal tDCS, post-tDCS MEP amplitudes under nicotine were not different from baseline values, but differed significantly from those under placebo medication (table S1). A trendwise reversal of the effects of anodal tDCS from facilitation to inhibition under nicotine did not reach statistical significance ($p > 0.073$). The excitability diminution induced by cathodal tDCS, as compared to baseline excitability, was also abolished under nicotine; however relative to the placebo medication condition nicotine induced only a trendwise change (Figure 2 A, B, table S1).

Effect of nicotine on PAS-induced plasticity

As shown by the post hoc tests, PAS induced a significant facilitation of MEP amplitudes following PAS-25 and excitability diminution following PAS-10, lasting for up to 90 or 120 minutes after stimulation (table S1) under placebo medication. Under nicotine, the facilitatory effects of PAS-25 remained significant as compared to the respective baseline MEP for up to 90 min after PAS. MEP amplitudes under nicotine did not differ versus the respective placebo medication condition for up to 90 min after PAS-25, but were significantly larger as compared to placebo 120 min after PAS-25, being in favor for a prolonged excitability enhancement accomplished by PAS-25 under nicotine. The missing difference of PAS-25 under nicotine relative to baseline excitability 120 min after placticity induction might be caused by the relatively large variability of the MEPs at this time point (see Table S1). The inhibitory effect of PAS-10 was abolished under nicotine. Consequently, the post hoc tests revealed no significant differences of the respective MEP amplitudes relative to baseline, but significant differences of the respective MEP amplitudes relative to those under placebo medication (figure 3A, B).

DISCUSSION

The present study shows that in healthy non-smoking individuals nicotine prominently affects neuroplasticity. Our data illustrate that (i) nicotine exposure slightly prolongs or at least preserves the synapse-specific cortical excitability enhancement induced by PAS-25, but abolishes the PAS-10 induced depression of cortical excitability (ii) the non-focal excitability enhancing after-effect of anodal tDCS and the excitability diminution caused by cathodal tDCS are both abolished (Figure 4). There is also a trendwise reversal of anodal tDCS - induced facilitation to inhibition under nicotine. Hence, we observe a focusing effect of nicotine on facilitatory neuroplasticity and an abolishment of inhibitory plasticity, the latter irrespective of the focality of stimulation.

Some of our observations match with those of previous studies. The enhancement or preservation of facilitatory plasticity induced by PAS-25 by nicotine is similar to the effect it had on intermittent theta burst stimulation (Swayne et al., 2009). The effect of nicotine on facilitatory plasticity is also comparable to the effect of the cholinesterase inhibitor rivastigmine on identical plasticity induction protocols, however its effects on inhibitory plasticity are at variance to those under global cholinergic enhancement under rivastigmine (Kuo et al., 2007).

Proposed mechanism of action

The focusing effect of nicotine on facilitatory plasticity, i.e. consolidating/preserving focal, PAS-induced, but diminishing non-focal tDCS-generated plasticity, is quite similar to that of global cholinergic enhancement via rivastigmine described in a previous study (Kuo et al., 2007). A likely explanation for this effect is the different impact of cholinergic activation on recurrent activation of afferent input to cortical neurons. It has been shown that excitatory glutamatergic synaptic transmission is suppressed by presynaptic inhibition at intrinsic,

recurrent synapses, but not at afferent fibre synapses via cholinergic activation (Hasselmo & Bower, 1992; Hasselmo et al., 1995; Vogt & Regehr, 2001). Since PAS induces plasticity by a combination of afferent somatosensory input with a motor cortical stimulus, while tDCS is thought to affect primarily excitability of cortical interneurons (Nitsche et al., 2005), it might be speculated that these specific aspects of the stimulation techniques caused the differences of the effects also in the present experiments. In accordance, in animal experiments an inhibitory effect of the activation of nicotinergic subreceptors on feedforward interneurons has has been shown to prevent LTP induction by inhibiting pyramidal neurons, whereas spike-timing dependent LTP was enhanced (Rosza et al., 2008; Yamazaki et al., 2005), although the latter effect was not shown in all studies (Couey et al., 2007). Due to the results of the present experiment, this effect of cholinergic activation on facilitatory plasticity in humans can likely be attributed to an impact of nicotinic receptors. Another possible explanation of the results is based on the enhancement of intracellular calcium concentration by nicotinic receptors, e.g. the alpha-7 subreceptor. Intracellular calcium is a key determinant of plasticity induction, and the after-effects of tDCS and PAS are calcium-dependent (Nitsche et al., 2003b; Stefan et al., 2002). The amount of intracellular calcium determines if inhibitory, facilitatory, or no plasticity is induced. A slight enhancement of intracellular calcium induces LTD, a large enhancement LTP (Lisman, 2001). Medium and very large concentrations of intracellular calcium induce no or convert plasticity, the latter possibly due to an activation of hyperpolarizing calcium channels (Misonou et al., 2004). Since tDCS induces facilitatory plasticity by tonic depolarization of neurons for some minutes, which might activate voltage-dependent calcium channels, whereas PAS is a phasic stimulation technique, which induces only short lasting depolarization, the amount of intracellular calcium increase caused by tDCS might be larger than that caused by PAS. Therefore, a further calcium enhancement via nicotinic receptor activation might enhance the calcium level above the concentration inducing LTP-like plasticity, and therefore result in an abolishment of

plasticity in case of tDCS, but not PAS. This effect on different kinds of facilitatory plasticity of nicotine might enhance the signal-to-noise ratio (Hasselmo and Barkai, 1995), which would facilitate the representation of meaningful, synchronous inputs and suppress non-meaningful inputs. Indeed, it has been recently suggested that nicotine improves memory performance via calcium-dependent mechanisms in animals (Biala & Kruk, 2009).

In contrast, nicotine abolished all kinds of inhibitory plasticity irrespective of the specific stimulation protocol. One possible explanation might be that the calcium-enhancing properties of nicotinic receptor activation here resulted in an intracellular calcium level too large to induce LTD-like plasticity. This mechanism of action would also explain the different effect of global cholinergic activation by rivastigmine on inhibitory plasticity, as described in a previous study, where this substance prolonged tDCS- and PAS-generated inhibitory plasticity (Kuo et al., 2007), because muscarinic receptors inhibit voltage-gated calcium channels (Brown, 2010), and thus might counteract the effects of nicotinic receptors in this case. The effects of nicotine on inhibitory plasticity obtained in the present study are not in accordance with some animal experiments, where nicotinic activation has been shown to be important for LTD induction (Partridge et al., 2002; Fujii & Sumikawa, 2001), however, the effect of nicotine on LTD seems to depend on the general proneness of the system to inhibitory plasticity (Alzoubi et al., 2007, 2008), which likely differs between animal preparations and in vivo studies in humans.

It should be mentioned that these mechanistic explanations of the results are hypothetical presently. Alternative explanations, such as the modification of NMDA receptor-dependent plasticity by nicotine-dependent alteration of GABAergic activity (Couey et al. 2007), cannot be ruled out. These hypotheses should be tested more directly in future studies, e.g. by altering the activity of nicotinic subreceptors, or calcium channels, in combination with nicotine exposure.

General remarks

The results of the present study demonstrate that nicotine clearly influences neuroplasticity in non-smoking individuals. Nicotine focuses facilitatory plasticity whereas it abolishes inhibitory plasticity. The effects differ from those of non-specific cholinergic activation. The focusing effect of nicotine on facilitatory plasticity might help to explain how this drug improves attention, working memory and long-term memory in animals and humans via enhancing the signal to noise ratio of plasticity. Also the abolition of inhibitory plasticity by nicotine might affect cognitive processes. First, it might shift the net balance of plasticity more into the direction of facilitatory plasticity, and therefore indirectly enhance cognitive performance further. Second, inhibitory plasticity, especially long-term depression (LTD) has been shown to be directly involved in certain forms of learning and memory formation. Collingridge and colleagues (2010) describe a role of LTD in hippocampal-based learning and memory formation, and recognition memory in perirhinal cortex. Since our findings show that nicotine abolishes LTD-like plasticity it could be speculated that nicotine might worsen LTD-dependent forms of learning and memory. However, an impairment of cognitive functions by nicotine has been rarely described (Toledano et al., 2010), thus further behavioral studies are needed to explore the cognitive effects of nicotine more systematically.

Some limiting aspects of this study should be mentioned. Blinding could have been somewhat compromised considering the fact that the experimenters were not blinded to the intervention. However, the data were collected by more than one investigator without notable difference in the results, which probably indicates low experimenter bias, and experimenters were not informed about hypotheses about expected outcomes of the experiments, which should have limited expectancy effects. Although the subjects did not complain of any sedation due to nicotine, the degree of alertness was not explicitly assessed and hence its effect on the measurements cannot be ruled out completely. We did not measure plasma concentrations of nicotine, thus it could be argued that inter-individual differences of the bioavailability of the

substance had an impact on the results. However, since we studied a fairly homogenous group of participants, and we induced plasticity during steady state drug concentration, we do not think that variability of plasma concentration of nicotine can explain the results. We studied the effect of only a single dosage of nicotine in the present experiments. Thus it cannot be ruled out that the effect of nicotine on plasticity differs dose-dependently, as shown for other neuromodulators, like dopamine (Monte-Silva et al. 2009, 2010). Moreover, since nicotine receptors are rapidly modified by chronic exposure, this study cannot discern between primary effects of nicotine on nicotinic receptors and secondary effects caused by receptor desensitization or upregulation. Moreover, it should not be taken for granted that the effects obtained on motor cortex plasticity, as in the current study, translate exactly to other cortices, where nicotinic receptor density, and subreceptor composition might differ (McGehee and Role, 1995; Gotti et el., 2009) Furthermore the results of a single dosage nicotine application in non-smokers, as performed here, might differ from the effects of nicotine in smokers who are chronically exposed to it. Future studies should address these aspects in larger detail.

Conclusion

The results of this study deliver clear evidence for an important role of nicotine in the formation of neuroplasticity, the likely basis of learning and memory formation, in humans. Via its focusing effect on facilitatory plasticity, nicotine might be an attractive candidate to enhance these processes in neuropsychiatric diseases accompanied by cognitive decline. The abolition of inhibitory plasticity by nicotine also could have a significant impact on some forms of learning and memory, and also affect addictive behavior to some extent. Moreover, its effect on plasticity might be an important mechanism for starting nicotine consumption, addiction and the high probability of relapse in smokers. Interestingly the effects of nicotine on plasticity share some of those of dopamine (Kuo et al., 2008), which might be an explanation for the frequent nicotine consumption in schizophrenia, in which dopaminergic

malfunctioning is an important pathologic mechanism. Clearly, more studies are needed to explore the exact role of nicotine in healthy humans and in those suffering from neuropsychiatric diseases to a larger degree. Moreover the results of this study are important in another aspect. Non-invasive brain stimulation techniques are increasingly used as scientific and therapeutic tools. The results of the present study show that the activity of the nicotinergic system might critically affect the effects of brain stimulation. This potentially important confounding factor should thus be taken into account in future studies using brain stimulation.

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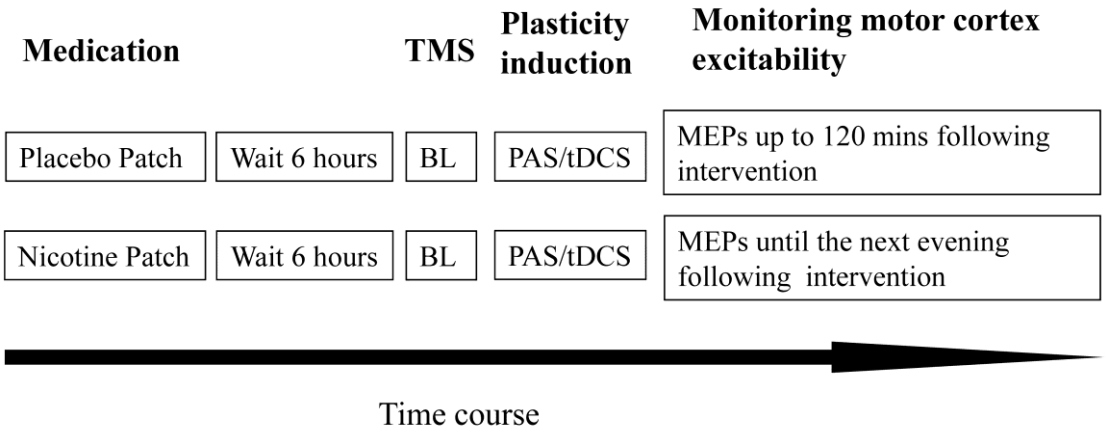
Table 1: Comparison of the subject characteristics of the four experiments.

Stimulation / Parameter	Anodal tDCS	Cathodal tDCS	PAS 10	PAS 25
Number of subjects	12	12	12	12
Number (%) of females	6 (50)	7 (58.33)	6 (50)	6 (50)
Age of subjects (mean ± SD) in years	24.3±1.1	26.9±3.5	25.9±2.1	24.5±1.3

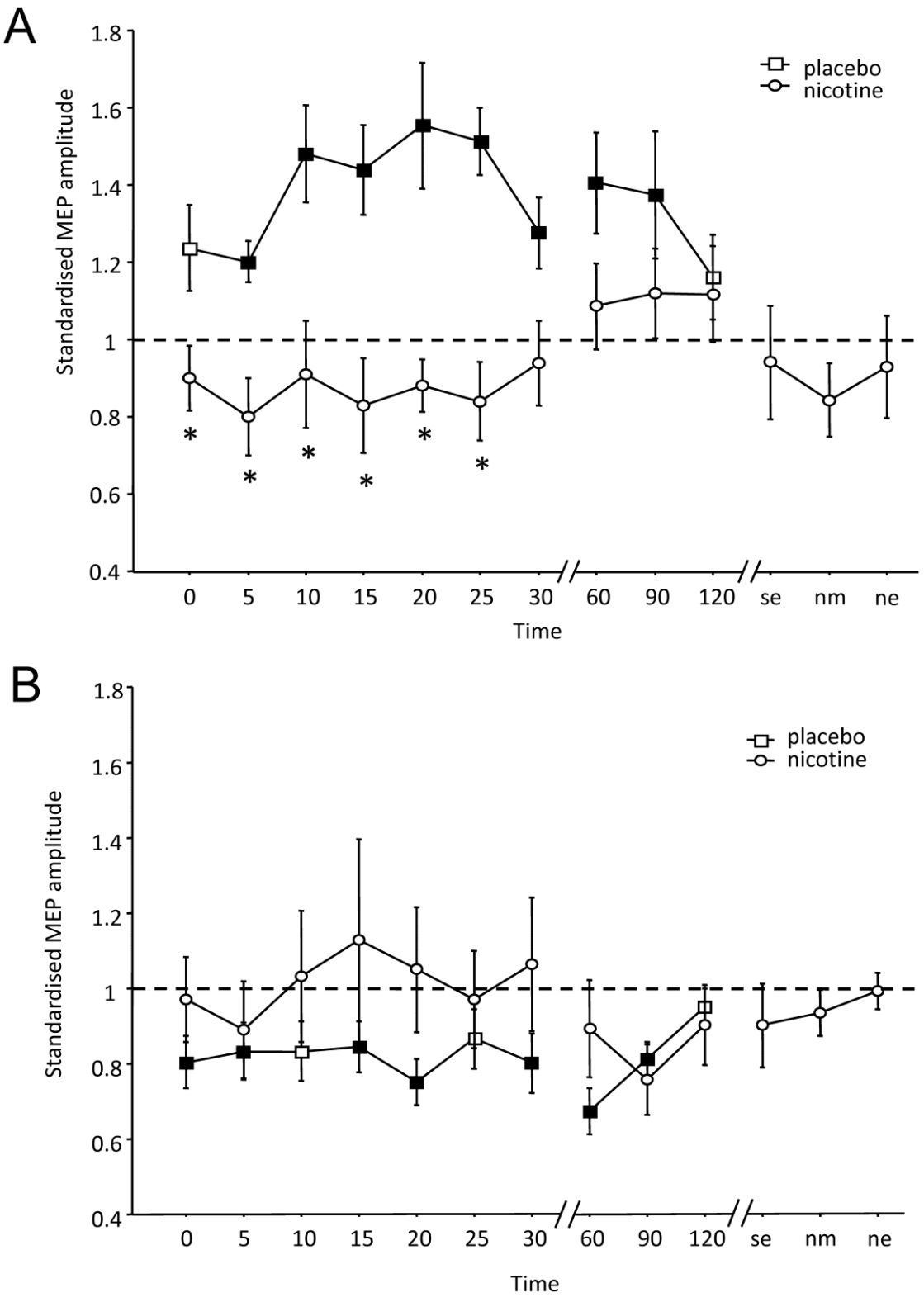
The table lists the age and gender characteristics of the subjects who participated in the four different categories of the experiment. Also the mean of the absolute baseline MEP amplitude values are shown.

Figure 1: Illustration of the experimental design.



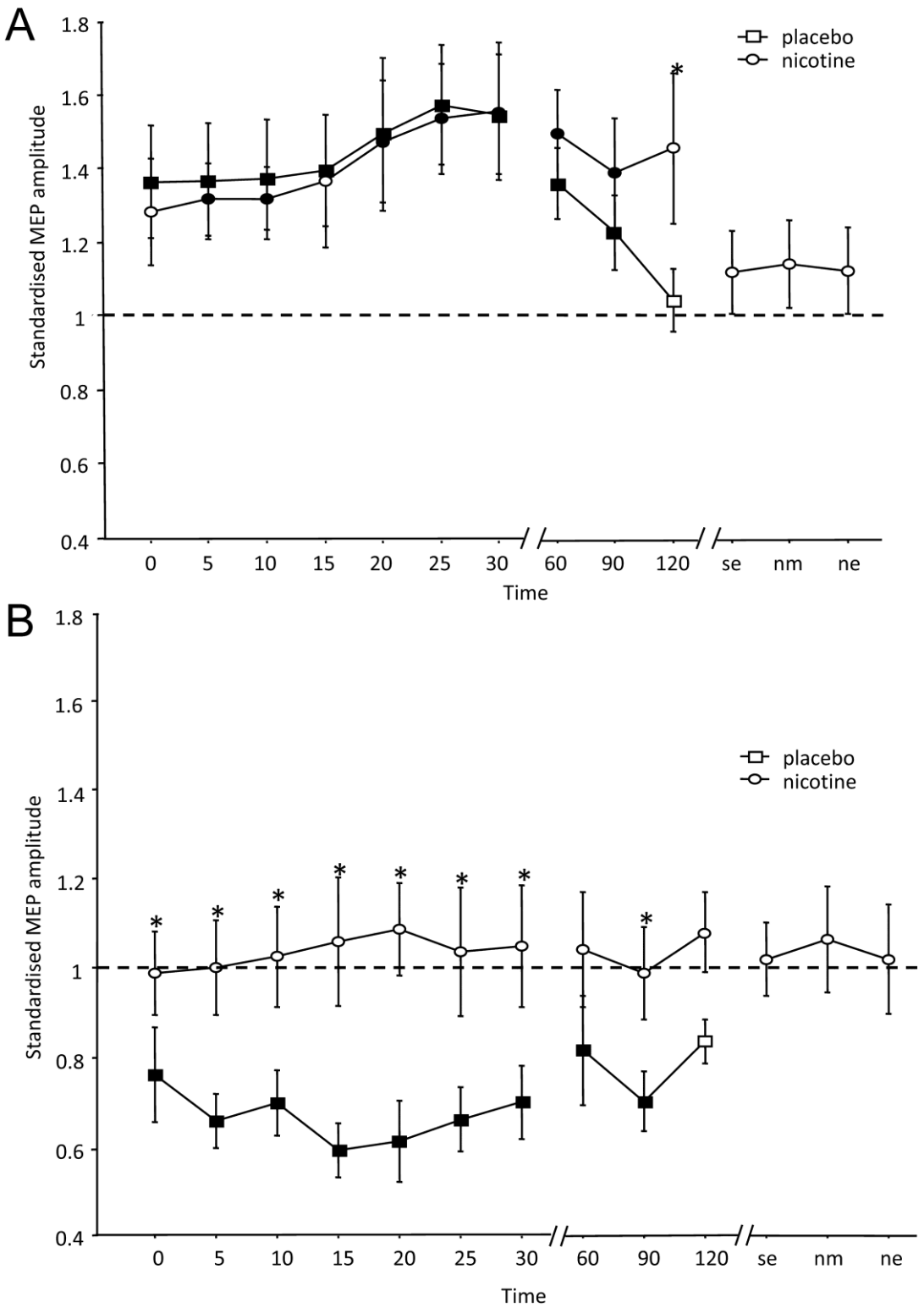
Each subject underwent two experimental sessions. At the beginning of the session, the subjects first received either a placebo or a nicotine patch. After 6 hours break, baseline (BL) motor evoked potentials (MEPs) of approximately 1 mV amplitude were recorded by single pulse transcranial magnetic stimulation (TMS) over the motor cortical representational area of the abductor digiti minimi muscle (ADM). One of the four stimulation protocols (anodal tDCS/cathodal tDCS/PAS-25/PAS-10) was then applied. After-measurements were made immediately following the stimulation and once every 5 min for the first 30 min, then once every 30 min for 120 min for both sessions. In addition, MEPs were also recorded the same evening, next morning and evening for the nicotine patch sessions.

Figure 2: Nicotinergic impact on transcranial direct current stimulation (tDCS) induced neuroplasticity.



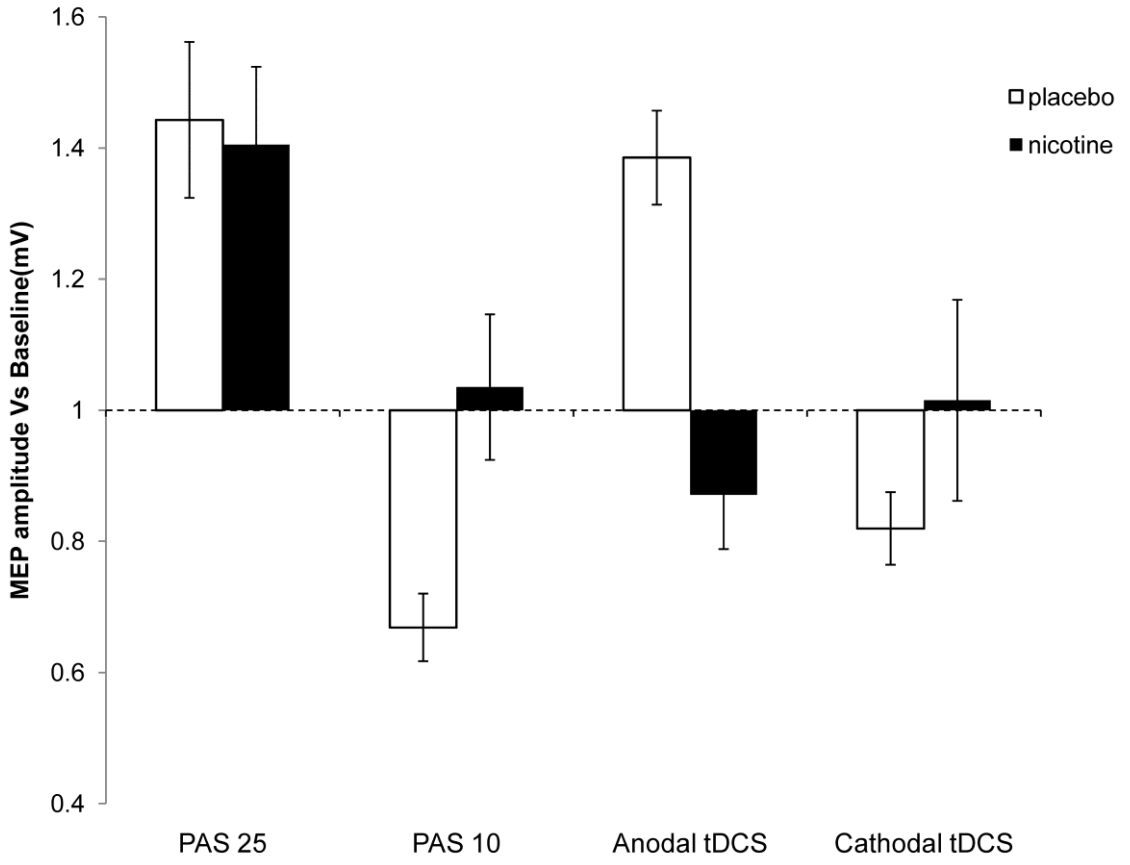
Shown are the graphs with MEP amplitudes standardized to baseline on the Y-axis plotted against various time points following the stimulation. (A) In the placebo condition (squares), anodal tDCS enhances motor cortex excitability until 90 minutes following stimulation. This effect of anodal tDCS is abolished in the nicotine condition (circles). (B) The cathodal tDCS-induced excitability diminution observed in the placebo condition (squares) is also abolished by application of nicotine (circles). Filled symbols indicate statistically significant deviations from baseline and asterisks indicate significant differences between the control and nicotine conditions (Student's t-test, paired, two-tailed, $p<0.05$). SE, same evening; NM, next morning; NE, next evening; MEP, motor evoked potential. Error bars indicate S.E.M.

Figure 3: Nicotinergic impact on paired associative stimulation (PAS) induced neuroplasticity.



The graphs show baseline-standardized MEP amplitudes on the Y-axis plotted at different time points following the stimulation. (A) In the placebo condition (squares), PAS-25 induced a clear increase of MEP amplitudes lasting for 90 minutes after stimulation. With nicotine (circles), the facilitatory after-effect of PAS-25 is slightly prolonged. (B) PAS-10 under placebo medication (squares) induced a prominent decrease of MEP amplitudes significant until 90 minutes after stimulation. Nicotine (circles) abolished the inhibitory after-effect of PAS-10. Filled symbols indicate statistically significant deviations from baseline and asterisks indicate significant differences between the control and nicotine conditions (Student's t-test, paired, two-tailed, $p<0.05$). SE, same evening; NM, next morning; NE, next evening; MEP, motor evoked potential. Error bars indicate SEM.

Figure 4: Effect of nicotine on focal/non-focal neuroplasticity induced in the human motor cortex until 30 minutes following stimulation.



Nicotine shows a focusing effect on facilitatory plasticity. Focal facilitatory plasticity is prolonged, whereas non-focal facilitatory plasticity is abolished under nicotine. Nicotine also abolishes any effect of excitability-reducing plasticity-generating protocols. Each bar represents the mean MEP amplitude until 30 minutes after stimulation standardized to the baseline. Error bars indicate S.E.M.

Supplementary information

TABLE S1: Results of post-hoc t-tests.

Shown are the results of the post-hoc t-tests (paired, two-tailed). MEP amplitudes at different time points were compared to the respective baseline MEP amplitude. Also, the MEP amplitudes at specific time points in the nicotine patch condition were compared with the MEP amplitudes at the corresponding time points in the placebo medication condition. Asterisks indicate statistically significant differences. tb – baseline; iPAS – PAS-10; np – nicotine patch; ePAS – PAS-25; cath – cathodal tDCS; anod – anodal tDCS.

	t	**df**	**Sig.(2-tailed)**
tb_iPAS - t0_iPAS	2.267	11	.045*
tb_iPAS - t5_iPAS	5.655	11	<.001*
tb_iPAS - t10_iPAS	4.099	11	.002*
tb_iPAS - t15_iPAS	6.755	11	<.001*
tb_iPAS - t20_iPAS	4.245	11	.001*
tb_iPAS - t25_iPAS	4.754	11	.001*
tb_iPAS - t30_iPAS	3.660	11	.004*
tb_iPAS - t60_iPAS	1.497	11	.163
tb_iPAS - t90_iPAS	4.427	11	.001*
tb_iPAS - t120_iPAS	3.351	11	.006*
tb_iPAS_np - t0_iPAS_np	.126	11	.902
tb_iPAS_np - t5_iPAS_np	-.008	11	.994
tb_iPAS_np - t10_iPAS_np	-.224	11	.827
tb_iPAS_np - t15_iPAS_np	-.411	11	.689
tb_iPAS_np - t20_iPAS_np	-.838	11	.420
tb_iPAS_np - t25_iPAS_np	-.258	11	.801

tb_iPAS_np - t30_iPAS_np	-.362	11	.724
tb_iPAS_np - t60_iPAS_np	-.319	11	.756
tb_iPAS_np - t90_iPAS_np	.112	11	.913
tb_iPAS_np - t120_iPAS_np	-.893	11	.391
tb_iPAS_np - t240_iPAS_np	-.232	11	.821
tb_iPAS_np - tnm_iPAS_np	-.540	11	.600
tb_iPAS_np - tne_iPAS_np	-.158	11	.877
tb_ePAS - t0_ePAS	-2.361	11	.038*
tb_ePAS - t5_ePAS	-2.292	11	.043*
tb_ePAS - t10_ePAS	-2.275	11	.044*
tb_ePAS - t15_ePAS	-2.582	11	.026*
tb_ePAS - t20_ePAS	-2.374	11	.037*
tb_ePAS - t25_ePAS	-3.512	11	.005*
tb_ePAS - t30_ePAS	-3.344	11	.007*
tb_ePAS - t60_ePAS	-3.721	11	.003*
tb_ePAS - t90_ePAS	-2.222	11	.048*
tb_ePAS - t120_ePAS	-.457	11	.656
tb_ePAS_np - t0_ePAS_np	-1.949	11	.077
tb_ePAS_np - t5_ePAS_np	-3.190	11	.009*
tb_ePAS_np - t10_ePAS_np	-3.721	11	.003*
tb_ePAS_np - t15_ePAS_np	-2.011	11	.070
tb_ePAS_np - t20_ePAS_np	-2.840	11	.016*
tb_ePAS_np - t25_ePAS_np	-3.575	11	.004*
tb_ePAS_np - t30_ePAS_np	-2.976	11	.013*
tb_ePAS_np - t60_ePAS_np	-4.057	11	.002*
tb_ePAS_np - t90_ePAS_np	-2.576	11	.026*

tb_ePAS_np - t120_ePAS_np	-2.200	11	.050
tb_ePAS_np - t240_ePAS_np	-1.032	11	.324
tb_ePAS_np - tnm_ePAS_np	-1.166	11	.268
tb_ePAS_np - tne_ePAS_np	-1.018	11	.330
t0_iPAS - t0_iPAS_np	-1.640	11	.129
t5_iPAS - t5_iPAS_np	-2.662	11	.022*
t10_iPAS - t10_iPAS_np	-2.823	11	.017*
t15_iPAS - t15_iPAS_np	-3.134	11	.010*
t20_iPAS - t20_iPAS_np	-3.525	11	.005*
t25_iPAS - t25_iPAS_np	-2.807	11	.017*
t30_iPAS - t30_iPAS_np	-2.499	11	.030*
t60_iPAS - t60_iPAS_np	-1.721	11	.113
t90_iPAS - t90_iPAS_np	-2.551	11	.027*
t120_iPAS - t120_iPAS_np	-2.130	11	.057
t0_ePAS - t0_ePAS_np	.347	11	.735
t5_ePAS - t5_ePAS_np	.223	11	.827
t10_ePAS - t10_ePAS_np	.264	11	.797
t15_ePAS - t15_ePAS_np	.114	11	.911
t20_ePAS - t20_ePAS_np	.065	11	.949
t25_ePAS - t25_ePAS_np	.132	11	.897
t30_ePAS - t30_ePAS_np	-.041	11	.968
t60_ePAS - t60_ePAS_np	-.812	11	.434
t90_ePAS - t90_ePAS_np	-.792	11	.445
t120_ePAS - t120_ePAS_np	-2.245	11	.046*
tb_cath - t0_cath	2.850	11	.016*
tb_cath - t5_cath	2.210	11	.049*

tb_cath - t10_cath	2.113	11	.058
tb_cath - t15_cath	2.255	11	.045*
tb_cath - t20_cath	4.090	11	.002*
tb_cath - t25_cath	1.695	11	.118
tb_cath - t30_cath	2.507	11	.029*
tb_cath - t60_cath	5.315	11	<.001*
tb_cath - t90_cath	4.127	11	.002*
tb_cath - t120_cath	1.031	11	.325
tb_cath_np - t0_cath_np	-.625	11	.545
tb_cath_np - t5_cath_np	.545	11	.597
tb_cath_np - t10_cath_np	-.004	11	.997
tb_cath_np - t15_cath_np	-.163	11	.874
tb_cath_np - t20_cath_np	.271	11	.791
tb_cath_np - t25_cath_np	.613	11	.553
tb_cath_np - t30_cath_np	-.093	11	.927
tb_cath_np - t60_cath_np	.691	11	.504
tb_cath_np - t90_cath_np	2.021	11	.068
tb_cath_np - t120_cath_np	1.708	11	.116
tb_cath_np - t240_cath_np	.889	11	.393
tb_cath_np - tnm_cath_np	.254	11	.804
tb_cath_np - tne_cath_np	.543	11	.598
tb_anod - t0_anod	-2.120	11	.058
tb_anod - t5_anod	-3.771	11	.003*
tb_anod - t10_anod	-3.788	11	.003*
tb_anod - t15_anod	-3.763	11	.003*
tb_anod - t20_anod	-3.391	11	.006*

tb_anod - t25_anod	-5.936	11	<.001*
tb_anod - t30_anod	-2.962	11	.013*
tb_anod - t60_anod	-3.096	11	.010*
tb_anod - t90_anod	-2.264	11	.045*
tb_anod - t120_anod	-1.456	11	.173
tb_anod_np - t0_anod_np	1.194	11	.258
tb_anod_np - t5_anod_np	1.979	11	.073
tb_anod_np - t10_anod_np	.655	11	.526
tb_anod_np - t15_anod_np	1.391	11	.192
tb_anod_np - t20_anod_np	1.776	11	.103
tb_anod_np - t25_anod_np	1.577	11	.143
t0_anod_np - t30_anod_np	-.335	11	.744
tb_anod_np - t60_anod_np	-.773	11	.456
tb_anod_np - t90_anod_np	-1.021	11	.329
tb_anod_np - t120_anod_np	-.945	11	.365
tb_anod_np - t240_anod_np	.339	11	.741
tb_anod_np - tnm_anod_np	1.569	11	.145
tb_anod_np - tne_anod_np	.488	11	.635
t0_cath - t0_cath_np	-1.729	11	.112
t5_cath - t5_cath_np	-.530	11	.606
t10_cath - t10_cath_np	-.770	11	.457
t15_cath - t15_cath_np	-.637	11	.537
t20_cath - t20_cath_np	-1.023	11	.328
t25_cath - t25_cath_np	-.329	11	.748
t30_cath - t30_cath_np	-1.017	11	.331
t60_cath - t60_cath_np	-1.482	11	.166

t90_cath - t90_cath_np	.119	11	.907
t120_cath - t120_cath_np	1.049	11	.317
t0_anod - t0_anod_np	3.447	11	.005*
t5_anod - t5_anod_np	2.970	11	.013*
t10_anod - t10_anod_np	3.197	11	.008*
t15_anod - t15_anod_np	4.303	11	.001*
t20_anod - t20_anod_np	3.666	11	.004*
t25_anod - t25_anod_np	5.376	11	<.001*
t30_anod - t30_anod_np	1.924	11	.081
t60_anod - t60_anod_np	1.592	11	.140
t90_anod - t90_anod_np	1.026	11	.327
t120_anod - t120_anod_np	.282	11	.783

2.2 Dose-dependent non-linear effect of L-DOPA on paired associative stimulation-induced neuroplasticity in humans.

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ABSTRACT

Dopamine is one of the major neuromodulators in the central nervous system, which is involved in learning and memory processes. A non-linear, inverted U-shaped dose-response curve of its effects on cognition has been observed in animal studies. The basis for this non-linear effect might be a similar effect of dopamine on neuroplasticity. Whereas it has been shown that dopamine affects paired associative stimulation (PAS) –induced plasticity, which might reflect learning-related processes to a larger degree than other non-invasive plasticity induction protocols in the human motor cortex in principle, its dose-dependency has not been explored so far. We studied the effect of different dosages of the dopamine precursor l-dopa on motor cortex plasticity induced by facilitatory and inhibitory PAS of the motor cortex in twelve healthy humans. They received 25, 100 or 200 mg l-dopa or placebo medication combined with either excitability-enhancing or -diminishing PAS. Cortical excitability level was monitored before and for up to two days after plasticity induction by assessment of transcranial magnetic stimulation (TMS)-induced motor evoked potentials (MEP). Low dose l-dopa abolished the after-effects of PAS while medium dose l-dopa prolonged facilitatory plasticity. High dose l-dopa reversed the excitability enhancement accomplished by facilitatory PAS to diminution. Thus the results show a clear non-linear effect of l-dopa

dosage on associative plasticity, different from that on non-focal plasticity. This might help to explain dopaminergic effect on cognition and could be relevant for understanding the pathophysiology and treatment of neuropsychiatric diseases accompanied by alterations of the dopaminergic system.

INTRODUCTION

Dopamine is a neuromodulator that influences the functions of other neurotransmitters and ion channels directly or indirectly (Abekawa et al., 2000; Briand et al., 2007; Grobin and Deutch, 1998; Rétaux et al., 1991). These effects are heterogeneous, depending on neuronal activity, dosage, sub-receptor specificity and brain region, amongst others (Seamans and Yang, 2004). Dopamine is involved in various neuropsychiatric diseases at least partly associated with cognitive impairment, such as Parkinson's disease, restless legs syndrome, Lewy body dementia, and schizophrenia (Iversen and Iversen, 2007; Molloy et al., 2005; Poewe et al., 2010; Trenkwalder and Paulus, 2010). In the cognitive domain, dopamine has been shown to improve attention (Nieoullon, 2002), working memory (Durstewitz and Seamans, 2008; Zahrt et al., 1997), learning and memory formation (Knecht et al., 2004; Molina-Luna et al., 2009; Shohamy et al., 2005). The likely neurophysiological foundation of the learning- and memory-modulating effects of dopamine is its effect on neuroplasticity, namely long term- potentiation (LTP), and -depression (LTD). Dopamine influences neuroplasticity in animals (Calabresi et al., 2007; Kung et al., 2007; Seamans and Yang, 2004) and humans (Kuo et al., 2008; Lang et al., 2008). Associative or spike-timing dependent plasticity (STDP) is a strong candidate mechanism for memory formation (Hebb, 1949; Letzkus et al., 2007). Associative, timing-dependent plasticity can be induced in humans by paired associative stimulation (PAS, Stefan et al., 2000) where combined somatosensory stimulation of a peripheral nerve and the motor cortex via transcranial magnetic stimulation (TMS) is performed. Interstimulus intervals which result in synchronous

activation of motor cortical neurons by somatosensory afferents and motor cortex TMS enhance cortical excitability, whereas asynchronous stimulation diminishes it (Stefan et al., 2000). These excitability alterations last for about an hour and are both NMDA receptor- and calcium-dependent (Stefan et al., 2002; Wolters et al., 2003).

Interestingly, PAS-induced plasticity is absent in a hypo-dopaminergic state in patients suffering from Parkinson´s disease off medication, but restituted by dopaminergic agents in them (Ueki et al., 2006). Likewise, dopamine is known to enhance PAS-induced facilitatory plasticity in healthy humans at a dosage of 100mg of the dopamine precursor levodopa (Kuo et al., 2008). This relates well with the positive effect of this drug on learning and memory formation (Floel et al., 2005a; Knecht et al., 2004; Shohamy et al., 2005). However, l-dopa did not improve performance in all studies testing its effects on cognition (Ghilardi et al., 2007; Gotham et al., 1988; Shohamy et al., 2006). This could be partly due to a non-linear dosage-dependent effect of l-dopa on cognition, and underlying neuroplastic phenomena. Animal studies have revealed an inverted U-shaped dose-response curve of dopamine on cognition (Granon et al., 2000; Seamans and Yang, 2004; Williams and Goldman-Rakic, 1995; Zahrt et al., 1997). However, only dosage-dependency of dopaminergic medication on non-focal plasticity (Monte-Silva et al., 2010) but not associative plasticity has been explored directly so far.

In the current study, we aimed to explore such a non-linear effect of l-dopa on associative plasticity in humans, using the human motor cortex as a model system.

MATERIALS AND METHODS

Subjects

Twelve healthy human volunteers (six females; age, 29.67 ± 8.04 years) participated in the study. All of them were right-handed according to the Edinburgh handedness inventory

(Oldfield, 1971). The eligible age of subjects to participate in the study was between 18 and 65 years. The subjects did not have any metallic implant in the body or any history of a neurological/psychiatric or chronic or acute medical disease. They took no acute/chronic medication during or up to two weeks before participating in the study. Pregnancy was ruled out in female subjects. All participants signed an informed consent form before participating in the experiment. The experiment conforms to the guidelines stated in the Declaration of Helsinki and was approved by the local Ethics Committee.

Monitoring motor cortex excitability

Motor cortex excitability was monitored by the peak to peak amplitudes of motor evoked potentials (MEP) generated by transcranial magnetic stimulation (TMS). Single magnetic pulses were delivered from a Magstim 200 stimulator (Magstim Company, Whitland, Dyfed, UK) at a frequency of 0.25 Hz. A figure-of-eight coil (diameter of one winding 70mm; peak magnetic field 2.2T) was held tangentially on the scalp at an angle of 45º to the mid-sagittal plane with the handle pointing laterally and posteriorly to deliver the pulses. This coil position induces a postero-anterior directed current flow in the brain. The exact point for TMS was the motor cortex representation of the right abductor digiti minimi (ADM) muscle. This 'motor hot spot', as determined by TMS, was defined as the point where a magnetic stimulus of constant, slightly suprathreshold intensity elicited consistently MEPs of the highest amplitude. Surface electromyography (EMG) electrodes (Ag-AgCl) were placed over the right ADM in a belly-tendon montage for recording the MEPs. The signals from the EMG electrodes were amplified (gain = 1000), band-pass filtered (2Hz – 2KHz), digitized at a frequency of 5KHz and stored in a laboratory computer for later offline analysis by Signal software and CED 1401 hardware (Cambridge Electronic Design, Cambridge, UK). The intensity of the magnetic stimulus required to elicit approximately 1mV MEP amplitudes (SI1mV) was determined. At this intensity, 25-30 MEPs were recorded before and at several

time points after the intervention. The change in the mean MEP amplitude over time was considered as measure of cortical excitability alterations caused by the intervention. Table 1 shows the mean values of the basic neurophysiological parameters measured during the experiment.

Pharmacological intervention

The subjects received low dose (25mg), medium dose (100mg) or high dose (200mg) l-dopa in combination with the dopamine decarboxylase inhibitor benserazide (one-fourth the dose of l-dopa) or a placebo medication at each experimental session. One hour before the intake of this medication, the subjects received a 20mg oral tablet of domperidone in order to counteract the systemic side effects of l-dopa. For the high dose sessions, the subjects were asked to take 20mg domperidone orally three times daily for two days before the experiment. The rationale for using the above-mentioned dosages of l-dopa is that these had prominent non-linear effects on another plasticity induction protocol in a recently published study (Monte-Silva et al., 2010).

Paired Associative Stimulation (PAS)

For PAS, an electrical pulse was delivered to the ulnar nerve at the wrist followed by a magnetic pulse to the motor hot spot of the ADM. The intensity of the electrical pulse was three times the sensory perceptual threshold, delivered from a Digitimer D185 multipulse stimulator (Digitimer, Welwyn Garden City, UK). The magnetic pulse had an intensity which resulted in MEP amplitudes of about 1mV (SI1mV). Both stimuli were separated by an interval of either 10 or 25 milliseconds, with the peripheral nerve pulse always followed by the TMS stimulus. These paired pulses were administered 90 times at a frequency of 0.05 Hz for 30 minutes over the motor hot spot of the ADM. Here, the inter-stimulus interval (ISI) determines the direction of plasticity that is induced. When the ISI is 10 milliseconds (PAS-

10), excitability diminution occurs, whereas an ISI of 25 milliseconds (PAS-25) induces excitability enhancement (Stefan et al., 2000; 2002; Wolters et al., 2003). The reason for these different effects is that in PAS –10 the somatosensory stimulus reaches the primary motor cortex some milliseconds before the TMS stimulus (asynchronous stimulation), whereas in case of PAS-25, both stimuli reach the motor cortex simultaneously and such synchronous activation results in facilitation at the synapse.

Course of the experiment

The study design was single-blinded, complete crossover, and placebo-controlled. Between the experimental sessions (eight sessions per subject), an interval of at least one week was essential to avoid interference effects.

The participants received domperidone or equivalent placebo medication before the start of the neurophysiological part of experiments, as outlined above. They were seated comfortably on a reclining chair with head and arm rests, and asked to relax completely. EMG electrodes were placed at the right ADM, and the motor cortex hotspot was determined. Both the position of the EMG electrodes and the motor hotspot were marked with a permanent skin marker in order to ensure their constant positioning throughout an experimental session. SI1mV was determined and at least 25 MEPs were recorded as baseline 1 at this stimulus intensity. Immediately after the baseline measurement, the participants received low/medium/high dose l-dopa or placebo medication. The combination of drug dose and PAS was given in a randomized order for all the subjects. Baseline 2 (25 MEPs) was obtained after one hour, because at this time l-dopa has reached its maximal plasma concentration (Crevoisier et al., 1987) and have prominent effects on brain function (Floel et al., 2005a; Kuo et al., 2008), to reveal an influence of the medication on cortical excitability. In case of any drug-induced MEP amplitude changes, another set of MEPs was recorded at the adjusted

SI1mV (baseline 3). Subsequently, either PAS-10 or PAS-25 was administered as described above. Following PAS, 25-30 MEPs were recorded at 0, 5, 10, 15, 20, 25, 30, 60, 90 and 120 minutes for all sessions. Since the after-effects of PAS have not been reported to last for more than 90 minutes, we recorded MEPs in the placebo condition only until 120 mins after cessation of stimulation. Further after-measurements were conducted the evening of the same day (se), next morning (nm), next afternoon (na), next evening (ne) and on the third day morning (3m) for all sessions except the placebo sessions (see Figure 1).

Data analysis and statistics

Individual mean MEP amplitudes for each subject for baseline 1, 2, 3 and each time point following intervention were calculated. The post-intervention MEP amplitudes were normalized to the mean baseline 3. In most of the individual measurements l-dopa did not alter baseline MEP amplitudes. In these cases, there was no need to change the S1mV and baseline 3 was identical to baseline 2. The normalized MEP amplitudes from all subjects were pooled together session-wise by calculating the grand average across subjects for each condition and time point.

A repeated-measures ANOVA was performed for the normalized data. MEP amplitude served as dependent variable. We included only the data until 120 minutes after PAS in the analysis, which were obtained from all sessions. PAS (PAS-10/ PAS-25) and drug (low dose/ medium dose/ high dose/ placebo) served as within-subjects factors. Mauchly's sphericity test was performed and Greenhouse-Geisser correction applied when necessary. If the ANOVA yielded significant results, we performed post-hoc comparisons using Student's t-test (paired, two-tailed, $p<0.05$, not adjusted for multiple comparisons). Here we compared (i) the mean MEP amplitudes at all time points after PAS versus baseline 3 and (ii) the means at a specific time point for the various drug conditions against the placebo medication condition. Baseline

MEP amplitudes of all drug/PAS combinations were compared by Student´s t-tests to exclude a priori differences between conditions, and baseline 1 and 2 MEP amplitudes to test for any influence of the drug alone on cortical excitability.

RESULTS

All except one male subject tolerated the experimental procedures well. This subject developed nausea and vomiting approximately 90 minutes after the intake of 200 mg l-dopa. We had to exclude the data of this participant from the analysis because of artifacts of MEP measures caused by insufficient relaxation. Mean absolute baseline MEP amplitudes and percentage of maximal stimulator output to achieve baseline amplitudes of about 1 mV did not differ significantly between sessions (Student's t-test, paired, two-tailed, $p>0.05$). Baseline MEP amplitudes were not affected by any of the drug dosages significantly (Student's paired t-test, two-tailed, $p>0.05$). There was no significant difference of baseline 3 between the different sessions (Student's paired t-test, two-tailed, $p>0.05$). Also, baseline 1 and baseline 3 did not differ significantly (Student's paired t-test, two-tailed, $p>0.05$) See Table 1.

The repeated-measures ANOVA resulted in significant main effects of drug dosage ($F(3,30) = 2.990$; $p = 0.047$) and PAS ($F(1,10) = 11.261$; $p = 0.007$). There was no significant effect of time though ($F(10,100) = 0.948$; $p = 0.426$). Two-way interactions of drug dosage X PAS ($F(3,30) = 12.182$; $p <0.001$) and PAS X time ($F(10,100) = 2.761$; $p = 0.005$) were significant. There was no significant interaction between drug dosage and time ($F(30,300) = 1.176$; $p = 0.247$). The three-way interaction of drug dosage X PAS X time was significant ($F(30,300) = 2.245$; $p<0.001$).

Dose-dependent effect of l-dopa on PAS-induced neuroplasticity

In the placebo medication condition, PAS-10 diminished excitability and PAS-25 enhanced it until at least 30 minutes following the stimulation. Under low dose (25mg) l-dopa, the excitability diminution induced by PAS-10 as well as the excitability enhancement induced by PAS-25 (Figure 2A) were diminished, that is, following both PAS-10 and PAS-25, there was no significant difference in the MEP amplitudes compared to baseline values. Furthermore, MEP amplitudes in the low dose condition differed significantly from those in the placebo condition at the initial time points after PAS-25 and at later time points after PAS-10. Following medium dose (100mg) l-dopa, the excitability changes induced by both PAS-10 and PAS-25 were preserved (Figure 2B). We observed that the excitability enhancement caused by PAS-25 was no longer significant between 60 and 120 min after PAS, but then recovered later and remained significantly enhanced until the afternoon of the next day after PAS, and thus for about 24h. More prominently enhanced and prolonged facilitation with 100 mg l-dopa was observed in a previous study by Kuo et al. (2008). The MEP amplitudes at identical time points did not differ significantly between the medium dose and placebo conditions. After intake of high dose (200mg) l-dopa, the excitability diminution induced by PAS-10 lasted longer compared to that under placebo medication, whereas the facilitatory after-effects of PAS-25 were converted into inhibition (Figure 2C). Such inhibition was significant compared to the baseline until 20 minutes following the stimulation. Differences between the MEP amplitudes in the high dose and placebo conditions were significant only for PAS-25.

DISCUSSION

The results of the present study reveal a non-linear effect of l-dopa dosage on associative plasticity in the human motor cortex. Whereas low dose l-dopa reduces or abolishes the after-

effects of both facilitatory as well as inhibitory PAS protocols, medium dosage of the drug prolongs PAS-25-generated facilitation and preserves inhibition resulting from PAS-10. In contrast, high dose l-dopa reverses PAS-25-induced facilitation into inhibition, and induces a trendwise prolongation of the inhibition induced by PAS-10.

These results are similar to those of the tDCS study (Monte-Silva et al., 2010) only for the low dose condition, but not for the medium and high doses. This is discussed in further detail below.

Proposed mechanisms of action

For the medium l-dopa medication, the results are in principal accordance with those of a former study with regard to the prolongation of the facilitatory after-effects of PAS. However, there are minor differences in the magnitude of the effects, most probably caused by interindividual variability due to the different groups of participants. The prolongation of facilitatory plasticity by l-dopa might be primarily caused by enhanced D1 receptor activation, because D2-like receptor block does not abolish this kind of plasticity (Nitsche et al., 2009). Specifically the NMDA receptor-enhancing function of moderate D1 activation (Seamans and Yang, 2004) is a likely candidate mechanism, since PAS is known to induce NMDA receptor-dependent plasticity (Stefan et al., 2002). In accordance to both, D1-, and D2-like activation (Monte-Silva et al., 2009; Nitsche et al., 2009), inhibitory plasticity was not enhanced or diminished by the medium l-dopa dosage. This does however not mean that dopamine does not affect inhibitory associative plasticity *per se*, as it was abolished by D2 receptor block (Nitsche et al., 2009). It is more likely that a balanced activation of both receptors is needed for this kind of plasticity.

The plasticity-diminishing or –abolishing effects of low dosage l-dopa medication is in accordance with a similar effect of low dosage ropinirole, a combined D2/D3 dopaminergic

agonist, on PAS-induced facilitatory plasticity (Monte-Silva et al., 2009). It is thus most probably caused by a minor activation of these receptors. The proposed mechanism of action is a preferential activation of presynaptic autoreceptors by low dose dopaminergic activation, which reduces dopamine release into the synaptic cleft (Schmitz et al., 2003; Yamada et al., 1980). This mechanism of action might also explain the plasticity-abolishing effect of low-dose l-dopa on inhibitory plasticity. However, this kind of plasticity was not affected by low dosage ropinirole. The reason for this might be that ropinirole as a dopaminergic agonist activates also postsynaptic D2 receptors independent from dopamine excretion, which is not the case for l-dopa. This minor activation of postsynaptic D2 receptors might have been sufficient to preserve inhibitory plasticity in case of low dose ropinirole application.

For high-dosage l-dopa, the conversion of the facilitatory after-effects of PAS-25 into inhibition is different to that of high-dose ropinirole, which abolished this kind of plasticity, when it was administered in high dosages, but did not convert it into inhibition. Thus the D1 receptor might have contributed to this effect. Here it is of importance that a high grade of D1 receptor activation in difference to low or medium D1 activation inhibits NMDA receptors (Seamans and Yang, 2004). Such an inhibition might reduce NMDA receptor activation to a level inducing LTD-like plasticity. The reason for this is that a low enhancement of intracellular calcium concentration induces LTD, while a larger enhancement generates LTP (Lisman, 2001), and NMDA receptor activity controls the amount of calcium influx. Alternatively it might be speculated that NMDA receptors are not inhibited by the level of dopaminergic activation accomplished by 200 mg l-dopa, but that a major enhancement of NMDA receptor activity will result in an intracellular calcium concentration sufficiently large to activate hyperpolarizing potassium channels (Misonou et al., 2004), which will convert facilitatory plasticity into inhibition. For the only trendwise effect of high-dosage l-dopa

medication on PAS-10-generated inhibition, this might hint for a larger range of dopaminergic activation compatible with inhibitory plasticity.

The results of the present study not only show some similarities with, but also differences from a recently conducted study, where the effects of identical l-dopa dosages on motor cortex plasticity induced by transcranial direct current stimulation (tDCS) were explored (Monte-Silva et al., 2010). tDCS induces plasticity by a tonic modulation of resting membrane potentials (Nitsche and Paulus, 2000; 2001; Nitsche et al., 2003; Nitsche et al., 2008). The after-effects of tDCS, like those of PAS, depend on NMDA receptor and calcium channel activity (Nitsche et al., 2003; 2004). However, in difference to PAS, plasticity induction by tDCS is thought not to be restricted to specific synaptic subgroups because of the relatively large electrodes which deliver the direct currents, and for the induction of after-effects, a tonic stimulation of some minutes is needed. For low dosage of l-dopa, also plasticity induced by tDCS was prevented. Since the induction of after-effects of tDCS, similar to those accomplished by PAS, need dopaminergic activity (Nitsche et al., 2006), this result is compatible with a primarily presynaptic effect of low dose l-dopa. However, for medium l-dopa medication, tDCS-induced facilitatory after-effects were converted into inhibition, thus mimicking the high-dosage l-dopa effects on PAS-25-induced plasticity. This pattern of results can be explained by a larger calcium increase induced by the less selective and tonic stimulation induced by tDCS, as compared to the more specific and phasic PAS stimulation procedure. Thus facilitatory tDCS in concert with medium l-dopa medication might have enhanced intracellular calcium sufficiently to activate hyperpolarizing potassium channels. For high-dose l-dopa medication, facilitatory as well as inhibitory plasticity were abolished, which could be explained by an NMDA receptor-inactivating effect of large D1 activation.

Taken together, a complex picture of dopaminergic effects on plasticity emerges. The results of the present and other studies suggest that the effect of l-dopa on plasticity depends on its dosage, sub-receptor specificity and the type of plasticity induced. Interestingly, a focusing effect of l-dopa on facilitatory plasticity as revealed by decreased non-focal tDCS – induced facilitation and preserved focal PAS – induced facilitation seems to be restricted to medium enhancement of dopaminergic activation.

General Remarks

In the present study, we aimed to explore the impact of l-dopa on focal associative plasticity as induced by PAS. Previous studies showed an improvement of cognitive performance in humans under 100mg (medium dose) l-dopa administration (Floel et al., 2005a; Knecht et al., 2004). However, some studies revealed heterogeneous effects of the drug on cognition (Cools et al., 2001; Gotham et al., 1988; Kulisevsky et al., 2000). Based on our results, it can be speculated that the non-uniformity of the effects of l-dopa on cognition might be partly explained by its non-linear dose-dependent effects on plasticity. This might be relevant because dopamine levels are altered in many neuropsychiatric conditions where cognitive performance is impaired (Floel et al., 2005b; Liepert et al., 2008). Alterations of plasticity in these patients might correlate with impaired cognition and restoring plasticity might probably help to regain cognitive performance. Some limitations of the present study should be mentioned. Since we did not measure the plasma levels of dopamine, we could not control for inter-individual variability caused by differences in the bioavailability of the drug. However, we would not expect a large interindividual variability, because we studied a fairly homogenous group of subjects. Blinding might have been somewhat compromised by different durations of the after-measures, but with the multitude of sessions (8 per subject) and blinded PAS protocols we do not think that an expectancy effect could have been relevant. We only studied young healthy subjects in our current study and it is important to be aware

that these results might not directly translate to elderly subjects or patient populations, in which the activity of the dopaminergic system might differ. Further studies are needed to explore the effects in these subject groups. Moreover, apart from other confounding factors like age (Floel et al., 2008), tobacco smoking (Lang et al., 2008), genetics (Cheeran et al., 2008), altered dopamine levels in patients should be borne in mind when brain stimulation protocols are applied for diagnostic or therapeutic purposes.

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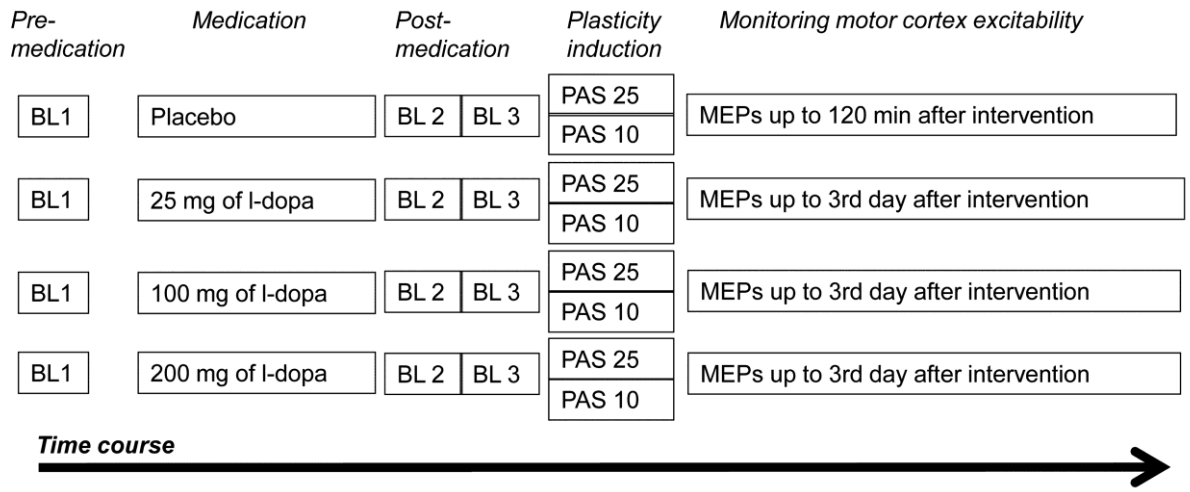
Table 1: Comparison of baseline TMS parameters for different l-dopa dosage conditions.

Drug dosage / Parameter	Placebo	25mg l-dopa	100mg l-dopa	200mg l-dopa
Baseline S1mV (% MSO)	47.64 ± 6.8	47.5 ± 6.8	47.68 ± 6.4	47.27 ± 6.8
S1mV after drug (% MSO)	47.82 ± 7.0	48.27 ± 7.3	48.64 ± 6.8	48.09 ± 6.7
Mean Baseline 1 (mV)	1.14 ± 0.09	1.14 ± 0.11	1.04 ± 0.08	1.09 ± 0.08
Mean Baseline 2 (mV)	1.08 ± 0.17	1.01 ± 0.35	1.01 ± 0.19	1.02 ± 0.22
Mean Baseline 3 (mV)	1.09 ± 0.10	1.14 ± 0.14	1.10 ± 0.11	1.15 ± 0.13

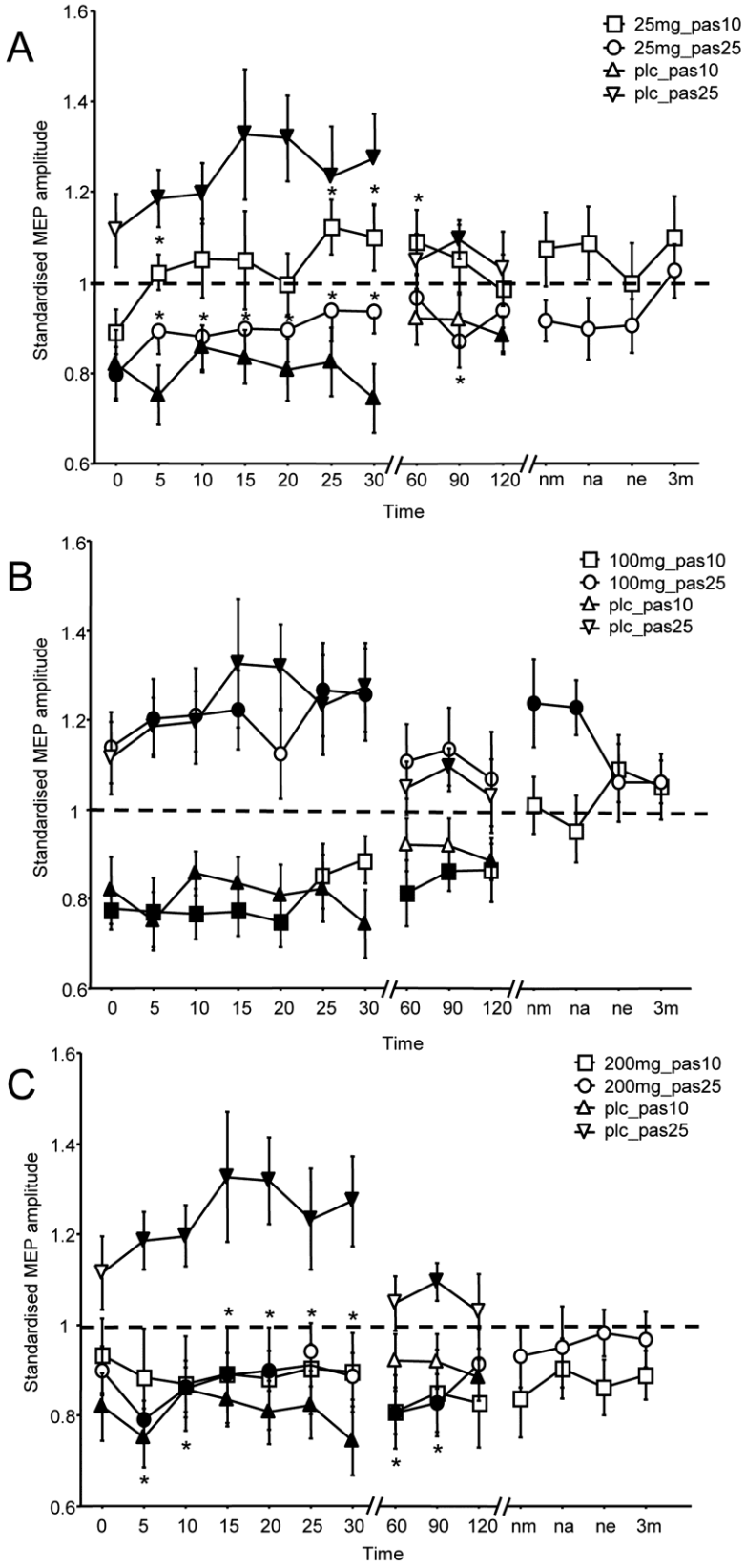
Shown are mean ± SD values of the baseline TMS parameters for the different l-dopa dosage conditions. There was no significant difference between the parameters across the different conditions (Student's t-test, paired, two-tailed, $p \leq 0.05$). MSO = maximum stimulator output.

Figure 1: Course of the experiment



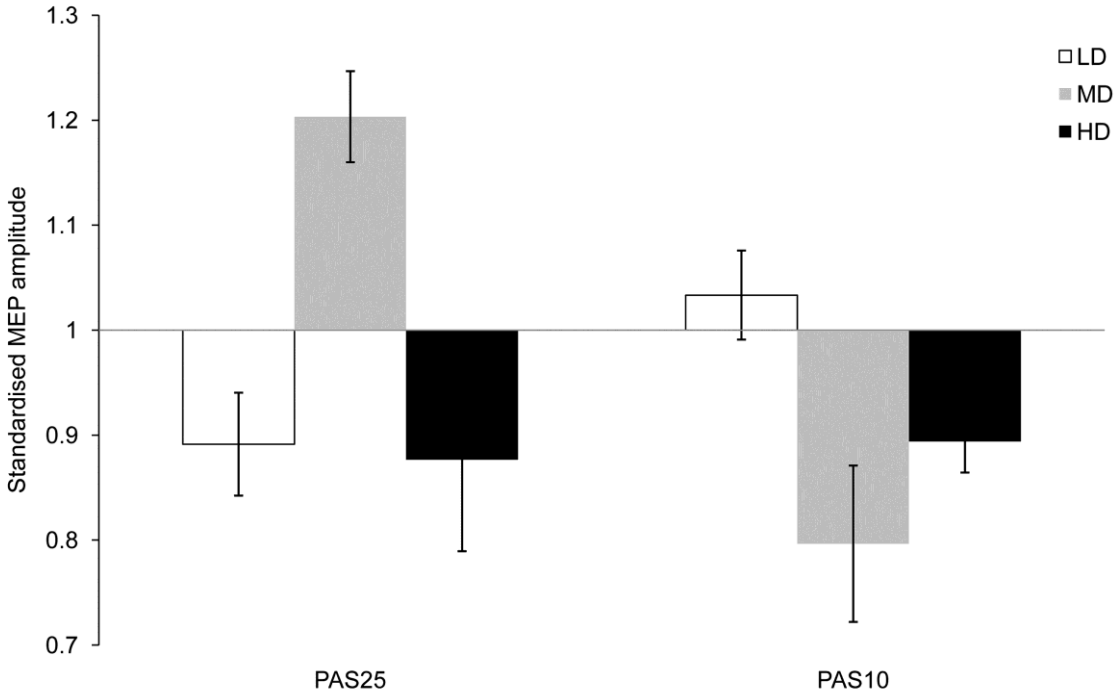
Motor evoked potentials (MEPs) elicited from single pulse transcranial magnetic stimulation (TMS) over the motor hotspot were recorded at 1mV intensity prior to drug intake (baseline 1- BL1). One hour after drug intake, baseline 2 (BL2) was recorded to look for an effect of the drug on cortical excitability. In case of any individual MEP alterations from BL1, baseline 3 (BL3) was recorded by adjusting the stimulator output in order to obtain a mean of 1mV. Then PAS-10 or PAS-25 was administered which was immediately followed by MEP after-measurements that lasted until 120 minutes. For all sessions except the placebo medication sessions, after-measurements were carried out until the third day morning following the stimulation.

Figure 2: Dose-dependent effect of l-dopa on PAS-induced neuroplasticity



The horizontal axis displays the time points (in minutes) of after-measurements during the experiment. Motor evoked potential (MEP) amplitudes standardized to the corresponding baseline values (mean ± SEM) are plotted on the vertical axis. The graphs show that under placebo medication facilitatory PAS-25 induces an excitability enhancement lasting for at least 30 minutes whereas PAS-10 diminishes excitability for a similar duration following the stimulation. **A)** Shown is the effect of 25mg (low dose) l-dopa on the PAS-induced after-effects. Low dose l-dopa reduces or abolishes the after-effects of both PAS-25 and PAS-10. **B)** shows that 100mg (medium dose) l-dopa prolongs facilitatory PAS-induced plasticity, but does not alter the excitability-reducing after-effects of PAS-10. **C)** 200mg (high dose) l-dopa reverses the facilitation induced by PAS-25 to inhibition while trendwise prolonging the inhibitory effect of PAS-10. Filled symbols indicate statistically significant deviation of the post-PAS values compared to the baseline. Asterisks indicate significantly different values in the l-dopa condition compared to the placebo medication conditions at the same time points after the respective PAS protocols (Student's t-test, paired, two-tailed, $p \leq 0.05$). nm = next morning; na = next afternoon; ne = next evening; 3m = third day morning.

Figure 3: Dose-dependent effect of l-dopa on cortical excitability of the human motor cortex until 30 minutes following PAS



Shown is the change of the standardized mean MEP amplitudes pooled for time points until 30 minutes following PAS. Both PAS-25 and PAS-10 show maximum excitability enhancement and diminution respectively following medium dose (100mg) l-dopa. Thus, optimal levels of PAS-induced after-effects are observed with 100 mg l-dopa. LD = low dose; MD = medium dose; HD = high dose.

Chapter 3 Discussion

3.1 Summary of findings

In the aforementioned studies, we explored specific aspects of the cholinergic and dopaminergic impact on stimulation-induced plasticity in healthy humans. The results show (a) a prominent impact of the nicotinic subreceptor on plasticity, and (b) a dose-dependent effect of dopaminergic activation on associative plasticity.

The first study on nicotinergic modulation of plasticity shows clearly that nicotine exposure in non-smoking healthy individuals has a focusing effect on facilitatory plasticity. It abolished non-focal faciltitatory plasticity induced anodal tDCS, but prolonged PAS-induced facilitation. Nicotine exposure, on the other hand, abolished both kinds of inhibitory plasticity that were explored in the current study, i.e. focal and non-focal plasticity. Thus, although the nicotinergic impact on facilitatory plasticity resembles that of non-specific cholinergic activation, its effect on inhibitory plasticity differs clearly. These effects of nicotine on plasticity might contribute to the effect of the substance on cognition and substance addiction. However, direct evidence for this association has not been obtained so far and should be explored in future studies.

In the second study we explored the dose-dependency of dopaminergic activation on associative neuroplasticity in healthy humans. The results showed that with low dose l-dopa, both focal facilitatory and inhibitory plasticity are abolished. With medium dose l-dopa focal facilitatory and inhibitory plasticity were preserved/prolonged. Administration of high dose l-dopa reversed the focal facilitatory after-effects to inhibition while prolonging inhibitory plasticity. Thus the results of this study show a clear dose-dependent non-linear effect of dopamine on associative plasticity. Interestingly, especially the effects of medium dosed l-

dopa on PAS differ clearly from those obtained by tDCS, favoring a focusing effect of this dosage with regard to facilitatory plasticity. Future studies have to show if these effects on plasticity correlate with learning and memory performance, and thus might explain the partially seemingly contradictory effects of l-dopa on these functions.

3.2 Conclusions

From the results of these studies, we conclude that –

(i) Neuromodulators clearly influence plasticity induced by non-invasive brain stimulation protocols.

(ii) Subreceptor specificity and concentration levels of neuromodulators are important factors that determine the impact of neuromodulators on plasticity.

(iii) Nicotinergic impact on facilitatory neuroplasticity is similar to the impact of non-specific cholinergic activation, that is, both show a focusing effect with regard to facilitatory plasticity.

(iv) Nicotinergic activation abolishes all kinds of inhibitory plasticity, different from the effect of non-specific cholinergic activation.

(v) L-dopa only at medium dosage (100mg) produces a focusing effect on facilitatory plasticity.

(vi) Future studies have to show if these effects of nicotinic and dopaminergic activation correlate with the impact of the respective substances on learning and memory formation.

(vii) Interactions of drug- or disease-related alterations of neuromodulator activity with the after-effects of brain stimulation protocols should be considered carefully before applying

plasticity-altering protocols to neuropsychiatric patients for therapeutic purposes, because the effects of brain stimulation might critically depend on neuromodulator level.

3.3 Future prospects

Our study exploring the impact of nicotinic receptor activation on neuroplasticity in humans gives clear evidence for the relevance of this receptor with regard to this mechanism. Future studies should explore the mechanisms of action of these effects in larger detail, e.g. by pharmacological interventions, especially exploring the contribution of nicotinic subreceptors and calcium channels, as well as using TMS to probe the impact of nicotine on excitatory and inhibitory cortical systems, e.g. by specific TMS protocols. Furthermore it will be interesting to explore a dosage-dependency of nicotinic receptor activation on plasticity, which might be non-linear, similar to the effects of dopaminergic activation. Although more is known about the impact of dopamine on plasticity in humans, especially with regard to subreceptors and dosage-dependency, also here we need to explore the mechanisms of action into larger detail, as mentioned above. Beyond experiments in humans, cellular, slice and in vivo animal experiments will help to understand the mechanisms of action of both neuromodulators on plasticity into larger detail, because they enable the exploration of biological effects on a level not accessible for experiments in humans.

Both, dopamine and nicotine, have been shown to alter not only neuroplasticity, but also learning and memory formation. Since neuroplasticity is thought to be an important neurophysiological basis for cognition, it is attractive to speculate that alterations of plasticity induced by these substances are the basis for the respective cognitive effects. Such a causal relationship has not been established at present, but will be an important aspect of future research.

Pathological alterations of neuromodulators are thought to be an important feature of various neuropsychiatric diseases, e.g. Alzheimer´s disease, Lewy body dementia, Schizophrenia, and others. These might affect neuroplasticity in specific ways associated with clinical symptoms, e.g. loss of memory and learning abilities in Alzheimers disease caused by reduced plasticity due to decline of the cholinergic system. It will be interesting to explore in future studies if therapeutic medication in these patients improves symptoms at least partly due to a re-establishment of plasticity. If this would be the case, plasticity induction via non-invasive brain stimulation might turn out as a biomarker for the clinical efficacy of the respective substances.

Taken together, we are still at the beginning of our understanding of the neurophysiological and functional effects of neuromodulators in the human central nervous system. However, the central involvement of these substances in various brain functions in health and disease makes studies in this area important and promising for both, improving our understanding of brain function, but also in developing new therapeutic strategies to treat patients suffering from diseases involving pathological alterations of neuromodulatory activity.

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Thirugnanasambandam N, Grundey J, Adam K, Drees A, Skwirba AC, Lang N, Paulus W, Nitsche MA. *Nicotinergic impact on focal and non-focal plasticity induced by non-invasive brain stimulation in non-smoking humans.* (accepted, Neuropsychopharmacology).

Fricke C, Seeber AA, **Thirugnanasambandam N**, Nitsche MA, Paulus W, Rothwell JC. *Time course of the induction of homeostatic plasticity generated by repeated transcranial direct current stimulation (tDCS) of the human motor cortex* (Under revision – J Neurophysiol.)

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Thirugnanasambandam N, Sparing R, Dafotakis M, Meister IG, Paulus W, Nitsche MA, and Fink GR. *Isometric contraction interferes with transcranial direct current stimulation (tDCS) induced plasticity – evidence of state-dependent neuromodulation in human motor cortex.* (Submitted)

Grundey J, **Thirugnanasambandam N**, Adam K, Drees A, Skwirba AC, Lang N, Paulus W, Nitsche MA. *Nicotinergic impact on focal and non-focal plasticity induced by non-invasive brain stimulation in smokers.* (manuscript under preparation)

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