# THE EFFECT OF PAIRED ASSOCIATIVE STIMULATION-INDUCED LONG-TERM POTENTIATION-LIKE PLASTICITY ON VISUOMOTOR LEARNING

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# ABSTRACT

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Brain adapts in response to motor learning by changing neuroanatomical and functional properties. One proposed model for these changes is long lasting enhancement of synaptic strength called long-term potentiation (LTP). LTP-like changes in motor cortex are possible to induce artificially with paired associative stimulation (PAS). Further, evidence exist that PAS can enhance specific types of motor learning. The aim of this research was to study the effect of PAS-induced LTP-like plasticity on early acquisition phase of brief visuomotor learning task.

A motor learning task was conducted three times following PAS. The learning performance was calculated as a magnitude of average error throughout the task. Transcranial magnetic stimulation was applied before (PRE) PAS and after each learning task to measure motor-evoked potentials (MEP) from soleus muscle. In addition, electrical nerve stimulation was used to measure the Hoffman reflex (h-reflex) and maximal compound muscle action potential (Mmax). The subjects were divided into three different groups: control (CON), responders (RES, PAS induced MEP facilitation) and non-responders (NON, PAS did not increase MEP size). All groups improved their performance between learning task 1 and 3 (L1 and L3). Only the RES group improved between L2 and L3. The improvement was significant compared with CON and almost significant with NON. MEP amplitudes following PAS increased in RES group; 138.0 ± 98.6 % (POST1), 227.8 ± 130.2 % (POST2, $p < 0.022$) and 228.4 ± 152.9 % (POST3, $p < 0.012$). In the NON group MEP amplitudes were 83.0 ± 35.2 % (POST1) 70.7 ± 21.7 % (POST2, $p < 0.020$) and 72.0 ± 15.5 % (POST3). There were no changes in CON compared with PRE measurement. Both the h-reflex and Mmax did not change significantly between conditions.

Thus, the learning performance did not differ between the groups when compared the total amount of learning (between L1 and L3). However, at the end of the learning task as the learning proceeded, PAS seems to have an beneficial impact on visuomotor learning task.

# TIIVISTELMÄ

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Aivojen hermostollisissa rakenteissa ja toiminnassa tapahtuu adaptaatiota motorisen oppimisen seurauksena. Eräs ehdotetuista malleista, johon muutokset voivat perustua on hermoliitoksen voimakkuuden pitkäaikainen tehostuminen eli pitkäkestoinen potentiaatio (LTP). Keinotekoisesti LTP:n kaltainen vaikutus voidaan tuottaa parillisella assosiatiivisella stimulaatiolla (PAS). Lisäksi tutkimustulokset osoittavat, että PAS voi parantaa tietyn tyyppistä motorista oppimista. Tämän tutkimuksen tarkoituksena on selvittää miten PAS:n tuottama LTP:n kaltainen plastisuus vaikuttaa visuomotorisen oppimisen alkuvaiheeseen.
Visuomotorinen oppimistehtävä suoritettiin kolme kertaa PAS:n jälkeen. Oppimistehtävä oli tarkkuustehtävä, jossa koehenkilön piti seurata esimerkkivoimakäyrää mahdollisimman tarkasti tuottamalla voima plantaarifleksiolla. Transkraniaalisella magneettisella stimulaatiolla tuotettiin motorisia herätepotentiaaleja (MEP) ennen PAS:ta ja jokaisen oppimistehtävän jälkeen. Lisäksi hermon sähköistästimulaatiota käytettiin Hoffman refleksin (h-reflex) ja maksimaalisen m-aallon mittaamiseen (Mmax). Koehenkilöt jaettiin kolmeen ryhmään: kontrolli (CON), vastaajat (RES; PAS tuotti MEP:n fasilitoitumista) ja ei-vastaajat (NON, PAS ei kasvattanut MEP:n kokoa).
Kaikki ryhmät paransivat suoritustaan oppimistehtävien 1 ja 3 välillä (L1 ja L3). Vain RES-ryhmä paransi suoritustaan välillä L2 ja L3. Suorituksen parantuminen oli merkitsevä verrattuna CON-ryhmään ja lähes merkitsevä verrattuna NON-ryhmään. MEP:n amplitudit PAS:n jälkeen olivat seuraavat: RES 138,0 ± 98,6 % (POST1), 227,8 ± 130,2 % (POST2, $p < 0,022$) ja 228,4 ± 152,9 % (POST3, $p < 0,012$), NON 83,0 ± 35,2 % (POST1) 70,7 ± 21,7 % (POST2, $p < 0,020$) and 72.0 ± 15,5 % (POST3). MEP:n koossa ei tapahtunut muutoksia CON-ryhmässä. Myöskään h-refleksissä tai Mmax:ssa ei tapahtunut merkitseviä muutoksia eri mittausajankohtien välillä.
Kaikissa kolmessa ryhmässä suoritus parani saman verran välillä L1 ja L3. Kuitenkin oppimistehtävän lopussa PAS:lla näyttää olevan suotuisa vaikutus visuomotoriseen oppimistehtävään.

# CONTENT

# ABBREVIATIONS

| | |
|---|---|
| AMPA | α-amino-3-hydroxyl-5methyl-4-isoxazolepropionic acid |
| BDNF | brain-derived neurotrophic factor |
| C | carboxyl |
| CaM | calmodulin |
| EEG | electroencephalography |
| EMG | electromyography |
| GABA | gamma-Aminobutyric acid |
| H-reflex | the Hoffmann reflex |
| ICF | intracortical facilitation |
| ISI | interstimulus interval |
| LICI | long-interval intracortical inhibition |
| LTD | long-term depression |
| LTP | long-term potentiation |
| M1 | primary motor cortex |
| MEP | motor-evoked potential |
| Mmax | maximal compound muscle action potential |
| MT | motor threshold |
| MVC | maximal voluntary contraction |
| N | amino |
| NMDA | N-Methyl-D-aspartic acid |
| PAS | paired associative stimulation |
| $PAS_{LTD}$ | depressive PAS |
| $PAS_{LTP}$ | facilitatory PAS |
| RMT | resting motor threshold |
| SD | standard deviation |
| SEP | somatosensory-evoked potential |
| SICF | short-interval intracortical facilitation |
| SICI | short-interval intracortical inhibition |
| tDCS | direct current stimulation |
| TMS | transcranial magnetic stimulation |

# 1 INTRODUCTION

Life is a continuous learning process. Every day we face challenges that demand motor learning. Our daily life challenges that require learning of new motor skills are for example: riding a bike, dancing, playing an instrument, playing games or driving a car. Many of these activities require combination of sensory feedback and movement. One example is while driving a car, the driver needs to adjust the speed of the car to maintain the car position. Thus, the driver has to push the accelerator according to visual feedback. Also, certain diseases or accident could impair motor functions and the patients have to relearn the skills of everyday living.

Non-invasive brain stimulations have become more and more popular recently in both clinical and research use. Several studies demonstrate that non-invasive brain stimulation are able to modulate brain plasticity and enhance motor learning (e.g. Boggio et al. 2006; Hamada et al. 2014; Jung & Ziemann 2009). One type of non-invasive brain stimulation is paired associative stimulation (PAS). PAS combines a peripheral neural stimulation with transcranial magnetic stimulation (TMS). It is assumed that PAS is able to modify synaptic strength and thus, modulate brain plasticity. (Stefan et al. 2000.)

The aim of the present study was to investigate the effect of PAS on visuomotor learning. The PAS induces long-term potentiation (LTP) -like plasticity in primary motor cortex (M1). The LTP-like plasticity induced by non-invasive brain stimulation has been shown to either interfere (Cantarero et al. 2013b) or enhance (Jung & Ziemann 2009) motor learning. The focus of the present study will be on the early acquisition phase of visuomotor tracking type of learning. In addition, most of the studies have focused on upper limb muscles and in the present study the focus will be on lower limb muscles.

# 2 MOTOR LEARNING

## 2.1 Brain Plasticity

Humans learn new skills and adapt their behavior throughout life. In response the human brain needs to be adaptable. The brain's capacity to change and to adapt is termed brain plasticity. (Thickbroom 2007.) Brain also adapts in response to motor learning processes by changing the neuroanatomical structure or functional recruitment patterns (Bezzola et al. 2012). The adult brain can experience reorganization as a result of learning. A simple repetitive motor activity however, does not induce reorganization (representational map plasticity). Instead, skill acquisition, learning or practice of a novel motor task does induce map plasticity. Thus, specific patterns of motor activity are required to produce functional motor cortex plasticity. Mechanisms for plasticity include uncovering latent or existing connections, activation of silent synapses, activity-dependent synaptic plasticity, or excitability changes in postsynaptic neurons. (Classen et al. 1998; Rioult-Pedotti& Donoghue 2003.) More specifically above mentioned mechanisms can occur due to modulation of synaptic transmission, co-operative changes in networks of neurons, neurotransmitter and ionic regulation, changes in the properties of individual neurons, nonsynaptic electrical communication, extra-neuronal effects or morphological and anatomical modifications. These mechanisms can occur simultaneously or in some serial order. (Thickbroom 2007.)

*Long-term potentiation (LTP) and long-term depression (LTD).* Plasticity of the synaptic transmission is not a recent finding. It was suggested already in the 40s that " When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A’s efficiency, as one of the cells firing B, is increased". This interaction is termed Hebb's law. (Hebb, 1949). This was later demonstrated in rabbits' hippocampus, in which high-frequency stimulation caused an increase in the efficacy of synaptic transmission and thus long-term potentiation (LTP) (Bliss &Lømo, 1973). Thus, LTP is long lasting enhancement of synaptic strength and proposed to be a model for learning and memory processes. On the contrary, LTD is a depression of synaptic strength and

also proposed to be involved in learning. (Dan & Poo, 2006; Iriki et al. 1989.) Properties of LTP include specificity (only the activated synapses show LTP), cooperativity (intensity threshold for induction) and associativity (weak input can be potentiated if activated concurrently with another synapse on the same cell) (Bliss & Collingridge 1993).

An important factor determining if LTP or LTD is produced is the temporal correlation between presynaptic input and postsynaptic spikes. A potentiation is traditionally induced when the postsynaptic spikes peak within 20 ms after presynaptic input and depression occurs when postsynaptic spikes are induced up to 20–100 ms before presynaptic input. There is a narrow transition between LTP and LTD that is ~ 1–5 ms. In addition, induction of plasticity requires multiple pairs of pre- and postsynaptic spiking. (Bi & Poo 1998; Feldman 2012.) Recently, a new temporal window for LTD induction was reported. By using the PAS-method the depression occurred when presynaptic spikes peaked 250 and 450 ms after postsynaptic spiking (Schabrun et al. 2013). However, the temporal rules for LTP and LTD induction are not fixed. The site of dendritic interaction with the afferent input determines the direction of plastic changes and interestingly different neuronal populations and activity state of the cortex also seem to have an effect on the directions of plastic changes induced with PAS. (Koch et al. 2013; Sjöström & Häusser 2006.) The temporal order is only one of the several factors that influences the induction of LTP and LTD. The other factors include synaptic cooperativity (Sjöström et al. 2001), firing rate (Sjöström et al. 2001), postsynaptic voltage (Markram et al. 1997), neuromodulators (Pawlak & Kerr 2008), gamma-Aminobutyricacid (GABA) ergic inhibition (De Beaumont et al. 2012; Elahi et al. 2012) and baseline synaptic weight (Bi & Poo 1998). The importance of each component varies across specific dendritic zones and activity regimens. (Feldman 2012.)

Both the LTP and the LTD are prevalent at excitatory synapses, however they can also occur in inhibitory synapses (Kullmann et al. 2012; Sjöström et al. 2001). Different forms of LTP and LTD exist and they are influenced by a large amount of factors, and in spite of the large body of research there is some uncertainty considering the detailed mechanisms underlying the LTP and LTD. However, numerous factors that mediate or modulate LTP and LTD have been uncovered. (Malenka & Bear 2004.) It is well recognized that distinct forms of LTP occur depending on the cell type, age or other experi-

mental conditions. If these factors are kept constant distinct forms of LTP still exist. Hippocampal LTP is the most widely studied type of LTP. The hippocampal LTP is divided into three discrete groups. Further, it has been observed that different stimulation protocols induce distinct forms of LTP. It appears that these distinct types of LTP affect differently on the phases of learning. However, it remains to be elucidated if different stimulation protocols induce distinct forms of LTP also in motor cortex. (Raymond 2007.)

Pharmacological studies have shown that PAS-induced timing dependent LTP and also LTD are N-Methyl-D-aspartic acid (NMDA) receptor-dependent (Stefan et al. 2002; Wolters et al. 2003). A calcium influx into postsynaptic spine is a trigger for LTP induction, and in NMDA receptor-dependent LTP the postsynaptic increase in calcium is mediated through activation of the NMDA receptor. Both presynaptic and postsynaptic activation are needed for LTP induction. This characteristic of LTP may be explained by the properties of NMDA receptor. In which presynaptic activity (glutamate ligation) and postsynaptic depolarization (release of voltage-gated magnesium block which allows calcium influx) are required. Thus, when sufficiently strong stimulus arrives to synapse, glutamate is released and it binds to NMDA and α-amino-3-hydroxyl-5methyl-4-isoxazolepropionic acid (AMPA) receptors. As a consequence the AMPA receptor generates excitatory post-synaptic potential releasing magnesium block from the NMDA receptor. This enables influx of large amounts of $N^{+}$ and $Ca^{2+}$ ions. (Feldman 2012; Malenka & Bear 2004; Thickbroom 2007) The influx of calcium is mediated by calmodulin (CaM). Calcium binds to CaM, which has two calcium binding lobes, a carboxyl (C) lobe and an amino (N) lobe. If there is a rapid increase in the postsynaptic calcium level, the C lobe binding occurs and on the contrary a slow increase in the calcium concentration induces calcium binding to N lobe. As a result of rapid increase in postsynaptic calcium level and thus C lobe binding, the number of AMPA receptors is increased. This means that stimulus of same strength will induce a stronger response and the synapse is "enhanced". On the contrary, Calcium binding to N lobe will decrease the number of AMPA receptors and permeability, which means that the synapse is said to be "depressed". Thus, a single messenger is responsible for triggering both LTP and LTD (Fig 1.). (Feldman 2012.)

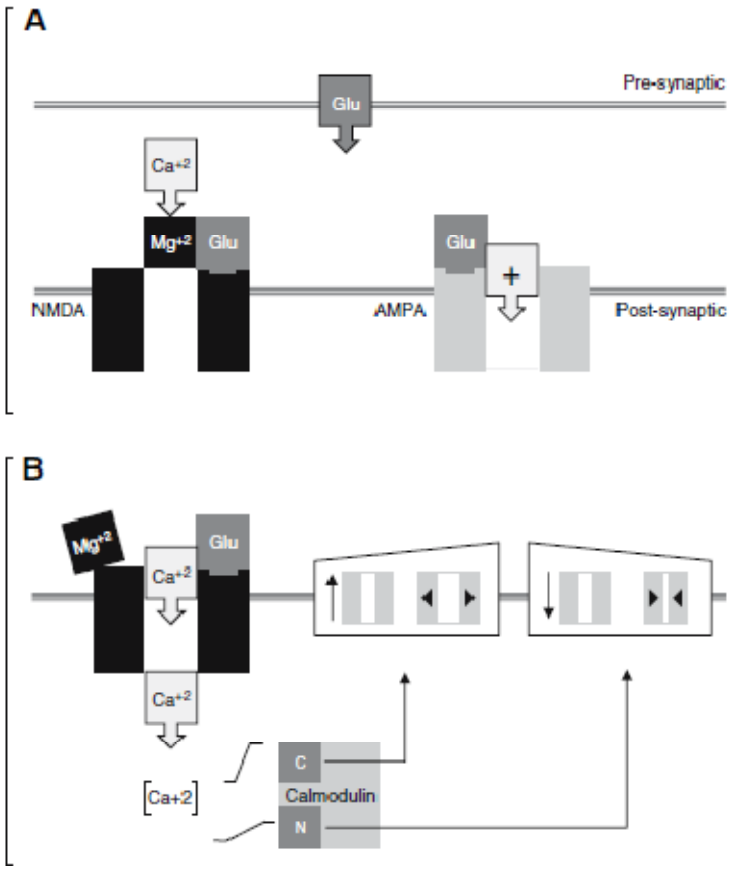



Figure 1. A model of NMDA receptor-dependent LTP and LTD. A.) A presynaptic glutamate binds to NMDA and AMPA receptors and opens channels in both receptors. However, the NMDA receptor stays blocked by magnesium. B.) Sufficiently strong stimulus releases magnesium block allowing calcium influx. Calcium binding to either C lobe or N lobe will cause changes in AMPA receptors. Whether the LTP or the LTD is induced depends of these AMPA receptor modulations. (Thickbroom 2007.)

## 2.2 Involvement of primary motor cortex (M1) in motor learning

The primary motor cortex (M1) is a part of cerebral cortex. It is a controller for different voluntary movements and it sends commands directly or indirectly (via interneurons) to motoneurons. More specifically, pyramidal cells of cortical layer V located in the M1 send their axons to spinal cord, where they can activate target motoneurons directly. These projections are called corticospinal neurons (corticospinal tract). In addition, these projections also activate interneurons in spinal cord and brain stem (indirect acti-

vation). These indirect pathways are rubrospinal, vestibulospinal and reticulospinal tracks. Corticospinal and rubrospinal tracks have somewhat overlapping functions in control of muscles and movements. Vestibulospinal and reticulospinal tracks have a function in posture regulation, mediating commands to initiate locomotion and perturbation corrections. Other brain areas such as basal ganglia, thalamus and cerebellum are also involved in motor control. (Grillner 2008.) Besides of its role in movement control, the M1 has been proposed to participate in motor learning. M1 has the intrinsic circuitry to enable reorganization and thus plasticity. The candidate substrate for plasticity in M1 is the extensive horizontal pathways that span the superficial layers II and III and layer V. However, to induce long-term persistent changes in horizontal connections, stable form of synaptic modification is required. LTP and LTD, which have also been demonstrated in M1, are mechanisms that can provide long-term synaptic modulation. The synaptic strength of the horizontal connections in M1 can be enhanced or depressed through activity-sensitive mechanisms. These modifications occur as a consequence of appropriate activity patterns. (Sanes& Donoghue 2000.)

The definition of motor learning is not unanimous and several different types of motor learning have been proposed to exist (Hardwick et al. 2013; Richardson et al. 2006; Sanes & Donoghue 2000; Seidler et al. 2012). For example, motor learning can be considered as "acquisition of new spatiotemporal muscle-activation patterns" or "combining of individually known movements to form a novel movement sequence" (Sanes & Donoghue 2000). Various types of motor learning tasks have reported to influence the M1. Both animal and human experiments have reported of learned modifications to representational maps of the M1. Single-cell activity from the M1 of two monkeys was recorded during perturbed reaching movement and it was found that cells changed their tuning properties during learning (Gandolfo et al. 2000). In humans a learning task involving rapid sequences of finger tapping showed changes of representational map of the M1 (Karni et al. 1995). Other types of motor learning tasks that have demonstrated involvement of M1 in motor learning include e.g.: a visuomotor gain adaptation task (Hamada et al. 2014), a skilled sequential reaching movement to visual target (Matsuzaka et al. 2007), a precise grip of an object (Nudo et al. 1996) and practicing already learned movements (Classen et al. 1998).

It is somewhat controversial to which extent the M1 participates in motor learning and how important the role of the M1 is at different types of motor learning. Several studies suggest that M1 has a role in acquisition of a novel motor skill (e.g. Hluštik et al. 2004; Hosp et al. 2011; Karni et al. 1995; Nudo et al. 1996), in addition it has been suggested that M1 also has a role in retention of the motor skill (e.g. Cantarero et al. 2013a; Karni et al. 1995; Muellbacher et al. 2002). Furthermore, after cortical lesions the M1 seem to have an important role in the recovery of previously acquired skills (Shmuelof & Krakauer 2011). On the contrary, some studies suggest that M1 does not have role in early phase of the motor skill adaptation (Cherian et al. 2013; Paz et al. 2005). Gandolfo et al. (2000) found variety of neuron types in monkey's motor cortex. Some of the neurons appear to have "dynamic" properties and some "memory properties". The neurons with "memory" properties might provide a necessary substrate for skill retention, 40% of responsive cells were classified as "memory cells". Whereas, about 16% were "dynamic cells", which may have a role in adaptation, but probably not in skill retention. This may explain some discrepancy in the results concerning the role of M1 in motor learning.

A learning of sequential motor behavior consists of combining of already known movements into new spatiotemporal sequences. It appears that M1 is involved in either acquisition or read-out of knowledge about movement sequences in motor-sequence learning. However, it seems that M1 does not have a key role in actual learning of sequential motor behavior. A sensory-motor association learning contains a previously known movement and sensory clue, but the relationship between the two stimuli is novel at the beginning of the task. The role of the M1 in sensory-motor association learning is contradictory; it may be that M1 participates in adjustments needed during repetition of actions rather than formation of associations between stimuli. (Sanes& Donoghue 2000.) A meta-analysis of motor learning imaging studies showed that during both sensory-motor association learning and motor-sequence learning, the M1 and the dorsal premotor cortex were consistently activated. However, the involvement of the M1 was suggested to occur mostly at the level of movement execution. In spite of this, the authors conclude that M1 may have involvement in motor learning through use-dependent mechanism. (Hardwick et al. 2013.) Thus, the role of M1 in motor learning is complex and it seems to vary between tasks.

## 2.3 A contribution of brain plasticity to motor learning

As seen above and in next chapter many studies demonstrate that brain plasticity is involved in motor learning. Martin et al. (2000) claim that to ensure the involvement of plasticity in learning, several criteria need to be confirmed. First criterion is *correlation*: "Is the expression of properties of LTP correlated with characteristics of learning?". Evidence exist that this criteria is fulfilled in the M1: Nudo et al. (1996) demonstrated that motor learning induces reorganization on representation maps in the M1. Further, significant correlation has been demonstrated between PAS-induced LTP-like plasticity and motor skill acquisition (Frantseva et al. 2008). Second criterion is *occlusion*: "Does saturation of LTP or LTD prevent the retrieval of old information of encoding of new memory traces" (Martin et al. 2000). It has been shown that motor learning impaired the subsequent LTP induction (Rioult-Pedotti et al. 2000). In addition, Ziemann et al. (2004) demonstrated that learning of a rapid thumb movement prevented subsequent PAS-induced LTP-like plasticity in M1, but enhanced LTD-like plasticity. Third criterion is *intervention*: "Does blockade or enhancement of the neural mechanisms responsible for LTP and LTD have commensurate effects on learning" (Martin et al. 2000). NMDA receptor-dependent LTP has been suggested to occur in M1. It was shown that NMDA receptor antagonist blocks training induced changes in humans (Bütefisch et al. 2000). Indirect evidence shows that induction of PAS before motor training enhanced performance (Jung & Ziemann 2009). Fourth criterion is *erasure*: "Does the reversal of LTP cause forgetting" (Martin et al. 2000). Muellbacher et al. (2002) showed that low-frequency repetitive stimulation of M1 disrupted the retention of the motor behavior improvements. Fifth criterion is *induction*: "is M1-dependent learning associated with the induction of LTP?" (Martin et al. 2000). This means that synaptic changes must occur during learning. Evidence for this in M1 has provided studies with primates. A stable reorganization of motor map was produced with artificial stimulation (Jackson et al. 2006). Thus, all the criteria to ensure the relationship between plasticity of the M1 and the motor learning are at least indirectly demonstrated. However, as stated above there are various types of motor learning, which may involve different systems. It may be that all five criteria mentioned above may not apply to every motor learning paradigm.

# 3 PAIRED ASSOCIATIVE STIMULATION (PAS)

Candidate mechanisms for learning are lasting changes in synaptic efficacy such as LTP and LTD. (Rioult-Pedotti et al. 2000.) LTP and LTD has been generated by pairing two stimuli. As mentioned earlier, the LTP is usually induced when presynaptic input occurs before postsynaptic cell fires. On the contrary, LTD is usually produced when the post synaptic spiking occurs before presynaptic activity. (Froemke et al. 2010.) Both LTP and LTD can be induced in human brain with paired associative stimulation (PAS). In which electrical stimulation of peripheral nerve is paired with transcranial magnetic stimulation (TMS). (Stefan et al. 2002.) PAS-induced plasticity develops rapidly (< 30 min) and is long lasting (> 60 min), yet reversible (Stefan et al. 2000).

## 3.1 Overview of the method

The paired associative stimulation is a method to induce plasticity in the human motor cortex (Stefan et al. 2000). With the method it is possible to induce either potentiation or depression of synaptic efficacy (LTP and LTD). As mentioned earlier, these are suggested to be cellular mechanisms underlying learning. (Rioult-Pedotti et al. 2000; Stefan et al. 2002; Wolters et al. 2003.) In the PAS method two consecutive stimuli are applied. An electrical stimulus is applied over peripheral nerve and TMS is applied over motor cortex (fig. 2). (Stefan et al. 2000.) TMS activates a variety of axons belonging to different populations of neurons. Some of the axons are local to the area of cortex under the coil, others project to or from the site of stimulation; some are excitatory, others inhibitory. (Rothwell 2011.) Further, TMS produces descending volleys in corticospinal neurons and the response can be measured as a motor-evoked potential (MEP) from EMG signal of a target muscle (Rossi et al. 2009). Peripheral nerve stimulation evokes signals that arrive at the somatosensory cortex and further reaches the motor cortex in a highly somatotopically organized fashion. Thus, afferent signals can modulate the motor cortical processes. (Classen et al. 2000.).

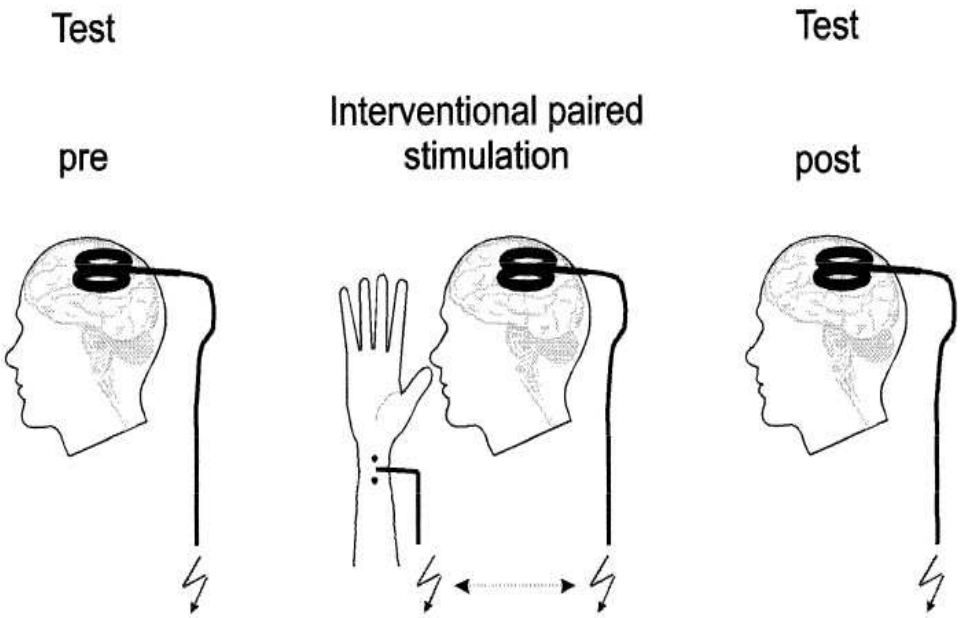



Figure 2.Modified from Stefan et al. (2000). Experimental design of the PAS protocol. The corticospinal excitability is measured with TMS before the induction of PAS for the baseline measurements. To measure the extend of PAS-induced plasticity the same measure is repeated after PAS. In PAS the electrical stimulation of nerve is followed by TMS.

The timing between the peripheral and the cortical stimuli is important factor in determining if the PAS intervention will induce potentiation or depression. If the afferent signal evoked by peripheral nerve stimulation reaches the motor cortex before TMS, PAS intervention usually produces a potentiation in the corticospinal excitability. On the contrary, a depression is often produced if the TMS is induced before the afferent signal reaches the motor cortex. (Wolters et al. 2003.) This type of plasticity is referred to as spike timing-dependent plasticity (Dan & Poo 2004). Several approaches have been used to determine interstimulus interval (ISI) between the peripheral stimulus and TMS. A fixed stimulus interval has been used in many studies (e.g. Fratello, et al. 2006) as well as MEP latency (e.g. Stinear & Hornby 2005). An another approach is to record the latency of somatosensory-evoked potential (SEP) (e.g. Kumpulainen et al. 2012; Mrachacz-Kersting et al. 2007). When stimulating the soleus muscle Kumpulainen et al. (2012) showed that optimal ISI for induction of LTP-like plasticity is SEP latency + 18ms. SEP latency was determined on the occurrence of the first negative peak P32.

This peak corresponds to the activations of the primary cortical somatosensory receiving area. (Kumpulainen et al. 2012.)

The paired stimulation (a pair of TMS and peripheral nerve stimulation) is usually repeated 90–225 times to induce changes in corticospinal excitability (Carson & Kennedy 2013). The PAS method in which a single peripheral stimulus is followed by a single TMS -stimulus is often used. Some studies have utilized a protocol in which single peripheral stimulus is replaced with trains of stimulation (e.g. 10 Hz trains of 1ms square waves for 500 ms and TMS is applied 25 ms after the onset of the train). (McKay et al. 2002; Ridding & Taylor 2001.) TMS intensity during the PAS protocol can be adjusted to produce MEPs of certain size (i.e. ~ 1.0mv) or at a designated percentage of resting motor threshold (RMT) (i.e. 120% of RMT). The intensity of the peripheral nerve stimulation is typically adjusted using perceptual threshold or motor threshold. (i.e 150% of motor threshold) (e.g. Kumpulainen et al. 2012; Stefan et al. 2000.) In addition, the rate of delivery of the stimulation pair varies between 0.02-0.5 Hz between different studies (Carson & Kennedy 2013).

A challenge with PAS is that the subjects' response is highly variable. There are several factors that affect to the response. Some of them are listed below. *The history of the synaptic activity* influence the response. The threshold for LTP/LTD induction is not fixed, instead it is flexible and depend on the previous synaptic activity. High activity preceding PAS increases the LTP threshold and on the contrary, low activity decreases the LTP threshold. The effects for LTD induction are the opposite. (Ridding & Ziemann 2010.) Motor learning has a similar effect, learning of a rapid novel movement prevented PAS-induced LTP-like plasticity but enhanced PAS-induced LTD-like plasticity (Ziemann et al. 2004). The interference of motor learning with PAS-induced plasticity is temporary. In a study by Rosenkranz et al. (2007), motor learning was demonstrated to modify the PAS-induced plasticity after the first day of learning. Subjects' performance continuously improved the following five days of learning, but during the fifth day the PAS-induced plasticity was not affected by motor training. This proposes that the early and the late phases of motor learning use different mechanisms. The authors of the study suggested that the early improvements of the task occurred through pre-existing connections and increasing the synaptic connections by LTP-like plasticity. However, by day five new synapses might have been formed. (Rosenkranz et al. 2007.)

In addition, *behavioral engagements* such as muscle contraction during PAS might also affect the response (Ridding & Ziemann 2010). The behavioral engagements influence the activity state of the cortex, which have been shown to be important factor in the induction of LTP (Koch et al. 2013).

Individual characteristics such as *age, sex, genetics, diseases* and *physical activity* may also influence the PAS-induced plasticity. Cirillo et al. (2009) found that participation in regular physical activity improves the response to PAS, and thus enhances neuroplasticity. The authors concluded that improved neuroplasticity may be beneficial for motor learning and neurorehabilitation. It seems that age determines the magnitude of motor cortical plasticity studied with PAS. The plasticity is large within young subjects and smaller in the elderly subjects. (Müller-Dahlhaus et al. 2008; Fathi et al. 2010.) However, in a study by Tecchio et al. (2008) the age dependency was present only in post menopausal women, not men. It is unclear to what extend gender influences the results of PAS. The gender affects on modulation of the short-term neuroplasticity when transcranial direct current stimulation (tDCS) is used. However, the difference between genders is not present in all tDCS protocols. (Chaieb et al. 2008.) There are also several diseases such as depression (Player et al. 2013), schizophrenia (Franzeva et al. 2008) and dystonia (Meunier et al. 2012) that influence the cortical plasticity and thus produce different response to PAS compared with healthy individuals. In addition to individual characteristics mentioned above, the plasticity in the motor cortex is at least partly determined by genetics. Brain-derived neurotrophic factor (BDNF) is involved in learning by facilitating LTP and mediating use-dependent plasticity. (Schinder & Poo 200). There are three variants of the BDNF, which all modulate differently motor cortex excitability and also the response to PAS is different between the genotypes (Cirillo et al. 2012; Kleim et al. 2006).

Other factors that have to take into account are *time of the day, attention* and *drugs*. It has been reported that PAS is more effective during afternoon than morning (Sale et al.2008). Stefan et al. (2004) studied the role of attention and noticed that when attention is focused to the target hand, PAS facilitated the corticospinal excitability. Interestingly, when the attention was directed towards non-target hand, the plasticity was completely blocked. Further, the authors demonstrated that the plasticity was greatest when the subjects viewed their target hand. Recently, Kamke et al. (2014) further examined

the role of the visual spatial attention. When visual attention was directed near thumb targeted by PAS, the LTP-like effects increased and LTD-like effects decreased compared with visual attention targeted on the other hand. The authors concluded that visual spatial attention has opposite effects on LTP- and LTD-like plasticity. In addition to factors mentioned above, drugs that affect the central nervous system can also influence the cortical synaptic plasticity. These are for example drugs that have influence on NMDA receptors or on GABAergic inhibition. (Ridding & Ziemann 2010.)

## 3.2 Mechanisms of PAS-induced changes

The mechanisms through which PAS influences the plasticity are not entirely known. With pharmacological interventions it is possible to modulate plasticity induced by PAS and get more knowledge about the mechanisms of PAS-induced changes. This can be done by targeting neurotransmitters and ion channels. Pharmacological intervention studies have revealed several systems that influence the non-invasive brain stimulation (NIBS)-induced (i.e. PAS-induced) plasticity. The systems that have been demonstrated to influence the NIBS-induced plasticity are the glutamatergic system, the GABAergic system, voltage-gated ion channels, the dopaminergic system, the cholinergic system, the serotonergic system and the adrenergic system. (Nitsche et al. 2012.) In addition to pharmacological studies it is important to know the neural circuits through which the PAS influences. Next few paragraphs focus on this part.

PAS seems to have an impact upon certain intracortical neural circuits. It is possible to investigate specific intracortical neural circuits by using paired pulse TMS. In this method, introduced by Kujirai et al. (1993), two consecutive stimuli are delivered through the same TMS coil. By adjusting the intensity of the stimuli and the interstimulus interval it is possible to investigate both either facilitatory or inhibitory circuits. (Kujirai et al. 1993; Valls-Solé et. al. 1992; Ziemann et al. 1996.) Intracortical inhibitory circuits are divided to a short-interval intracortical inhibition (SICI) and a long-interval intracortical inhibition (LICI). These inhibitions are mediated by different cell populations and are detectable at different interstimulus intervals. Paired-pulse TMS is needed to produce both of these inhibitions, but ISI is around 1–5 ms for SICI and 50–200ms for LI-

CI. Intracortical facilitation (ICF) can be also divided to two parts. ICF occurs at ISIs of 8-30 ms and short-interval intracortical facilitation (SICF) at ISIs of 1-5 ms. (Fisher et al. 2002; Ilić et al. 2002; Ziemann et al. 1996.)

SICI is suggested to be, at least in part, an effect of $GABA_A$ergic inhibition within the motor cortex (Hanajima et al. 1998). The effect of PAS on SICI is not consistent (Murase et al. 2010; Russmann et al. 2009; Singh et al. 2014). Thus, it is possible that the state of interneuronal networkinfluences the efficacy of the intervention and further the SICI measurements (Carson & Kennedy 2013). Inhibitory phenomenon occurring at longer interstimulus intervals is less known. Sanger et al. (2001) demonstrated that different cell populations mediate SICI and LICI. Werhahn et al. (1999) suggested that LICI is related to $GABA_B$ receptor dependent inhibitory postsynaptic potentials (IPSP). The facilitating PAS protocol decreases LICI when the intensity of the cortical stimulus is moderate (Meunier et al. 2012; Russmann et al. 2009). Another measurement of cortical inhibition is cortical silent period. It is considered to reflect GABAergic inhibition. There are indications that the late part of cortical silent period is infected by both $GABA_A$ and $GABA_B$ receptors. (Inghilleri et al. 1993; Nakamura et al. 1997.) In several studies facilitative PAS protocols have induced an elongation of cortical silent period (e.g. Cirillo et al. 2009; Stefan et al. 2000) or no change (Di Lazzaro et al. 2011).

The facilitatory effect to the test stimulus is referred to ICF and it is thought to be influenced by glutamatergic interneurons, NMDA receptors and $GABA_A$ activity (Reis et al 2008). When the facilitating PAS protocol has been conducted, no changes in ICF have been detected (e.g. Di Lazzaro et al. 2011; Roy et al. 2007). SICF is proposed to occur mainly at cortical level, and probably reflects interactions within the circuits involved in production of I-waves in the corticospinal tract. (Tokimura et al. 1996.) It has been reported that SICF increases at short ISIs (0.8–2.0 ms) following paired stimulation (Ridding & Taylor 2001). A Stimulation of afferent nerve preceding the transcranial magnetic stimulation causes a diminution in MEP amplitude at specific ISIs. To a some degree GABAergic inhibitory interneurons are responsible of this phenomenon. (Alle et al. 2009; Sailer et al. 2002.) However, effects of PAS to this phenomenon are contradictory (e.g. Meunier et al. 2012; Stefan et al. 2002). In addition to impact that the PAS has on intracortical neural circuits, there are evidence that some changes in excitability may occur at the level of the spinal cord. These changes may depend upon alteration of des-

cending inputs to presynaptic interneurons acting on the 1a pathway, or changes in presynaptic networks at the spinal level. (Carson & Kennedy 2013.)

## 3.3 PAS and motor learning

Two types of approaches have been used when studying the connections between the PAS and the motor learning. First, the motor learning task has been conducted prior to induction of the PAS and the changes in PAS-induced plasticity have been compared to non-motor learning control group. Secondly, the PAS has been induced prior to the motor learning and the changes in motor learning compared to control group have been monitored. (Jung & Ziemann 2009; Ziemann et al. 2004)

Applying the first approach, Ziemann et al. (2004) showed that practicing rapid thumb movements that resulted learning, prevented PAS-induced LTP-like plasticity and on the contrary, learning enhanced LTD-like plasticity. This type of interaction was not present when equivalent amount of slow thumb movements were repeated. It was concluded that motor practice resulted in a shift in threshold of LTP/LTD induction. Thus, giving a support to the view that the LTP-like mechanisms are involved in motor learning in human motor cortex. (Ziemann et al. 2004.) Stefan et al. (2006) had similar findings; after motor learning the PAS-induced LTP-like plasticity was abolished, but the LTD-like plasticity remained unaffected. As mentioned above in chapter 2.1, the motor learning appears to interfere with the PAS-induced plasticity only a certain period of time. It was concluded that lack of interference during the fifth day of training may have been caused by transfer of motor memories to new synaptic connections. (Rosenkranz et al. 2007.)

Recently, other non-invasive brain stimulation -methods have been applied to study further interaction between motor learning and occlusion of the LTP-like plasticity. It seems that when the motor training-induced occlusion of the LTP-like plasticity is disrupted, the skill retention is impaired. In other words, the motor practice-induced occlusion of the LTP-like plasticity is important for skill retention. (Cantarero et al. 2013a.) Müller-Dahlhaus & Ziemann (2014) conclude that homeostatic metaplasticity is in-

volved in the LTP-like plasticity in human motor cortex and it is a limiting factor for learning when same neural networks are involved. It has been also demonstrated that both the excitatory corticospinal pathway and the inhibitory intracortical neural circuits are regulated by homeostatic metaplasticity (Murakami et al. 2012). Homeostatic metaplasticity refers to a bidirectional plasticity, which can result both, the LTP and the LTD. The plasticity is affected by the history of synaptic activity. The threshold for LTP and LTD induction is not stable, in a presence of recent high level of postsynaptic activity the threshold for LTP-like plasticity is increased and controversially decreased for LTD-like plasticity. (Bienenstock et al. 1982 according to Murakami et al. 2012.)

The second approach was applied by Jung & Ziemann (2009). They studied how LTP- and LTD-like plasticity induced by PAS influences learning of rapid thumb movements (acceleration). Two motor practice sessions were conducted in two different experiments. In the experiment 1 the first motor practice was done immediately after the PAS protocols and in the experiment 2, the motor practice was delayed and started 90 minutes following PAS. In both experiments, the depressive PAS ($PAS_{LTD}$) significantly enhanced learning compared to the facilitatory PAS ($PAS_{LTP}$) and to the control PAS (which did not produce changes in MEP size). In the experiment 1 $PAS_{LTP}$ also facilitated learning, compared to control PAS. These results support the view that, in certain conditions, the enhancement of learning following the PAS occurs through homeostatic interactions. However, when the motor practice is done immediately after the PAS, the nonhomeostatic mechanisms may influence, this was shown by the enhanced learning after $PAS_{LTP}$ induction. (Jung & Ziemann 2009.) Rajji et al. (2011) studied the effect of facilitatory PAS on learning of rotary pursuit motor task. The PAS did not result in enhanced motor learning immediately after the protocol, but resulted in enhanced motor learning at one week following the PAS, when compared to control group. The authors concluded that the delayed enhancement of the motor learning might be due to the complex nature of rotary pursuit task. The task is a complex motor task, which is believed to involve also other systems in brain than only motor cortical system. (Rajji et al. 2011.) Both of these studies (Jung & Ziemann 2009; Rajji et al. 2011) used a motor learning task in which the contribution of both agonists and antagonists muscles are essential for the improved performance. One of the properties of LTP is specificity which means that only the activated synapses show LTP. PAS protocol typically is targeted to specifically activated certain muscles. To my knowledge none of the studies which investigate the

effect of PAS on motor learning has used a motor learning task in which antagonist muscles are not used during the motor learning task.

The dose-response of facilitatory PAS was recently studied. The number of stimulus pairs influences the cortical plasticity. In addition, the interaction between the PAS and the motor learning depends also on the number of stimulus pairs and the state of cortical excitability prior to motor training. However, there were no changes in motor learning following different number of stimulus pairs in this study. Thus, the motor learning, measured as a accuracy of the performed task, was not affected by the number of stimulus pairs. (Elahi et al. 2012.) In addition to factors mentioned above, the direction of the TMS current affects critically to the induced plasticity and motor learning. It was demonstrated that activation of posterior-anterior inputs instead of anterior-posteriorinputs can modulate model-free motor learning. (Hamada et al 2014.)

# 4 THE PURPOSE OF THE STUDY

Motor learning involves several brain areas including M1. Mechanisms responsible of motor learning include long-term potentiation and depression of synaptic efficacy. It has been previously demonstrated that PAS is able to induce LTP-like plasticity in the human M1. Further studies have demonstrated that PAS-induced LTP-like plasticity can enhance subsequent motor learning through nonhomeostatic interactions.

The aim of this study was to examine the effect of PAS-induced LTP-like plasticity on brief visuomotor learning task performed with plantar flexors. Thus, the focus was on early acquisition phase of motor learning. The learning task consisted of adjustment of isometric plantar flexion force according to visual cue. The learning performance was calculated as a magnitude of average error throughout the task. The isometric plantar flexion was chosen as a motor task, because it does not require the activation of antagonist muscles. Thus, the improvement in performance depend on the agonist/synergist activation. The motor learning in this study is defined as a short-term acquisition of visuomotor task which results in enhanced performance (Cirillo et al. 2011). It was hypothesized that PAS-induced LTP-like plasticity will induce improved motor learning compared with control group.

# 5 METHODS

## 5.1 Subjects

Twenty healthy volunteers (8 females, 12 males; age = 26.6 ± 4.3; height = 174.4 ± 8.7; weight = 75.5 ± 12.5; mean ± standard deviation [SD]) participated in this study. Subjects were informed verbally and in writing of the experimental procedures and associated risks. All the subjects were in good health. The procedures used in this study were approved by the university ethics board and the recommendations contained in the declaration of Helsinki were followed. A written informed consent was obtained for each participant.

## 5.2 Experimental design

The measurements were conducted during two separate sessions. The first session consisted of the PAS, learning tasks, TMS and electrical stimulation -measurements. The second session was conducted two weeks after the first session. The second session included only two learning tasks separated with 15 minute break and it was considered as a control measurement for learning task. The subjects performed a short warm-up before the measurements at both sessions. In addition maximal voluntary contraction (MVC) was determined after warm-up. Three isometric plantar-flexion MVCs were performed and the highest value was considered as MVC. The control measurement for learning task was conducted two weeks after the measurements, since it has been shown that motor learning prior PAS may prevent subsequent PAS-induced LTP-like plasticity (Ziemann et al. 2004). During the first session, measurements of MEP, maximal compound muscle action potential (Mmax) and the Hoffmann reflex (H-reflex) preceded the PAS (PRE). The PAS was followed by five minutes break during which the subjects could move their legs in order to avoid numbness. After the break a learning task 1(L1) was conducted. There was a 15 minutes break between the consecutive learning tasks (three learning tasks), during these breaks MEP, Mmax and H-reflex were measured (POST1, POST2 and POST3) (Fig 3.). During the measurement subjects were seated in

a custom-made ankle dynamometer (University of Jyväskylä) with following angles: hip 110°, knee, 180° and ankle 90°.

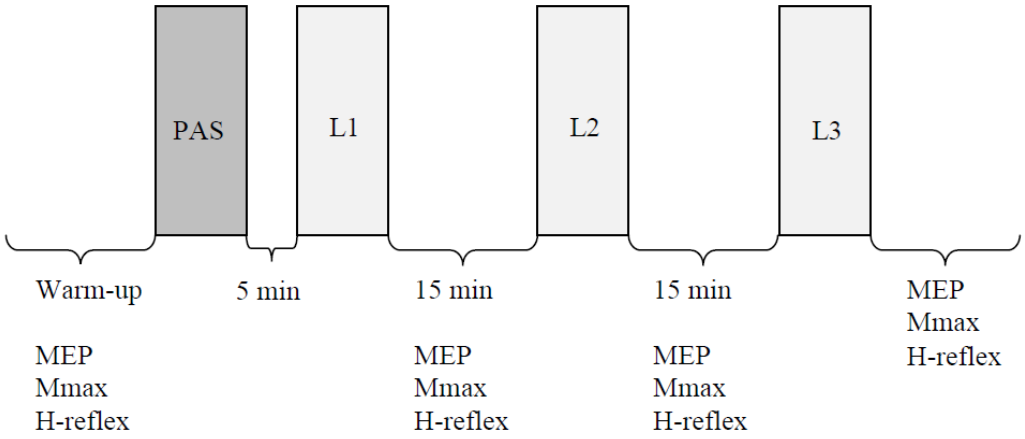



Figure 3. Illustration of the protocol of the first session. The PRE measurements preceded the PAS, which was followed by three learning tasks (L1, L2 and L3). Between the learning tasks (during the 15 min break) and after the third task, measurements of MEP, Mmax and H-reflex were conducted.

## 5.3 PAS

PAS consisted of 200 electrical stimuli delivered to the posterior tibial nerve at popliteal fossa, paired with a single TMS pulse of the soleus area over the contralateral M1. The rate of paired stimulation was 0.2 Hz. Subjects were asked to count the electrical stimuli and after every 20 stimuli produce a slight plantar-flexion force (Stefan et al. 2004). The ISI between electrical stimuli and TMS was SEP latency + 18ms (Kumpulainen et al. 2012). The SEP latency was determined at the first session before warm-up. SEPs were recorded with two conventional electroencephalography (EEG) needle electrodes inserted into the skin 2 cm behind and 5 cm in front of the vertex. The amount of traces that were recorded and averaged was 200 (amplification: 100,00; filtering: 1–500Hz; Neuropack Four Mini, MEB-5304 K, Nihon Kohden, Tokyo Japan). The occurrence of the peak P32 was determined. The measure of cortical potentials were evoked by a sti-

mulation of tibial nerve. A circular cathode was placed over the tibial nerve at popliteal fossa (Unilect, Ag/AgCl, Unomedical Ltd.,Redditch, UK) and the anode above the patella (V-trodes electrodes, Mettler Electronics corp., Anaheim, USA). During the PAS the TMS was delivered at the intensity of 120 % of the RMT and electrical stimuli was deliverer 150 % of motor threshold (MT), which was defined as a minimal intensity that produced a muscle twitch in the soleus muscle.

## 5.4 Learning task

The participants performed the same visuomotor learning task three times (L1, L2 and L3). Before each learning task the subjects were shown the force line they had to follow during the actual task. During the learning task subjects were asked to follow the force line as precisely as possible. The force line consisted of several peaks, and the highest peak was 60 % of the participants MVC (Fig 4.). A length of a single task was 25 seconds. Subjects were seated in a ankle dynamometer (University of Jyväskylä) and the force was produced with right leg (isometric plantar-flexion). Force was collected with 16 bit AD board (CED 1401, Cambridge Electronic Design, Cambridge, UK). MVC was determined following warm-up as a highest value of three trials.

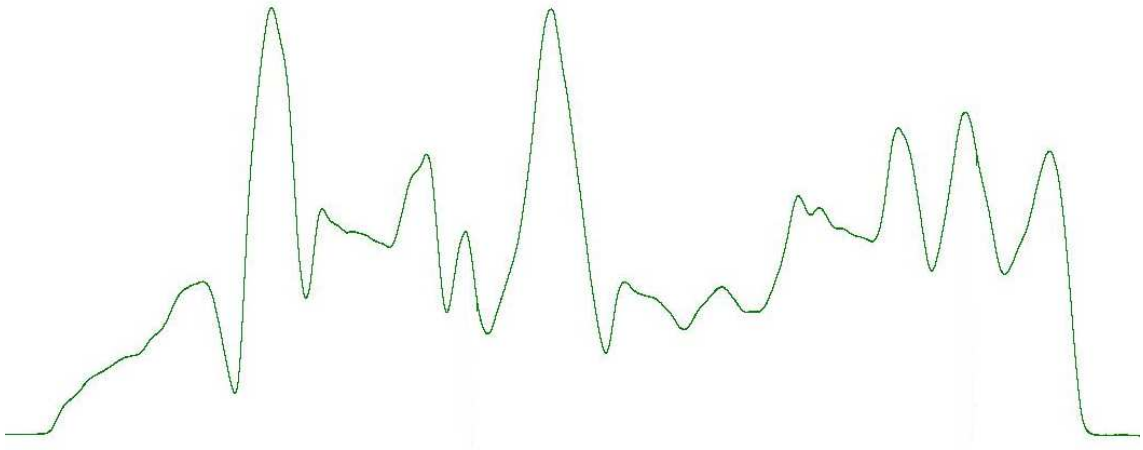


Figure 4.The force line of the learning task.

## 5.5 Procedures

*TMS.* TMS was delivered (posterior-anterior current) using a 9-cm double batwing coil attached via a BiStim unit to a Magstim $200^2$ stimulator (Magstim, Whitland, UK). A single pulse was applied over the left M1 to preferentially activate the soleus muscle. The position was marked on a tightly fit swim cap. Position and orientation was held with custom-made coil holder and rubber bands. The RMT was defined as the minimum intensity that elicit three out of five MEPs which amplitude was minimum 50µV. Intensity of stimulation for MEP measurement was 120 % of the RMT. The soleus muscle was relaxed during the TMS measurement and subjects were asked to count silently backwards from 100 during stimulation (Kumpulainen et al. 2012).

*Electrical stimulations*. In order to produce the Mmax, a rectangular pulse of 1ms was delivered. The cathode was placed over the posterior tibial nerve at popliteal fossa and the anode above the patella (as in SEP measurement). The site of the stimulating electrode was determined to produce greatest response in soleus and minimal response in tibial anterior muscle. Stimulus intensity was increased gradually until it reached plateau. The stimulus intensity used in the actual measurements was set 120 % above this. As in the Mmax measurements, a rectangular pulse of 1ms was delivered to tibial nerve to elicit h-reflex.

*Electromyography (EMG) recordings.* For EMG measurements two types of electrode arrangements were used. A pseudomonopolar setup was used to record MEPs and bipolar setup to record Mmax and H-reflex. The pseudomonopolar electrode (10 mm diameter, Blue Sensor N, N-00-S, Ambu A/S, Denmark) was placed over soleus and the reference electrode (Unilect, Ag/AgCl, Unomedical Ltd., Redditch, UK) over tibia. The bipolar electrodes were placed over soleus and antagonist tibialis anterior. The EMG electrodes were attached to soleus and tibialis anterior according to SENIAM recommendations (Hermens et al. 2000). The reference electrode (Unilect, Ag/AgCl, Unomedical Ltd., Redditch, UK) was placed over the head of tibia. The skin under the electrodes was shaved, part of the dead skill cells were abraded and cleaned with alcohol. The interelectrode distance (bipolar setup) was 20 mm and the resistance between the electrodes was <2 kΩ. The EMG signals were amplified (x1000) and filtered (10–1000Hz)

(NL900D & NL844, Digitimer Ltd., Hertfordshire, UK.). EMG and force were sampled at 2 kHz with a data acquisition system (CEDPower 1401 with Spike2 software, Cambridge ElectronicsDesign Limited, UK).

## 5.6 Data analysis

Peak-to-peak amplitudes of ten MEPs, three Mmax and three h-reflex were analyzed and averaged after each learning task and during PRE measurements (POST1, POST2, POST3 and PRE) according to Martin et al. (2006). All three were measured form soleus muscle. The h-reflex were normalized to the preceding m-wave. The results concerning h-reflex are expressed H/M -value. For each participant It was made sure that the h-reflex measured was on the ascending part of the h-reflex curve. This is necessary in order to detect facilitation or inhibition since the last motoneurons recruited are sensitive to the changes (Pierrot-Deseilligny & Burke 2005). There were quite large differences between subjects at the point (as a percentage of Mmax) in which the h-reflex curve reached its peak. Thus, a variation in m-wave preceding h-reflex was 10-20% of Mmax. However, for each participant a point from the ascending curve was selected e.g. 15% of Mmax and the H/M -value was calculated from three successful trials in which the preceding m-wave was e.g. 15% ± 2.5% of Mmax. MVC was defined as a peak value of three trials.

The target force line and force trace produced by a subject were compared to define the learning task performance. Continuously throughout the learning task an average tracking error between the two traces were calculated. The average tracking error of each learning task was normalized to MVC value and further normalized to average tracking error of the second session. Averaging to the second session values (control values) was performed in order to minimize possible differences between the groups. At the second session two learning trials were averaged. Based on the subjects' response to PAS intervention, they were divided into two groups (responders and non-responders). Subjects who had increased (>1) MEP amplitude after third learning task, compared with PRE measurements, were placed on the responder group (RES, n=8). In addition, the subjects who did not experience MEP facilitation (after third learning task) were placed to non-

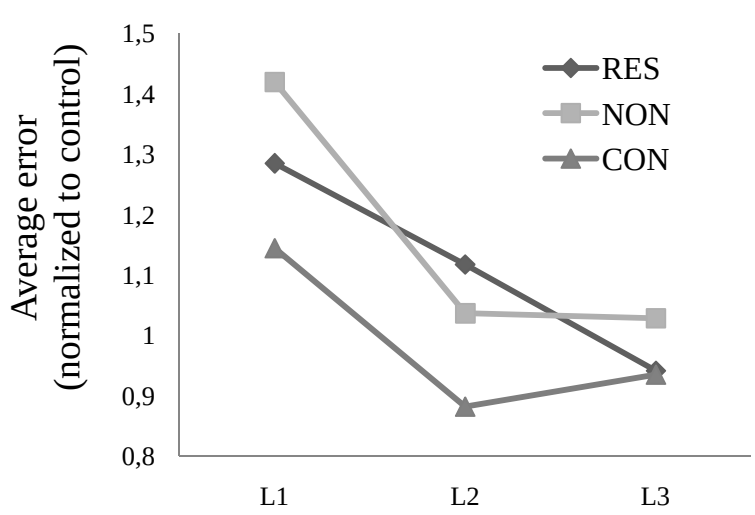
responder group (NON, n=6). This division into RES and NON groups was done because when targeting soleus muscle PAS may not cause MEP facilitation in all subjects (Kumpulainen et al. 2012). Third group in the current study was control group (CON, n=6), instead of PAS intervention the subjects in the control group sat in the same position equivalent time than subjects in the PAS intervention group. Subjects in the CON group did not also have any stimulations during that time.

## 5.7 Statistical analysis

All data are presented as mean ± SD. The results were analyzed using IBM SPSS statistics 22 -program. The normality of the variables was tested with Shapiro-Wilk test. Repeated measures ANOVA was used with normally distributed variables (h-reflex, Mmax and learning task) and the non-parametric Friedman's repeated-measures ANOVA by ranks was used when appropriate (MEP). Normally distributed results were compared with repeated-measures ANOVA with LSD post hoc. If the sphericity was violated, the Grenhouse-Geisser correction factor was applied to compensate for non-sphericity. To define the differences between groups for MEPs the Kruskal-Wallis test was applied. Pearson correlation coefficients were calculated between MEP amplitudes and learning tasks. The significance level was set at $p < 0.05$.

# 6 RESULTS

*Learning task.* An average tracking error normalized to control values for RES were 1.28 ± 0.22, 1.11 ± 0.27 and 0.94 ± 0.21 for the L1, L2 and L3, respectively (Fig 5.). A decrease in the tracking error was significant between L1/L3 ($p < 0.001$) and L2/L3 ($p < 0.015$). There was a tendency for decreased error between L1/L2 ($p < 0.068$). NON group task values for L1, L2 and L3 were 1.41 ± 0.38, 1.03 ± 0.27 and 1.02 ± 0.28. Significant changes were only detected between L1/L3 ($p < 0.02$), the changes were not significant between L2/L3 ($p < 0.90$), but there was slight decrease in the error between L1/L2 ($p < 0.054$). The average errors for CON were 1.14 ± 0.21, 0.88 ± 0.10 and 0.93 ± 0.17 during the L1, L2 and L3 respectively. CON group exhibit significant changes between L1/L2 ($p < 0.014$) and L1/L3 (0.010), on the contrary there were no changes between L2/L3 ($p < 0.470$). A statistically significant interaction between group and task was found for L2/L3 between RES and CON ($p < 0.021$) and near significant interaction between RES and NON for the L2/L3 ($p < 0.068$). In addition, learning task performance did not correlate with MEP amplitudes when analyzed among groups or all subjects together .



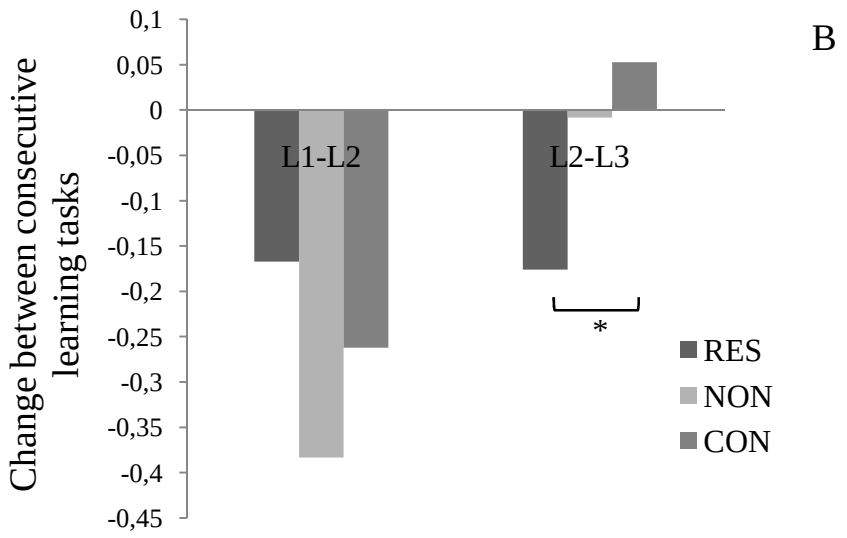



Figure 5. A) Average errors during the three consecutive learning tasks. B) Change in average errors between consecutive learning tasks L1-L2 and L2-L3. Asterisk represent a significant ($p < 0.05$) difference between RES and CON groups. Data expressed as mean.

*MEP amplitudes.* The MEP amplitudes normalized to PRE values for the RES group were; 138.0 ± 98.6 % (POST1), 227.8 ± 130.2 % (POST2, $p < 0.022$) and 228.4 ± 152.9 % (POST3, $p < 0.012$). In the NON group MEP amplitudes were 83.0 ± 35.2 % (POST1) 70.7 ± 21.7 % (POST2, $p < 0.020$) and 72.0 ± 15.5 % (POST3). There were no changes in CON compared with PRE measurement, 112.1 ± 47.4, % 100.2 ± 25.7 % and 91.6 ± 20.4 % (POST1, POST2 and POST3, respectively). MEPs were significantly larger in POST3 condition in RES -group when compared to NON ($p < 0.017$) and CON ($p < 0.020$). In addition, MEPs in RES -group were significantly larger in POST2 condition compared with NON (<0.017) and almost significantly larger than CON ($p < 0.053$). However, there were no changes between the groups in POST1. Fig. 6. shows the MEP amplitudes normalized to PRE values.

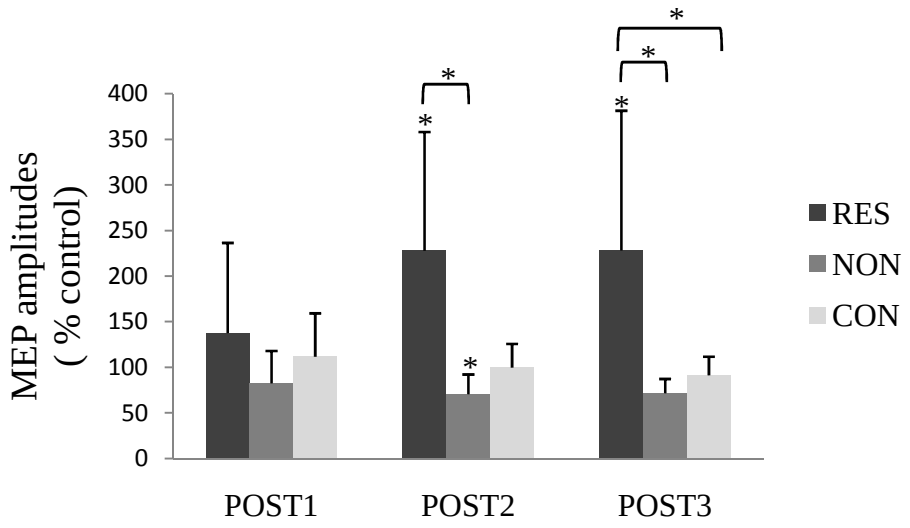



Figure 6. The effect of PAS on MEP values. The increase in MEP values between PRE-POST2 and PRE-POST3 was significant for RES, and there was a small, but significant decrease between PRE-POST2 for NON. Asterisks represent significant ($p < 0.05$) differences between PRE and POST measurements, and in addition between groups. Data presented as mean ± SD.

*Mmax*. Mmax did not change significantly between the conditions and in addition there were no significant differences between the groups. The mean amplitudes were 6.26 ± 2.33 (PRE), 6.37 ± 2.88 (POST1), 6.28 ± 3.02 (POST2) and 6.12 ± 3.08 mV (POST3) for RES group. For the NON group mean values were 6.17 ± 1.21 (PRE), 6.69 ± 1.58 (POST1), 6.74 ± 1.50 (POST2) and 6.94 ± 1.47 mV (POST3). Values for the CON group were 5.79 ± 2.29 (PRE), 6.33 ± 2.75 (POST1), 6.47 ± 2.85 (POST2) and 6.40 ± 2.82 mV (POST3).

*H-reflex*. The average H/M values for the RES were 0.62 ± 0.31 (PRE), 0.59 ± 0.27 (POST1), 0.62 ± 0.28 (POST2) and 0.60 ± 0.28 (POST3), and for the NON were 0.63 ± 0.13 (PRE), 0.59 ± 0.17 (POST1), 0.61 ± 0.16 (POST2) and 0.60 ± 0.11 (POST3). In addition mean values for CON were 0.67 ± 0.30 (PRE), 0.62 ± 0.35 (POST1), 0.60 ± 0.32 (POST2) and 0.60 ± 0.32 (POST3). No significant effects were found neither for condition nor group.

# 7 DISCUSSION

The present experiment demonstrates that the overall extent of learning was similar for all groups. Thus, all groups improved their performance through L1 to L3 in a similar manner. However, only the RES group improved between L2 and L3 and the improvement was significantly different compared with CON and almost significantly different compared with NON (group * task interaction). PAS did not have an effect on the peripheral and spinal excitabilities as shown by h-reflex and Mmax measurements. In addition the motor learning task did not elicit changes in h-reflex and Mmax or in corticospinal excitability measured with MEPs from the soleus muscle.

Previous findings show that PAS-induced LTP-like plasticity either improve (Jung & Ziemann 2009) or do not affect (Rajji et al. 2011) motor learning immediately after PAS. Other non-invasive brain stimulation studies also have contradictory results, demonstrating either enhanced (Antal et al. 2008) or not changed (Agostino et al. 2007; Agostino et al. 2008) motor learning after facilitatory brain stimulation. Improved learning after LTP induction has been proposed to result from nonhomeostatic mechanisms such as LTP-induced blockade of LTD or nonsaturated LTP-induced facilitation of learning (Jung & Ziemann 2009). It might be that moderate increase in background excitability may lower the threshold at which the synapse efficacy is strengthened (Antal et al. 2008). On the contrary, if the increase in the background excitability is large the synapses might approach the upper limit of synaptic modification range, and further strengthening through LTP would be blocked (Rioult-Pedotti et al. 2007). If PAS would induce large synaptic enhancement in the synapses involved in motor learning, the subsequent motor learning would be impaired. In the current study the overall learning performance was not reduced following PAS. Thus, occlusion of LTP did not probably occur. Interestingly the participants in RES group did improve their performance between L2 and L3 compared with CON group. Increased background excitability might have lowered the threshold for synaptic strengthening. Thus, it is possible that acquisition of brief visuomotor learning occurs through nonhomeostatic interactions.

MEP amplitudes has been demonstrated to increase after visuomotor learning (Cirillo et al. 2011). The increased amplitudes following learning are thought to occur due to LTP-like changes in synaptic efficacy, thus reflecting use-dependent plasticity (Bütefisch et al. 2000). In this study, the MEP amplitudes did not increase after motor learning in CON group. However, there was a significant improvement in the learning task between L1 and L3. It appears that the improvements after the brief visuomotor learning task ( 3 * 25 seconds) are not due to changes in corticospinal track. Previously it has been shown that several brain areas are involved in motor learning. The importance of each area seem to vary between different types of learning tasks. (Hardwick et al. 2013.) In simple repetitive learning tasks M1 appears to have more important role than in complex sensimotor learning task. (Hardwick et al 2013; Platz et al. 2012). In addition, TMS does not seem to disrupt the initial error reduction phase of reaching movement learning, instead it appears to impair the motor learning when the performance has plateaud (Orban de Xivry et al. 2011). Thus, it can be speculated that improved performance in the present study was, at least partly, due to other cortical and subcortical networks than M1 and corticospinal track.

Even though increase MEP amplitudes following learning are thought to reflect LTP-like plasticity, as stated above, there might be no association between the change in MEP amplitude and the magnitude of motor learning (Cirillo et al. 2011). Also in this study there were no correlation between motor learning and MEP amplitude. During the initial phase of motor learning the errors are large, but the reduction in errors occurs rapidly. It has been suggested that the excitability of active M1 decreases after an error (Amengual et al. 2013). Thus, during the initial phase when the amount of errors is large, the excitability of M1 might be alternating between correct and erroneous responses. Further, this could explain why the MEP amplitude measured following the learning task did not associate with the learning performance.

In previous studies concerning leg muscles, the h-reflex has not been reported to change as a result of PAS (Mrachacz-Kersting et al. 2007; Roy et al 2007). In alignment with these studies, there were no change in the h-reflex amplitude following PAS in the current experiment. In addition, similarly with the present study, the Mmax has not been reported to change due to PAS (Wolters et al. 2003). Thus, this supports the assumption of cortical origin of PAS-induced after effects.

The learning task was relatively short in the present study ( 3* 25 seconds). Currently it is not known how and when different plasticity mechanisms (e.g. LTP) involved in learning are used as learning evolves. However, learning probably results from multiple plasticity mechanisms that might operate at different sites and times. (Yang & Lisberger 2014.) It is unclear if the LTP-like plasticity was one of the mechanisms responsible of improved performance in the current study. Previous studies have found that a simple motor learning task preceding PAS prevents PAS-induced LTP-like plasticity (Ziemann et al. 2004). Thus, it can be concluded that LTP-like mechanism contribute to motor learning in M1, and the LTP-like plasticity induced by both the PAS and motor learning influence through mutual neural circuits. However, in the current study the overall learning performance was similar between groups. It is possible that a longer training period should have been required to induce interaction between PAS and overall motor learning. Interestingly the RES group improved their performance significantly between L2/L3 when NON and CON did not experience performance improvements. In addition the improvement was significantly larger than CON group. It may be that during the later part of the motor learning task, the plasticity mechanisms responsible of the performance improvement involved similar neural circuits as PAS.

Previous studies investigating the effect of PAS on motor learning (Jung & Ziemann 2009; Rajji et al. 2011) used a motor learning task in which activation of antagonist muscles was important. In the present study learning task consisted of adjustment of isometric plantar flexion force according to visual cue. Thus, antagonist muscles did not have a significant role in the motor learning. According to specificity property of the LTP (only the activated synapses show LTP) it might have been possible that the effect of PAS on motor learning would have been stronger than in studies mentioned above. However, in the current study PAS seemed to have an effect on learning only at the end of the task. Probably other factors in addition to PAS were responsible of the improved performance, as mentioned above.

There are some limitations in the current study. First, the amount of subjects per group is relatively small, and larger sample would be needed. Secondly, there was no sham-condition for the experiment. Thus, it may be possible that condition (PAS or control) may have affected results. However, the subjects were not aware that the main purpose of the study was to investigate the effect of PAS on motor learning. Instead it was stated

that "The main purpose of this study is to examine if corticospinal and motor cortex excitability changes differently when paired-associative stimulation (PAS) precedes motor learning compared with motor learning without PAS". In addition, the participants were divided into two groups based on their response to PAS, neither did the subjects nor the experimenters know which group participants belonged during the measurements. Collection and analysis of the data is rather automated, thus the experimenters will unlikely have a significant influence on the results. Third, the results of this study are limited to plantar flexion and visuomotor learning (tracking), and do not give information about other types of learning tasks or motor tasks.

In summary, the present findings show that PAS-induced LTP-like plasticity did not improve visuomotor learning immediately after PAS intervention. However, as the learning proceeds PAS might have a beneficial effect on learning. This might be due to delayed effect of PAS, which was shown as higher MEP values in POST2 and POST3 than in POST1. Visuomotor learning occurs through many different mechanisms. In the current study visuomotor learning (CON group) did not affect MEP amplitudes. Thus, other cortical and subcortical networks than corticospinal track may have been responsible of the improved performance.

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