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Paired Associative Stimulation Fails to Induce Plasticity in Freely Behaving Intact Rats

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Manuscript Title Page

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Abbreviated Title (50 character maximum)

PAS Fails to Induce Plasticity in Behaving Rats

List all Author Names and Affiliations in order as they would appear in the published article

Windsor Kwan-Chun Ting[1], Maxime Huot-Lavoie[1], Christian Ethier[1*]

[1]Centre de Recherche CERVO, Département de psychiatrie et de neurosciences, Université Laval

Author Contributions:

WT wrote the first draft of the manuscript. WT, MHL and CE conducted the experiments. WT and CE designed the study, analyzed data and critically revised subsequent drafts to the manuscript. MHL provided critical revisions to the manuscript for intellectual content. All authors approved the final version of the paper for submission.

Correspondence should be addressed to (include email address)

*Corresponding Author:

Dr. Christian Ethier

Centre de Recherche CERVO

Département de psychiatrie et de neurosciences, Université Laval

2601 Chemin de la Canardière, Québec, QC; G1J 2G3

christian.ethier@fmed.ulaval.ca

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Conflict of Interest

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NA

Abstract

Paired Associative Stimulation (PAS) has been explored in humans as a non-invasive tool to drive plasticity and promote recovery after neurological insult. A more thorough understanding of PAS-induced plasticity is needed to fully harness it as a clinical tool. Here, we tested the efficacy of PAS with multiple inter-stimuli intervals in an awake rat model in order to study the principles of associative plasticity. Using chronically implanted electrodes in motor cortex and forelimb, we explored PAS parameters to effectively drive plasticity. We assessed changes in corticomotor excitability using a closed loop, EMG-controlled cortical stimulation paradigm. We tested eleven PAS intervals, chosen to force the coincidence of neuronal activity in the rats' motor cortex and spinal cord with timings relevant to the principles of Hebbian spike-timing-dependent plasticity. However, despite a relatively large number of stimulus pairings (300), none of the tested intervals reliably changed corticospinal excitability relative to control conditions. Our results question PAS effectiveness under these conditions.

Significance Statement

Paired Associative Stimulation (PAS) can be applied non-invasively to modulate corticomotor plasticity in humans. However, our understanding of how we can use paired stimuli to produce the greatest beneficial reshaping of corticomotor connections *in vivo* is still rudimentary. We completed a systematic study varying inter-stimulus intervals between cortical and muscle stimulation in a freely-behaving rat PAS model, following the principles of spike-timing dependent plasticity (STDP). Crucially, our experiments have *not* demonstrated that the STDP model is effective *in vivo* using our PAS protocol. We discuss several other factors in addition to the inter-stimulus interval which may play a larger role in driving plasticity, and potential ways that the field can approach future work.

Introduction

Spike Timing as a Driver of Synaptic Plasticity

Seminal studies on synaptic plasticity have led to the development of the spike-timing-dependent plasticity (STDP) model (Markram et al., 1997; Bi and Poo, 1998), which is an extension of the Hebbian postulate (Hebb, 1949) (Figure 1). Whether synaptic potentiation (Long Term Potentiation, LTP) or depression (LTD) occurs is contingent upon the pattern of firing activity in the pre- and post-synaptic neurons (Cooper, 2005). These concepts led to the development of stimulation-based neuromodulation methods aimed at conditioning cortical and spinal motor circuits to promote motor recovery after neurological lesions.

[Figure 1 near here]

However, the STDP hypothesis as it applies to larger circuits such as the corticomotor system is contingent upon certain assumptions, one being that principles derived from *in vitro* studies at the synaptic level remain sound when applied to higher level systems *in vivo*. Beyond the complexity of the system's anatomy, ongoing patterns of neural activity (spontaneous or behaviour related) may interfere with the fine-tuned firing patterns which STDP putatively requires, introducing variability into the equation. Hence, the field would benefit from more systematic study of STDP at the systems level in conjunction with ongoing neuronal activity.

Non-invasive Paired Associative Stimulation (PAS) in Humans

In humans, Paired Associative Stimulation (PAS) using transcranial magnetic stimulation and transcutaneous peripheral nerve stimulation is a non-invasive method used to modulate the excitability of corticomotor connections and facilitate the recruitment of targeted muscles, based on Hebbian STDP principles. The first clear demonstration of PAS was designed to promote plasticity at the cortical level

(Stefan et al., 2000). The results indicated that topographically specific and sustained (30-60 min) increases in excitability of the motor system were possible through non-invasive PAS in humans, by carefully timing cortical stimulation with somatosensory signals afferently propagated towards the cortex. PAS utility was subsequently reproduced in the spinal circuits (Taylor and Martin, 2009), by timing peripheral nerve stimulation so that antidromic potentials in motoneurons reached the cell bodies in the spinal cord shortly after the arrival of TMS-induced corticospinal volleys. Since that time, several studies have attempted to validate this phenomenon with mixed success, and studied PAS to drive plasticity in the neural circuits controlling upper and lower limbs of humans (Carson and Kennedy, 2013; Suppa et al., 2017) .

PAS has demonstrated potential as a therapeutic intervention to strengthen residual circuits after spinal cord injury and promote functional recovery (Bunday and Perez, 2012; Urbin et al., 2017; Bunday et al., 2018). Studies have also employed modified PAS protocols with mixed success in improving functional recovery after neurovascular insult, both in animals (Shin et al., 2008) and in humans (Castel-Lacanal et al., 2007; Castel-Lacanal et al., 2009; Rogers et al., 2011; Cho et al., 2016; Ferris et al., 2018; Palmer et al., 2018; Tarri et al., 2018a). PAS initially showed great promise for rehabilitation, however enthusiasm for this approach has been tempered by lack of experimental rigor and inconsistent results (Alder et al., 2019). PAS has shown to have a very high inter-subject variability (Sale et al., 2007; McGie et al., 2014; Tarri et al., 2018b), its effects are strongly dependent on mindful, persistent attention on the target limb (Stefan et al., 2004) or even failed to induce any consistent plastic effects (McGie et al., 2014).

PAS in Animals

Animal models are being developed to obtain a more robust and systematic evaluation of PAS effectiveness and underlying mechanisms. A few studies in rats have thus far shown that PAS could drive changes in corticomotor excitability toward both forelimb and hindlimb muscles (Shin et al., 2008;

Mishra et al., 2017; Zhang et al., 2018). However, most studies have been performed under anesthesia, which can itself modulate plasticity (Yang et al., 2011; Huang and Yang, 2015), or using non-invasive methods in restrained animals. An animal model with chronically implanted electrodes allowing for a systematic study of the effectiveness of PAS in freely-moving subjects did not exist thus far.

We aimed at developing such a model to perform a robust evaluation of PAS effectiveness in a context where stimulation is applied during naturally ongoing neuronal activity. We applied PAS in freely-behaving rats with chronically implanted cortical and intramuscular electrodes (Figure 2A). By holding other parameters constant (stimulation amplitude, frequency, number of pulses), we tested a wide range of inter-stimulus intervals, hypothesizing that certain timings would result in corticomotor potentiation, but failed to significantly modulate corticomotor excitability as predicted by the STDP model.

Methods

Animals and Surgical Preparation

All animal procedures were performed in accordance with the [Author University] animal care committee's regulations. Nine Long Evans rats and one Sprague-Dawley rat (all male) were housed under 12/12 inverse daylight cycle with food and water available *ad libitum*. Animals were individually housed to prevent implant damage. 150 experimental sessions were planned in ten animals during the dark (active) phase of their daylight cycle (coinciding with our work day), to investigate the effectiveness of 15 inter-stimulus intervals (ISI), including control stimulation protocols. However, due to rare implant failure, some ISIs were not tested in all the rats. All ISI conditions were tested in a minimum of five animals, and an average of seven to eight (Extended Data for Figure 4-1). Our PAS intervention typically targeted the *extensor carpi radialis (ECR)* muscle. However, in some animals, we used pairs of EMG

wires implanted in more proximal locations (*Biceps* or *Trapezius muscles*). The distribution of muscles tested within the full dataset is shown in Extended Data for Figure 4-2.

[Figure 2 near here]

Chronic PAS Implantation Surgery

During aseptic surgeries performed under isoflurane anesthesia, rats were implanted with 1 mm x 1 mm custom square arrays of four 80/20 platinum-iridium electrodes, each 75 microns in diameter and an approximately impedance of 20 kOhms. The electrodes were inserted 1.5 mm deep into the caudal forelimb area (CFA) of the primary motor cortex (M1) by a stereotaxic craniectomy, centered at 1 mm anterior and 3.5 mm lateral relative to bregma, and the surrounding exposed dura matter was covered with silicone gel for protection (Figure 2B). This allowed us to perform intracortical stimulation using an isolated constant current stimulator (model 2100, A-M Systems Inc.). Three pairs of PFA-coated multi-stranded stainless-steel wire electrodes (A-M Systems Inc.) were inserted into the contralateral *ECR*, *biceps brachii* and *trapezius* muscles (the latter two serving as alternative muscles in case the ECR electrode failed). EMG and cortical electrodes were pre-soldered to either an InVivo1 MS12P or a SAMTEC 2x7 connector, which were secured to the skull with dental cement and six bone screws as anchors. A posterior skull screw served as the ground electrode for cortical monopolar stimulation. An additional reference electrode for the EMG measurement was embedded subcutaneously in the upper back. Animals recovered undisturbed for a week after implantation prior to testing and were given time to familiarize themselves with being connected. EMG electrodes were then tested for recording quality, and two electrodes in each cortical array with the lowest stimulation intensity required for target MEPs were determined prior to data collection (barring electrode failure, the same two were used for all the experiments for that rat).

Study Design and Experimental Paradigm

We used a repeated-measures randomized block design (same rat tested on all ISI conditions in a randomized order) to test the effect of STDP Timing Condition on the change in integral of the averaged MEP response after the PAS experiment.

We tested nine rats with chronic implants (the implant for one rat failed prior to data collection). Each rat was to be tested once in each condition. To account for possible order effects inherent to a within-subjects design, the order of testing conditions was randomly assigned using the *randperm* function in MATLAB (Extended Data for Figure 4-1). One condition (ISI -15 ms) was added at the end for six rats which had all data collection completed, based on another study (Zhang et al., 2018) which showed a promising timing condition and was published while data collection was in progress. For rats with whom data collection had not started yet, a re-randomization was performed to integrate this new condition. For each rat, each test was separated by roughly 24 hours, to minimize carryover effects between previous paired stimulation interventions. *A posteriori* analyses verified that there was no cumulative effect of PAS (Extended Data for Figure 4-4).

We tested four control conditions: three PAS controls, involving (1) cortical stimulation only, (2)-peripheral stimulation only, and (3) no stimulation, as well as (4) one extra-long ISI timing control involving paired stimulation of the motor cortex and the contralateral peripheral muscle offset by +505 ms. We reasoned that if timing during paired stimulation was the driving factor behind plasticity, and not the pairing of stimulation *per se*, this condition should have a null effect comparable to the previous control conditions.

Each experiment followed a fixed schedule (Figure 3 inset). After connecting the rat to the hardware interface, we completed three 5 minute “probes” to assess the corticomotor excitability prior to the PAS intervention (see following section). The probe was completed when 30 stimulations were delivered, or

5 minutes had elapsed, whichever came first. Probes were separated by 10 minutes each. After each PAS intervention, three post-PAS probes were completed in the same manner to assess excitability of the corticomotor system after paired stimulation. We allotted 2 minutes for wire switching and software changes, immediately before and after each PAS intervention.

Probe Assessment of Corticomotor Excitability

To assess corticomotor excitability before and after PAS, we compared the size of MEPs obtained from cortical stimulation using a closed-loop stimulation protocol (Figure 2C). As demonstrated by Darling et al. (2006), cortical stimulation during a low level of muscle contraction (5% or 10% of their maximal voluntary contraction) reduces MEP variability, compared to fully relaxed conditions. Drawing inspiration from this, we designed a protocol to stimulate during low levels of muscle contraction in the target muscle. To this end, the EMG activity in the target muscle was continuously measured, and cortical stimulation was triggered in real-time if the activity reached within [2 12] standard deviations above the baseline (defined as the mean value of the rectified EMG signal measured over two seconds when the limb was fully relaxed, during sleep or sustained rest behavior with no weight-bearing in each rat), and if the EMG activity was on the rising phase (contraction was being initiated during free behaviour as opposed to when the muscle was relaxing from a previously larger contraction). The baseline calibration was performed on each rat prior to data collection and recalculated as necessary if we suspected a change in baseline noise. This allowed us to customize the excitability assessment to each rat to adjust for slight differences in electrode placement or impedance across days. However, the EMG assessment window was never changed within a PAS experiment. The 2 to 12 SD range above baseline effectively restricted the conditions for stimulation within a low to moderate level of voluntary activation of the corticospinal system achieved during free behavior (walking/grooming/exploring). This approach provides the means to stimulate under consistent conditions of corticospinal activity, in an animal model where behavioural instructions cannot be clearly provided such as in human studies. To

do this, we used the *envelope* function in MATLAB, which calculated the peak envelope of the filtered data with a moving spline over the downsampled local maxima of the previous 32 data points. Cortical stimulation was contingent upon the EMG envelope crossing the pre-determined activity threshold. The variability was still high albeit reduced after applying the closed-loop stimulation protocol, so we averaged all three baseline measurements during the statistical analysis to obtain an overall assessment of corticomotor excitability prior to the PAS intervention. We recorded from different muscles, depending on the location where we obtained the best quality MEPs (Extended Data for Figure 4-2). We always used the same muscle involved in the PAS intervention to provide the closed-loop control for the corticomotor excitability assessments. When available, we chose the ECR as the PAS target muscle, but fell back on the biceps or trapezius respectively if electrode malfunctions or failure to evoke MEPs in the more distal muscles prevented their use. In one rat, we used a monopolar EMG recording configuration resulting in an EMG signal contaminated with crosstalk from cardiac activity. We manually adjusted the upper and lower limit of the EMG window enabling probe stimulation, in a manner that better reflected a low amplitude muscle contraction.

Electrophysiological Data Acquisition and Stimulation Configuration

Independent paired electrical stimulation protocols were achieved through two A-M Systems (Sequim, WA, USA) 2100 stimulators, each connected to separate pins on an *InVivo1* (Roanoke, VA, USA) commutator through a custom-made breakout board interface. Multi-channel recording was made possible by routing the EMG signal into a Brownlee Precision Model 440 (Santa Clara, CA, USA) Instrumentation Amplifier. Within this unit, a signal gain of 100, a bandpass filter between 50 Hz and 1.0 kHz was used for EMG, and bandpass of 1-300Hz for LFP signals. A 60Hz notch filter was applied. This output signal was split in two, with one copy being routed into a Powerlab 8/sp unit by AD Instruments (Colorado Springs, CO, USA), and further processed with a 10 Hz highpass filter before being saved. The second copy was routed into a National Instruments (Austin, TX, USA) Digital to Analog Converter (DAC)

SCB-68A system, which was operated via custom MATLAB software. We used the DAC system and MATLAB software to initiate all probe and PAS stimulation protocols, via the trigger input ports on the A-M systems stimulators.

Latency Measurements and Sign Convention for Spike Timing Experiments

To confirm the conduction latencies, we completed a series of acute experiments under anaesthesia, in rats with a similar weight and size to those used for chronic implants. First, to measure the antidromic conduction time in motoneurons between the muscle and the spinal cord, we performed an acute experiment under urethane anaesthesia to record and stimulate between the spinal cord and the ECR muscle, respectively. We exposed the dorsal spinal cord between the C4 and C6 regions by performing a laminectomy, and deafferented the C3 to C7 segments by cutting the dorsal roots, to isolate antidromic propagation instead of conduction along afferent sensory fibers. With the dura intact, we inserted a tungsten electrode, 127 µm in diameter in the C5 region ipsilateral to the right forelimb, 1.0 mm lateral to the midline. We also inserted a pair of EMG electrodes in the right *extensor carpi radialis* using the same method as in the chronic implants. Stimulation of the spinal cord C5 region using single pulses led to an isolated wrist extension in the rat's forelimb, verifying the location of the ECR motoneuron pool for efferent connections (Tosolini and Morris, 2012). Following this, we stimulated the EMG electrodes and recorded local field potentials (LFPs) from the electrode site in C5. Filtering parameters for the LFP recording included a bandpass of 1Hz-300Hz, with a 60Hz notch filter and a gain of 100. Data was averaged across 200 stimulations. We determined the antidromic motoneuronal propagation delay to be 3 ms (Extended Data for Figure 2-1A).

With an average MEP latency of 12 ms for ECR deriving from cortical stimulation the intact animal, and a 3 ms peripheral efferent conduction time, we estimated a latency of 9 ms for a cortical stimulation-induced descending volley to reach motoneurons, including synaptic integration time.

In a second acute experiment with a different animal under Ketamine/Xylazine anesthesia, we measured the time a neuronal volley requires to reach the cortex after muscle stimulation. In an intact animal, we recorded local field potential (LFP) responses in M1 following intramuscular stimulation (Extended Data for Figure 2-1B). We postulated that the peak of the initial negative inflection in the local field potential from contralateral muscle stimulation reflected the time at which the greatest neural activity is observed amongst the post-synaptic neurons in the cortex. Again, we inserted a pair of EMG electrodes in the right *extensor carpi radialis*, then inserted one platinum-iridium electrode 1.5 mm dorso-ventrally into M1, centered at the array coordinates of the rats involved in the PAS experiments. A reference electrode about 1 mm lateral from the first was positioned on the surface of the dura. Both cortical electrodes were connected by a common ground at the skull screw, and local field potentials (LFPs) were measured by calculating the voltage differential between the cortical electrodes with the same LFP recording parameters above. Stimulation was delivered to the EMG electrode in the right ECR through bipolar single pulses with a 0.2 ms duration, repeated at 0.5 Hz. Data was averaged across 360 stimulations. The afferent latency from ECR stimulation to the peak of the cortical evoked potential was 16 ms (Extended Data for Figure 2-1B).

Using these conduction latencies, we chose a set of stimulus intervals which would result in various pre- and post-synaptic timings relevant to the rules of spike-timing-dependent plasticity, either at the cortical and/or spinal levels. The full list of ISI conditions tested can be found in Extended Data for Figure 4-1. Our experimental design and results followed the convention that a **positive latency** means the

periphery was stimulated **after** the cortex by that time difference. These stimulation offsets lead to physiological offsets calculated at the levels of the spinal cord and cortex; positive latencies result in pre-synaptic activity that preceded post-synaptic activity at the specified location.

PAS Intervention

We used a PAS protocol of 300 paired stimulations to the motor cortex and designated peripheral muscle, using single pulses of biphasic electrical stimulation 0.2 ms in duration, separated by 0.5 Hz. We note that this is on the higher end in terms of number of paired stimulations compared to previous protocols, and is delivered at a higher frequency – but we reasoned, in the absence of evidence otherwise, that any effect that may be present due to paired stimulation should be enhanced using this slightly more intensive protocol.

Cortical stimulation intensity was set at 1.25 times the threshold for a MEP and muscle stimulation at 1.5 times the threshold to elicit a visible twitch (the mean motor threshold across all experiments was 790 µA for cortical stimulation and 1.8 mA for muscle stimulation). Thresholds were operationally defined as the minimal stimulation intensity required to induce a response more than 50% of the time. All PAS experiments were completed in the animals' home cage with a modified cover that enabled us to pass the tethering cable during free behavior (consisting mostly of walking, grooming, and exploring, sometimes sleeping).

MEP measurement

Raw EMG data were saved and processed offline in LabChart Version 7 and custom scripts written in MATLAB. We plotted all individual responses for each cortical stimulation and manually excluded trials for which there was significant excessive movement artifact, and/or lack of EMG signal (this was rare and the most likely reason was due to an intermittent connection with a faulty cable, which was

repaired or replaced promptly). The resulting set of verified MEPs for each probe were collected for further analysis.

MEP amplitudes were initially quantified with three different methods: (1) the peak-to-peak value of individual EMG responses (the literature standard), (2) the mean value of the integral of individual rectified EMG responses, measured over a tailored time window following stimulation, and (3) the integral of the averaged rectified EMG responses, over the same time window. Every individual response to cortical stimulation was first manually screened to exclude any EMG traces containing large movement artifacts or other obvious contamination. In pilot analyses (unpublished data) we assessed qualitatively that the calculation method did not much impact the normalized changes in the MEPs, so we proceeded with taking the integral of the average response for the probe (method 3 above). We reasoned that this approach was most effective in capturing both unimodal and multimodal MEP responses. This decision was made prior to the pooled study data analysis. In summary, the MEP values reported here were thus calculated by first rectifying the filtered EMG signal, then averaging the activity from all stimuli within a probe post-screening, and then calculating the integral of the resulting signal (Figure 3). The software described in the paper is freely available online at [URL redacted for double-blind review] (Windows 10). The code is available as Extended Data, if required.

[Figure 3 near here]

Statistical Analysis

Statistical analysis and visualization were completed using SAS Software, version 9.4 for Windows, Minitab 18 for Windows, and R 3.6.1/RStudio 1.2.5019 for Windows. We completed a mixed design ANOVA with repeated measures *on the normalized data* to test the main effects of ISI Timing Condition (CONDITION) with fifteen levels (one for each of 11 timings and four control conditions) as well as PAS Probe (SESSION) with three levels (2, 17 and 32 min after PAS). We also tested for any interaction effects

between CONDITION and SESSION. A random effect on the rat was used to account for the randomized block design. The level of significance for the mixed ANOVA was fixed at $p < 0.05$. Type III Fixed Effects are reported in Table 1, obtained through the Restricted Maximum Likelihood (REML) Estimation method. Data from one rat in the ISI +6 condition was removed from the statistical analysis because of poor data quality (very few MEPs in each probe). Normality was assessed on standardized residuals using graphical methods.

Results

Failure of Spike Timing to Modulate Cortical and Spinal Plasticity

We tested a wide range of STDP-relevant intervals between cortical and peripheral stimuli (ISI conditions), in a randomized fashion for each rat and used an EMG-controlled closed-loop method to measure pre- and post-intervention MEPs. We found no significant modulation of corticospinal excitability using our PAS intervention *in vivo*. We analyzed the MEP amplitudes obtained from cortical stimulation probes before and after each PAS intervention using a mixed model ANOVA on the normalized data. Probe time (SESSION) was considered a repeated-measures fixed factor, and ISI Condition (CONDITION) was the second fixed factor. We included a SESSION x CONDITION interaction term in the statistical model, and a random factor for the rat accounting for the randomized design. We did not find a significant effect of our PAS intervention for any of the timings we tested (Figure 4). Statistically, there was no significant interaction[a] between SESSION (pre- and post-intervention MEPs) and CONDITION ($F (28, 251) = 0.53$, $p = 0.98$)), meaning that there was no ISI condition for which the intervention resulted in a statistically significant change in corticospinal excitability over assessment time. There was no main effect of ISI CONDITION[b], $F (14, 254) = 1.56$, $p = 0.09$, Table 1b), independent of the time at which the MEP was measured post-intervention, indicating that the ISI condition did not have a significant effect on the MEP amplitude. There was no significant main effect of SESSION[c], F (2,

251) = 0.08, p = 0.921, Table 1c. Qualitatively, our PAS protocol did not induce changes in MEP size consistent with STDP in individual rats (Extended Data for Figure 4-3). In summary, our statistical analyses did not support the efficacy of PAS under these conditions.

[Figure 4 near here]

373 **Control Experiments**

374 Four different control protocols where we did cortical/muscle stimulation in isolation, no stimulation,
375 and maintained a large offset between paired stimuli, respectively did not significantly alter
376 corticomotor excitability (Figure 4, conditions to the right of vertical dotted line). Interestingly, we noted
377 a trend towards a depressive effect for the cortical stimulation only (mean ratio post/pre = 0.87) and ISI
378 +505 ms stimulation (0.84) conditions, but less so for the muscle stimulation only (0.94) and the no-
379 stimulation conditions (0.95).

380

381 **Discussion**

382 There exists a mixed literature on human PAS and several variations of the original protocol (Stefan et
383 al., 2000), with some convincing reports demonstrating its effectiveness for inducing at least transient
384 changes in corticomotor excitability (Taylor and Martin, 2009; Bunday et al., 2018), and others showing
385 ineffective interventions or highly subject-dependent results (Muller-Dahlhaus et al., 2008; McGie et al.,
386 2014). Our own results support the latter findings. Our PAS protocol, with parameters inspired by typical
387 interventions in humans, was ineffective at modulating plastic changes in corticospinal excitability.
388 There was no significant interaction between fixed factors, leading us to conclude that our PAS protocol
389 was ineffective overall in potentiating corticospinal connections.

390 **PAS Parameter Space**

391 Setting aside spike timing, the entire parameter space for a PAS intervention protocol is vast, with no
392 known physiological principles guiding a specific combination of stimulation intensity, frequency and/or
393 number of repetitions over another. Consistent with most studies, we chose above-threshold but sub-
394 maximal stimulation amplitudes (1.5x and 1.25x motor threshold for muscles and cortex respectively).

We decided on a PAS protocol with a number of paired stimulations (300) and stimulation frequency (0.5 Hz) on the higher end compared to most other published protocols (Suppa et al., 2017). We reasoned that if anything, this would enhance any PAS effects. It would be possible but counterintuitive that these differences reduced the likelihood of inducing plastic changes.

Overall Depressive Trend

We observed that MEPs after PAS interventions were generally smaller than the average of the baseline measurements. This trend was also present for control conditions involving cortical stimulation alone, but less so when the rats received no stimulation or muscle stimulation only in place of PAS. These observations can be apprised given the evidence that single pulses of peripheral electrical stimulation are insufficient to change corticomotor excitability with or without coincident voluntary contraction in humans (Saito et al., 2014), and that higher frequencies are needed for supraspinal effects (Grospretre et al., 2017). These results indicate that the probes themselves had no effects, but that all stimulation interventions involving cortical stimulation induced a trend towards an LTD-like effect. The effect was not statistically significant, but it would be consistent with depression of motor cortical excitability observed after low-frequency (1 Hz) TMS in humans (Chen et al., 1997). A small decrease in cortical excitability would have reduced any LTP-like effects and enhanced LTD-like effects induced by the PAS protocol as predicted by STDP. In other words, a general dampening cortical effect induced by the slow repetition of our stimulus pairs could prevent us from detecting any LTP-like effect but would presumably make PAS-induced LTD-like effects even more prominent. As our statistical analysis did not reveal any significant changes in MEP sizes in either direction, we conclude that our PAS intervention did not induce plastic changes following STDP rules.

Closed-loop Assessment

Another plausible explanation for our negative results is the high intrinsic variability observed in the MEP responses of our rats during free behaviour. Our EMG-based closed-loop pre- and post-intervention assessment probes were specifically designed to assess the excitability of the corticomotor system at relatively similar, low levels of EMG activity (approximately 5-15% of maximum EMG amplitude observed under free behavior). The aim was to minimize MEP variability, by avoiding stimulating in different conditions of corticomotor excitability, such as during a strong voluntary contraction or during reciprocal inhibition acting on the recorded muscle. However, the PAS intervention itself was not completed in an EMG dependent manner, because we could not record and stimulate muscles simultaneously with our setup. Perhaps applying the closed-loop approach to the paired stimulation as well, would have allowed for a more systematic and reproducible recruitment of neuronal elements, thereby leading to more reliable PAS effects.

Stimulation Models and Specificity

In our chronic PAS model, we inserted electrodes directly into the target muscle, and validated this approach in an acute experiment to verify that electrical stimulation of the muscle fiber was sufficient to generate antidromic volleys back-propagating to the deafferented spinal cord. Compared to direct nerve stimulation, intramuscular stimulation may result in a small difference in the relative timing of stimulation-induced antidromic motoneuron activation and orthodromic afferent activity. In addition, direct nerve stimulation can recruit a larger number of fibers of all modalities, not limited to a specific target muscle, but including all motor and sensory fibers traveling in the nerve at the chosen stimulus location. These differences in peripheral fiber recruitment may have contributed to the apparent inconsistency between our results and that of others showing the effectiveness of PAS using electrical stimulation in rodents (Mishra et al., 2017).

With respect to the PAS literature, we can hypothesize there may be intrinsic differences in MEP variability between non-invasive (TMS, the standard technique for PAS in humans) and invasive (Intracortical Microstimulation, ICMS) stimulation methods due to different circuits being recruited. ICMS, although having a greater spatial and temporal resolution than non-invasive methods of neural activation such as TMS, is also non-specific in the sense that it activates all types of neurons and other cell types. The exact recruitment patterns of the cortical circuits are of very little theoretical importance for PAS interventions targeting spinal circuits, as long as a corticospinal volley occurs in a timely manner relative to peripheral stimulation. Therefore, especially since it was previously used successfully in rats (Mishra et al., 2017), it would be surprising if our use of ICMS was factor explaining our negative results.

Some electrophysiology studies have suggested there are both monosynaptic and polysynaptic connections onto rats' corticospinal motoneurons (Elger et al., 1977; Liang et al., 1991; Hori et al., 2002), but more recent work has suggested the rat corticospinal tract is exclusively polysynaptic (Alstermark et al., 2004). Although this is a major physiological difference between rats and primates, we believe that this is not a critical factor to explain differences between our negative results and successful human PAS. In addition to the variable conduction time between individual fibers, a polysynaptic pathway will increase the temporal spread of action potentials in a stimulation-induced volley. Furthermore, the ascending afferent sensory pathway in humans is polysynaptic (Abraira and Ginty, 2013), and yet PAS is still effective when TMS is timed with the arrival of afferent volleys in the cortex (Stefan et al., 2000). By the same token, we expected that a polysynaptic descending pathway would not prevent us from timing the descending volley with antidromic motoneuron activation.

Opposing Plastic Changes Along the Corticomotor Pathway

Thinking along these lines, however, the ISI timing offset of the paired stimulation dictates the target location of plasticity. In an ideal world, the effects will be localized only to one target area. However,

462 since the corticomotor contains multiple synaptic connections, any given ISI condition predicted to
463 induce LTP-like changes at one site according to Hebbian STDP (the motor cortex for example), could
464 lead to LTD-like effects at the second site (the spinal cord for instance), and vice versa. In rats, we
465 estimated the interval between PAS-induced pre- and post-synaptic activity at the cortex and the spinal
466 cord for given cortical and peripheral stimulation intervals. These opposing effects are reflected in the
467 lack of situations where LTP-like effects (numbers shaded in red regions) can be predicted at both spinal
468 and cortical levels (Figure 4). This competition between potentiation and depression at different
469 locations may reduce the PAS effectiveness to induce a net increase in corticospinal excitability. Due to
470 the non-invasive nature of human PAS experiments, this can potentially be an explanatory factor for the
471 variance in PAS effectiveness observed in the clinical data. This issue can be dissected in animal models
472 with terminal experiments, but addressing this issue *in vivo* will require advances in our stimulation
473 methods to be simultaneously non-invasive, yet highly spatially specific. The goal here would be to
474 isolate the bookends of the paired stimulation just bounding the targeted synapses. That would be a
475 seminal advance in addressing the utility of PAS *in vivo*.

476 **Seeking the Perfect Storm**

477 Voluntary effort itself has been shown to be a necessary driver for potentiation in humans for specific
478 PAS protocols (Kujirai et al., 2006), with two proposed mechanisms being the reduction of intracortical
479 inhibition networks coincident with contraction, or the facilitatory effect of attention via the activation
480 of memory systems (Stefan et al., 2004), but this is contradictory to earlier cited findings that PAS works
481 well under anaesthesia in animals (despite the differences among species). The effect of known
482 neuromodulators on PAS, such as dopamine, should not be underestimated particularly because of its
483 direct role in mediating neuronal potentiation (Yagishita et al., 2014), and its broader implications in
484 maintaining attention (Suppa et al., 2017). Additional factors influencing PAS effectiveness are
485 numerous, and may include even the time of day – a study in humans showed that PAS sessions

performed in the afternoon were significantly potentiated in one study, whereas sessions completed in the morning did not (Sale et al., 2007). In that paper, variance was attributed to circadian effects and specifically the inhibitory effect of cortisol on plasticity. These examples drive home the point that our knowledge of what coincident factors are required to induce LTP-like potentiation remains limited, and based on our study, future studies should likely *not* be restricted to simple application of Hebbian principles; it may not be enough.

PAS has also been reported to exhibit high variance depending on the subject being tested. McGie et al. (2014) conducted a non-invasive PAS study in humans, with the goal of comparing different paired stimulation protocol frequencies (McGie et al., 2014). Tarri et al. (2018) studied the effect of PAS in humans as a therapeutic adjunct to stroke using a randomized double-blind controlled approach (the CIPASS Trial) (Tarri et al., 2018b). Both groups reported high between-subject variability in PAS outcomes but found no consistent effect of PAS targeting spinal circuits, attributing the variability observed to individual factors such as the lesion size / location, and different rehabilitation intensiveness, both influencing the physiological capacity available for PAS effects. Importantly, the degree of muscle facilitation can vary greatly even *within the same participants* across repeated PAS sessions (Tarri et al., 2018b). These studies emphasize the mercurial nature of PAS effectiveness even within individuals, and the highly stereotyped/specialized conditions necessary for consistent beneficial effects to become apparent. It may turn out that a conjunction of multiple concurrently acting factors is necessary in order to facilitate PAS potentiation under free behavior in animals.

Conclusion

In conclusion, our data does not support the effectiveness of PAS in promoting plasticity through the Hebbian STDP model in freely behaving rodents. Our initial goal was to develop a clinically relevant animal model for paired stimulation which would have allowed more detailed studies and optimize

interventions. Although the model itself was developed successfully, this series of experiments suggested that an open-loop PAS intervention in a freely moving animal is not effective to reliably drive plasticity in the corticospinal system. Our results highlight the complexity of associative plasticity and demonstrate that forced coincidence of neuronal activity is not sufficient to reliably potentiate corticospinal excitability. Future research will need to investigate whether other variations in the PAS parameter space, reduction of interference from ongoing neuronal activity or manipulations of neuromodulators may be required to drive corticospinal potentiation more reliably. This will determine whether PAS indeed has potential as an interventional measure for modulating corticomotor plasticity.

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614

615 Figure and Table Legends

616 **Figure 1: Spike-Timing-Dependent Plasticity (STDP) Rules.** At the synaptic level, but to a lesser extent at
617 the systems level, it has been demonstrated that the relative timing of activity between the pre-synaptic
618 neuron and post-synaptic neuron is crucial for plasticity. When pre-synaptic activity repetitively occurs
619 within several milliseconds prior to post-synaptic activity, Long-Term Potentiation (LTP, red) is induced.
620 When the timing is reversed, Long-Term Depression (LTD, blue) is induced. The potential for LTP or LTD
621 decreases as the time window between the pre- and post-synaptic activity at the synapse increases
622 (Song et al., 2000).

623

624 **Figure 2: Experimental Showcase.** A: Rats were chronically implanted with three pairs of subcutaneous
625 stainless steel microwires to stimulate and record from the Extensor Carpi Radialis (ECR), Trapezius
626 (Trap), and Biceps (Bi) muscles contralateral to the cortical array. PAS = Paired Associative Stimulation.
627 B: Dorsal view of the rat brain, showing where we inserted the 2x2 platinum-iridium electrode array in
628 the caudal forelimb area (CFA) of M1. Coordinates are anterior (A) and lateral (L) relative to Bregma (B).
629 C: Corticomotor excitability was assessed before and after PAS using closed-loop, EMG-controlled motor
630 cortical stimulation. The upper envelope (red) of the EMG signal was calculated in pseudo real-time on
631 the computer controlling data acquisition and stimulation using MATLAB's envelope function. Cortical
632 stimulation was invoked when the envelope rose within 2 to 12 standard deviations above the mean
633 signal (green horizontal lines) for at least 50 ms. The minimum time between stimulations was 1 second.
634 D: We know from our and previous studies under anaesthesia that it takes approximately 9 ms and 3 ms
635 for signals issued from cortical and peripheral stimulation to arrive at the spinal cord, respectively.
636 Peripheral stimulation of afferent fibers result in a volley of motor cortical activity after 16 ms. The pre-
637 and post-synaptic activity offset at the levels of spinal cord and motor cortex for different inter-stimulus

intervals were calculated based on these conduction latencies. **Extended Data for Figure 2-1: Spinal and Cortical Evoked Potentials from Peripheral Stimulation. A:** the latency of the deepest trough response at 3 ms in the C5 region of the spinal cord, averaged across 200 stimulations. Note that a DC offset in the baseline signal was manually adjusted here. **B:** measuring the latency of the deepest trough response at 16 ms in the cortex, averaged across 360 stimulations.

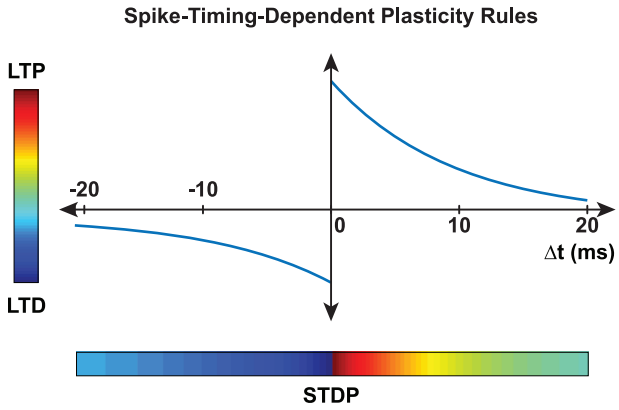
Figure 3: Example Experiment-Level Result, MEPs recorded in the Right ECR of one rat, obtained from electrical cortical stimulation for each probe. Shaded areas in light blue and red indicate 1 standard deviation about the mean. Inset: Each session began with three 5-minute probes in which we performed closed-loop EMG-dependent cortical stimulation to assess baseline MEP amplitudes, each separated by 10 minutes. The PAS session itself, involving 300 pairs of stimuli to the cortex and the muscle at a rate of 0.5 Hz, took about 10 minutes. This was followed by three post-PAS probes so we could assess corticospinal excitability up to 30 minutes after paired stimulation for each inter-stimulus interval. After each experiment, we manually verified all MEPs using custom software and excluded traces with movement artifacts or noisy EMG signals.

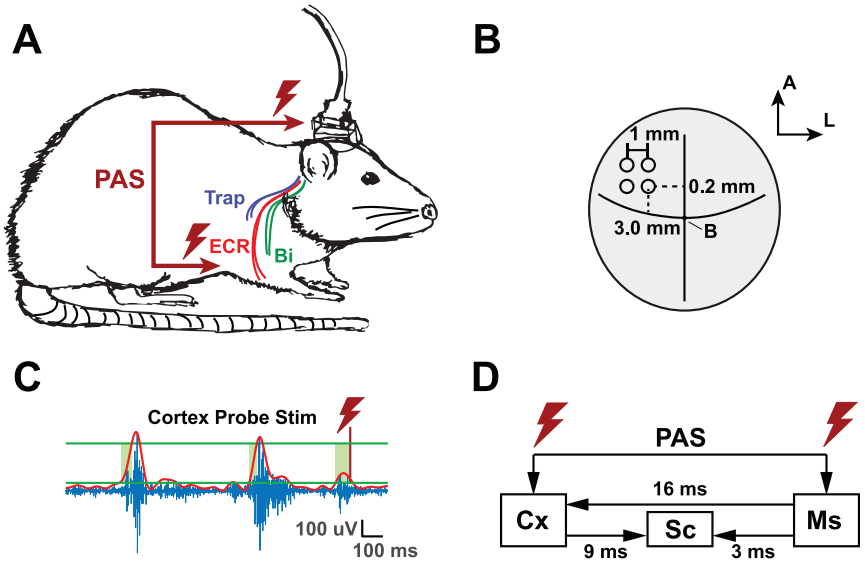
Figure 4: PAS does not significantly potentiate MEP responses *in vivo.* Grouped bar plot, depicting that for Post 1 (2 min after PAS, light red), Post 2 (17 min after PAS, medium red), and Post 3 (32 min after PAS, dark red) sessions, there were no significant differences between STDP experimental conditions and control conditions. Error bars are 95% Confidence Intervals about the mean. The horizontal reference line marked in red signifies no change between that post condition and the baseline average. Control conditions are shown to the right of the vertical dotted black line, to separate them from the ISI conditions tested to the left (Cx = Cortical Stimulation Only, Ms = Muscle Stimulation Only, No = No

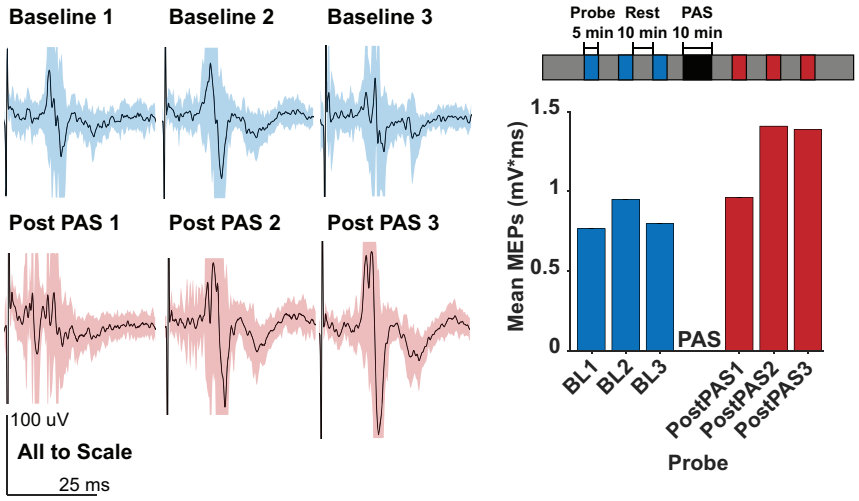
Stimulation). ISI refers to the latency between stimulation of the Cortex and the Muscle. Here, positive numbers refer to muscle stimulation occurring after cortical stimulation. “Spinal Cord” numbers are the estimated latencies between the arrival of the descending volley onto the motoneurons in the spinal cord and the arrival of the antidromic action potentials evoked from muscle stimulation (positive if orthodromic arrives before antidromic); “Motor Cortex” numbers are the estimated latencies between the arrival in M1 of peripheral stimulation-induced afferent activity and the motor cortex stimulation (positive if peripheral afferent signal arrives before cortex stimulation signal). The colors on the horizontal bars at the bottom indicate conditions expected to induce LTP-like effects (red), LTD-like effects (blue) or no significant modulation (green) based on the Hebbian STDP model. **Extended Data for Figure 4-1: Study Design for Chronic Experiments** and *a priori* Randomization Order (number within each cell). The final sample sizes for each PAS condition are written to the left. Sessions used in the final dataset are shaded in grey. **Extended Data for Figure 4-2: Muscle Distribution**. Distribution of muscles used as PAS target (ISI Conditions, Latencies are Stimulation Offsets). * = PAS muscle stimulation component was between one EMG electrode and reference. **Extended Data for Figure 4-3: No Individualized Effect of PAS.** Analogous plot to Figure 4, but paneled by rat, demonstrating that there was no effect consistent with STDP from our PAS intervention even on the level of individual animals. **Extended Data for Figure 4-4**: **No Cumulative Effect of PAS**. Baseline MEP amplitude on days immediately following a PAS session was not correlated to MEP changes in that session. The “Within-Session MEP Ratio” is defined as the normalized change in averaged corticomotor excitability across a given PAS intervention WITHIN an experimental day (N), and the “Next-Day Baseline MEP Ratio” is defined ibid but BETWEEN the baseline average on day N, and the baseline average on day N + 1 (consecutive calendar day). While processing the data BETWEEN days, we ensured that there were no changes in cortex stimulation intensity, muscle stimulation intensity, the electrode leads used for both sites, the type of EMG recording (mono- or bi-polar), and the EMG range for closed-loop stimulation. If

any of these parameters changed between days, those data points were excluded. This ensured full homogeneity in the stimulation conditions used to calculate the appropriate quotients. All of the above parameters were held constant by design within a particular experimental day. By testing the correlation between these two ratios, we could directly assess whether the change induced by any particular PAS protocol on a given day is related to the change in baseline excitability across days. The two variables were not strongly correlated ($r = 0.18$) and the relationship was not significant ($p=0.21$), even after winsorization to remove outliers ($p=0.19$), confirming there was no carry-over effect of our PAS intervention.

Table 1 Legend: Mixed Effects Model Analysis, Fixed Effects (Type III) using the Restricted Maximum Likelihood Estimation (REML) Method







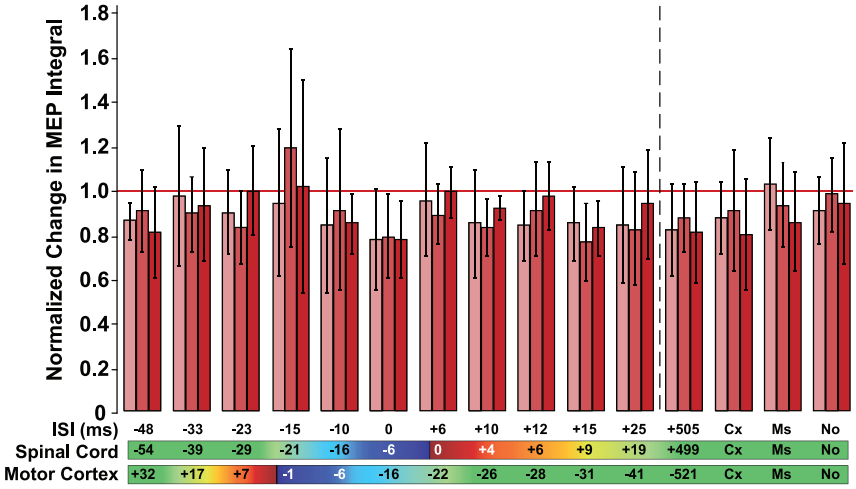


Table 1: Summary of Statistical Analyses

Figure	Type of Test	Term	Data Structure	DF Num	DF Den	F-Value	P-Value
3	Mixed Effects ANOVA	a. Session*Condition	Model Residuals Normal	28.00	250.92	0.53	0.976
		b. Condition		14.00	253.60	1.56	0.092
		c. Session		2.00	250.92	0.08	0.921