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

Windsor Kwan-Chun Ting[1], Maxime Huot-Lavoie[1,2], Christian Ethier[1]*
[1]Centre de Recherche CERVO, Département de psychiatrie et de neurosciences, Université Laval
[2]Centre de recherche de l'Hôtel-Dieu de Lévis, CISSS Chaudière-Appalaches, Lévis, QC, Canada.

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 the phenomenon of PAS-induced plasticity is needed to fully harness it as a clinical tool. Here, we tested a PAS model in awake rats in order to study the principles of associative plasticity. Using chronically implanted electrodes in motor cortex and forelimb, we explored PAS parameters to effectively change corticomotor excitability, assessed using closed loop, EMG-controlled cortical stimulation. We tested eleven stimulation 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.

Keywords:
Paired Associative Stimulation (PAS); Spike-Timing-Dependent Plasticity (STDP); Hebbian; Plasticity; Motor Cortex; Spinal Cord; Rodent Models

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Figure 1: Illustration depicting PAS, with diagram illustrating conduction latencies used in the STDP Experiments. Inset: Schematic of electrode array used in chronic experiments, overlaid on top of a contour of rat brain dorsal view.
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***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

Introduction

Spike Timing as a Driver of Synaptic Plasticity

Donald Hebb first postulated that coincident activity between active neurons could induce a change in the strength of their connecting synapses (Hebb 1949). The propensity to strengthen or weaken synapses based on coincident activity could be a model of learning, paving a mechanistic foundation for connecting biology with behaviour. At the outset however, it was clear that Hebbian learning principles would need to be further refined to accurately describe biological phenomena at the synapse. Seminal studies in spike-timing-dependent plasticity (STDP) (Markram, Lubke et al. 1997, Bi and Poo 1998) have been conducted to develop models of this plasticity at the synaptic level. Whether synaptic potentiation (Long Term Potentiation, LTP) or depression (Long Term Depression, LTD) occurs is contingent upon the pattern of firing activity in the pre- and-post-synaptic neurons (Cooper 2005).

The Hebbian STDP model posits that activity in the pre-synaptic neuron should occur prior to activity in the post-synaptic neuron for facilitation to occur at the target synapse (Feldman 2012). One advantage of the Hebbian STDP model is that accidental coincidences in pre- and post- synaptic firing do not significantly change the strength of the synapse, and uncorrelated activity will lead to a weakening of the connection (Song, Miller et al. 2000). It suggests a method of neuromodulation, making conceivable the artificial restructuring of cortico-cortical and cortico-spinal circuits involved in motor control towards more beneficial and functional connections by inducing repetitive paired activity at a desired set of synapses. This may even represent a framework for harnessing beneficial plasticity after cerebrovascular or traumatic insult.

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. Hence, although STDP has been well-studied at the synaptic scale, to really harness this model effectively, we need to study it more systematically at the systems level in conjunction with ongoing neuronal activity.

Non-invasive Paired Associative Stimulation (PAS) in Humans

Paired Associative Stimulation (PAS) is a non-invasive method of modulating the excitability of the motor cortex and downstream corticospinal connections to facilitate the recruitment of targeted muscles, based on Hebbian STDP principles. The idea is that repeated coincidences of stimulation-induced activity in targeted areas promotes strengthening of useful neural connections within the sensorimotor system.

The first clear demonstration of PAS in humans was designed to promote plasticity at the cortical level (Stefan, Kunesch et al. 2000). Stefan et al. timed Transcranial Magnetic Stimulation (TMS) with the cortical arrival of afferent activity induced by electrical stimulation of the median nerve, which elicited an increase in cortical excitability. This was assessed by comparing resting motor evoked potential (MEP) amplitude from TMS stimulation in the abductor pollicis brevis (APB) muscle of healthy humans, before and after intervention. The results indicated that topographically specific and sustained (30-60 min) increases in excitability of the motor system was possible through non-invasive PAS in man, by carefully timing cortical stimulation with somatosensory signals afferently propagated towards the cortex.

Taylor and Martin performed the first study demonstrating in humans that PAS could also be used to induce a plasticity in the spinal circuits (Taylor and Martin 2009). They demonstrated that by timing peripheral nerve stimulation so that antidromic potentials in motoneurons reached the cell bodies in the spinal cord one millisecond after the arrival of TMS-induced corticospinal volleys, they could induce a sustained potentiation of the corticomotor circuits controlling the biceps brachii muscle. Since that time, several studies have attempted to validate this phenomenon with mixed success, and studied human PAS to target plasticity in the neural circuits controlling upper and lower limbs of man; see (Carson and Kennedy 2013, Suppa, Quartarone et al. 2017) for reviews on the subject.

PAS is seen as a potential tool to strengthen residual circuits after neurological injury and promote functional recovery. In humans, PAS has been studied as a potential therapeutic intervention. Bunday et al. (2012) studied the potential to use PAS to reshape functional corticospinal connections in human patients with spinal cord injury (Bunday and Perez 2012). Pairing TMS over the motor cortex and Peripheral Nerve Stimulation (PNS) of the ulnar nerve, they were able to potentiate the MEPs and maximal voluntary contraction force obtained in the first dorsal interosseous muscle using an STDP-based protocol. The effectiveness of a subsequently modified PAS protocol in increasing voluntary force output was replicated in the tibialis anterior hindlimb muscle of the participants as well (Urbin, Ozdemir et al. 2017). Subsequent research has extended modified PAS protocols with mixed success in improving functional recovery after neurovascular insult, both in animals (Shin, Han et al. 2008) and in humans (Castel-Lacanal, Gerdelat-Mas et al. 2007, Castel-Lacanal, Marque et al. 2009, Rogers, Brown et al. 2011, Cho, Sabathiel et al. 2016, Ferris, Neva et al. 2018, Palmer, Wolf et al. 2018, Tarri, Brihmat et al. 2018). PAS initially showed great promise for rehabilitation, however enthusiasm for this approach has been dampened by inconsistent results. PAS was shown to have a very high inter-subject variability (Sale, Ridding et al. 2007, McGie, Masani et al. 2014, Tarri, Brimhat et al. 2018), its effects are strongly dependent on mindful, persistent attention on the target limb (Stefan, Wycislo et al. 2004) or even failed to induce any consistent plastic effects (McGie, Masani et al. 2014).

Invasive PAS in Animals

To better characterize the effects of PAS and clarify its potential as a therapeutic tool, research groups have more recently begun investigating this approach in animal models. Mishra et al. (2017) performed cervical epidural stimulation in conjunction with stimulation in the rodent motor cortex, to create sustained increases in biceps MEPs arising from cortical stimulation (Mishra, Pal et al. 2017). However, these experiments were done under anaesthesia, leaving open the possibility of pharmacological effects of Ketamine/Xylazine preparations (Yang, Chang et al. 2011, Huang and Yang 2015) and thereby reducing the ecological validity of the results. Non-invasive PAS in restrained, awake rats can drive potentiation of the *hindlimb* corticospinal circuits using stimulation intervals between cortex and tibial nerve of 5 and 15 ms (Zhang, Sui et al. 2018). A non-invasive model under anaesthesia has also been used to investigate PAS for therapeutic improvements after stroke, using TMS over the rat cortex and coincident peripheral nerve stimulation using electrodes inserted into the contralateral soleus hindlimb muscle (Shin, Han et al. 2008), finding that specific interstimulus-intervals could potentiate MEP amplitudes. Clearly, there has been a convergence of research in recent decades, from both clinical and fundamental sciences towards applying timed stimulation to the motor system to promote plasticity.

Objective

The project aimed to establish an effective animal PAS paradigm in the *forelimb* using an awake and invasive rat model, to better understand the fundamental mechanisms of associative plasticity and ultimately to improve interventions in humans for neurorehabilitation.

Hypotheses and Predictions

We hypothesized that we could use PAS to modulate corticomotor excitability toward target contralateral muscles in the forelimbs of rats. Further, based on Hebbian principles we predicted that LTP-like plasticity would be induced with spike timing conditions where the pre-synaptic descending volley arrived at the level of the spinal cord a few milliseconds *prior to,* or *at the same time as* the antidromic post-synaptic volley arising in motoneurons from peripheral stimulation of the target muscles. By holding other PAS parameters constant (stimulation amplitude, frequency, number of pulses), we tested PAS protocols in a randomized trial and predicted that different stimulation intervals would induce systematic changes in corticomotor excitability as predicted by the STDP model.

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 2). Statistically, there was no significant interaction between SESSION (pre- and post-intervention MEPs) and CONDITION ($F(28, 245) = 0.53$, $p > 0.05$), Table 1), 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 a significant main effect of ISI Condition however, $F(14, 248) = 1.74$, $p = 0.049$), independent of the time at which the MEP was measured post-intervention, indicating that the ISI condition had a barely significant effect on the MEP amplitude. However, a two-way Dunnett's test used *a posteriori* to make the relevant pairwise comparisons between the no stimulation control condition and all other STDP and PAS control conditions, while controlling for family-wise error rate at 5% did not detect significant differences. There was no significant main effect of SESSION, $F(2, 245) = 0.12$, $p > 0.05$. In summary, our statistical analyses did not support the efficacy of PAS under these conditions.

Control Experiments

Four different control protocols where we did cortical/muscle stimulation in isolation, no stimulation, and maintained a large offset between paired stimuli, respectively did not significantly alter corticomotor excitability (Figure 2, conditions to the right of vertical dotted line). Interestingly, we noted a trend towards a depressive effect for the cortical stimulation only (mean ratio post/pre = 0.86), muscle

stimulation only (0.93), and ISI +505ms stimulation (0.85) conditions, but not in the control condition for which rats received no stimulations at all in place of a PAS intervention (0.98).

Discussion

There exists a mixed literature on human PAS and several variations of the original protocol (Stefan, Kunesch et al. 2000), with some convincing reports demonstrating its effectiveness at inducing at least transient changes in corticomotor excitability (Taylor and Martin 2009, Bunday, Urbin et al. 2018), and others showing ineffective interventions or highly subject-dependent results (Muller-Dahlhaus, Orekhov et al. 2008, McGie, Masani et al. 2014). Our own results support the latter findings. Our PAS protocol, with parameters inspired by typical interventions in humans, was ineffective at modulating plastic changes in corticospinal excitability. There was no significant interaction between fixed factors, and the critical comparisons between the hypothesized potentiating ISI conditions with the no stimulation control condition were all non-significant, leading us to conclude that our PAS protocol was ineffective overall in potentiating corticospinal connections.

PAS Parameter Space

Setting aside spike timing, the entire parameter space for a PAS intervention protocol is vast, with no known physiological principles guiding a specific combination of stimulation intensity, frequency and/or number of repetitions over another. Consistent with most studies, we chose above-threshold but sub-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, Quartarone 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.

Neuromuscular Fatigue

We observed that MEPs after PAS interventions were generally smaller than the average of the baseline measurements. This trend was surprising 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, Sugawara et al. 2014), and that higher frequencies are needed for supraspinal effects (Grospretre, Gueugneau et al. 2017). This trend was also present for control conditions involving cortical or muscle stimulation alone, but not when the rats received no stimulation in place of PAS. These results indicate that the probes themselves had no effects, but that all stimulation interventions induced a low level of spinal and/or supraspinal fatigue, in a manner similar to that of a sustained fatiguing muscle contraction, which has been shown to evoke a reduction in TMS-induced MEP size (McKay, Tuel et al. 1995, Pitcher and Miles 2002). Finally, the physiological recruitment order of motoneurons using electrical stimulation is also inversed compared to natural activation patterns (Hennings, Kamavuako et al. 2007), so this may have contributed to the fatigue-like responses we observed throughout.

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. While we attempted to reduce this through our closed-loop stimulation design, enabling us to probe the corticomotor system only when the pooled

208 motoneuron output (EMG) was within a fixed range of activity, it nevertheless remained high. Our EMG-
209 based closed-loop pre- and post- intervention assessment probes were specifically designed to assess
210 the excitability of the corticomotor system at relatively similar, low levels of EMG activity (approximately
211 5-15% of maximum EMG amplitude observed under free behavior). The aim was to minimize MEP
212 variability, by avoiding stimulating in different conditions of corticomotor excitability, such as during a
213 strong voluntary contraction or during reciprocal inhibition acting on the recorded muscle. However, the
214 PAS intervention itself was not completed in an EMG dependent manner, because we could not record
215 and stimulate muscles simultaneously with our setup. Perhaps applying the closed-loop approach to the
216 paired stimulation as well, would have allowed for a more systematic and reproducible recruitment of
217 neuronal elements, thereby leading to more reliable PAS effects.

218 **Stimulation Models and Specificity**

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

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

239 Some electrophysiology studies have suggested there are both monosynaptic and polysynaptic
240 connections onto rats' corticospinal motoneurons (Elger, Speckmann et al. 1977, Liang, Moret et al.
241 1991, Hori, Carp et al. 2002) , but more recent work has suggested the rat corticospinal tract is
242 exclusively polysynaptic (Alstermark, Ogawa et al. 2004). Although this is a major physiological
243 difference between rats and primates, we believe that this is not a critical factor to explain differences
244 between our negative results and successful human PAS. In addition to the variable conduction time
245 between individual fibers, a polysynaptic pathway will increase the temporal spread of action potentials
246 in a stimulation-induced volley. The ascending afferent sensory pathway in humans is polysynaptic
247 (Abraira and Ginty 2013), and yet PAS is still effective when TMS is timed with the arrival of afferent
248 volleys in the cortex (Stefan, Kunesch 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, since the corticomotor contains multiple synaptic connections, any given ISI condition predicted to induce LTP-like changes at one site according to Hebbian STDP (the motor cortex for example), could lead to LTD-like effects at the second site (the spinal cord for instance), and vice versa. In rats, we estimated the interval between PAS-induced pre- and post-synaptic activity at the cortex and the spinal cord for given cortical and peripheral stimulation intervals. These opposing effects are reflected in the lack of situations where LTP-like effects can be predicted at both spinal and cortical levels, represented by numbers in green (Figure 2). This competition between potentiation and depression at different locations may reduce the PAS effectiveness to induce a net increase in corticospinal excitability. Due to the non-invasive nature of human PAS experiments, this can potentially be an explanatory factor for the variance in PAS effectiveness observed in the clinical data. This issue can be dissected in animal models with terminal *ex vivo* experiments, but addressing this issue *in vivo* will require advances in our stimulation methods to be simultaneously non-invasive, yet highly spatially specific. The goal here would be to isolate the bookends of the paired stimulation just bounding the targeted synapses. That would be a seminal advance in addressing the utility of PAS *in vivo*.

Seeking the Perfect Storm

Voluntary effort itself has been shown to be a necessary driver for potentiation in humans for specific PAS protocols (Kujirai, Kujirai et al. 2006), with two proposed mechanisms being the reduction of intracortical inhibition networks coincident with contraction, or the facilitatory effect of attention via the activation of memory systems (Stefan, Wycislo et al. 2004), but this is contradictory to earlier cited findings that PAS works well under anaesthesia in animals (despite the differences among species). The effect of known neuromodulators on PAS, such as dopamine, should not be underestimated particularly because of its direct role in mediating neuronal potentiation (Yagishita, Hayashi-Takagi et al. 2014), and its broader implications in maintaining attention (Suppa, Quartarone et al. 2017). Additional factors influencing PAS effectiveness are 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, Ridding 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, Masani 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, Brimhat et al. 2018). 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, Brimhat et al. 2018). 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.

Methods

Animals and Surgical Preparation

All animal handling and experimentation were performed in accordance with institutional review board (Comité de Protection des Animaux de l'Université Laval), and guidelines from the Canadian Council on Animal Care. Nine Long Evans rats and one Sprague-Dawley rat (all male) were housed under 12/12 inverse light-day cycle with food and water available *ad libitum*. 150 experimental sessions were planned in ten animals, to investigate the effectiveness of 15 inter-stimulus intervals (ISI), including controls. 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 (Supplementary Table 1). Our PAS intervention typically targeted the *extensor carpi radialis* 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 Supplementary Table 2.

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. The array was inserted into the caudal forelimb area (CFA) of the primary motor cortex (M1) by a stereotaxic craniectomy, centered at 1mm anterior and 3.5mm lateral relative to bregma, and the surrounding exposed dura matter was covered with silicone gel for protection. Three pairs of PFA-coated multi-stranded stainless-steel wire electrodes (A-M Systems Inc.) were inserted into the contralateral *extensor carpi radialis (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. See the Figure 1 inset for a visual schematic of the electrode array as well as the implant location. Animals recovered undisturbed for a week after implantation prior to testing and were given time to familiarize themselves with being connected prior to data collection.

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 ten rats with chronic implants. 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. One condition (ISI -15ms) was added at the end for six rats which had all data collection completed, based on another study (Zhang, Sui 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.

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 PAS control conditions.

Each experiment followed a fixed schedule (see Figure 3). 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. 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] SD above the baseline (defined as the mean value of the rectified EMG signal measured over two seconds when the limb was fully relaxed), and the if 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). We thought that restricting the conditions for stimulation to a low-moderate level of voluntary activation of the corticospinal system achieved during free behavior (walking/grooming/exploring) would reduce the baseline MEP variability observed with cortical

371 stimulation (Darling, Wolf et al. 2006). To do this, we used the *envelope* function in MATLAB, which
372 calculated the peak envelope of the filtered data with a moving spline over the local maxima of the
373 previous 32 data points. Cortical stimulation was contingent upon the EMG envelope crossing the pre-
374 determined activity threshold. The variability was still high albeit reduced after applying the closed-loop
375 stimulation protocol, so we averaged all three baseline measurements during the statistical analysis to
376 obtain an overall assessment of corticomotor excitability prior to the PAS intervention. We recorded
377 from different muscles, depending on the location where we obtained the best quality MEPs
378 (Supplementary Table 2). We always started with the most distal forelimb muscles (the extensors) and
379 moved proximally for each rat, as necessary. In one rat, we used a monopolar EMG recording
380 configuration resulting in an EMG signal contaminated with crosstalk from cardiac activity. We manually
381 adjusted the upper and lower limit of the EMG window enabling probe stimulation, in a manner that
382 better reflected a low amplitude muscle contraction.

383 *Electrophysiological Data Acquisition and Stimulation Configuration*

384 Independent paired electrical stimulation protocols were achieved through two A-M Systems (Sequim,
385 WA, USA) 2100 stimulators, each connected to separate pins on an *InVivo1* (Roanoke, VA, USA)
386 commutator through a custom-made breakout board interface. Multi-channel recording was made
387 possible by routing the EMG signal into a Brownlee Precision Model 440 (Santa Clara, CA, USA)
388 Instrumentation Amplifier. Within this unit, a signal gain of 100, a bandpass filter between 50 Hz and 1.0
389 kHz was used for EMG, and bandpass of 1-300Hz for LFP signals. A 60Hz notch filter was applied. This
390 output signal was split in two, with one copy being routed into a Powerlab 8/sp unit by AD Instruments
391 (Colorado Springs, CO, USA), and further processed with a 10 Hz highpass filter before being saved. The
392 second copy was routed into a National Instruments (Austin, TX, USA) Digital to Analog Converter (DAC)
393 SCB-68A system, which was operated via custom MATLAB software. We used the DAC system and
394 MATLAB software to initiate all probe and PAS stimulation protocols, via the trigger input ports on the
395 A-M systems stimulators.

396 *Latency Measurements and Sign Convention for Spike Timing Experiments*

397 To confirm the conduction latencies, we completed a series of acute experiments under
398 ketamine/xylazine and urethane anaesthesia, in rats with a similar weight and size to those used for
399 chronic implants. We performed an acute laminectomy under urethane anaesthesia to measure the
400 antidromic conduction time in motoneurons between the muscle and the spinal cord. We exposed the
401 dorsal spinal cord between the C4 and C6 regions. With the dura intact, we inserted a tungsten
402 electrode, 127 um in diameter in the C5 region ipsilateral to the right forelimb, 1.0 mm lateral to the
403 midline. We also inserted a pair of EMG electrodes in the right *extensor carpi radialis* using the same
404 method as in the chronic implants. Stimulation of the spinal cord C5 region using single pulses led to an
405 isolated wrist extension in the rat's forelimb, verifying the location of the ECR motoneuron pool for
406 efferent connections (Tosolini and Morris 2012). The dorsal spinal cord was deafferented between C3
407 and C7, to isolate antidromic propagation instead of conduction along afferent sensory fibers. Following
408 this, we stimulated the EMG electrodes and recorded local field potentials (LFPs) from the electrode site
409 in C5. Filtering parameters for the LFP recording included a bandpass of 1-300Hz, with a 60Hz notch
410 filter applied and a gain of 100. Data was averaged across 200 stimulations. We determined the afferent
411 latency to be 3 ms (Supplementary Figure 1A).

412 To measure the time a neuronal volley requires to reach the cortex after muscle stimulation, we
413 recorded local field potential (LFP) responses in M1 following intramuscular stimulation (Supplementary
414 Figure 1B). We postulated that the peak of the initial negative inflection in the local field potential from
415 contralateral muscle stimulation reflected the time at which the greatest neural activity is observed
416 amongst the post-synaptic neurons in the cortex. We inserted a pair of EMG electrodes in the right
417 *extensor carpi radialis*, then inserted one platinum-iridium electrode 1.5 mm DV into M1 centered at the
418 array coordinates for the other rats. A second electrode about 1 mm lateral from the first was
419 positioned on the surface of the dura. Both cortical electrodes were connected by a common ground at
420 the skull screw, and local field potentials (LFPs) were measured by calculating the voltage differential
421 between the cortical electrodes with the same LFP recording parameters above. Stimulation was
422 delivered to the EMG electrode in the right ECR through bipolar single pulses with a 0.2 ms duration,
423 repeated at 0.5 Hz. Data was averaged across 360 stimulations. The afferent latency from ECR
424 stimulation to cortical evoked potential was 16 ms (Supplementary Figure 1B). With an average MEP
425 latency of 12 ms for ECR and a 3 ms travel time from muscle to spinal cord, we estimated a latency of 9
426 ms for a cortical stimulation-induced descending volley to reach motoneurons, including synaptic
427 integration time. These latencies are reported in Figure 1.

428 Using these conduction latencies, we chose a set of stimulus intervals which would result in various pre-
429 and post-synaptic timing relevant to the rules of spike-timing-dependent plasticity, either at the cortical
430 and/or spinal levels. The full list of ISI conditions tested can be found in Supplementary Table 1. Our
431 experimental design and results followed the convention that a **positive latency** means the periphery
432 was stimulated **after** the cortex by that time difference. These stimulation offsets lead to physiological
433 offsets calculated at the levels of the spinal cord and cortex; positive latencies result in pre-synaptic
434 activity that preceded post-synaptic activity at the specified location.

435 *PAS Intervention*

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

442 Cortical stimulation intensity was set at 1.25 times the threshold for a MEP and muscle stimulation at
443 1.5 times the threshold to elicit a visible twitch. Thresholds were operationally defined as the minimal
444 stimulation intensity required to induce a response more than 50% of the time. All PAS experiments
445 were completed in the animals' home cage with a modified cover that enabled us to pass the tethering
446 cable, during free behavior (consisting mostly of walking, grooming, and exploring, sometimes sleeping).

447 *MEP measurement*

448 Raw EMG data was saved and processed offline in LabChart Version 7 and custom scripts written in
449 MATLAB (codebase at https://github.com/ethierlab/PAS). We plotted all individual responses for each
450 cortical stimulation and manually excluded trials for which there was significant excessive movement
451 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, repaired or replaced promptly). The resulting set of verified MEPs for each probe were collected for further analysis (Figure 4).

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 4).

Statistical Analysis

Statistical analysis and visualization were completed using SAS Software, version 9.4 for Windows, and Minitab 18 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. *Post-hoc* pairwise comparisons between ISI conditions were completed using Dunnett's Test with the family-wise error rate also set at 0.05. Data from one rat in the ISI +6 condition was removed from the analysis because of poor data quality (very few MEPs in each probe). Normality was assessed on standardized residuals using graphical methods.

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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.

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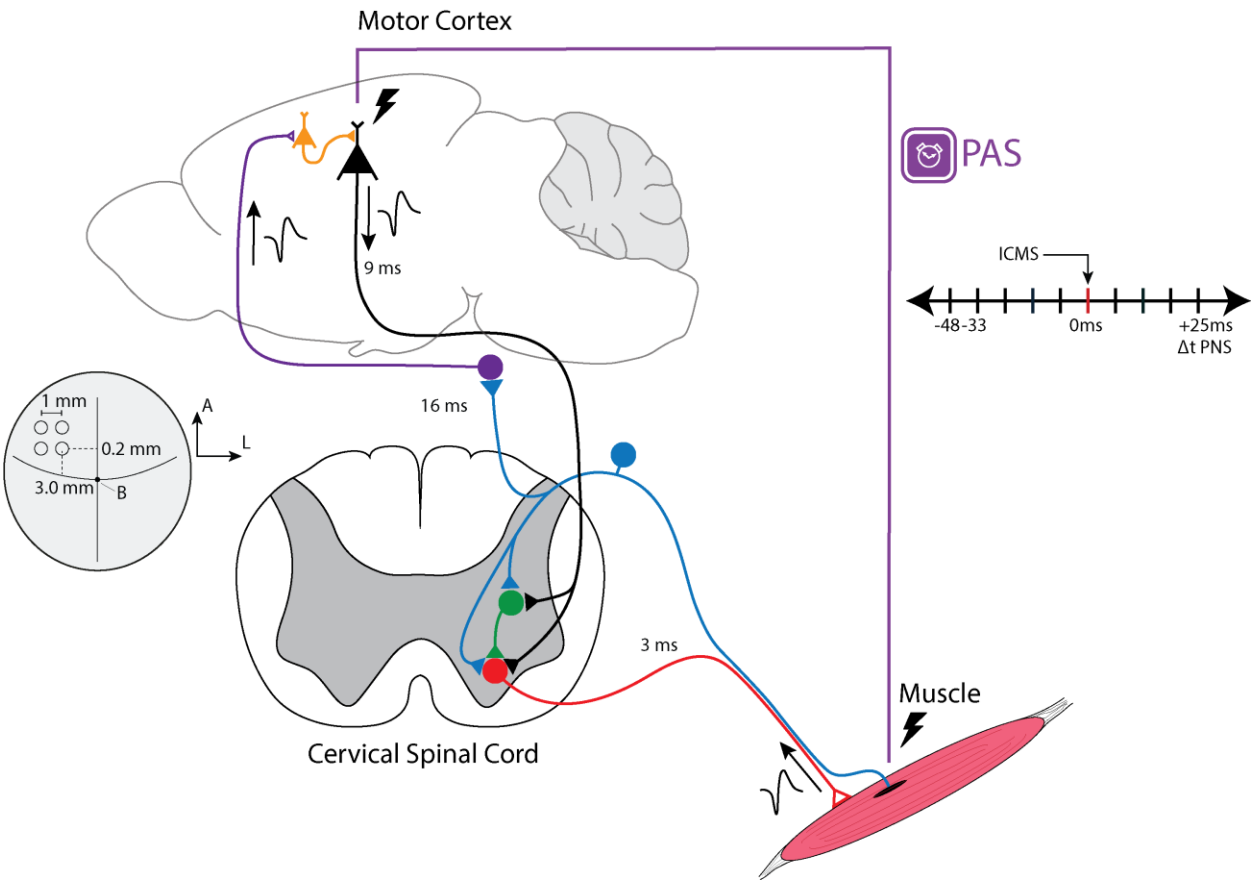


Figure 1: Spinal Cord and Cortical PAS. To induce plasticity at the level of the spinal cord, we timed the descending action potentials from cortical ICMS to arrive at the spinal cord with a set latency before or after the arrival of the ascending antidromic action potentials coming from peripheral stimulation. We know from our and previous studies under anaesthesia that it takes approximately 9 ms and 3 ms for both signals to arrive at the spinal cord, respectively. To induce plasticity at the level of the cortex, we stimulated the periphery first and waited for 16 ms for the signal to cause pooled action potentials in the motor cortex. Timing offsets for subsequent cortical stimulation were calculated based on these conduction latencies. **Inset:** Dorsal view of the rat brain, showing where we inserted the platinum-iridium electrode array in the caudal forelimb area (CFA) of M1. The medial posterior electrode was positioned 3.0 mm ML (left), 0.2 mm AP, and -1.5 mm DV. All other leads were 1 mm anterior (A) and lateral (L) from this reference electrode, as illustrated.

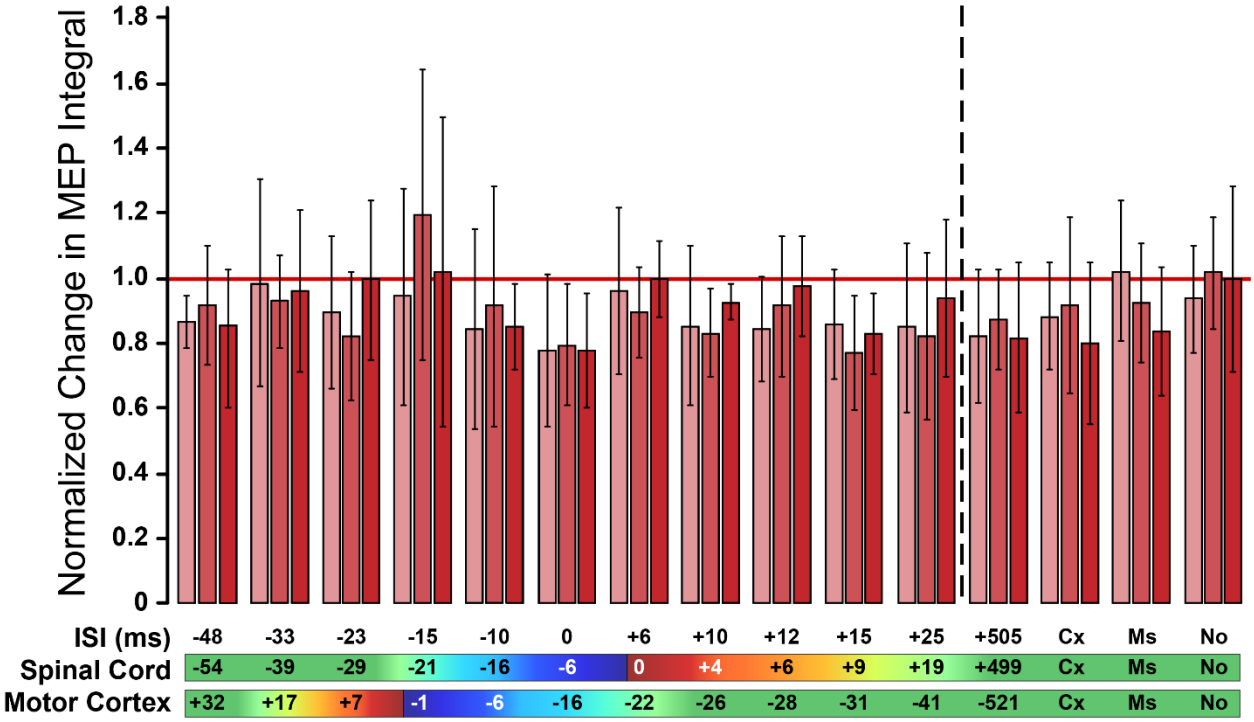


Figure 2: PAS does not significantly potentiate MEP responses *in vivo.* Main effect plot, depicting that for Post 1 (2 min after PAS), Post 2 (17 min after PAS), and Post 3 (32 min after PAS) sessions, there were no significant differences between STDP experimental conditions and control conditions. 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 line, to separate them from the ISI conditions tested to the left (Cx = Cortical Stimulation Only, Ms = Muscle Stimulation Only, No = No Stimulation). Stimulation (ISI) Offset refers to the latency between stimulation of the Cortex and the Muscle. Positive numbers refer to muscle stimulation occurring after cortical stimulation. Delta Spinal Cord is the estimated latency 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; Delta Motor Cortex is the estimated latency between the arrival in M1 of peripheral stimulation-induced afferent activity and the motor cortex stimulation. Red indicates conditions we predicted to induce LTD-like effects, green indicates PAS conditions we predicted would induce LTP-like effects.

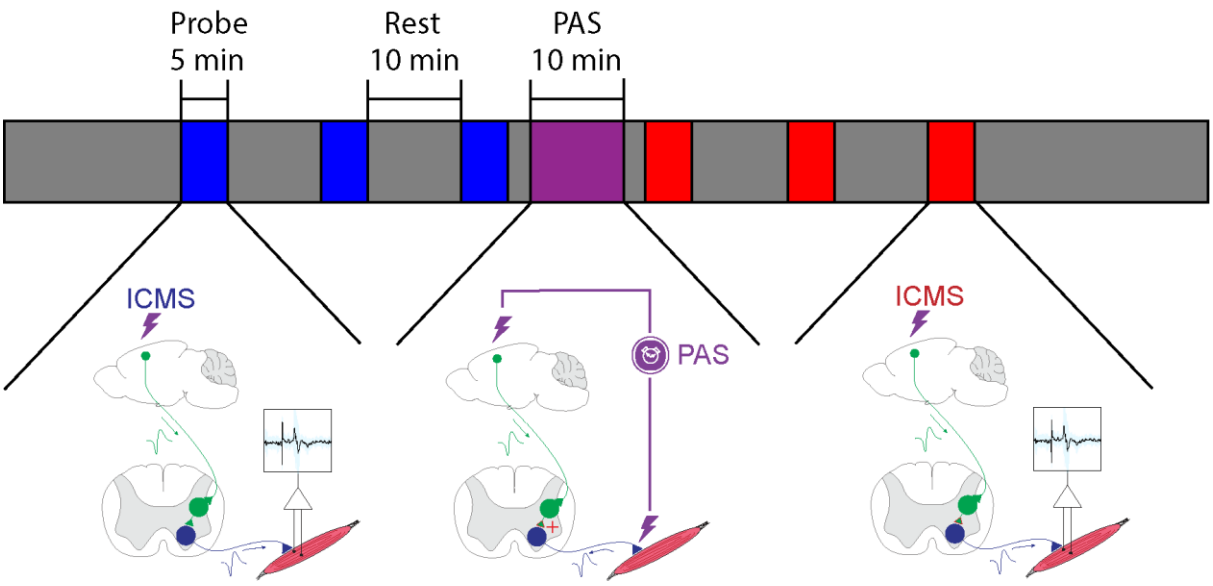


Figure 3: Experimental Design. A: Each session began with three 5-minute probes in which we performed closed-loop EMG-dependent cortical stimulation to assess baseline MEPs amplitude, each separated by 10 minutes. The PAS session itself, involving 300 pairs of stimuli to the cortex and the muscle at 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.

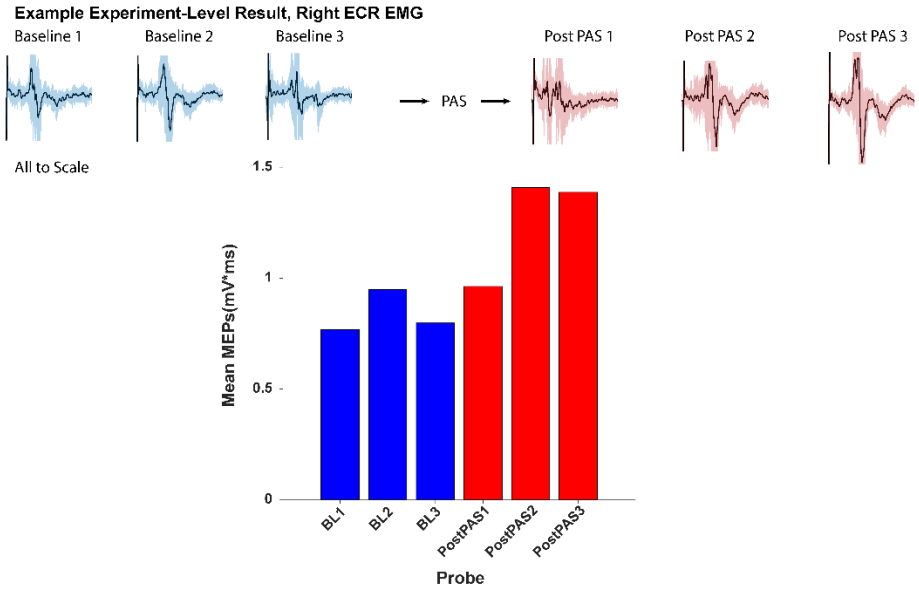
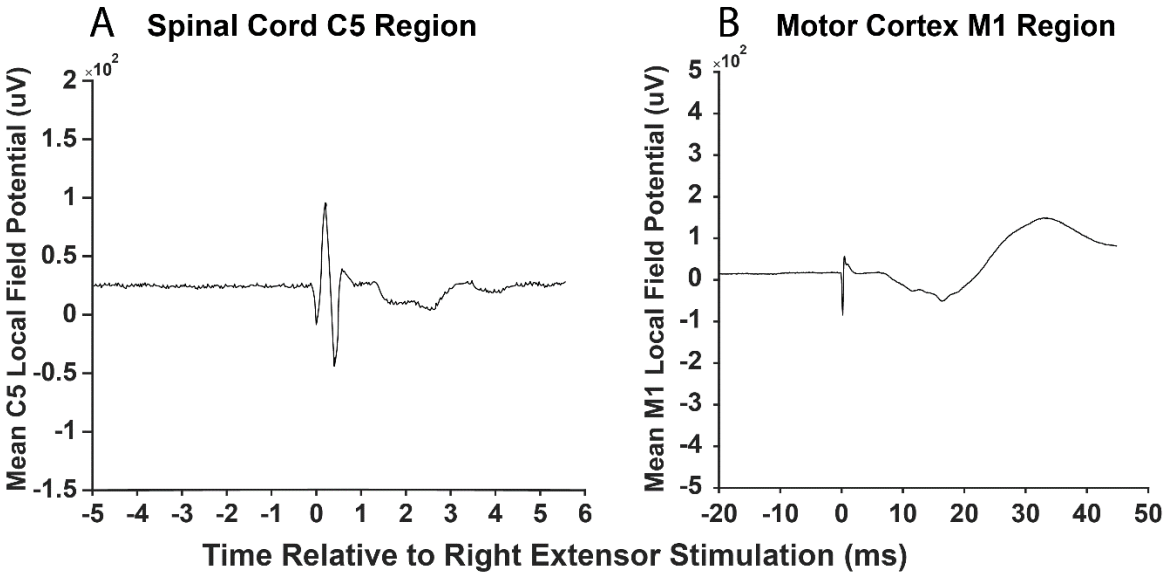


Figure 4: Representative Experiment-Level Result, MEPs recorded in the Right ECR of one rat, obtained from electrical cortical stimulation. Shaded area in blue indicates 1 SD about the mean.

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

Term	DF Num	DF Den	F-Value	P-Value
Session	2.00	245.42	0.12	0.886
Condition	14.00	247.91	1.74	0.049
Session*Condition	28.00	245.42	0.53	0.975

Supplementary Tables and Figures



Supplementary Figure 1: Spinal and Cortical Evoked Potentials from Peripheral Stimulation, demonstrating in **A:** the latency of the deepest trough response at 3 ms in the C5 region of the spinal cord, averaged across 200 stimulations, and in **B:** measuring the latency of the deepest trough response at 16 ms in the cortex, averaged across 360 stimulations.

Supplementary Table 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.

Chronic PAS Experimental Matrix											
Condition		Rat									
		1	2	3	4	5	6	7*	8	9	10
ISI Condition											
STDP -23 (N = 7)		14	14	3	1	3	10	3	11	4	1
STDP -33 (N = 8)		9	2	1	11	6	9	4	15	14	15
STDP -48 (N = 7)		7	4	6	6	11	7	13	5	9	6
STDP -15 (N = 5)		15	15	15	15	15	15	2	3	1	14
STDP -10 (N = 6)		12	1	12	14	7	12	8	10	11	10
STDP 0 (N = 6)		1	12	11	12	13	14	5	2	10	8
STDP +6 (N = 8)		2	6	9	7	9	5	7	9	5	13
STDP +10 (N = 8)		6	9	2	4	14	6	15	13	6	5
STDP +12 (N = 7)		10	5	13	10	12	4	1	8	7	12
STDP +15 (N = 7)		8	11	5	3	1	1	12	14	13	9
STDP +25 (N = 7)		13	8	7	13	4	3	14	4	12	11
STDP Timing Control +505 (N = 6)		11	3	8	9	2	13	9	12	15	7
STDP Controls											
Cortex Only Stimulation (Cx) (N = 7)		5	7	14	5	10	2	10	1	3	2
Muscle Only Stimulation (Ms) (N = 6)		3	10	4	8	5	11	11	7	2	3
No Stimulation Control (No) (N = 8)		4	13	10	2	8	8	6	6	8	4

* Implant Failure Prevented Data Collection

810 **Supplementary Table 2: Muscle Distribution** (ISI Conditions, Latencies are Stimulation Offsets). * = PAS
811 muscle stimulation component was between one EMG electrode and reference.

Rat/Muscle	*Extensor Carpi Radialis*	*Biceps*	*Trapezius*
Rat 1	-10, 0, +6, +10, +12, +15, +25, +505. -48, -33, -23. Ms, No, Cx.	---	---
Rat 2	---	---	-10, 0, +6, +10, +12, +15, +25, +505*. -15, -23, -33*, -48*. Cx, Ms, No.
Rat 3	+6	---	+10 -33
Rat 4	-23 No		
Rat 5	---	---	-10, 0, +6, +10, +12, +15, +25, +505. -15, -23, -33, -48. Cx, Ms, No.
Rat 6	+6*, +10*, +12*, +15*, +25*. -33*, -48*. Cx*, No*.	---	---
Rat 7 (implant failure before useful data could be collected)	---	---	---
Rat 8	-10*, -0*, +6*, +10*, +12*, +15*, +25*, +505*. -15*, -23*, -33*,	---	---

	-48*. Cx, Ms*, No*.		
Rat 9 (NB: Manual Range Translation During Closed Loop Due to Heartbeat).	---	-10*, 0*, +6*, +10*, +12*, +15*, +25*, +505*. -15*, -23*, -33*, -48*. Cx*, Ms*, No*.	---
Rat 10	-10, 0, +6, +10, +12, +15, +25. -15, -23, -33, -48. Cx, Ms, No.	---	---