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Associative plasticity in the human motor cortex is enhanced by concurrently targeting separate muscle representations with excitatory and inhibitory protocols

Marc R. Kamke,[1] Abbey S. Nydam,[1] Martin V. Sale,[1] and Jason B. Mattingley[1,2]
[1]*Queensland Brain Institute, The University of Queensland, Brisbane, Queensland, Australia; and* [2]*School of Psychology, The University of Queensland, Brisbane, Queensland, Australia*

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Kamke MR, Nydam AS, Sale MV, Mattingley JB. Associative plasticity in the human motor cortex is enhanced by concurrently targeting separate muscle representations with excitatory and inhibitory protocols. *J Neurophysiol* 115: 2191–2198, 2016. First published February 10, 2016; doi:10.1152/jn.00794.2015.—Paired associative stimulation (PAS) induces changes in the excitability of human sensorimotor cortex that outlast the procedure. PAS typically involves repeatedly pairing stimulation of a peripheral nerve that innervates an intrinsic hand muscle with transcranial magnetic stimulation over the representation of that muscle in the primary motor cortex. Depending on the timing of the stimuli (interstimulus interval of 25 or 10 ms), PAS leads to either an increase (PAS_{25}) or a decrease (PAS_{10}) in excitability. Both protocols, however, have been associated with an increase in excitability of nearby muscle representations not specifically targeted by PAS. Based on these spillover effects, we hypothesized that an additive, excitability-enhancing effect of PAS_{25} applied to one muscle representation may be produced by simultaneously applying PAS_{25} or PAS_{10} to a nearby representation. In different experiments prototypical PAS_{25} targeting the left thumb representation [abductor pollicis brevis (APB)] was combined with either PAS_{25} or PAS_{10} applied to the left little finger representation [abductor digiti minimi (ADM)] or, in a control experiment, with PAS_{10} also targeting the APB. In an additional control experiment PAS_{10} targeted both representations. The plasticity effects were quantified by measuring the amplitude of motor evoked potentials (MEPs) recorded before and after PAS. As expected, prototypical PAS_{25} was associated with an increase in MEP amplitude in the APB muscle. This effect was enhanced when PAS also targeted the ADM representation but only when a different interstimulus timing (PAS_{10}) was used. These results suggest that PAS-induced plasticity is modified by concurrently targeting separate motor cortical representations with excitatory and inhibitory protocols.

paired associative stimulation; transcranial magnetic stimulation; plasticity; motor cortex; motor evoked potential

PAIRED ASSOCIATIVE STIMULATION (PAS) is a technique for inducing changes in excitability of the human cortex that outlast the procedure. In its prototypical form PAS involves repeatedly pairing electrical stimulation of a peripheral nerve that innervates an intrinsic hand muscle with transcranial magnetic stimulation (TMS) over the representation of that muscle in the primary motor cortex (M1). When the paired stimuli are applied with an interstimulus interval (ISI) of 25 ms (PAS_{25}), such that the afferent signal from the peripheral nerve stimulation reaches motor cortex just prior to the TMS pulse depolarizing motor output cells, an increase in excitability results (Stefan et al. 2000). Employing an ISI of 10 ms (PAS_{10}) reverses the temporal order of inputs to motor cortex, resulting in reduced excitability (Wolters et al. 2003). These bidirectional changes in excitability are believed to reflect synaptic plasticity, underpinned by long-term potentiation (LTP)-like and long-term depression (LTD)-like processes (Carson and Kennedy 2013; Muller-Dahlhaus et al. 2010). Here we report for the first time the effect of concurrently targeting separate motor cortical representations on PAS-induced plasticity. An initial report of this work has been presented in abstract form (Kamke et al. 2015).

Plasticity induced by the PAS procedure is often described as being topographically specific, but evidence suggests that this specificity is relative rather than absolute. In the seminal study describing the technique, the median nerve-innervated abductor pollicis brevis (APB; thumb) muscle representation was targeted with PAS (Stefan et al. 2000). After PAS_{25} the largest increase in excitability, as indexed by the amplitude of motor evoked potentials (MEPs) elicited by single-pulse TMS, was found in the APB muscle representation. However, an increase in excitability was also found in the representations of the ulnar nerve-innervated abductor digiti minimi (ADM) of the little finger and of the upper arm biceps brachii (Stefan et al. 2000). While these latter effects were numerically smaller in magnitude than those in the target (APB) muscle, there was no statistical difference between the plasticity effects induced in the two hand muscle representations (APB and ADM). Numerous other studies have reported a similar pattern of PAS_{25}-induced LTP-like effects in both targeted and nontargeted hand muscle representations (Cheeran et al. 2008; Potter-Nerger et al. 2009; Quartarone et al. 2003; Rosenkranz and Rothwell 2006), while further studies have observed an increase in excitability in the nontargeted representation that was not statistically reliable (e.g., Fratello et al. 2006; Weise et al. 2006). Thus, rather than inducing effects that are strictly limited to the targeted muscle representation, excitability-enhancing PAS_{25} seems to induce LTP-like plasticity on a topographically spatial gradient (for review, see Carson and Kennedy 2013).

In contrast to the spatial gradient of effects induced by excitability-enhancing PAS protocols, excitability-diminishing PAS_{10} may induce qualitative differences in the targeted and nontargeted muscle representations. For example, in a recent study investigating the influence of spatial attention on PAS-induced effects we reported the expected increase in excitabil-

Address for reprint requests and other correspondence: M. R. Kamke, The Queensland Brain Institute, Bldg. 79, Univ. of Queensland, St Lucia, Queensland, 4072, Australia (e-mail: m.kamke@uq.edu.au).

ity of the targeted APB representation following PAS_{25} and decreased excitability following PAS_{10} (Kamke et al. 2014). Both procedures, however, were associated with an increase in excitability of the nontargeted ADM representation. Three further studies have reported the expected excitability-diminishing effects in the targeted APB representation following PAS_{10} (in normal control participants) but an increase in excitability in the nontargeted ADM (Weise et al. 2006, 2011, 2013). Some authors, however, have failed to find a change in excitability of a nearby nontargeted muscle representation after PAS_{10} (Potter-Nerger et al. 2009; Russmann et al. 2009).

Although the mechanisms underpinning the spread of PAS-induced effects to nontarget muscle representations are unknown (Weise et al. 2013), it is clear that the afferent and efferent connectivity of M1 provides a substrate for those effects. Specifically, outputs from multiple (spatially segregated) M1 neurons converge onto single motor neuron pools, and those from single M1 neurons also diverge onto multiple motor neuron pools (for review, see Schieber 2001), providing an anatomical basis for a topographical spread of PAS-induced effects. Furthermore, somatosensory inputs to M1 have a scattered and intermingled distribution (Schieber 2001), such that sensory information arising from any one digit may reach multiple representations in M1. This latter organization explains why serial afferent inhibition, in which stimulation of a peripheral nerve suppresses MEPs at short latencies, can be induced in both the median nerve-innervated APB and the ulnar nerve-innervated first dorsal interosseous (FDI) muscle with stimulation of either the median or ulnar nerve (Fischer and Orth 2011). Thus the anatomical and functional connectivity of sensorimotor networks provide a basis for the spread of PAS-induced effects to nontarget muscle representations.

The present study investigated whether simultaneously targeting PAS to two separate intrinsic hand muscle representations would boost the excitability-enhancing effects. It was hypothesized that an additive, excitability-enhancing effect should be produced in the APB representation by simultaneously targeting PAS_{25} to the APB and PAS_{25} or PAS_{10} to the ADM. More specifically, prototypical PAS_{25} induces an LTP-like increase in excitability of both the target and nearby nontarget muscle representations, as reviewed above. Thus we predicted that applying PAS_{25} to the ulnar nerve-innervated ADM representation would boost the effects induced by PAS_{25} simultaneously targeting the median nerve-innervated APB. Second, because PAS_{10} also leads to an increase in excitability of nontargeted muscle representations, applying PAS_{10} to the ADM simultaneously with PAS_{25} to the APB should also boost plasticity in the APB representation.

MATERIALS AND METHODS

Participants. Fifty-seven neurologically healthy volunteers participated in the study. Twelve participants successfully completed each of *experiment 1* [mean age 22.6 ± 0.6 (SD) yr, range 20–27 yr; 7 women, 5 men; 11 right handed], *experiment 2* (24.1 ± 1.3 yr, range 20–33 yr; 9 women, 3 men; 12 right handed), *experiment 3* (24.4 ± 1.3 yr, range 20–35 yr; 5 women, 7 men; 9 right handed), *experiment 4* (mean age 22.9 ± 1.2 yr, range 20–33 yr; 7 women, 5 men; 11 right handed), and *experiment 5* (mean age 21.3 ± 2.0 yr, range 18–25 yr; 8 women, 4 men; 10 right handed, 2 ambidextrous). Three volunteers participated in two experiments. Data from four additional volunteers were removed before analysis (2 from *experiment 2* and 1 each from *experiments 3* and *5*) as their baseline MEPs were >2.5 SD from the whole group mean (all were in fact >3 SD from the mean). Fourteen additional participants underwent initial assessment but were excluded before PAS because of either *1*) an inability to evoke MEPs of at least 0.5 mV in both the APB and ADM muscles with the TMS coil placed over a single location (10 participants) or *2*) failure to isolate activity from the peripheral nerve stimulation in the targeted muscle (i.e., a motor response isolated to the APB for median nerve stimulation and to the ADM for the ulnar nerve; 4 participants). These screening criteria were essential *1*) to ensure that any difference in the size of PAS-induced effects in the two muscles could not simply be attributed to differences in baseline excitability (especially floor effects) and *2*) for isolating any effects to stimulation of one or the other peripheral nerve. All participants were screened for contraindications to TMS (Rossi et al. 2009), including consumption of neuroactive medications and illicit drugs, and provided fully informed, written consent. Experimental procedures were approved by the Medical Research Ethics Committee of The University of Queensland (no. 2012000747).

TMS and electromyography. Participants sat comfortably with both hands resting on cushions placed on a desk in front of them. Surface electromyography (EMG) was recorded from the APB and ADM of the left hand with 24-mm-diameter disposable Ag-AgCl electrodes (Kendall H124SG by Covidien) in a belly-tendon montage (see Fig. 1). Reference electrodes were placed over the proximal phalanx of the thumb and little finger (for the APB and ADM, respectively). Note that we also recorded responses from the left FDI, but because it was not possible to achieve baseline responses in this muscle that were of magnitude similar to the APB and ADM (see below), only descriptive data are reported for this muscle. For the FDI the reference electrode was placed over the proximal phalanx of the index finger. A common reference electrode was placed on the elbow. Raw signals were amplified (×1,000) and filtered (20-2,000 Hz; 50-Hz notch filter) with a Digitimer NeuroLog system (Digitimer, Welwyn Garden City, UK) and digitized (5,000 Hz) with Power 1401 hardware and Signal software (v5; Cambridge Electronic Design, Cambridge, UK). Throughout the experiment EMG activity was monitored online with a digital oscilloscope, and participants were prompted to relax if activity was observed.

TMS was applied to the right motor cortex with a Magstim 200^2 stimulator and a 70-mm figure-eight coil (no. 9925-00; Magstim, Whitland, UK). The site for stimulation (hot spot) was defined as the point on the scalp over the motor cortex that elicited the most consistent and largest-amplitude MEPs from both the APB and ADM muscles with a slightly suprathreshold intensity [~105–110% of resting motor threshold (rMT)]. The TMS coil was held tangentially on the scalp with the handle pointing posteriorly and laterally at ~45° to the sagittal plane. Once defined, an infrared neuro-navigation system was used to maintain the position and orientation of the coil over the motor hot spot (Visor 1 by ANT Neuro, Enschede, The Netherlands). Threshold (rMT) was determined with a staircase procedure (two-down, one-up from a suprathreshold intensity) and was defined as the lowest intensity required to evoke an MEP of ≥50-μV amplitude (peak to peak) in at least 5 of 10 consecutive trials. Cortical excitability, probed before and after PAS, was measured with the TMS test intensity, which elicited mean MEPs of ~1 mV in both the APB and ADM muscles before PAS. Across experiments this equated to an average TMS intensity of 124–137% rMT for both muscles.

PAS procedure. As shown in Fig. 1, peripheral nerve stimulation was delivered with bar electrodes (30-mm electrode spacing) attached at the level of the wrist. The cathode was located proximal to the body, and stimulation (200-μs pulse width) was delivered with a constant-current stimulator (DS7A; Digitimer). During PAS, peripheral nerve stimulation was delivered at motor threshold intensity, which was defined as the lowest intensity at which a muscle twitch (EMG response clearly above the background noise level but typically > 200 μV) was elicited on at least 50% of trials (~2–3 times the perceptual threshold).

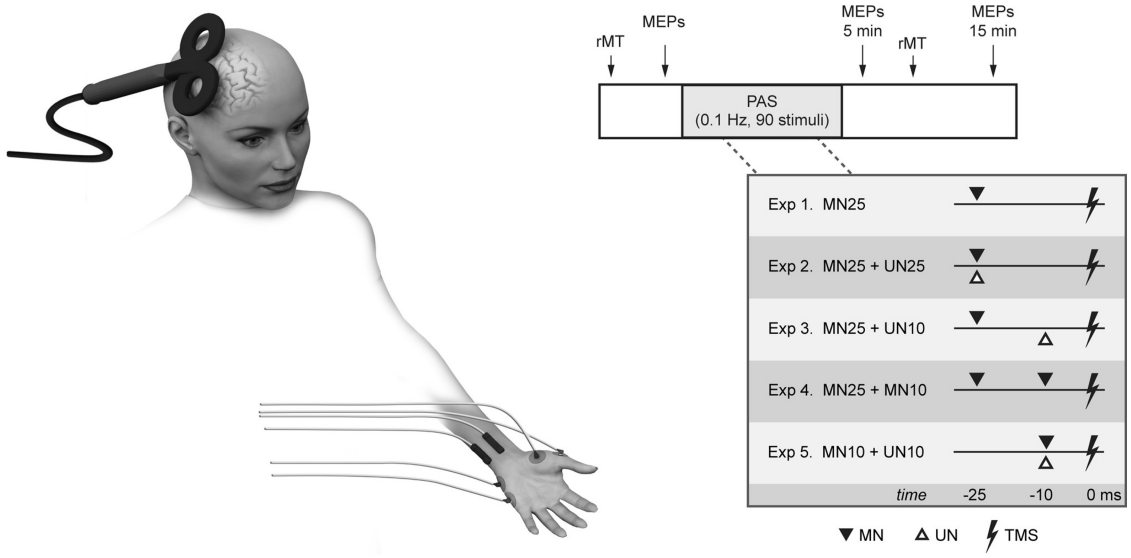

Fig. 1. Experimental design. Plasticity was induced in the motor cortex with paired associative stimulation (PAS). As shown on *left*, the technique repeatedly paired transcranial magnetic stimulation (TMS) over right motor cortex with stimulation of the ulnar (UN) and/or median (MN) nerve at the left wrist. PAS-induced effects were investigated by measuring motor evoked potentials (MEPs) and resting motor threshold (rMT) with single-pulse TMS before and after the procedure (*top right inset*). In different experiments PAS was delivered to 1 nerve or concurrently to both nerves at 1 or 2 interstimulus intervals, as shown in table at *bottom right*.

As described previously, PAS consisted of 90 TMS pulses delivered at a rate of 0.1 Hz and paired with the peripheral electrical stimulation (Kamke et al. 2012, 2014). In the present experiments, however, the peripheral stimulation consisted of two stimuli delivered to the same or different nerves. As shown in Fig. 1, *bottom right*, in the first four experiments PAS involved median nerve stimulation delivered 25 ms before the TMS pulse. The key variable of interest in all experiments was the change in the amplitude of MEPs recorded from the APB by this PAS protocol. In *experiment 1* this stimulation was given alone in the prototypical PAS procedure (PAS_{MN25}). In *experiments 2* and *3*, peripheral stimulation was delivered to both the median and ulnar nerves: In *experiment 2* both nerves were stimulated 25 ms before the TMS pulse ($PAS_{MN25+UN25}$), whereas in *experiment 3* median nerve stimulation was applied 25 ms before and ulnar nerve 10 ms before TMS ($PAS_{MN25+UN10}$). To examine the spatial specificity of the effects produced by targeting the median and ulnar nerves at different timings in *experiment 3*, the median nerve was stimulated at both 25 ms and 10 ms before TMS in *experiment 4* ($PAS_{MN25+MN10}$). Finally, to confirm the requirement for both spatial and temporal variance in the protocols, in *experiment 5* both the median and ulnar nerves were stimulated 10 ms before TMS ($PAS_{MN10+UN10}$; see Fig. 1).

Cortical excitability was probed before PAS and at 5 and 15 min after the procedure. At each time point 21 TMS pulses were delivered with an ISI of 6 ± 1 s. rMT, which provides an index of alterations in resting membrane potential, was also determined before and after PAS (see Fig. 1, *top right*), separately for the APB and ADM. It should be noted that although we have previously shown an influence of attention on PAS-induced effects (Kamke et al. 2012, 2014), we deliberately did not manipulate attention in the present study. Our aim was to investigate whether a novel twin-nerve PAS procedure impacts changes in cortical excitability without the overt influence of attention. Importantly, there is no reason to expect that the direction or level of attention varied systematically between the different experiments.

Data analysis. PAS-induced effects were assessed by comparing mean MEP amplitude before and after the procedure. At all time points MEPs elicited by the first of the 21 test pulses were discarded from the analysis, as were trials with EMG activity in the 200 ms prior to the onset of the TMS pulse. On the basis of this criterion <4.2% of the data was excluded for either muscle in any experiment. For the targeted APB representation, changes in MEP amplitude were examined with a two-way ANOVA with the within-subject factor TIME (baseline, post 5 min, post 15 min) and the between-subject factor PAS (PAS_{MN25}, $PAS_{MN25+UN25}$, $PAS_{MN25+UN10}$, $PAS_{MN25+MN10}$, $PAS_{MN10+UN10}$). A two-way ANOVA was also used to assess changes in rMT, with the factors TIME (pre-PAS, post-PAS) and PAS (PAS_{MN25}, $PAS_{MN25+UN25}$, $PAS_{MN25+UN10}$, $PAS_{MN25+MN10}$, $PAS_{MN10+UN10}$). The same analyses were conducted separately for the ADM. In all cases significant effects were followed up with two-tailed *t*-tests and the Bonferroni correction for multiple comparisons. An α level of 0.05 was adopted as the criterion for statistical significance. In cases of nonsphericity, the Greenhouse-Geisser correction was used. Data analysis was carried out with SPSS (v22; IBM).

RESULTS

There were no adverse reactions to TMS.

Peripheral nerve stimulation and rMT. The mean intensities used for stimulation of the median and ulnar nerves are shown in Table 1. It can be seen that the intensity used for median nerve stimulation was similar across the experiments, but ANOVA revealed a significant variation (PAS: $F_{4,55} = 2.868$, $P = 0.031$, $\eta_p^2 = 0.17$). Follow-up analysis indicated that this effect was driven solely by a marginal difference between the $PAS_{MN25+UN25}$ and $PAS_{MN25+UN10}$ conditions (Bonferroni adjusted $P = 0.030$; all other adjusted P values > 0.15). ANOVA also revealed a significant variation in the intensity used for ulnar nerve stimulation (PAS: $F_{2,33} = 3.430$, $P = 0.044$, $\eta_p^2 = 0.17$) that was driven by a difference between the $PAS_{MN25+UN10}$ and $PAS_{MN10+UN10}$ conditions (Bonferroni adjusted $P = 0.040$; all other adjusted P values ≥ 0.5). It should be noted, however, that all nerve stimulation was delivered at the individual's motor threshold, such that differences in absolute intensity do not necessarily indicate variance in the strength of the ascending volleys. Table 1 also shows mean rMT for the APB and ADM muscles. It can be seen that baseline rMT varied little across the experiments. For the APB,

Table 1. *Mean intensity for electrical nerve stimulation and resting motor threshold before and after PAS*

	Electrical Nerve Stimulation, mA		Resting Motor Threshold, % stimulator output			
			APB		ADM	
	Median	Ulnar	Pre-PAS	Post-PAS	Pre-PAS	Post-PAS
Experiment 1: PAS_{MN25}	11.95 (1.79)		41.58 (1.73)	42.33 (1.81)	41.50 (1.77)	41.83 (1.68)
Experiment 2: $PAS_{MN25+UN25}$	7.40 (1.12)	10.07 (0.87)	44.18 (1.05)	44.45 (1.37)	44.09 (1.09)	43.18 (1.13)
Experiment 3: $PAS_{MN25+UN10}$	15.48 (2.41)	12.57 (1.87)	39.50 (1.40)	38.67 (1.41)	39.83 (1.77)	39.92 (1.89)
Experiment 4: $PAS_{MN25+MN10}$	12.00 (2.39)		44.50 (2.01)	44.67 (1.91)	44.25 (1.90)	43.25 (1.55)
Experiment 5: $PAS_{MN10+UN10}$	9.02 (0.95)	7.94 (0.65)	42.92 (2.66)	43.00 (2.82)	42.83 (2.89)	42.42 (2.65)

Values are means (SE). APB, abductor pollicis brevis; ADM, abductor digiti minimi.

ANOVA failed to reveal any difference in rMT after PAS or across the baseline measures (all $P > 0.25$). Similarly, there were no differences in rMT for the ADM (all $P > 0.17$).

PAS-induced effects in APB. Mean MEP amplitudes recorded from the APB muscle are presented in Fig. 2*A* for the five PAS protocols. It can be seen that, as expected, after prototypical PAS_{MN25} MEPs increased in amplitude. It is also clear from Fig. 2*A* that this effect was enhanced in the $PAS_{MN25+UN10}$ protocol but was not evident after the other protocols. ANOVA confirmed a significant difference in MEPs over time (TIME: $F_{2,110} = 9.430$; $P < 0.001$, $\eta_p^2 = 0.15$) and confirmed that overall amplitudes varied across the four experiments (PAS: $F_{4,55} = 2.860$, $P = 0.032$, $\eta_p^2 = 0.17$). Importantly, the changes in MEP amplitude following PAS varied across the different protocols (TIME × PAS: $F_{8,110} = 3.900$, $P < 0.001$, $\eta_p^2 = 0.22$). Follow-up one-way ANOVAs for each protocol revealed a significant difference across the time intervals only for $PAS_{MN25+UN10}$ ($F_{2,22} = 12.787$, $P < 0.001$, $\eta_p^2 = 0.54$; all other $P > 0.12$). Planned comparisons between baseline MEPs and the two post-PAS time points revealed that MEP amplitude increased after $PAS_{MN25+UN10}$ at both the 5 ($t_{11} = -3.160$, $P = 0.009$) and 15 ($t_{11} = -4.973$, $P < 0.001$) min measures (adjusted $\alpha = 0.025$). Removal of left-handed participants did not alter the significance of any of these effects.

To explore individual differences in the PAS-induced effects, MEP data for each participant were normalized to their pre-PAS (baseline) level and averaged over the two post-PAS time points. The resultant PAS-induced changes in MEP amplitudes are shown in Fig. 2*B*. It can be seen that there was substantial variability in the magnitude and direction of MEP changes across individuals. It is also evident that one participant in each of the PAS_{MN25} and $PAS_{MN25+UN10}$ experiments had large responses ("outliers"). Removal of those individuals did not alter the statistical significance of any of the preceding analyses. To determine whether the change in MEP amplitude in the $PAS_{MN25+UN10}$ condition differed statistically from the other experiments, exploratory post hoc *t*-tests were used to compare the data presented in Fig. 2*B*. For this analysis, the two individuals with very large responses (identified above) were removed. The analysis revealed that the mean change in MEP amplitude following $PAS_{MN25+UN10}$ was larger than in all other experiments (all $P \leq 0.002$; adjusted $\alpha = 0.012$).

PAS-induced effects in ADM. Although PAS-induced alterations in MEP amplitude in the APB were the primary variable of interest, changes were also expected in the ADM. Figure 3*A* presents the mean MEP amplitudes for the ADM recorded before and after PAS. It can be seen that MEPs in the ADM follow a trend similar to that of the APB (Fig. 2*A*), albeit smaller in magnitude. Specifically, the largest change in MEP

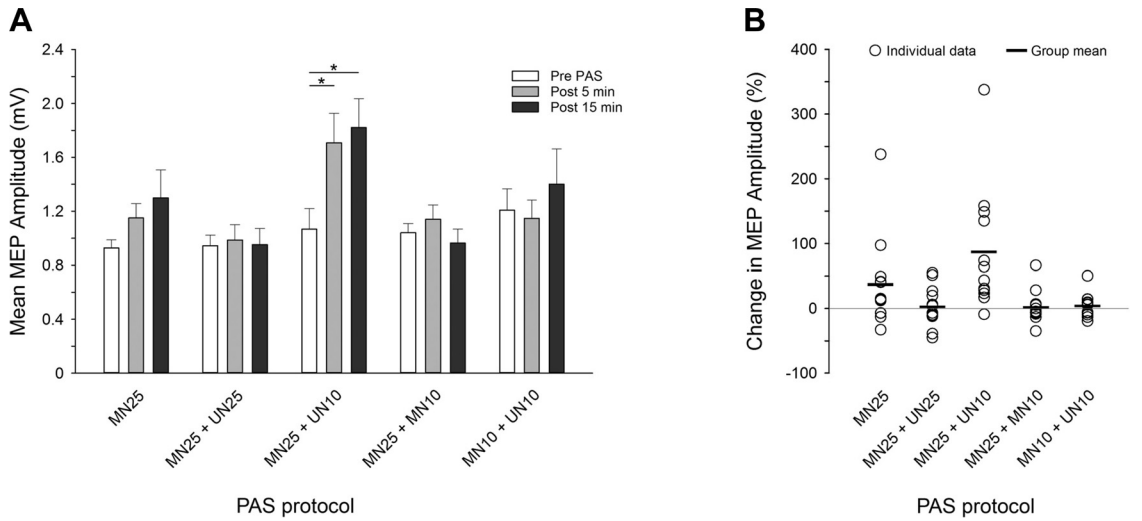

Fig. 2. Mean MEP amplitude in the APB before and after PAS and individual differences in the PAS-induced effects. *A*: after prototypical PAS (PAS_{MN25}) mean MEPs increased in the APB. This effect was larger, and reached statistical significance, after PAS that concurrently targeted the median and ulnar nerves with different timings ($PAS_{MN25+UN10}$). *$P < 0.05$. Error bars show SE. *B*: change in MEP amplitude (relative to baseline) averaged across the 2 post-PAS time points is shown for each participant (circles). Horizontal lines show the mean response for each experiment.

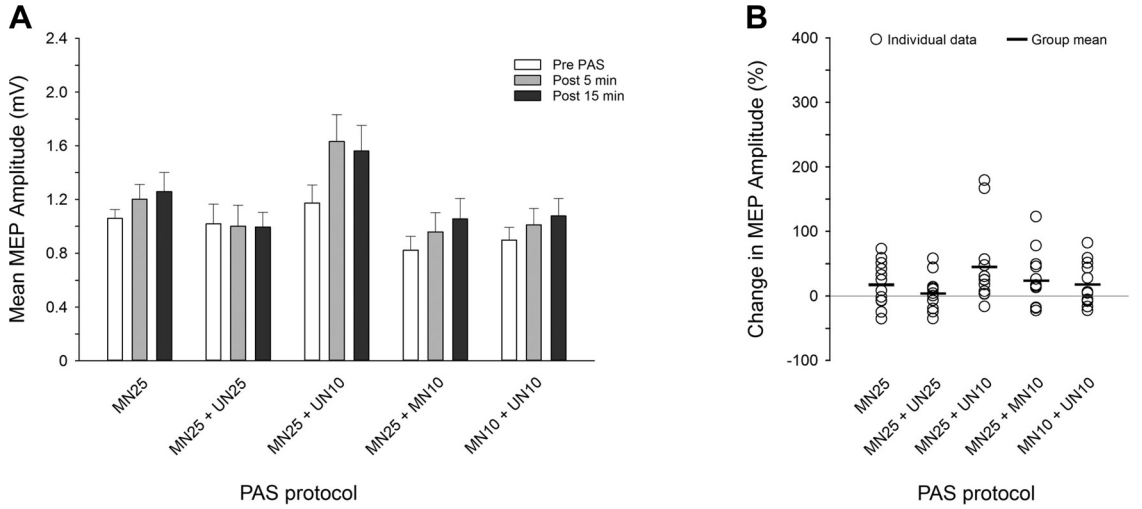

Fig. 3. Mean MEP amplitude in the ADM before and after PAS and individual differences in the PAS-induced effects. *A*: after PAS mean MEPs in the ADM showed a pattern of increases similar to those in the APB (Fig. 2*A*). Overall, MEP amplitudes increased significantly after PAS, and exploratory analysis revealed a significant increase in the $PAS_{MN25+UN10}$ condition. Error bars show SE. *B*: PAS-induced changes in MEP amplitudes in the ADM showed interindividual variability similar to those seen in the APB (Fig. 2*B*). This variability is even evident in cases where the ADM was not targeted by peripheral nerve stimulation (i.e., PAS_{MN25} and $PAS_{MN25+MN10}$).

amplitudes occurred after the $PAS_{MN25+UN10}$ procedure. ANOVA confirmed that there was a significant difference in MEP amplitudes over time (TIME: $F_{2,110} = 8.448$, $P < 0.001$, $\eta_p^2 = 0.13$) and across the four experiments (PAS: $F_{4,55} = 3.006$, $P = 0.026$, $\eta_p^2 = 0.18$). Unlike for the APB, however, the change in MEP amplitude after PAS in the ADM did not vary across the different protocols (TIME × PAS: $F_{8,110} = 1.434$, $P = 0.190$). To follow up the main effect of TIME, MEPs were combined across the five PAS protocols and planned comparisons confirmed that overall PAS led to an increase in MEP amplitude at both the 5 min ($t_{59} = -2.830$, $P = 0.006$) and 15 min ($t_{59} = -3.772$, $P < 0.001$) post-PAS time points (adjusted $\alpha = 0.025$). Exploratory one-way ANOVAs for each protocol, however, revealed a significant difference across the time intervals only for $PAS_{MN25+UN10}$ ($F_{2,22} = 3.816$, $P = 0.038$, $\eta_p^2 = 0.26$) and a marginal effect for $PAS_{MN25+MN10}$ ($F_{2,22} = 3.390$, $P = 0.052$, $\eta_p^2 = 0.24$; all other $P > 0.12$). Finally, as shown in the normalized MEP data presented in Fig. 3*B*, there was large interindividual variability in response to PAS in the ADM, even when that muscle was not targeted by nerve stimulation (i.e., PAS_{MN25} and $PAS_{MN25+MN10}$).

A pattern of effects similar to those found in the ADM was also observed in the ulnar-innervated FDI (data not shown). Specifically, there was an average increase in post-PAS MEPs of 29% in the FDI for $PAS_{MN25+UN10}$ but only 2–12% across the other four experiments. In that muscle, however, mean baseline MEPs varied in amplitude between 1.3 and 2.3 mV across experiments (cf. 0.8–1.2 mV for APB and ADM; see Fig. 2*A* and Fig. 3*A*). For this reason, and because baseline responses from the FDI may have been close to ceiling in various participants (>3 mV), inferential analysis was not performed.

DISCUSSION

The PAS procedure is a well-established method for inducing changes in the excitability of sensorimotor networks. Here, as expected, after administration of a standard PAS procedure targeting the median nerve with an ISI of 25 ms (PAS_{MN25}) MEPs in the APB were enhanced. The size of this effect (average 37%; see Fig. 2*B*) was similar to that we reported previously with the same PAS protocol (when attention was not controlled; Kamke et al. 2014) but failed to reach statistical significance. In this context it is noteworthy that the efficacy of prototypical PAS for inducing reliable effects at the group level has been questioned by similar findings (Lopez-Alonso et al. 2014; Muller-Dahlhaus et al. 2008; Murase et al. 2015). In an attempt to boost PAS-induced responses, we tested whether an additive, excitability-enhancing effect may be produced by applying PAS_{25} or PAS_{10} to the ADM simultaneously with PAS_{25} targeting the APB. We found that when ulnar nerve stimulation with an ISI of 10 ms was added to prototypical PAS_{25} targeting the median nerve ($PAS_{MN25+UN10}$) the increase in MEPs was enhanced (average 87% increase). As shown with prototypical PAS, this increase in MEP amplitude was not associated with a change in rMT, suggesting that alterations in membrane potentials do not account for the effect (Stefan et al. 2000; Wolters et al. 2003). In contrast to our predictions, however, after PAS targeting the median and ulnar nerves with an ISI of 25 ms ($PAS_{MN25+UN25}$), there was no change in MEPs in the APB. There was also no change in MEPs after peripheral stimulation that targeted the median nerve at two time points ($PAS_{MN25+MN10}$) or that targeted both the median and ulnar nerves with an ISI of 10 ms ($PAS_{MN10+UN10}$). These results suggest that inputs that are both spatially and temporally segregated can converge to alter PAS-induced effects.

At first sight the demonstration that PAS-induced plasticity is boosted by targeting separate motor cortical representations with different timings is difficult to reconcile with notions of how PAS is believed to work. Changes in cortical excitability induced by PAS are most commonly attributed to spike timing-dependent plasticity (STDP; e.g., Muller-Dahlhaus et al. 2010). In that model, the timing of the arrival in motor cortex of the afferent signal (from peripheral nerve stimulation) is critical in determining the direction of plasticity effects. When the signal

reaches motor cortex just before the TMS pulse (PAS_{25}) an increase in excitability is induced (an LTP-like effect; Stefan et al. 2000), whereas the later arrival of the signal (PAS_{10}) results in decreased excitability (an LTD-like effect; Wolters et al. 2003). Based on this notion, $PAS_{MN25+UN10}$ should lead to an increase in MEP amplitude in the APB but a decrease in the ADM. This was not the case, as MEPs in the ADM showed a pattern of increases similar to those in the APB (Fig. 3). It is indeed difficult to account for this finding in terms of STDP, but it is noteworthy that various factors, such as dopamine (Fresnoza et al. 2014; Ilic et al. 2012) and attention (Kamke et al. 2014), can influence not only the magnitude but also the direction of PAS-induced plasticity effects. Clearly these demonstrations alone do not explain our present results, but they do suggest that the findings may be amenable to an explanation in terms of STDP.

It has been suggested that, rather than revealing STDP, the timing dependence of PAS-induced effects simply reflects a window over which increased levels of network activity can be achieved (e.g., Carson and Kennedy 2013; Thickbroom 2007). That is, PAS induces activity-dependent plasticity through a spatial and temporal summation of inputs that converge to increase overall levels of network activity (Thickbroom 2007). The results of the present study are perhaps easier to explain in terms of such activity-dependent plasticity, as PAS targeting two nerves should increase activity levels through spatial convergence. More specifically, PAS_{MN25} causes an influx of calcium at one (APB) synapse, leading to LTP. The local spread of calcium, however, also induces plasticity in neighboring synapses not targeted by the procedure: Nearby synapses also undergo LTP, whereas those at more distant sites on the dendrite (where the calcium concentration is lower) experience LTD (e.g., Shouval et al. 2002). If inputs from the ulnar nerve terminate close to those more distal sites, the weak calcium entry (from PAS_{MN25}) will be enhanced (by PAS_{UN10}) and result in LTP. The overall effect will be enhanced LTP-like plasticity in the APB representation. Concomitantly, calcium concentration in synapses representing the ADM may be boosted and reach the threshold for LTP. Interestingly, PAS_{10} has been associated with a decrease in cortical inhibition (as measured with TMS and the short-interval intracortical inhibition protocol; Di Lazzaro et al. 2011; Russmann et al. 2009), which could also act to boost excitability in the APB representation. It will be interesting for future studies to determine whether the changes in cortical inhibition normally associated with PAS are altered with the twin-nerve protocol reported here. It will also be of interest to determine whether the $PAS_{MN25+UN10}$ protocol alters not only the magnitude of PAS-induced plasticity but also its duration.

The notion that spatial and temporal convergence led to enhanced effects after $PAS_{MN25+UN10}$ can also explain why there was no advantage with $PAS_{MN25+MN10}$ over the prototypical procedure. Specifically, there is no spatial convergence of inputs when two pulses target the same nerve. Similarly, there was no chance for a temporal summation of inputs with the $PAS_{MN25+UN25}$ and $PAS_{MN10+UN10}$ protocols. The notion that spatial and temporal convergence is important for enhancing PAS-induced effects, however, does not readily explain why we did not find any evidence of PAS-induced effects in *experiments 2*, *4*, and *5*. It is possible that additional mechanisms, such as mutual surround inhibition induced by PAS in the two muscles (Belvisi et al. 2014), homeostatic processes arising because of overall increased levels of network activity (Nitsche et al. 2007), or even mutual inhibition arising from different plasticity mechanisms (Strigaro et al. 2014) impacted those protocols. It is also possible that the plasticity effects were delayed in those protocols, but this seems unlikely as PAS-induced effects typically begin to manifest shortly after the cessation of the protocol and we did not even find a numerical increase in amplitude by 15 min after PAS in *experiments 2* and *4* or a decrease in *experiment 5*. In this context, given that PAS_{25} and PAS_{10} appear to involve different inhibitory circuits that differentially affect targeted and nontargeted muscle representations (for review, see Carson and Kennedy 2013), it would be interesting to investigate the evolution of changes in excitability during our novel PAS protocols. This could be achieved by interspersing the PAS protocol with TMS alone, or even paired-pulse TMS, to measure short-interval intracortical inhibition. Understanding the evolution of PAS-induced changes in excitability in the APB and ADM representations could help to reveal the mechanisms underpinning the effects reported here.

Although our results are consistent with the suggestion that only signals that are spatially and temporally segregated will enhance PAS-induced effects, it is possible that our different protocols simply altered the strength of the afferent input. In *experiment 4* ($PAS_{MN25+MN10}$), the MN_{10} pulse would have been delivered during a period of axonal recovery from MN_{25} stimulation. Based on evidence from stimulation of cutaneous afferents in the median nerve of humans, an ISI of 15 ms (i.e., the delay between MN_{25} and MN_{10} stimulation) falls close to the crossover point between the axonal supernormal and subnormal periods (Ng et al. 1987). Thus it could be expected that the afferent volley from the MN_{10} pulse, if altered at all, may have been slightly stronger. It is possible that such an increase in the strength of sensory stimulation could enhance PAS-induced plasticity (Carson and Kennedy 2013), but we found no change in MEPs in that experiment. As well as changes in the periphery, however, our protocols could have led to suppression of afferent input at more central sites. Specifically, numerous studies have shown that the somatosensory evoked potential elicited by median nerve stimulation is attenuated by preconditioning with another stimulus, an effect attributed to inhibition of the afferent input at cortical and/or subcortical levels (Greenwood and Goff 1987). In particular, the amplitude of the (electric and magnetic) N20 component of the somatosensory evoked potential, around which PAS is based (e.g., Muller-Dahlhaus et al. 2010), is substantially attenuated at ISIs similar to those used in our twin-pulse studies (e.g., Greenwood and Goff 1987; Hoshiyama and Kakigi 2001). Such suppression, however, occurs with conditioning of either the median or ulnar nerve (Okajima et al. 1991). Thus, while suppression of the N20 could potentially account for the lack of any plasticity effects in our $PAS_{MN25+MN10}$ experiment, it does not explain why we found the largest PAS-induced effects with the $PAS_{MN25+UN10}$ protocol. Similarly, the N20 elicited by simultaneous stimulation of the median and ulnar nerves has been shown to be similar in characteristics to the arithmetic sum of the N20 evoked by median and ulnar nerve stimulation alone (Okajima et al. 1991). This result suggests that a reduction in the N20 is unlikely to explain the absence of PAS-induced effects in the $PAS_{MN25+UN25}$ or $PAS_{MN10+UN10}$ con-

ditions. In summary, our results are not readily explained by interactions in the periphery or more central structures acting to directly enhance or suppress sensory inputs arising from the nerve stimulation.

Another potential explanation for our results is that the additive effects found in the $PAS_{MN25+UN10}$ condition were due to targeting an area in M1 that was intermediate between the APB and ADM representations. One argument against this possibility, however, is that only a single muscle representation was targeted in previous PAS studies that showed a change in excitability of both the targeted and nontargeted muscle representations (see introduction). Moreover, motor maps scale with TMS intensity (van de Ruit and Grey 2016), meaning that the same population of cells can be stimulated, even when the coil is moved away from the center of a map, simply by increasing the intensity. Moving the coil away from the optimal hot spot does not result in stimulation of a different population of cells (such as those at the "edge" of a representation; Thickbroom et al. 1998). Furthermore, it has been shown that the centers of gravity of the APB and ADM muscle representations in healthy control subjects have a mean Euclidean distance of only 3.6 ± 1.4 mm with stimulation with an intensity of 130–140% rMT (Weise et al. 2012). This is a fraction of the size of the motor map for the APB representation, which at 130% rMT has a mean size of >2,500 mm^2 (van de Ruit and Grey 2016). Importantly, we adjusted the TMS intensity to evoke an average baseline MEP of ~1 mV in the APB and ADM of each volunteer. This procedure ensured that the muscle representations in M1 were activated to a degree similar to that evoked in previous studies (that also employed a 1 mV criterion). We also disqualified participants in whom we could not evoke an average MEP of at least 0.5 mV in both the APB and the ADM using the single hot spot. This procedure would have excluded participants whose APB and ADM representations may have been more spatially separated. It is also noteworthy that across all experiments the stimulation intensity required to induce a 1-mV response (average of 124–137% of rMT), as well as baseline rMTs (range 39.5–44.5% machine output) were typical for PAS studies. Thus the hot spot used in our study is unlikely to have had a significant impact on the results.

The present results therefore provide preliminary evidence suggesting that simultaneously targeting PAS to two separate intrinsic hand muscle representations with different time intervals alters plasticity induction in sensorimotor networks. Although this observation does not shed light on the mechanisms underpinning the spread of PAS-induced effects reported in numerous studies, it seems likely that the effect involves multiple mechanisms, potentially including both activity-dependent plasticity and STDP. Indeed, recent evidence showing that pairing (trains of) peripheral nerve stimulation with bursts of transcranial alternating current stimulation induces LTP-like plasticity supports the idea that multiple cellular pathways may mediate the responses induced by PAS protocols (McNickle and Carson 2015). More generally, the present results also highlight that PAS-induced effects are tied to the organization of sensorimotor networks and, as well as suggesting a new method to enhance LTP-like plasticity, warn against a simple conceptualization of the technique as inducing plasticity that is strictly limited to the targeted muscle.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.R.K., M.V.S., and J.B.M. conception and design of research; M.R.K. and A.S.N. performed experiments; M.R.K. and A.S.N. analyzed data; M.R.K., M.V.S., and J.B.M. interpreted results of experiments; M.R.K. and A.S.N. prepared figures; M.R.K. and A.S.N. drafted manuscript; M.R.K., A.S.N., M.V.S., and J.B.M. edited and revised manuscript; M.R.K., A.S.N., M.V.S., and J.B.M. approved final version of manuscript.

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