

1 **Within-subject consistency of paired associative** 2 **stimulation as assessed by linear mixed models.**

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Abstract

27 Objective

28 Paired associative stimulation (PAS) is a TMS paradigm used to induce long-term potentiation in the
29 human cortex. Little is known about the within-subject consistency of PAS-induced effects. We
30 determined PAS-induced effects and their consistency in healthy volunteers between two PAS
31 sessions. Additionally, we assessed the benefit of applying linear mixed models (LMMs) to PAS data.

32

33 Methods

34 Thirty-eight healthy volunteers underwent two identical PAS sessions with a >1 week interval. During
35 each session, motor evoked potentials (MEPs) were assessed once before PAS induction and 3 times
36 after at 30 min intervals.

37

38 Results

39 We did not detect any significant potentiation of MEP size after PAS induction. However, MEP size
40 during PAS induction showed significant potentiation over time in both sessions (LR(1) = 13.36,
41 $p < 0.001$). Nevertheless, there was poor within-subject consistency of PAS-induced effects both during
42 (ICC=0.15) and after induction (ICC=0.03-0.08). Additionally, statistical model selection procedures
43 demonstrate that LMMs are more appropriate than conventional longitudinal models for estimating
44 PAS-induced effects (LR(34) = 214.73, $p < 0.001$).

45

46 Conclusion

47 PAS-induced effects are more pronounced during than after induction, have a low within-subject
48 consistency in any phase of the measurement, and are best estimated with LMMs. The implication of
49 our study is that PAS is an inappropriate method to assess the evolution of brain plasticity over time
50 periods longer than the PAS measurement itself.

51 **Introduction**

52 Synaptic plasticity is a fundamental process in our central nervous system, because it is essential for
53 learning and memory [1, 2]. In addition, plasticity deficits are important in the etiology of
54 neurocognitive disorders [3, 4]. Synaptic plasticity is conventionally measured with invasive
55 techniques, such as field electrophysiology, which cannot readily be performed in human subjects.
56 Non-invasive extracranial brain stimulation techniques, such as transcranial magnetic stimulation
57 (TMS), have emerged in the last two decades, enabling measuring plasticity-like effects in human
58 subjects. Specific TMS paradigms have been shown to produce long-term potentiation (LTP) as well
59 as long-term depression (LTD) like effects, resembling the plasticity effects seen using
60 intraparenchymal electrophysiological studies in animal models.

61
62 Paired associative stimulation (PAS) is one of the TMS paradigms used to induce long-term plasticity
63 [5]. PAS is typically applied by pairing median nerve stimulation (MNS) with magnetic stimulation of
64 the contralateral hand area of the primary motor cortex (M1). Consistent with the fundamental
65 properties of spike-timing dependent plasticity (STDP) [7], when MNS precedes magnetic
66 stimulations by 25ms, PAS stimulation induces a long-term increase in excitability of the M1 hand
67 area that can be observed as an increase of motor-evoked potentials (MEPs) in the contralateral hand.
68 In contrast, if the MNS precedes the magnetic stimulation by 10ms, the result is a long-term
69 depression effect [6]. The resemblance to STDP is further strengthened by pharmacological studies
70 showing that PAS is dependent on the function of the *N*-methyl-D-aspartate (NMDA) receptor, known
71 to be essential for many forms of long-term synaptic plasticity [7].

72
73 Because of the temporal similarity of PAS results to classical STDP experiments in rodents, PAS has
74 emerged as a potentially very useful proxy for studying long-term synaptic plasticity in human
75 subjects. However, PAS produces highly variable results in humans [8, 9], which is often attributed to
76 the challenge of achieving similar levels of standardization as for animal experiments. Environmental
77 factors, lifestyle factors, experimental conditions and even genetic determinants have been suggested

78 to influence the magnitude of the PAS-induced plasticity (for review see Ridding et al. (2010) and
79 Wischniewski et al. (2016)) [10, 11]. However, these factors only explain between-subject variability,
80 whereas little is known about the within-subject consistency.

81
82 Besides inter- and intra-individual variability, PAS studies show variable effect sizes between
83 laboratories as well [9, 11]. In addition to optimizing experimental procedures, some types of
84 variability might be possible to account for by appropriate statistical modeling. PAS measurements
85 generate relatively complex data, combining both repeated measures as well as a nested data structure
86 (i.e. multiple MEP size assessments per time point). In the last decades, linear mixed models (LMMs)
87 have emerged as a statistical method that is specifically suited to handle such a data structure, reducing
88 the chance of both false-positive and false-negative results [12, 13]. Additionally, LMMs are excellent
89 for estimating reproducibility measures in the form of intra-class correlations. However, the utility of
90 LMMs for the analysis of PAS data has not yet been demonstrated.

91
92 In this study, we examined healthy volunteers using two identical PAS sessions with an interval of at
93 least 1 week, in order to determine the within-subject consistency between the two sessions. Using
94 linear mixed model (LMM) analyses, we estimated PAS-induced effects and PAS consistency.

95

96

97 **Materials and Methods**

98 **Subjects**

99 Thirty-eight out of 61 subjects were included in this study (reasons for exclusion are summarized in
100 Table S1 Table), who were recruited by advertising in the local community and on a Dutch research
101 subject-recruitment website. Subjects were included if aged 18-40, right-handed according to the
102 Edinburgh Handedness Inventory [14], in good health, medication free (excluding contraceptives) and
103 able and willing to give written informed consent. Subjects were excluded if they were women
104 lactating or pregnant, had a history of psychiatric illness and/or treatment, had a history of

105 neurological illness or did not meet the international safety guidelines considering TMS [15, 16]. All
106 subjects underwent the Wechsler Abbreviated Scale of Intelligence (WASI) [17] to determine their
107 intelligence quotient (IQ) [18] for descriptive purposes. This study was approved by the Medical
108 Ethical Review Board of the Erasmus MC Rotterdam in 2013, requiring study procedures to comply
109 with the latest version of the Declaration of Helsinki.

110

111 **Fig 1. Schematic representation of the PAS paradigm.**

112 (A) Schematic of one PAS session, in which the PAS induction is preceded by a baseline measurement
113 consisting of 20 TMS stimulations. To measure the change in MEP-size as of PAS induction, this
114 measurement is repeated 3 times with 30 min intervals. (B) Experimental setup during PAS induction,
115 where median nerve stimulation (MNS) at 3 times the sensory threshold (ST) precedes transcranial
116 magnetic stimulations (TMS) delivered at the stimulation intensity 1mV (SI1mV) by 25 ms. Motor
117 evoked potentials (MEPs) are measured using electromyography (EMG) of the abductis pollicis brevis
118 muscle. C Example traces of single MEPs before and after PAS induction.

119

120 **Electromyography**

121 Muscle activity was recorded from the left abductor pollicis brevis (ABP) muscle with
122 electromyography (EMG), using Ag-AgCl electrodes in a belly-tendon montage. EMG signals were
123 amplified using a universal amplifier (ANT Neuro, Enschede, The Netherlands) and digitalized at
124 5kHz for later offline analysis using Visor2 XT software (ANT Neuro, Enschede, The Netherlands).
125 During measurements, a continuous EMG signal and trigger related EMG epochs were plotted at real
126 time for online analysis, while applying a 50Hz notch filter and a 20-2000Hz bandpass filter.

127

128 **Transcranial magnetic stimulation**

129 Subjects were invited in the afternoon between 12 and 5.30 PM [19], and were asked to not perform
130 intense physical activities 24 hours prior to the measurement and to not smoke nicotine cigarettes or
131 drink coffee on the day of the measurement. They were seated in a comfortable chair with their left

132 arm resting on a pillow and were told to maximally relax their left hand during the measurement.
133 Magnetic stimulations were applied using a figure-of-eight coil with an inner diameter of 27mm and
134 outer diameter of 97mm, connected to a MagPro X100 with MagOption TMS device (MagVenture,
135 Farum, Denmark). The coil was held tangentially to the left primary cortex and diverging 45° from
136 midline. The electric field subsequently created in the cortex had a posterior to anterior direction.

137

138 To find the optimal position of the coil in order to maximally activate the ABP (the hotspot), TMS
139 stimulations were randomly placed around a predefined reference point. This reference point was
140 defined as the location at 10% of the ear-to-ear span lateral to Cz over the right hemisphere. Data on
141 coil location and position at every stimulation was collected using a neuronavigation system (ANT
142 Neuro, Enschede, The Netherlands), allowing a precise spatial definition of the hotspot and precise
143 determination of the angle and distance errors of every stimulation relative to the hotspot. All TMS
144 procedures hereafter described are performed at the hotspot.

145

146 The resting motor threshold (RMT) was determined using a maximum-likelihood threshold hunting
147 procedure [20]. For this procedure, a MEP with a peak-to-peak amplitude of $\geq 50\mu\text{V}$ with an onset
148 within a timeframe of 20-50ms post stimulation was considered a MEP. Subsequently, the stimulation
149 intensity 1mV (SI1mV) was determined, defined as the percentage of maximal stimulation output
150 (%MSO) of the TMS device that resulted in a mean MEP of 0.8 - 1.2 mV after 10 stimulations at
151 0.1Hz.

152

153 **Paired associative stimulation**

154 Subjects underwent two identical paired associative stimulation (PAS) sessions at least one week
155 apart. First, baseline cortical excitability was assessed by applying a train of 20 magnetic stimulations
156 at the SI1mV at 0.1Hz. Second, the PAS induction phase followed, consisting of 200 paired
157 stimulations at 0.25Hz with each pair consisting of electric MNS followed by magnetic stimulation
158 with an inter stimulus interval of 25ms. Third, after this plasticity induction phase, the cortical

159 excitability measurement at baseline was repeated at three time points: immediately (Post 1), 30
160 minutes (Post 2), and 60 minutes (Post 3) after completing PAS induction (Fig 1B).

161

162 MNS during the PAS-induction was applied with a strength of three times the sensory threshold, using
163 a bipolar bar electrode connected to a constant current stimulator (model DS7A; Digitimer Ltd.,
164 Letchworth Garden City, UK) (Fig 1A). In case this stimulation intensity surpassed the pain threshold,
165 it was lowered to a painless but clearly noticeable level. The subject's attention level was standardized
166 as much as possible by applying four randomly timed electric stimuli during PAS induction to the
167 middle phalanx of the left thumb, and instructing participants upfront of PAS induction to focus their
168 attention on their left thumb, to count the number of thumb stimuli sensed and to report this number
169 after PAS induction [21]. These electric stimulations were administered at two times the sensory
170 threshold using a double ring electrode connected to a constant current stimulator (Micromed S.p.A,
171 Mogliano Veneto, Italy).

172

173 **MEP-analysis**

174 The EMG signal for every magnetic stimulation applied was stored for offline analysis as epochs of -
175 300ms to +300ms surrounding the TMS trigger (Fig 1C). Using software programmed in LabVIEW
176 (National Instruments, Austin, TX, US) pre-MEP noise, the maximal peak-to-peak amplitude and
177 MEP onset were determined using a seven-step data processing procedure:

- 178 1. Signals were linearly detrended.
- 179 2. The average amplitude value of the -300ms to -20ms before the TMS trigger was subtracted
180 to create a zero-baseline.
- 181 3. To prevent ringing due to filtering, the stimulation artefact was removed between -2ms to
182 +4ms surrounding the TMS trigger, followed by linear interpolation. For MEPs obtained
183 during PAS induction, the stimulation artefact of the MNS was removed similarly.
- 184 4. Signals were filtered using both a 20-2000Hz bandpass filter and a 50Hz-notch filter.

- 185 5. Noise was determined on a time window of -25ms to +15ms surrounding the TMS trigger
186 that was depleted of baseline wandering by subtracting a 2nd-order polynomial fit. It was
187 defined as a peak-to-peak amplitude of >50 μ V or an SD of >15. Signals meeting these criteria
188 were discarded for further statistical analysis.
- 189 6. The maximal peak-to-peak amplitude of every MEP was determined within a 20-48ms time
190 frame following the TMS trigger.
- 191 7. The MEP onset was determined within an 18-30ms time frame following the TMS trigger.

192

193 **Statistical analysis**

194 Statistical analyses were performed using R version 3.3.3 [22] and the nlme package for mixed model
195 analysis specifically [23]. Session specific subject characteristics were compared using paired t-tests
196 for normally distributed data (RMT and SI1mV), a Wilcoxon Signed Rank test for non-normal
197 continuous data (starting time of TMS measurement), a Chi-square test for categorical data (attention
198 score) and LMMs for data related to individual MEPs (angle and distance error). Furthermore, we
199 used LMMs to estimate PAS-induced changes of MEP size, their correlations with baseline MEP size,
200 and intraclass correlations (ICCs). For these LMMs, the dependent variable was MEP size, which was
201 log₂-transformed to better fit the assumption of normally distributed residuals. In addition, these
202 LMMs were adjusted for log₂-transformed angle and distance error.

203

204 We built Model 1 to estimate PAS-induced effects on MEP size at each time point *after induction*
205 (Post 1, Post 2 and Post 3) within each session. This LMM included time point (categorical), session,
206 and their interaction. The random effects included subject specific random effects for each time point
207 in each session separately. An unstructured covariance matrix for the random effects was used (Model
208 1a) and was tested against the more restrictive compound symmetry structure (Model 1b), using a
209 likelihood ratio (LR) test. Subsequently, the LR test was also used to assess the main effects of fixed
210 effects.

211

212 Model 2 was built to estimate PAS-induced effects *during PAS induction*. This LMM included
213 stimulus number (continuous), session and their interaction. Stimulus number was regarded as
214 continuous time variable, as stimulations were equally spaced by 4 seconds in all PAS experiments.
215 The model included subject specific random effects for stimulus number and session interaction and
216 session. The eventual model was selected in three steps using likelihood ratio tests. First, we started
217 out with a model using both natural cubic splines for stimulus number with three degrees of freedom
218 and an unstructured covariance matrix (Model 2a). Second, to investigate the correlation structure, we
219 tested Model 2a against a model with a compound symmetry structure (Model 2b). Last, to test
220 whether the relation between MEP size and stimulus number was non-linear, Model 2a was tested
221 against a model with a linear fit (Model 2c), using a LR test. After model selection, the LR test was
222 also used to determine the main effects of fixed effects.

223

224 As a measure of within-subject consistency we calculated ICCs from LMMs that included session as
225 an additional nesting level in the random effects. For the ICC of PAS-induced effects after induction,
226 fixed effects and subject specific random effects of time point (categorical) were used (Model 3). To
227 estimate the ICC of PAS-induced effects during PAS-induction over time, fixed effects as well as
228 subject specific slopes for stimulus number (continuous time variable) were included (Model 4). Since
229 the models used to calculate ICCs contained random effects for the respective time variables, the
230 variation partition method was used [24]. 95% confidence intervals (95% CIs) for each ICC were
231 estimated using 500 bootstrap samples.

232

233

234 **Results**

235 **Session characteristics**

236 Thirty-eight individuals (22 women; median age 23, range 19-38; mean IQ 107±10SD) underwent two
237 PAS sessions, which were spaced at least 1 week apart (median days between sessions was 14, IQR:
238 4). As displayed in Table 1, median starting time was significantly earlier in session 1 than in session

239 2, whereas both sessions did not differ in terms of baseline RMT, SII_{mV} or the level of attention
 240 during PAS induction. Additionally, the angle error and distance error relative to the hotspot did not
 241 differ between sessions either. The estimated means of both errors over each time point were minimal,
 242 with the upper limit of the 95% CIs not exceeding 5° for the angle error and 2mm for the distance
 243 error. There was, however, a significant effect of time point (including PAS induction) on both angle
 244 error (LR(8) = 22.41, p = 0.004) and distance error (LR(8) = 18.55, p = 0.018).
 245

Table 1. Session characteristics and comparisons.

Characteristic	Session 1	Session 2	Statistic	P-value
RMT at baseline, mean (±SD), %MSO	48.5 (±10.3)	48.3 (±9.1)	t(37) = 0.17	0.87
SII _{mV} , %MSO	61.4 (±14.4)	60.1 (±14.7)	t(37) = 1.10	0.28
Start time, median (IQR), hh:mm	12:44 (12:28-13:08)	15:23 (15:00-15:41)	Z=719	<0.0001
Attention score, n (%)			LR(2) = 3.74	0.15
<4 stimuli reported	10 (29)	14 (38)		
4 stimuli reported	10 (29)	15 (41)		
>4 stimuli reported	15 (43)	8 (22)		
Angle error, estimated mean [95%CI]*, degrees			LR(5) = 5.26**	0.39
Baseline	2.69 [1.75, 4.16]	1.67 [1.11, 2.51]		
Induction	2.95 [1.99, 4.35]	2.11 [1.47, 3.01]		
Post1	2.07 [1.39, 3.08]	1.86 [1.12, 3.08]		
Post2	2.23 [1.41, 3.54]	1.29 [0.89, 1.87]		
Post3	1.83 [1.15, 2.91]	1.09 [0.81, 1.46]		
Distance error, estimated mean [95%CI]*, mm			LR(5) = 3.85**	0.57
Baseline	1.01 [0.84, 1.23]	0.87 [0.68, 1.12]		
Induction	1.11 [0.95, 1.29]	0.91 [0.79, 1.04]		
Post1	1.10 [0.97, 1.26]	0.91 [0.79, 1.06]		
Post2	1.20 [0.96, 1.50]	1.30 [1.02, 1.65]		

Post3	1.31 [1.02, 1.68]	1.34 [1.03, 1.74]
* Estimated using a LMM with the log2 transformed error as dependent variable and time point, session and their interaction as fixed effects.		
** Main effect of session estimated by comparing LMMs using a likelihood ratio test		

246

247 PAS-induced effects post induction

248 We determined the PAS-induced effect on MEP size at each post-induction measurement in each
249 session. After filtering out MEPs with a noisy baseline, 5212 MEPs out of the originally 6080 MEPs
250 recorded could be used for statistical analysis, resulting in the exclusion of one complete session of
251 one subject. Plotting the individual trajectories of MEP size after induction (Fig 2A) indicates that
252 PAS-induced changes of MEP size were highly variable. It is, therefore, not surprising that a model
253 with an unstructured covariance matrix provided a better fit than one with a compound symmetry
254 matrix (LR(34) = 214.73, $p < 0.001$), and was therefore selected as the appropriate matrix to estimate
255 PAS-induced effects in our data.

256

257 The estimated mean of baseline MEP size of session 1 (0.54 mV; 95%CI [0.43, 0.68]) did not differ
258 from that of session 2 (0.61 mV; 95%CI [0.53, 0.71]) (LR(4)=2.26, $p=0.689$). MEP size changed
259 significantly over time (LR(6) = 16.23; $p = 0.013$), which was mainly driven by a negative effect on
260 MEP size in Post 3 in session 2 (Table 2), instead of a positive effect on MEP size as one would
261 expect when performing PAS. PAS-induced effects did not differ between sessions, as the interaction
262 between time point and session was not significant (LR(3) = 1.93; $p=0.586$). The absence of this
263 interaction is reflected by the similar profile of MEP size over time illustrated in Fig 2B. There was a
264 moderate positive correlation between individual baseline MEP size and the PAS-induced effect at
265 each time point in session 1 ($r_{\text{Post 1}} = 0.68$, $r_{\text{Post 2}} = 0.57$, $r_{\text{Post 3}} = 0.52$) and a poor positive correlation in
266 session 2 ($r_{\text{Post 1}} = 0.23$, $r_{\text{Post 2}} = 0.21$, $r_{\text{Post 3}} = 0.30$).

267

Table 2. Fixed effects of PAS induction on MEP size per post-induction time point and session estimated by linear mixed effect modelling.

Session 1	Session 2
-----------	-----------

Variable	β , %	95%CI, %	t (5165)	P-value	β , %	95%CI, %	t (5165)	P-value
Post 1	+16.83	[-9.05, 50.07]	1.22	0.22	-3.99	[-26.58, 25.55]	-0.30	0.77
Post 2	-10.00	[-30.92, 17.24]	-0.78	0.43	-23.92	[-42.44, 0.55]	-1.92	0.05
Post 3	-14.84	[-35.65, 12.69]	-1.12	0.26	-31.65	[-47.92, -10.30]	-2.74	0.006

268

269

270 **Fig 2. PAS-induced effects for session 1 and 2 separately.**

271 Subjects underwent two identical PAS sessions spaced >1 week apart, with session 1 displayed in red
272 and session 2 in blue. (A) The mean change in MEP size over time per session (black line) plotted
273 over the individual line plots (colored lines). (B) The mean change in MEP size over time for both
274 sessions, where dots represent means of individual medians and bars represent their standard error. (C)
275 Linear regression lines through all MEPs during PAS induction per session (black lines) plotted over
276 the linear regression lines through MEPs per individual (colored lines). (D) The change of MEP size
277 over time during the PAS induction, with every dot representing the mean MEP size over all
278 participants for that stimulation number. Lines are fitted linear regression lines per session.

279

280 **Potentiation during PAS induction**

281 We, additionally, took a novel approach to determine the PAS-induced effect on MEP size by
282 assessing this effect during PAS induction next to post induction alone. For this analysis, 9360 MEPs
283 were available out of the 15200 MEPs recorded, due to filtering out MEPs with a noisy baseline. The
284 exclusion of these MEPs resulted in the exclusion of one entire session for 9 subjects and the
285 exclusion of both sessions for 4 subjects. Viewing the individual trajectories of MEP size development
286 again indicates that there was high inter-individual variability (Fig 2C), which is reflected by the
287 superior fit of the model with an unstructured covariance matrix to one with a compound symmetry
288 covariance matrix (LR(8) = 525.31, $p < 0.001$). The development of MEP size over time appeared to
289 be linear (Fig 2D), supported by the fact that a model with a cubic fit was not superior to one with a
290 linear fit (LR(4) = 2.69, $p = 0.612$). Therefore, a model with an unstructured covariance matrix and a
291 linear fit was selected as the appropriate model to estimate PAS-induced effects.

292

293 The estimated mean of MEP size at the start of PAS induction in session 1 (0.43 mV, 95%CI [0.27,
294 0.59]) did not differ from that in session 2 (0.44 mV, 95%CI [0.29, 0.66]) (LR(2) = 0.967, $p = 0.617$).

295 There was a main effect of time (LR(1) = 13.36, $p < 0.001$), as a result of a significant positive
296 increase of MEP size over time in both session 1 (+132%, 95%CI [+51%, +258%]) and session 2
297 (+79%, 95%CI [+19%, +169%]). However, there was no evidence of this time effect being different
298 between sessions (LR(1) = 0.87, $p = 0.35$), reflected by the similar slope of the MEP size development
299 in Fig 2D. There was a moderate negative correlation between MEP size at the start of PAS induction
300 and the change in MEP size over time for session 1 ($r = -0.51$) and a weak negative correlation for
301 session 2 ($r = -0.41$).

302

303 **Consistency of PAS-induced effects**

304 The within subject consistency of PAS-induced effects between the two sessions was small: Post 1 had
305 an ICC of 0.08 (95%CI [0.03, 0.16]); Post 2 had an ICC of 0.07 (95%CI [0.03, 0.14]); and Post 3 had
306 an ICC of 0.03 (95%CI [0.01, 0.09]) (Fig 3). Furthermore, the PAS-induced effects during induction
307 showed a similarly poor within-subject consistency (ICC = 0.15; 95%CI [0.05, 0.35]) (Fig 3), despite
308 their significant potentiation at group level. The ICC of baseline MEP size before induction was poor
309 (ICC = 0.02; 95%CI [<0.01 , 0.04]), as well as at the start of PAS induction (ICC = 0.24; 95%CI =
310 [0.04, 0.42]). Nonetheless, the SI1mV did have a good within-subject consistency (ICC = 0.88; 95%CI
311 [0.83, 0.96]), as did the RMT at different time points (ICC_{Baseline} = 0.85, 95%CI [0.77, 0.92]; ICC_{Post 1}
312 = 0.83, 95%CI [0.79, 0.90]; ICC_{Post 2} = 0.85, 95%CI = [0.79, 0.92]; ICC_{Post 3} = 0.85, 95%CI [0.78,
313 0.92]).

314

315 **Fig 3. Intra-individual correlation of PAS-induced effects.**

316 Scatterplots per phase during or after induction illustrating the correlation between individual PAS-
317 induced effects (dashed slopes) of session 1 against session 2. The slopes plotted are derived from the
318 models used to calculate the ICC of PAS-induced effects within subject and between session.

319

320

321 **Discussion**

322 To our knowledge, we performed the largest study reported to date aimed at quantifying the within-
323 subject consistency of PAS-induced effects. Additionally, this is the first PAS-TMS study that
324 quantifies the change in MEP size during PAS induction over time and the first using a linear mixed
325 model analysis approach. We performed two identical PAS sessions in one group of healthy
326 volunteers, resulting in pronounced potentiation over time during PAS induction, which was not
327 consistent within subjects. PAS-effects after induction did not show the expected potentiation, and
328 these effects were not consistent within subjects either. Additionally, we demonstrated that a linear
329 mixed model with an unstructured covariance matrix provides the best model fit for our PAS data.

330

331 **PAS-induced effects during and after induction**

332 The absence of post-induction potentiation in our study, and even the negative trend towards the last
333 post-induction measurement, is certainly not in line with most PAS studies (for review see [11]), for
334 which a few factors in our study could be responsible. First, our baseline MEP size is lower than the
335 baseline of around 1mV shown in most PAS studies. In addition, we found a poor to moderate positive
336 correlation between baseline MEP size and the PAS induced effects at each post-induction time point,
337 suggesting that individuals with a low baseline were prone to show lower PAS-induced effects.
338 Combined, these two observations could indicate that our stimulation intensity during induction was
339 relatively lower than most PAS studies, possibly causing our PAS-induced effects to be smaller.
340 Nonetheless, this hypothetical interaction between stimulation intensity and potentiation effect has
341 never been specifically studied and relatively lower stimulation intensities that are still supra-threshold
342 should still be able to potentiate MEP size, as double activation of the cortex by TMS and MNS is
343 conserved. Second, coil placement during experiments could have been inaccurate. However, as angle
344 and distance error of the coil position relative to the hotspot were minimal and carefully corrected for
345 in our model, this is unlikely the case in our study. Last, the known compromising effect of sleepiness

346 on MEP size could be a contributor [25]. As our subjects remained seated and were not allowed to
347 perform any type of physical activity or specific types of mental activity between post-induction time
348 points, it is plausible that subjects became increasingly sleepy during this phase of the experiment.
349 Unfortunately, we cannot support this speculation with actual measures of sleepiness during
350 experiments, as these were not assessed.

351
352 Nevertheless, we did find a significant increase of MEP size during PAS induction that shows striking
353 resemblance to the increase in excitatory post synaptic potentials seen in STDP experiments in rodents
354 [26]. From these animal studies, we know that the potentiation during plasticity induction correlates
355 with the potentiation after induction. However, whether our observed potentiation of MEP size during
356 PAS induction is indeed a proxy for NMDA-dependent LTP as well, should be confirmed by studies
357 using a sham-stimulation control and preferably also placebo-controlled studies with an NMDA-
358 receptor antagonist. It is noteworthy, however, that in our study MEP size at the start of PAS induction
359 showed a negative correlation with PAS-induced effects during PAS induction. As MNS during paired
360 stimulations has a known acute inhibitory effect on MEP size, also known as short-latency afferent
361 inhibition [27, 28], lower MEP size at the start of induction could indicate successful paired
362 stimulations and, therefore, be related to a more prominent PAS-induced potentiation.

363

364 **The advantage of using linear mixed models for PAS data**

365 Our results provide insight in the potential advantage of LMMs for analyzing PAS data over
366 conventional analysis methods. Most importantly, we show that using an unstructured covariance
367 matrix provides a better model fit than using a compound symmetry matrix. First, our finding at least
368 demonstrates that PAS data can greatly benefit from a model of which the covariance matrix can be
369 flexibly designed and suggests that the compound symmetry matrix is unsuited for PAS data. As the
370 compound symmetry assumes that the correlations of MEP size between each time point are equal,
371 this result is actually not surprising for data sampled in a dynamic biological system such as the
372 human brain. Since conventionally used RM-ANOVA is restricted to a compound symmetry matrix,

373 LMMs are therefore more appropriate for the analysis of PAS data and are likely to deliver more
374 reliable results. Consequently, future consistent implementation of LMMs for analyzing PAS data will
375 improve the reproducibility between PAS studies.

376

377 **Consistency of PAS-induced effects**

378 We show that PAS-induced effects, both during and after PAS induction, have poor intra-individual
379 consistency. However, one could argue that the lack of significant post-induction potentiation
380 compromises the validity of the consistency levels in this study. Nonetheless, we were able to show
381 significant potentiation during induction, which showed similar low consistency. Additionally, a lack
382 of significant potentiation at group level does not warrant that at individual level, as can be
383 appreciated from the individual traces in Fig 2A, and the low consistency found in this study indicates
384 that the subset of individuals showing potentiation were largely different in each session. It is,
385 therefore, unlikely that the consistency would be much higher in case of significant potentiation at
386 group level, which is in line with previous results reported by Fratello et al. (2006): in their study, they
387 also performed two identical PAS sessions within one group of healthy volunteers (n=18) and found
388 equally poor intra-individual consistency, despite finding significant PAS-induced potentiation both
389 PAS sessions [29].

390

391 The low consistency of PAS does not seem to hold for methods that measure brain plasticity in
392 humans in general, as intermittent theta burst stimulation (iTBS) was found to produce reasonably
393 consistent measures of brain plasticity with an ICC of 0.53 [30]. Another paradigm, anodal
394 transcranial direct current stimulation (aTDCS), showed moderate to no intra-individual consistency in
395 two separate studies [31, 32]. The contrast between the within-subject consistency between iTBS and
396 PAS can be understood from animal studies, suggesting that plasticity induction protocols based on
397 low frequency stimulations, such as STDP, are much more vulnerable to metaplasticity than high
398 frequency paradigms such as theta burst stimulation [33]. This is in line with findings in human TMS
399 studies, also reporting a vulnerability of PAS to metaplasticity [34, 35]. Altogether, this implies that

400 PAS is probably much more dependent on highly dynamic time-varying factors than iTBS, resulting in
401 a lower within-subject consistency of PAS.

402

403 In our view, the inconsistency of PAS-induced plasticity has major implications for its use. As our
404 results have revealed that PAS-induced effects cannot be seen as a trait, dividing subjects into PAS
405 responders and PAS non-responders or even selecting subjects based on previous PAS results seems
406 generally unsuitable. Moreover, our results suggest that PAS has limited value in longitudinal studies,
407 for example, in studies monitoring drug effects over longer periods of time or in patient follow-up
408 studies characterizing disease progression.

409

410 **Conclusion**

411 Our results demonstrate that PAS-induced effects have a high intra-individual variability and that
412 PAS-induced potentiation of MEP size seems to be most pronounced at the end of the induction phase
413 opposed to after induction. Additionally, our results support the use of LMMs for analyzing PAS data.
414 The main implication of our results is that, due to its low consistency, PAS is unsuitable to track the
415 development of human brain plasticity over longer periods of time.

416

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421

422

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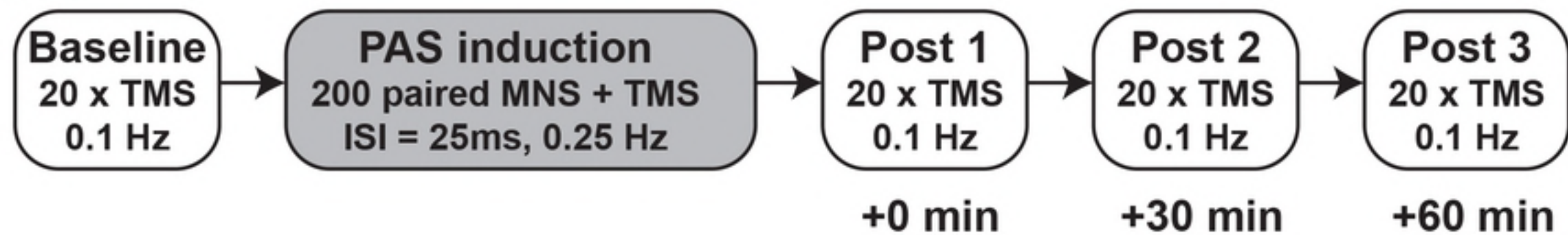
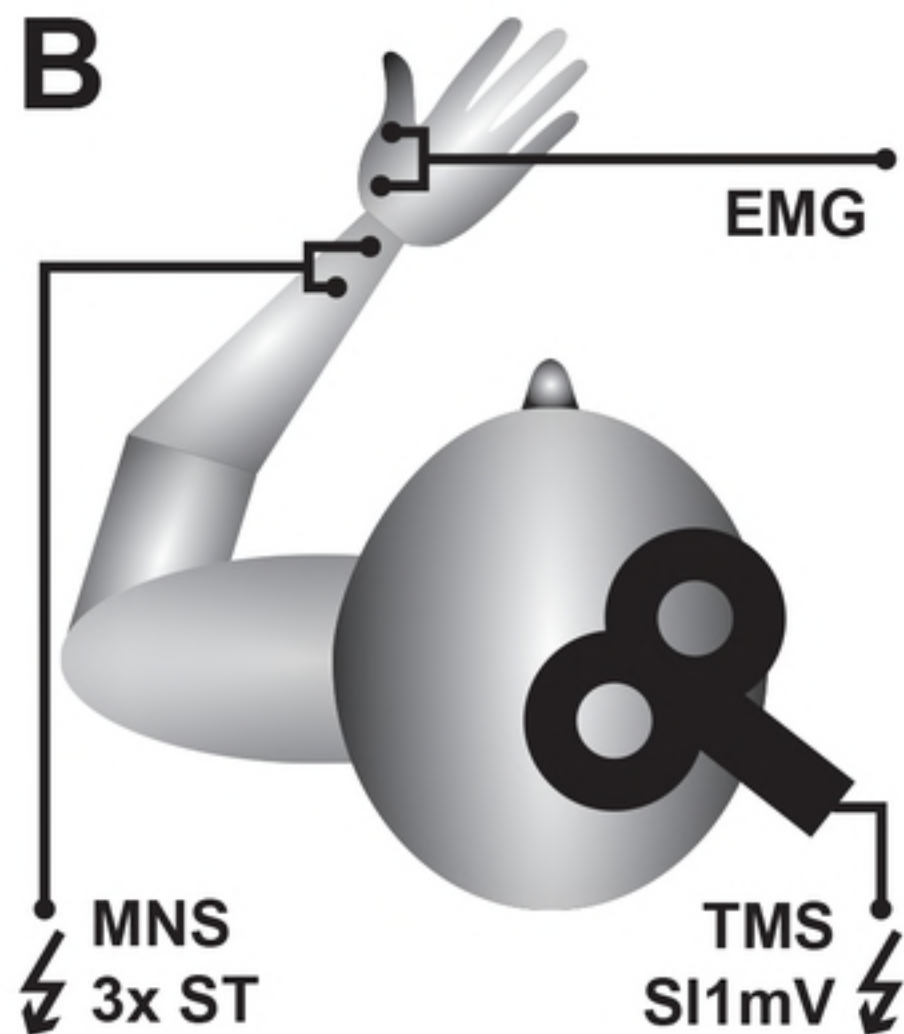
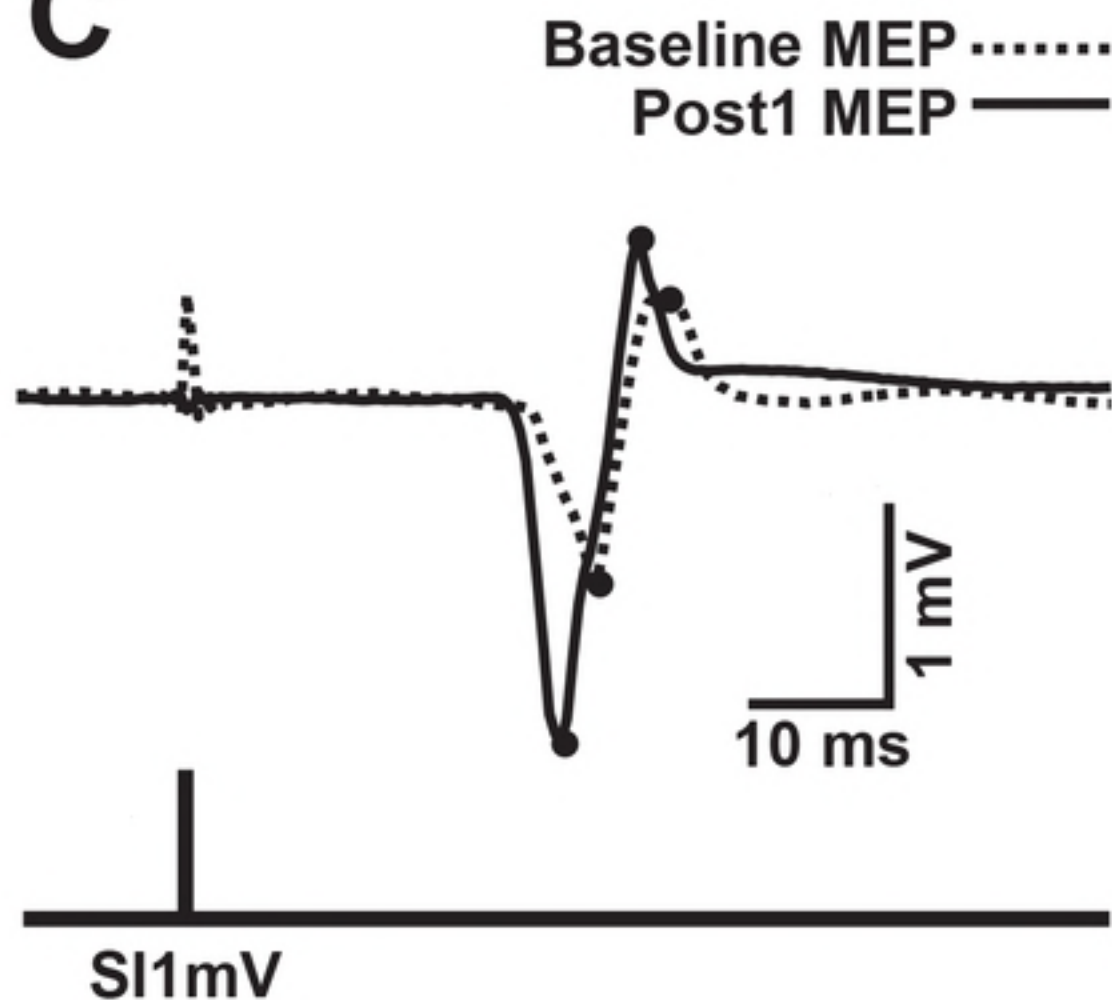
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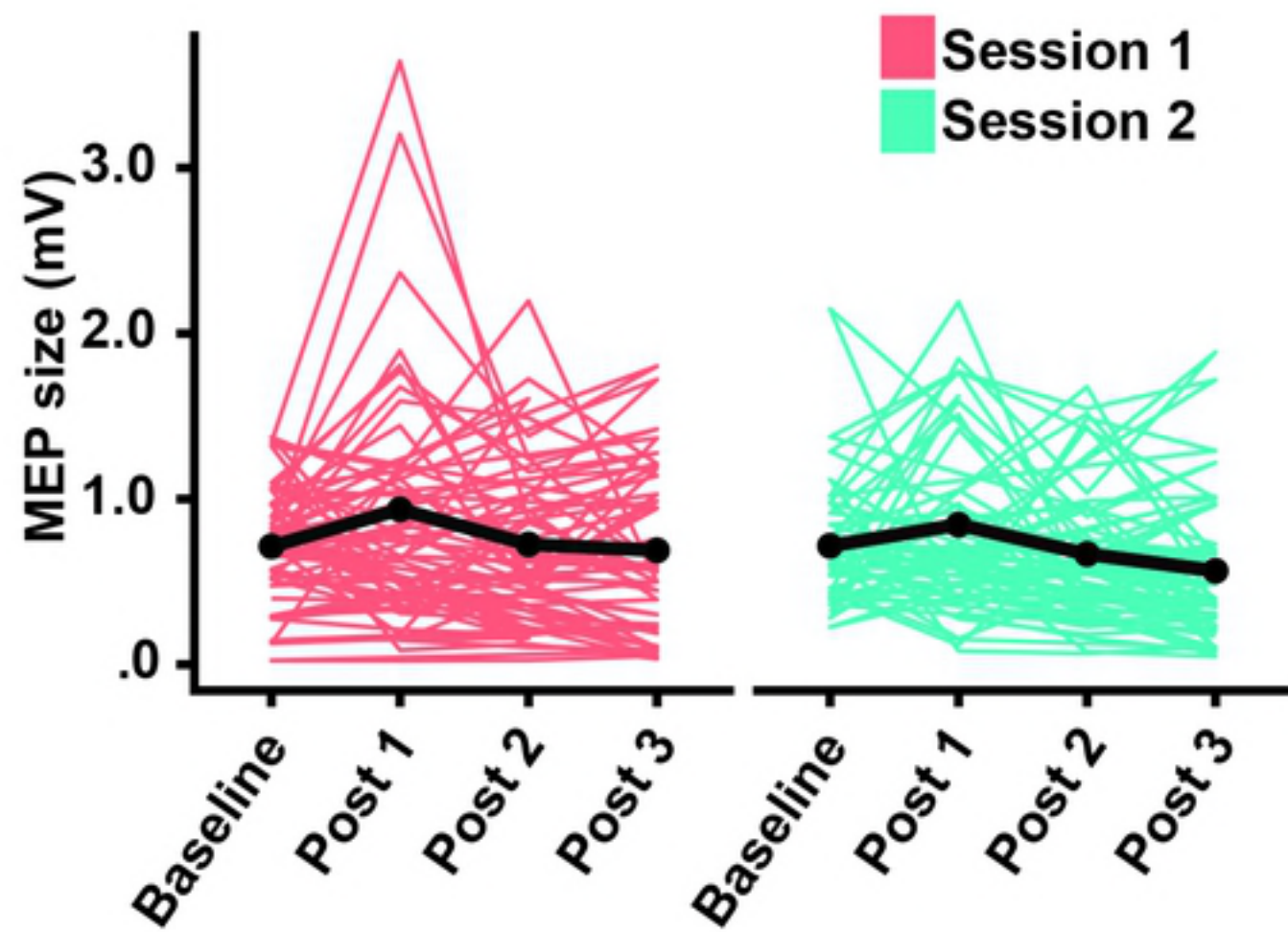
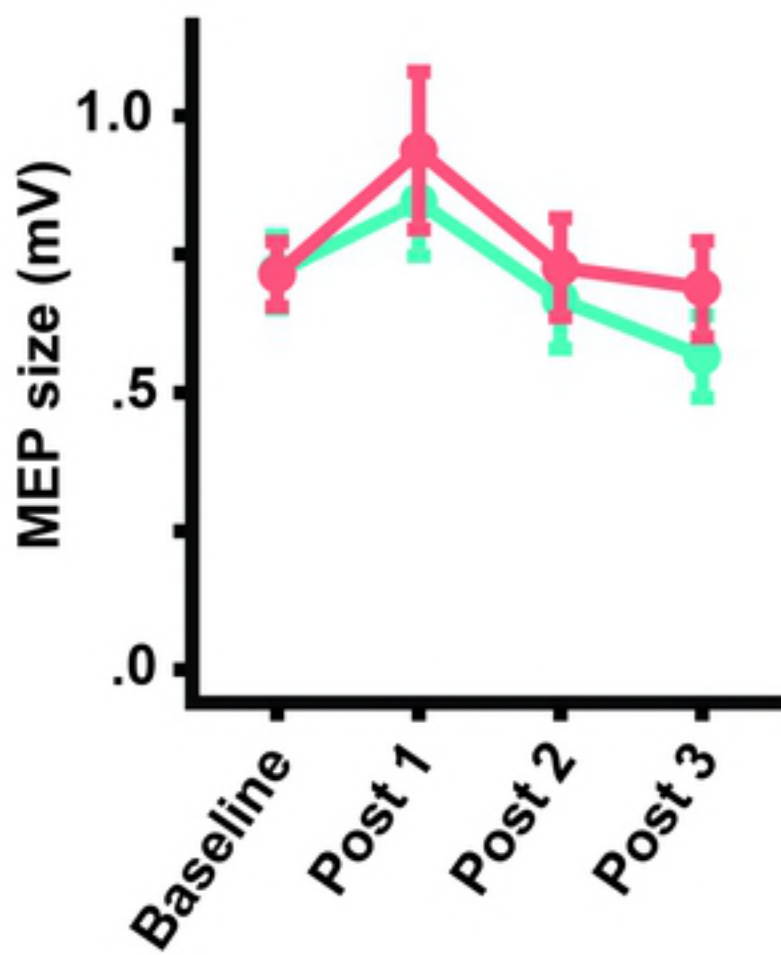
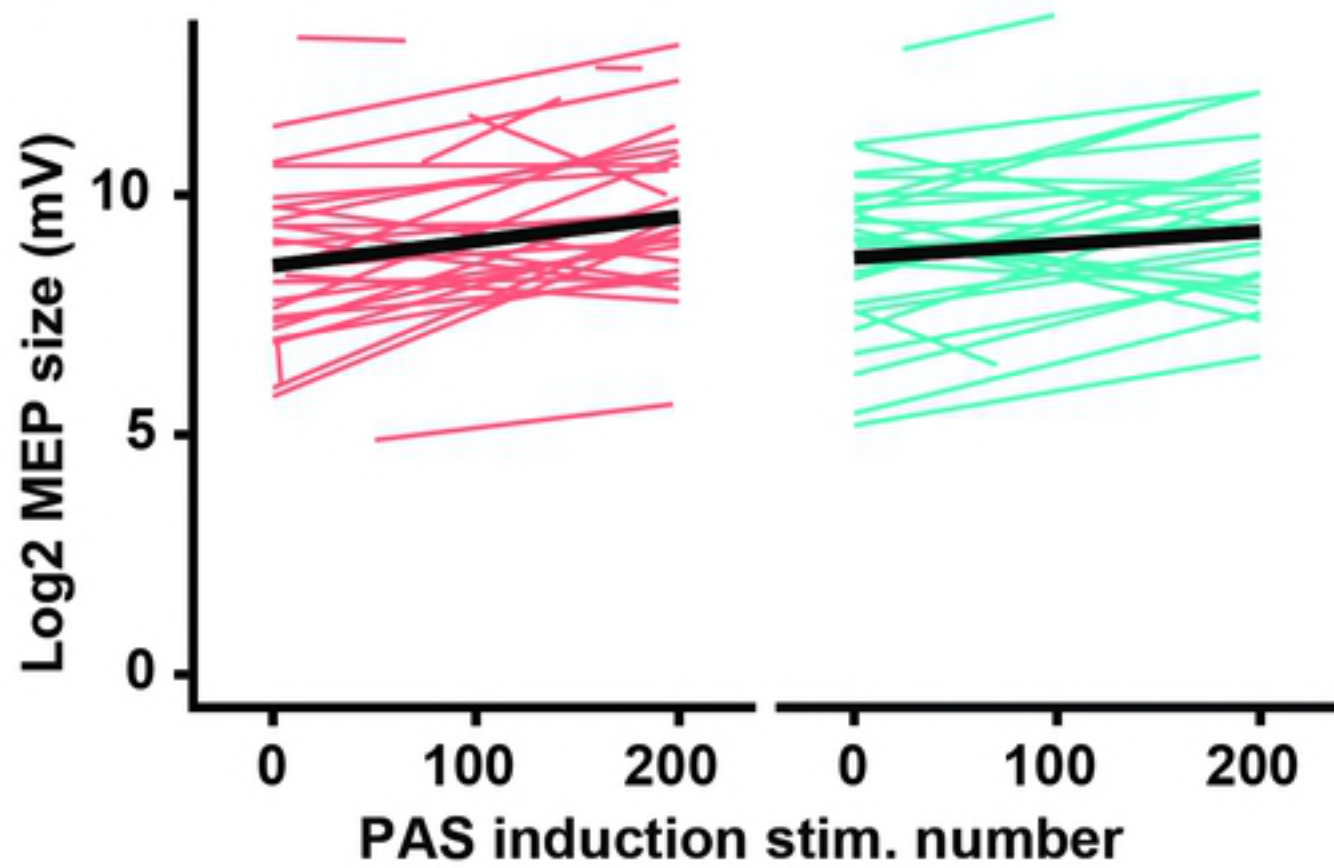
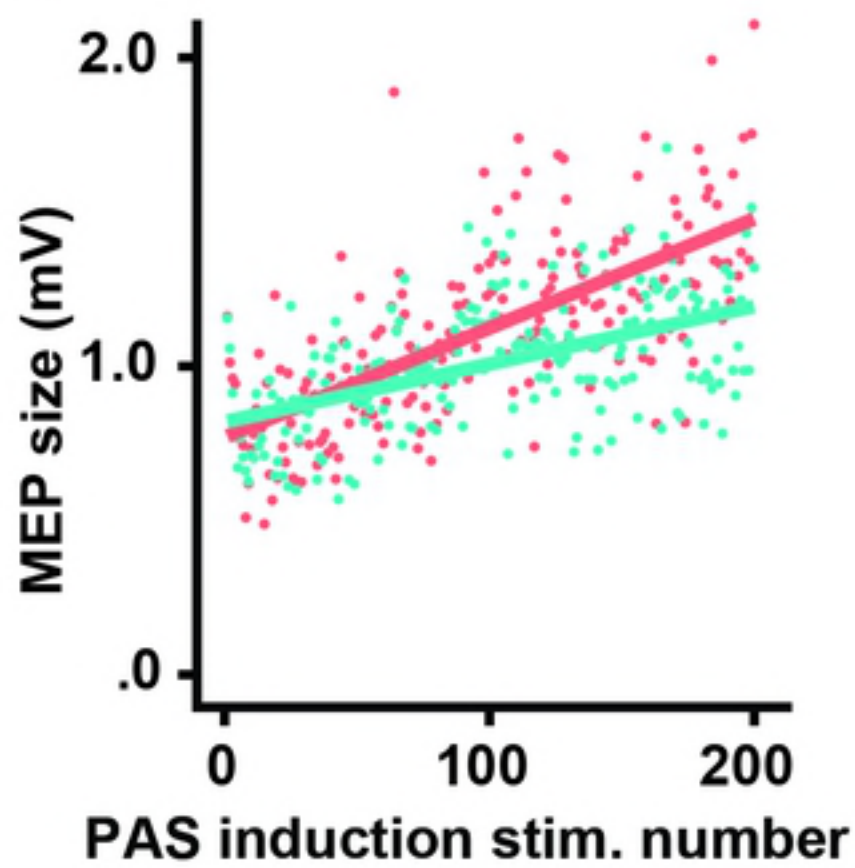
518 **Supporting Information**

519 **S1 Table. Reasons for excluding recruited individuals before entering the study.**

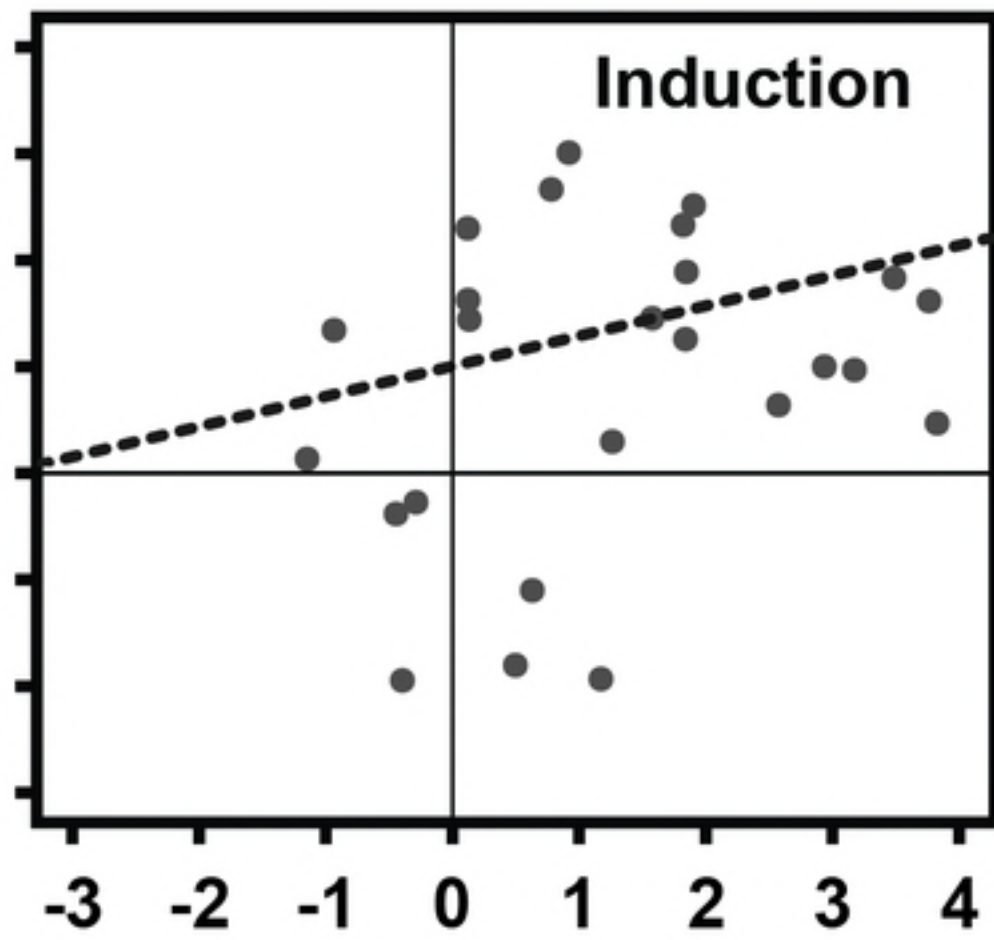
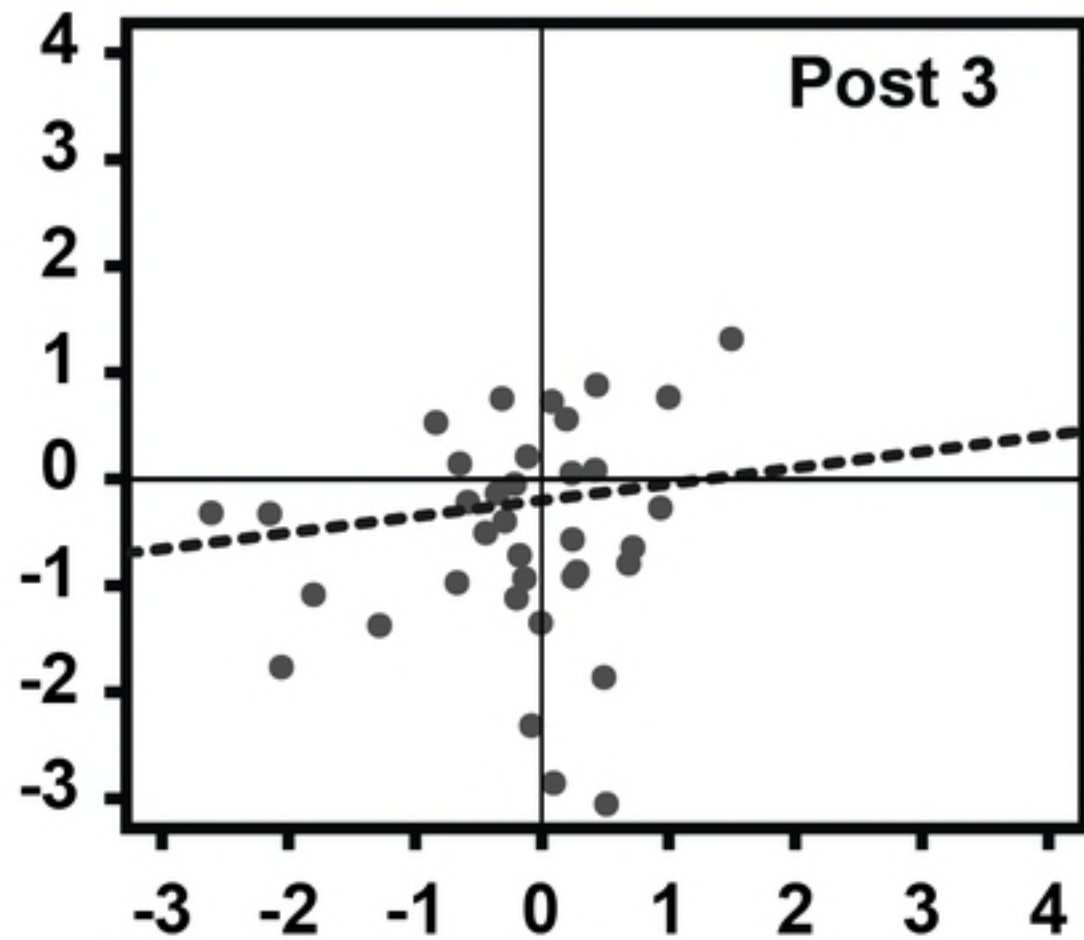
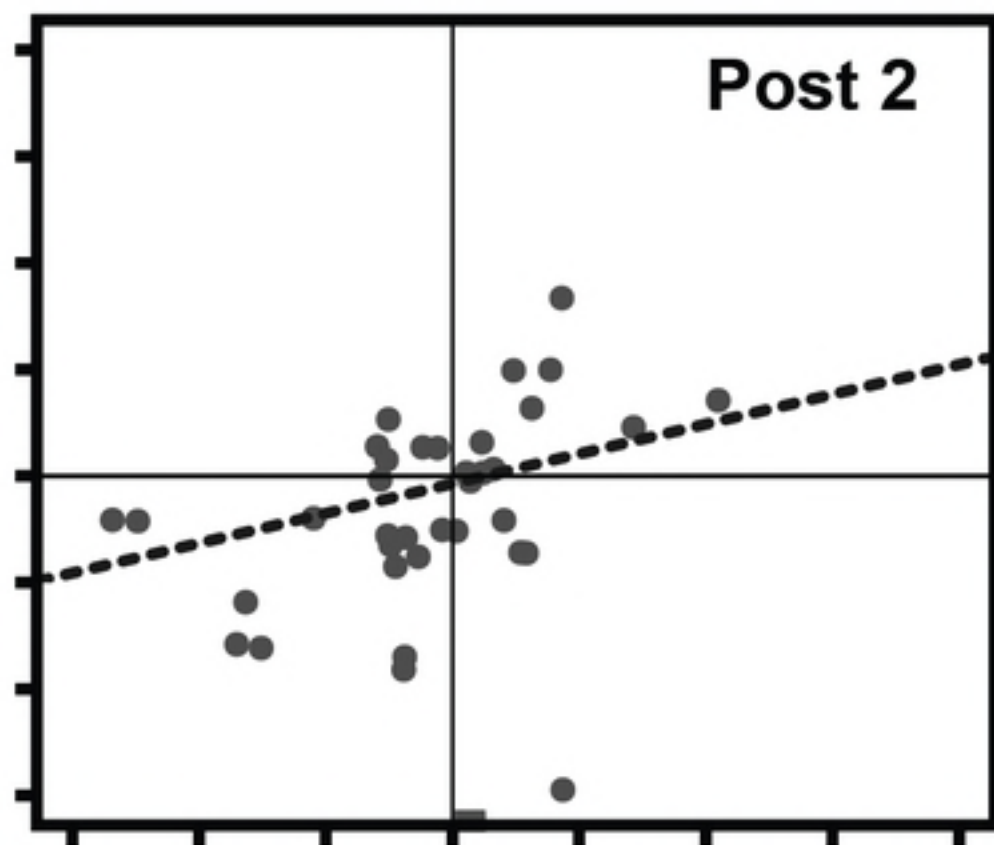
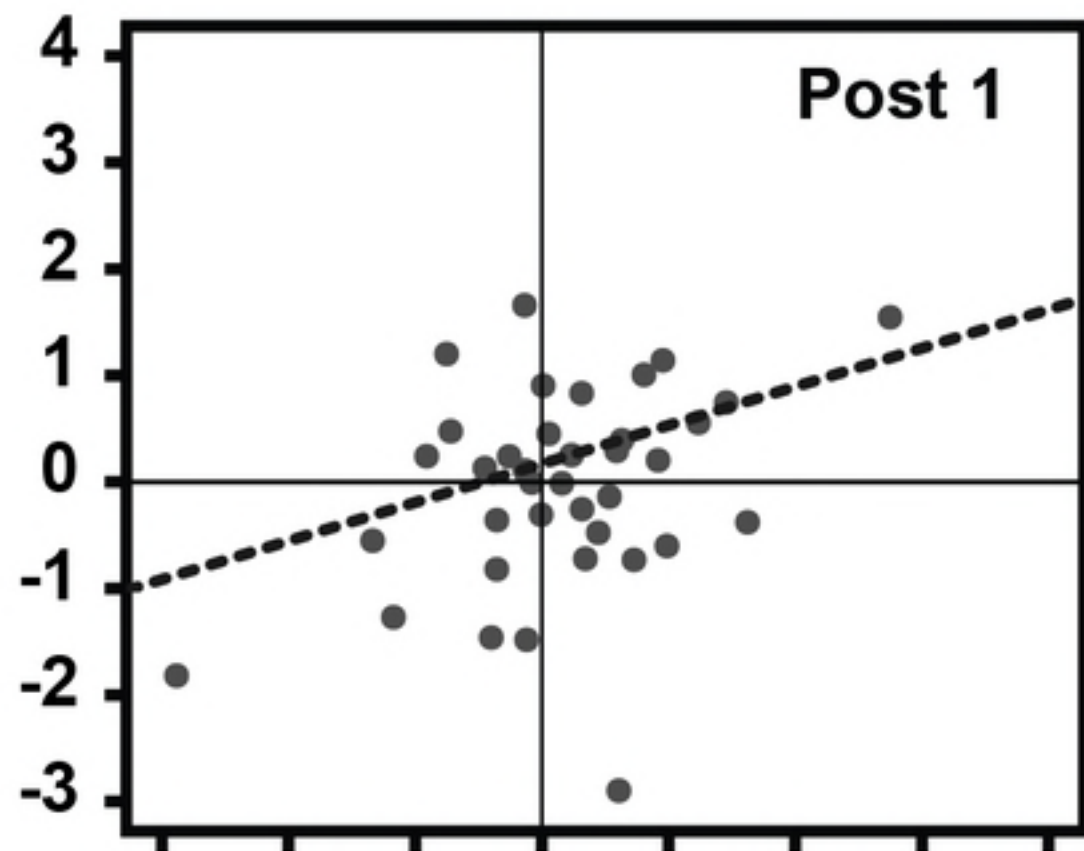
520 **S1 Dataset. Full dataset of all variables reported.** RDA-file containing all data on all variables
521 reported in this manuscript. This file can be opened and used with the open source R or RStudio
522 software [22].

523 **S1 Syntax. Syntax for all linear mixed models reported.** Syntax written in the R-language,
524 containing all linear mixed models that are used to generate results for this manuscript. This file can
525 be opened and used with the open source R or RStudio software [22].

A**B****C**

A**B****C****D**

Slope Session 2



Slope Session 1