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Supplemental vitamin B-12 enhances the neural response to sensory stimulation in the barrel cortex of healthy rats but does not affect spontaneous neural activity

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Abbreviations: B-12, Vitamin B-12; CON: control; CSD, current source density; EEG, electroencephalography; GABA, γ -aminobutyric acid; LFP, local field potential; MUA, multi-unit activity; PPR, paired-pulse ratio; PSD, power spectral density; SE, standard error.

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1 **Abstract**

2 **Background:** Although vitamin B-12 (B-12) is known to contribute to the structural and
3 functional development of the brain, it is unclear if B-12 supplementation has any beneficial
4 effect in healthy populations in terms of enhanced neurological status of the brain or
5 improved cognitive function.

6 **Objectives:** We investigated the effect of dietary supplementation of B-12 on the cortical
7 neural activity of well-nourished young adult rats and tested the hypothesis that B-12
8 supplementation in healthy rats may reduce sensory evoked neural activity due to enhanced
9 inhibition.

10 **Methods:** Female Lister Hooded rats weighing between 190g to 265g (2 to 4 months old)
11 were included in the study. The experimental group was fed with B-12 (Cyanocobalamin)
12 enriched water at a concentration of 1mg/L, and the control (CON) group with tap water for 3
13 weeks. Animals were then anaesthetised and cortical neural responses to whisker
14 stimulation were recorded *in vivo* using a multi-channel micro-electrode, from which local
15 field potentials (LFPs) were extracted.

16 **Results:** Somatosensory evoked LFP was enhanced 25% in the B-12 group ($4.13 \pm 0.24 \text{mV}$)
17 compared with the CON group ($3.30 \pm 0.21 \text{mV}$) ($P=0.02$). Spontaneous neural activity did not
18 differ between groups; frequency spectra at each frequency bin of interest did not pass the
19 cluster-forming threshold at the 5% significance level.

20 **Conclusions:** These findings do not provide evidence supporting the hypothesis of
21 decreased neural activity due to B-12 supplementation. As the spontaneous neural activity
22 was unaffected, the increase in somatosensory evoked LFP may be due to enhanced
23 afferent signal reaching the barrel cortex from the whisker pad, indicating that B-12
24 supplemented rats may have enhanced sensitivity to sensory stimulation compared to the
25 CON group. We suggest that this enhancement might be the result of lowered sensory
26 threshold, although the underlying mechanism has yet to be elucidated.

27 **Key words**

28 Local field potential, rat barrel cortex, vitamin B-12, dietary supplementation, sensory
29 threshold, GABA.

30 **1. Introduction**

31 Vitamin B-12 (B-12) is an essential nutrient, vital for the maintenance of blood and nervous
32 system function. It is a cofactor in the biosynthesis of methionine, a precursor for S-
33 adenosyl-methionine in the brain. S-adenosyl-methionine is a major methyl donor for
34 numerous central nervous system methylation reactions involving neurotransmitters, and
35 plays a crucial role in myelin methylation [1-4].

36 Given the critical biochemical role that B-12 plays in human metabolic processes and in the
37 synthesis of neurotransmitters, a recent study [5] investigated whether dietary
38 supplementation with a yeast extract rich in B-12 could alter neural activity produced by
39 visual patterns in the brains of healthy subjects. Using electroencephalography (EEG), the
40 researchers observed a reduction in the steady state visual evoked potential for the
41 intervention group compared to the placebo group, and it was speculated that B-12
42 supplementation in healthy subjects might lead to increased concentration of the inhibitory
43 neurotransmitter γ -aminobutyric acid (GABA), which in turn could modulate cortical
44 excitation and inhibition. We will refer to this as the 'GABA hypothesis'.

45 The primary objective of the current study was to investigate whether dietary
46 supplementation with B-12 has a significant effect on cortical neural activity. A set of *in vivo*
47 electrophysiological experiments were conducted to examine the effect of B-12
48 supplementation on cortical neural activity of healthy rats (2-4 months old) without B-12
49 deficiency. If B-12 supplementation could lead to increased global GABA concentration in
50 the brain, the likely effect would be a reduction in both the task-evoked and the spontaneous
51 neural activity [6]. Thus somatosensory evoked local field potential (LFP) as well as

52 spontaneous neural activity in rats with and without B-12 intervention were collected and
53 analysed. By examining the temporal dynamics of the evoked LFP, it was possible to assess
54 how B-12 supplementation may have modulated components of synaptic excitation and
55 inhibition in the LFP profile [7, 8]. To further investigate possible mechanisms underlying the
56 observed changes in evoked LFP responses, we used paired-pulse stimulation to compare
57 the sensory adaptation characteristics of the LFP between the diet groups, as sensory
58 adaptation has been shown to be related to the intensity of stimulation [9].

59 **2. Materials and Methods**

60 All experiments were carried out in accordance with United Kingdom Home Office
61 regulations (Animals (Scientific Procedures) Act, 1986) and approved by the Research
62 Ethics Committee at the University of Reading, UK.

63 **2.1 Animals and diets**

64 A total of 29 female Lister Hooded rats weighing between 190g to 265g (2 to 4 months old)
65 were included in the study. The strain, gender and age of the rats were chosen based on our
66 previous work [8, 10]. This choice allowed us to re-use some of our previous data when
67 making comparisons between CON and B-12 rats. Rats were housed in a temperature-
68 controlled room with a 12-h light:dark cycle with *ad libitum* access to food and water, and
69 were allowed to acclimatise to the animal room conditions and husbandry procedures for 3
70 days prior to the start of the feeding programme which lasted for 3 weeks.

71 All rats were fed with standard commercial food (Rat and Mouse No.3 Breeding, RM3(E),
72 801066, Special Diets Services, UK. The proximate composition: Moisture: 10.00%, Crude
73 Oil: 4.25%. Crude Protein: 22.39%. Crude Fibre: 4.21%. Ash: 7.56%. Nitrogen Free Extract:
74 51.20%), which has a B-12 (Cyanocobalamin) concentration of 26.78 μ g/kg, including
75 17.75 μ g/kg supplemented B-12 from manufactured sources. Although this is less than the
76 recommended dietary allowance (RDA) at 50 μ g/kg diet for rats [11], it is close to the B-12
77 concentration in standard feeds used in other studies, and is well above the B-12

78 concentration in feeds deficient in B-12 [12, 13]. This was confirmed by the analysis of B-12
79 concentration in serum samples (see Results).

80 The CON group (n=14) was fed with fresh water, while the B-12 group (n=15) was fed with
81 B-12 (Cyanocobalamin, Sigma-Aldrich, UK) enriched water. B-12 was added to water
82 incrementally for the first 3 days of the feeding programme at 25%, 50% and 75% of the final
83 concentration, which was 40 times the RDA for rats [11]. Assuming the daily intake of food
84 and water for adult rats to be approximately 5g/100g and 10mL/100g body weight
85 respectively [14, 15], we estimated the RDA of B-12 to be 0.25µg/100g of rat's body weight.
86 To provide 100% RDA of B-12 through water, the B-12 concentration would be 0.25µg/10mL
87 water. Thus the final concentration for the intervention was set at $40 \times 0.25\mu\text{g}/10\text{mL} =$
88 $10\mu\text{g}/10\text{mL}$ water, or 1mg/L water. We chose 40 x RDA to be the final concentration in
89 order to ensure the effectiveness of B-12 supplementation for this study. Dosages much
90 higher than this have been used in both rodents and humans without evidence of adverse
91 health effects [16, 17]. Note that fresh water (without B-12) was not supplied to the B-12
92 group.

93 ***2.2 Surgery, neural recording and sample collection***

94 For detailed experimental procedures, the reader is directed to our previous publications [8,
95 18, 19] for reference. They are briefly reviewed below.

96 Following 3 weeks of supplementation, animals were weighed, anaesthetised and operated
97 on following our laboratory's standard surgical procedures. Stimulation was in terms of brief
98 electric current pulses which were applied to the right whisker pad, and the neural activity of
99 the contralateral barrel cortex was recorded via a 16-channel multi-laminar micro-electrode
100 inserted perpendicular to the cortical surface of the barrel cortex. The neural signals thus
101 recorded were typically low-pass filtered below ~500Hz [20] to produce the LFP which
102 reflected changes in extra-cellular potentials with respect to a reference potential, and was
103 primarily the weighted sum of post-synaptic activities of the local pyramidal neural

104 population. During whisker stimulation, LFP became more negative as positive currents
105 flowed from extracellular space into intracellular space to depolarise principal neurons. The
106 amplitude of the LFP deflection is approximately proportional to the strength of the
107 stimulation [21], and the LFP deflection during the initial timeframe (1~2ms from the onset of
108 the deflection) represents solely the excitatory post-synaptic activity of the local pyramidal
109 neural population [7, 8].

110 A minimum of 100 trials were collected per animal with an inter-trial-interval of at least 5s. All
111 neural data were sampled at 24.41 kHz. Stimulus intensity of 1.2mA was used for all
112 animals. After initial analysis which revealed a larger evoked LFP amplitude in the B-12
113 supplemented group compared with the CON group (see Results), an additional
114 experimental condition with a stimulus intensity of 1.6mA was added to four rats in the CON
115 group to investigate if the evoked LFP response under stronger stimulus intensity without B-
116 12 supplementation could result in similar amplitude increases. The 1.6mA intensity is the
117 strongest intensity previously tested without causing changes in either blood pressure or
118 heart rate of rats under the adopted experimental paradigm [21-23]. The LFP data from
119 these rats were then combined with an existing data set (n=4) collected from previous
120 experiments conducted in our laboratory using identical experimental protocols and stimulus
121 intensities [8]. Thus the total number of rats (in the CON group) subjected to both 1.2mA and
122 1.6mA stimulus intensities was eight.

123 Finally for a subset of subjects (n=9/group), additional paired-pulse stimulation at 1.2mA was
124 used to investigate if sensory adaptation characteristics could be altered by B-12
125 supplementation. Stimulus parameters were kept the same for each pulse, while the inter-
126 pulse-interval was set as 200ms, with the inter-trial-interval set as 10s.

127 At the end of each experiment, the rat was terminated by cervical dislocation. Blood
128 collected via cardiac puncture was centrifuged at 3030 x g for 6 minutes at room
129 temperature, and serum was then collected and stored at -80°C for further analysis. Brains
130 were extracted, weighed and stored.

131 Note that only 26 (n=13/group) out of the 29 rats in the study provided usable neural data
132 due to premature death or damage during surgery. Also serum was successfully collected
133 from 21 (CON, n=10; B12, n=11) rats only. Serum samples were analysed for cobalamin (B-
134 12) concentration using the Immulite/Immulite 1000 Systems VB Vitamin B12 (Siemens
135 Healthcare Diagnostics Products Ltd) at the Pathology and Diagnostic Laboratories of the
136 Royal Veterinary College based in Hertfordshire, UK. The device uses a solid-phase,
137 competitive chemiluminescent enzyme immunoassay which has an intraassay imprecision
138 (mean \pm standard deviation) of 1308 ± 77 (pg/mL). The standard protocol for the quantitative
139 measurement of B-12 in serum, as detailed in the manufacture's user guide, was followed.

140 ***2.3 Data pre-processing and parameter Estimation***

141 Neural recordings from the micro-electrode were first pre-processed using our laboratory's
142 standard procedure [8]. Briefly, stimulus artifact was removed, data were zero-meant at
143 baseline and low pass filtered. Inverse Current Source Density (spline iCSD [24]) analysis
144 was performed to locate the layer IV sink [25] for each data set, and the CSD data were then
145 used to align both the CSD and the LFP data according to their sink locations across
146 animals, with the common sink placed 600 μ m below the pial surface. We used the re-
147 aligned LFP time series at channel 7, where the sink was located, to represent the evoked
148 neural activity to whisker pad stimulation, as this channel was located in the cortical layer
149 which was targeted by thalamocortical afferents, with thalamus acting as a relay to deliver
150 tactile response to whisker stimulation to the barrel cortex [26-28]. The evoked LFP was
151 calculated by averaging over 100 trials for each animal. The first negative deflection
152 observed in the evoked LFP was referred to as N1.

153 In order to compare evoked LFP across groups, the following parameters were extracted
154 after pre-processing to smooth and align the data: (i) the onset of N1, which was defined as
155 the time at which N1 exceeded -0.1mV, with the stimulus onset time assigned as zero; (ii)
156 the initial slope of N1, which was defined as the slope from 2~25% of the N1 peak
157 amplitude; (iii) the peak amplitude of N1, and (iv) the latency of the N1 peak.

158 For paired-pulse analysis, the amplitude of N1 of the second pulse was also extracted and
159 the paired-pulse ratio (PPR) was calculated within each trial from

$$160 \quad \text{PPR} = \frac{\text{Amp(N1 of second pulse)}}{\text{Amp(N1 of first pulse)}}$$

161 **2.4 Frequency domain analysis**

162 To investigate possible mechanisms giving rise to differences in evoked LFP responses
163 across diet groups, we checked the anaesthetic levels during the recording period to ensure
164 that they were not significantly different between groups, as it has been shown that sensory
165 evoked LFP is sensitive to the level of anaesthesia, and that the anaesthetic level is
166 reflected in the resting state PSD within the frequency range 1~8Hz [29, 30]. Thus the
167 resting state PSD below 8Hz was used to compare the anaesthetic depth between groups.
168 To compute the resting state PSD for each trial, we used the resting state LFP data 0.9~4.9s
169 post stimulation, down-sampled the data to 10KHz, and calculated PSD in Matlab™ via
170 Welch's method (Hamming window, 50% overlap).

171 It is also well known that sensory evoked neural activity is closely influenced by spontaneous
172 activity in the same cortical region [31-33]. One possible explanation for differences in
173 evoked LFP responses between diet groups could be changes in spontaneous subthreshold
174 activity and/or spontaneous spiking activity due to B-12 supplementation. Thus we extended
175 the resting state PSD calculation to include frequencies up to 3000Hz to cover both the
176 subthreshold (8~500Hz) neural activity and the multi-unit activity (MUA, 500~3000Hz) [20].

177 **2.5 Statistical analysis**

178 Throughout the analysis, the significance level α was set at 0.05. Group analysis was
179 performed to compare various measurements and parameters extracted from field potential
180 recordings between the two diet groups using the two-tailed two-sample Student's t-test
181 under the assumption that the sampling distribution of the mean was normally distributed.
182 Parameters were presented as mean \pm standard error (SE). To compare the N1 amplitude in

183 response to two stimulus intensities applied to the same rat, the two-tailed paired-sample
184 Student's t-test was used.

185 To compare the ratios of brain weight to body weight across the groups, the non-parametric
186 Wilcoxon rank-sum test was used to test for equal medians, as the ratio of two normally
187 distributed variables is no longer normally distributed.

188 For comparison of PSDs over the frequency range 1~3000Hz, a non-parametric cluster
189 correction procedure [34, 35] was used to determine significant clusters across the
190 frequency range while controlling for multiple comparisons. This involved conducting an
191 independent two-sample t-test at each frequency bin (width=1Hz) to compare responses in
192 the CON and the B-12 groups. Tests that were significant at $P<0.05$ were aggregated into
193 clusters across adjacent frequency bins. The summed t-statistic for each cluster was
194 compared to a null distribution generated by resampling the data from the largest cluster
195 10,000 times with randomly assigned group labels, and recalculating the summed t-statistic.
196 Clusters which fell outside of the empirical 95% confidence intervals of the null distribution
197 were considered significant.

198 Finally, we assumed no significant bias in the weight of the rats between the two groups at
199 the start of the feeding programme. This was reasonable based on the fact that all rats,
200 weighing between 175 ~ 224g, were purchased from the same source (Charles River, UK)
201 on 8 occasions (4 pseudorandom occasions per diet group) across a 20-month period. It
202 should be noted that rats were only weighed once immediately prior to surgery.

203 **3. Results**

204 ***3.1 B-12 serum concentration, body and brain weights***

205 The serum cobalamin concentration was 98% greater in the B-12 group compared with the
206 CON group ($P<0.01$) (**Figure 1A**). The concentration in the CON group was within the
207 normal range for rats [13, 36, 37], thus confirming that they were not deficient in B-12. There

208 was no significant difference between the final body weight (**Figure 1B**) and brain weight
209 (**Figure 1C**), and the brain/body weight ratio (**Figure 1D**) between the two diet groups. Thus
210 our results suggest that, assuming no significant weight difference across diet groups at the
211 start of the feeding programme, B-12 supplementation did not significantly change body
212 weight, brain weight, or the ratio between them.

213 **3.2 Amplitude of evoked LFP was increased by B-12 supplementation**

214 Along the cortical depth, the B-12 group had a larger LFP response (**Figure 2A**) and a
215 correspondingly stronger sink/source pair (**Figure 2B**) compared to the CON group. These
216 were reflected in the brighter blue colour associated with the B-12 group images. Time
217 series of the evoked LFP responses in the layer IV sink are displayed in **Figure 2C**. The
218 amplitude of N1 for the B-12 group was 25.2% larger than that of the CON group ($P=0.02$)
219 (**Figure 2D**), while the latency of the N1 peak for the B-12 group was significantly shorter
220 than the CON group ($P=0.03$) (**Figure 2E**). In addition, the initial slope of N1 for the B-12
221 group was significantly steeper than the CON group ($P<0.01$) (**Figure 2F**), however the
222 onset of N1 was not significantly different ($P=0.39$) (**Figure 2G**). Together these
223 characteristics suggested that the dynamics of the evoked LFP response for the B-12 group
224 were faster, reflected in the steeper initial slope, and stronger, in terms of the N1 amplitude,
225 compared to the CON group. However the onset of N1 was not significantly different
226 between the diet groups, with the important implication that B-12 supplementation for 3
227 weeks did not significantly change the transmission speed of the afferent neural signal
228 arriving at the barrel cortex from the whisker pad.

229 **3.3 Sensory adaptation was weakened by B-12 supplementation**

230 Sensory adaptation characteristics of neural responses were investigated using the paired-
231 pulse stimulus paradigm, results of which are shown in **Figures 3A** and **3B** for CON and B-
232 12 groups respectively. The PPR for the B-12 group was 21.9% higher than the CON group
233 ($P=0.04$) (**Figure 3C**), indicating that the second pulse was significantly less adapted for the

234 B-12 group than the CON group. Therefore, despite a higher amplitude of the first evoked
235 LFP pulse in the B-12 group compared to that of the CON group, the recovery of the second
236 pulse (200ms apart) was faster in the B-12 group.

237 **3.4 Resting state power spectral density (PSD) analysis**

238 There was a clear overlap of PSDs between individuals in the CON group and those in the
239 B-12 group, indicating no significant difference in either the depth of anaesthesia (**Figure**
240 **4A**), or the subthreshold and MUA neural activity (**Figure 4B**) between the two diet groups.
241 The nonparametric cluster correction analysis showed that, across all frequency bins, the
242 maximum absolute t-statistic was 1.84, less than the critical t-value of 2.06 (n=13/group,
243 degree of freedom=24) for significance at the 5% level, further confirming that there was no
244 significant difference in PSDs between the two diet groups in the frequency range
245 1~3000Hz.

246 **3.5 Effect of stimulus intensity**

247 For rats without B-12 supplementation, the evoked LFP amplitude to the 1.6mA stimulation
248 was 13.9% higher than that to the 1.2mA stimulation ($P<0.01$) (**Figure 5A**). For comparison,
249 we re-plotted the LFP responses of CON and B-12 groups to the 1.2mA stimulation (**Figure**
250 **5B**). As stated previously in Section 3.2, the N1 amplitude for the B-12 group was 25.2%
251 higher than that of the CON group.

252 **4. Discussion**

253 To the best of our knowledge, this is the first study to show that healthy rats supplemented
254 with B-12 demonstrate an increase in sensory evoked synaptic activity in the somatosensory
255 cortex. We discuss here possible mechanisms underlying the observed phenomena and
256 their implications for future research.

257 **4.1 B-12 supplementation and the myelin sheath**

258 It is well-known that B-12 plays a crucial role in myelin methylation. Recent research further
259 suggests that the myelin sheath is more than an inert insulating membrane structure [38-40].
260 A study on rat somatic sensorimotor system has shown that the structure of myelin sheath in
261 the spinal cord underwent changes throughout the aging process [41]. Furthermore
262 myelination properties have been shown to be regulated by neuronal activity and the
263 environment [42, 43]. It is therefore plausible that in young adult rats, as used in our study,
264 myelination properties such as myelin sheath length and/or thickness could be altered within
265 a 3-week period, and that the increased neural response described here could be the result
266 of strengthened myelination of neurons in B-12 rats. However, the onset of the N1 deflection
267 in our data across the diet groups did not differ significantly (Figure 2G), suggesting that the
268 neuron conduction velocity was not changed by the supplementation. However, we also
269 recognise that there is a minimum difference of onset that could be detected in our
270 measurement at a 5% level of significance. This can be estimated using the two-sample t-
271 statistic:

$$272 \quad \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s_1^2/n_1 + s_2^2/n_2}} = t_{n_1+n_2-2, 0.05}$$

273 With the standard errors of onset shown in Figure 2G (CON: SE=0.08ms; B-12: SE=0.06ms;
274 n=13/group), we found this difference to be 0.21ms. In other words, if the onset difference
275 between the two groups was 0.21ms or less, we would not be able to detect it at a 5% level
276 of significance.

277 **4.2 B-12 supplementation and the ‘GABA hypothesis’**

278 We are not aware of any study providing evidence linking dietary supplementation of B-12 to
279 changes in GABA in the brain. However using intracerebroventricular infusion, Ikeda et al
280 [44] investigated the effect of B-12 on circadian pace-making in rodents and found that B-12
281 infusion significantly increased the content of GABA in the suprachiasmatic nucleus of the
282 hypothalamus, while the content of the excitatory neurotransmitter glutamate in the same
283 region was significantly decreased. The authors speculated that B-12 may modulate the

284 metabolism of GABA and glutamate by facilitating glutamic acid decarboxylase activity. In
285 addition, a recent human study investigated the effect of dietary intervention of a yeast
286 extract substance on steady state visual evoked potentials (VEPs), and found reduced
287 neural responses in the diet group compared to the placebo group [5]. As the yeast
288 substance was richer in B-12 in comparison to the placebo substance, the researchers
289 suggested that the observed reduction could be the result of increased GABA concentration
290 in the brain due to dietary supplementation of B-12.

291 Direct comparison of our study to the above human study is not possible, not least because
292 the stimulus paradigms used in the two studies were very different. However if dietary
293 supplementation with B-12 increased the global GABA concentration in the brain, the likely
294 effect on the spontaneous as well as task-evoked neural activity would be a reduction in both
295 [6]. PSD analysis of our data during resting state showed no significant difference between
296 the two groups over the frequency range 1~3000Hz (Figure 4). In addition, the evoked LFP
297 for the B-12 group showed significantly increased amplitude and faster temporal dynamics.
298 Both of these observations could be taken as evidence against the GABA hypothesis.

299 GABA concentration in rat brain can be measured using techniques such as Gas
300 Chromatography-Mass Spectrometry, immunohistochemistry and magnetic resonance
301 spectroscopy. We plan to conduct some of these tests for our future studies.

302 ***4.3 B-12 enhanced LFP may implicate enhanced sensitivity to sensory stimulation***

303 Figure 2F showed that the initial slope of N1 for the B-12 group is steeper than that of the
304 CON group. Based on our previous study [8], this suggests faster excitatory post-synaptic
305 activity for the B-12 group. On the other hand we didn't observe significant difference in the
306 resting state neural activity between the groups. The scenario is analogous to the barrel
307 cortex responding to whisker stimulation with two levels of intensity, the stimulus with
308 stronger intensity will evoke a higher LFP response amplitude than that evoked by the lower
309 intensity stimulus, while the resting state LFP will be unaffected by stimulus strength [8, 21,

310 23]. In other words, the enhanced LFP response for the B-12 group could be due to
311 enhanced thalamo-cortical afferent signal, suggesting that B-12 supplementation in well-
312 nourished rats may have enhanced the sensitivity of neurons to sensory stimulation in the
313 lemniscal pathway linking peripheral nerves in the whisker pad to neurons in the thalamus.
314 This is further supported by our results on sensory adaptation. The mechanism underlying
315 sensory adaptation and stimulus strength was studied in detail by Ganmor et al [9] who
316 demonstrated that stronger whisker stimulation produced weaker sensory adaptation in the
317 somatosensory cortex of rodent. They pinpointed the source of this weaker adaptation to
318 neurons in the brainstem trigeminal complex and argued that such coding strategy may be
319 used to discriminate stimulus intensities during adaptation in order to counterbalance the
320 effect of short-term synaptic depression in the thalamus and subsequently in the cortex.
321 Based on their work, our observed weaker sensory adaptation in the B-12 group could be
322 due to neurons in the brainstem responding more strongly to the same stimulus compared to
323 the CON group, with faster recovery and subsequently less adaptation to the second
324 stimulus. Further experiments will be needed to confirm this by using a wider range of
325 stimulus intensities with neural recordings from the thalamus and the brainstem of both diet
326 groups.

327 ***4.4 B-12 supplementation and sensory threshold***

328 Changes in sensory evoked potentials have been linked to changes in sensory threshold.
329 Lund et al [45] found that, post-surgery, sensory threshold to cutaneous electrical stimulation
330 was increased, while the peak-to-peak amplitudes of somatosensory evoked potentials were
331 decreased significantly. A more recent study on pain-threshold and aggressiveness found
332 that individuals who more often behave aggressively had a higher pain threshold, and
333 aggressiveness was negatively correlated to the amplitude of pain-related evoked potentials
334 [46]. In auditory research, evoked potentials have been found to correlate with auditory
335 signal detection, specifically the amplitude of auditory evoked potentials associated with
336 correctly detected signals were found to be much higher than those corresponding to falsely

337 reported signals, undetected signals or correctly reported non-signals [47]. Furthermore,
338 sensory thresholds can be lowered by training. Using the human visual system, Skrandies et
339 al [48] demonstrated that sensory threshold decreased during repeated presentation of
340 visual hyper-acuity stimuli. This was accompanied by significantly larger amplitude of VEPs.
341 Interestingly, they also observed significantly shorter peak latency in VEPs post training,
342 which agrees with the shorter N1 peak latency for the B-12 group (Figure 2E).

343 Based on this body of literature, we suggest that B-12 supplementation may have the effect
344 of lowering the sensory threshold, thus enhancing the sensitivity of neurons to sensory
345 stimulation.

346 **4.5 Future work**

347 The conclusion of the study is limited by several shortcomings. One is that the concentration
348 of GABA in the brain was not measured, thus we were unable to confirm if the sensory
349 evoked LFP difference between the diet groups was related to the different levels of GABA
350 concentration. The other is that we did not measure B-12 concentration in the brain,
351 although its concentrations in serum samples were obtained. Furthermore, cognitive
352 correlates of B-12 supplementation were not assessed. We plan to incorporate these
353 measurements in future studies to further elucidate the role that B-12 may play in shaping
354 the neurological and cognitive functions of the brain.

355 Dietary supplementation of B12 is inexpensive and non-toxic. If it can be demonstrated to
356 slow down age-related cognitive decline through increased responsiveness to sensory
357 stimulation, it will have significant impact on the well-being of older people, and generate
358 considerable economic as well as public health benefits.

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363 The authors' contributions to the manuscripts are as follows: D.T.F., M.B-H., Y.Z. and D.H.B.
364 conceived the project. Y.Z., M.B-H. and C.W. designed the research. S.K., Y.H., M.B-H. and
365 Y.Z. collected data. M.B., X.W., K.K., I.S. and A.B. provided essential materials. Y.Z., S.K.,
366 M.B-H., D.H.B. analysed data. Y.Z. wrote the manuscript. D.T.F., C.W., A.B., M.B-H., D.H.B.
367 and I.S. contributed to the editing of the manuscript. Y.Z. had primary responsibility for the
368 final content. All authors have read and approved the final manuscript.

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Figure legends

FIGURE 1. Serum cobalamin concentration (A), final body (B) and brain weights (C), and the brain/body weight ratio (D) in adult female rats that did not or did consume B-12 for 3 weeks. Values are means \pm SEs. For serum samples, CON, n=10; B-12, n=11. For body and brain weights, CON, n=14; B-12, n=15. Asterisks indicate different from CON: $**P < 0.01$. B-12, vitamin B12; CON, control.

FIGURE 2. Neural responses and associated parameters in adult female rats that did not or did consume B-12 for 3 weeks. (A) Mean LFPs displayed as images for the two diet groups. Cortical depth is along the vertical axis, with the top of the image being 200 μ m below pia mater. Time is along the horizontal axis, with the black triangle indicating stimulus onset. (B) Similar to (A) but CSD of the two diet groups. (C) Mean evoked LFP time series of the two diet groups. Shadows indicate SE. Stimulus onset is at t=0. (D) The amplitude, (E) the latency, (F) the initial slope, and (G) the onset of N1. Values are means \pm SEs, n=13/group. Asterisks indicate different from CON: $*P < 0.05$; $**P < 0.01$. B-12, vitamin B12; CON, control; CSD, current source density; LFP, local field potential.

FIGURE 3. Mean LFP responses to paired-pulse stimulation in adult female rats that did not (A) and did (B) consume B-12 for 3 weeks. Shadows indicate SE. Stimulus onsets are at t=0 and t=200ms respectively. (C) PPR of the two diet groups. Values are means \pm SEs, n=9/group. Asterisks indicate different from CON: $*P < 0.05$. B-12, vitamin B12; CON, control; LFP, local field potential; PPR, paired-pulse ratio.

FIGURE 4. Mean resting state PSD in the frequency range 1~8Hz (A), and 8~3000Hz (B) of adult female rats that did not or did consume B-12 for 3 weeks. Individual subject's PSD are also displayed, n=13/group. B-12, vitamin B12; CON, control; PSD, power spectral density.

FIGURE 5. (A) Mean LFP responses to whisker stimulation at intensities 1.2mA (n=13) and 1.6mA (n=8) respectively for adult female rats that did not consume B-12. (B) Mean LFP

responses of the two diet groups at the same stimulus intensity of 1.2mA, n=13/group. Error bars indicate SEs. B-12, vitamin B12; CON, control; LFP, local field potential.

Figure 1

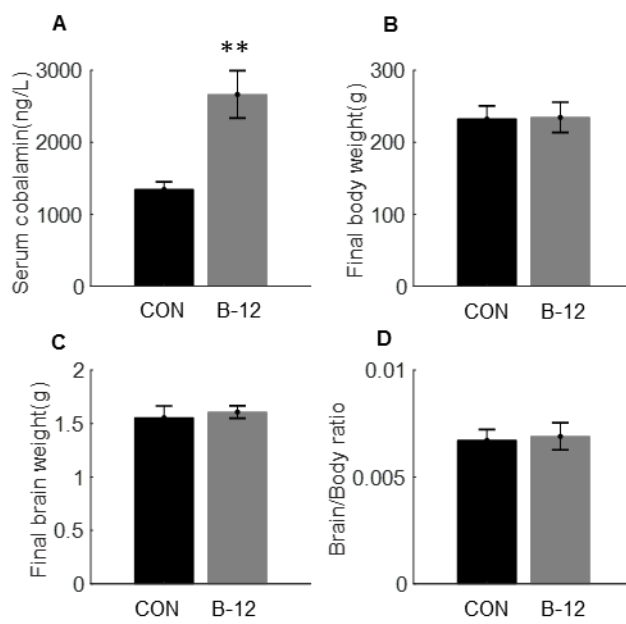


Figure 2

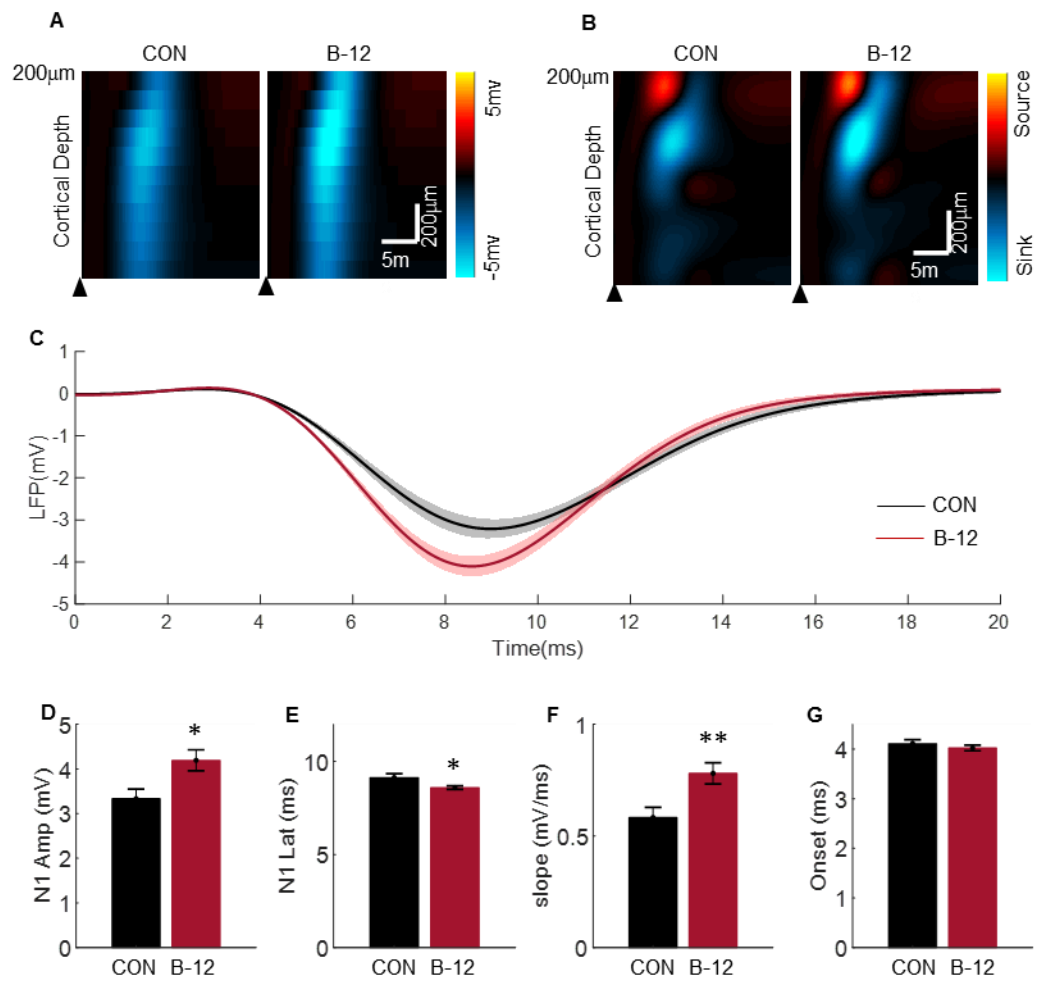


Figure 3

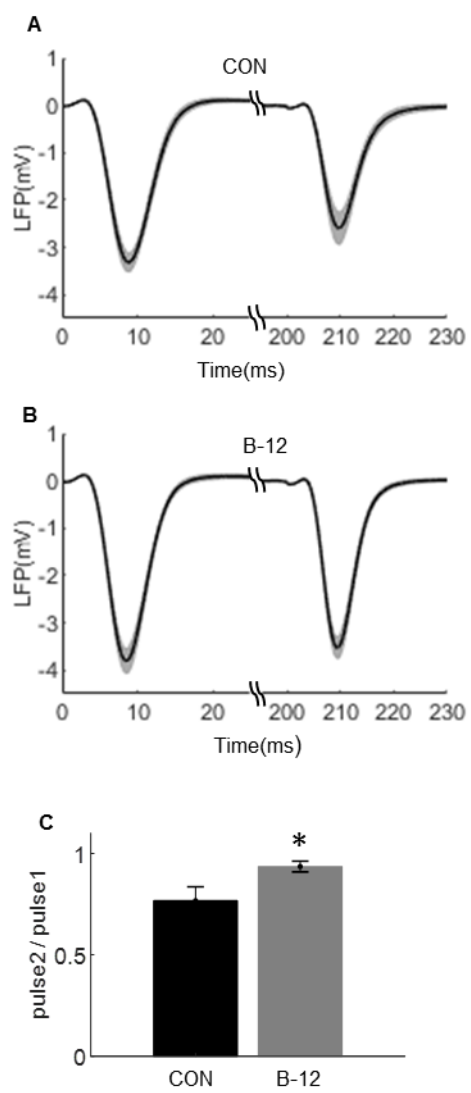


Figure 4

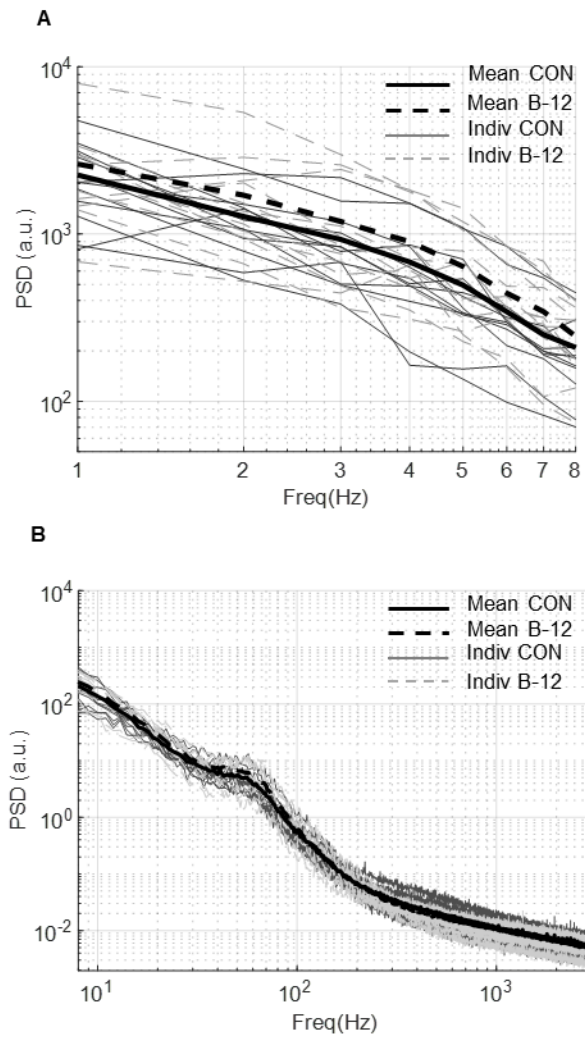


Figure 5

