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Afferent Input Induced by Rhythmic Limb Movement Modulates Spinal Neuronal Circuits in an Innovative Robotic In Vitro Preparation

Original

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note finali coverpage

(Article begins on next page)

1 **Afferent input induced by rhythmic limb movement modulates spinal neuronal circuits**
2 **in an innovative robotic *in vitro* preparation**

3

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13

14 *Running title:* passive exercise on spinal circuits

15

16

17 *Keywords:* spinal cord, locomotor patterns, motoneuron, dorsal afferents

18 **Abbreviations**

19	5-HT: 5-hydroxytryptamine, serotonin
20	AMPA: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
21	ANOVA: analysis of variance
22	BIKE: Bipedal Induced Kinetic Exercise
23	CAP: compound action potential
24	CC: current clamp
25	CCF: cross-correlation function
26	CPG: central pattern generator
27	DR: dorsal root
28	DRDRP: dorsal root – dorsal root potential
29	DRVRP: dorsal root – ventral root potential
30	FFT: fast Fourier transform
31	FL: fictive locomotion
32	GABA: γ -aminobutyric acid
33	I-V: current-voltage
34	l: left
35	L: lumbar
36	NMDA: N-methyl-D-aspartate
37	P: postnatal
38	r: right
39	R_m : membrane resistance
40	RMS: root mean square
41	SCI: spinal cord injury
42	SD: standard deviation
43	sPSC: spontaneous post-synaptic current
44	T: thoracic
45	Th: threshold
46	VC: voltage clamp
47	V_m : membrane potential
48	V_{off} : offset voltage
49	VR: ventral root

50 **Afferent input induced by rhythmic limb movements modulates spinal neuronal circuits**
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52

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54

55 **Abstract**

56 Locomotor patterns are mainly modulated by afferent feedback, but its actual contribution to
57 spinal network activity during continuous passive limb training is still unexplored. To unveil
58 this issue, we devised a robotic *in vitro* setup (Bipedal Induced Kinetic Exercise, BIKE) to
59 induce passive pedaling, while simultaneously recording low-noise ventral and dorsal root
60 (VR and DR) potentials in isolated neonatal rat spinal cords with hindlimbs attached. As a
61 result, BIKE evoked rhythmic afferent volleys from DRs, reminiscent of pedaling speed.
62 During BIKE, spontaneous VR activity remained unchanged, while a DR rhythmic
63 component paired the pedaling pace. Moreover, BIKE onset rarely elicited brief episodes of
64 fictive locomotion (FL) and, when trains of electrical pulses were simultaneously applied to a
65 DR, it increased the amplitude, but not the number, of FL cycles. When BIKE was switched
66 off after a 30-minute training, the number of electrically-induced FL oscillations was
67 transitorily facilitated, without affecting VR reflexes nor DR potentials. However, 90-minutes
68 of BIKE no longer facilitated FL, but strongly depressed area of VR reflexes and stably
69 increased antidromic DR discharges. Patch clamp recordings from single motoneurons after
70 90-minute sessions indicated an increased frequency of both fast- and slow-decaying synaptic
71 input to motoneurons. In conclusion, hindlimb rhythmic and alternated pedaling of different
72 durations affects distinct dorsal and ventral spinal networks by modulating excitatory and
73 inhibitory input to motoneurons. These results suggest defining new parameters for effective
74 neurorehabilitation that better exploits spinal circuit activity.

75 **Introduction**

76 In the spinal cord, dedicated neuronal networks, known as Central Pattern Generators (CPGs),
77 drive limb locomotion (Kiehn, 2006), as demonstrated by the alternating hindlimb movements
78 induced by neurochemicals in the *in vitro* isolated neonatal spinal cord with legs attached
79 (Kiehn and Kjaerulff, 1996; Klein and Tresch, 2010).

80 The CPG-driven movement of limbs stretches muscles and joint capsules and activates
81 cutaneous receptors to generate an afferent feedback to the spinal cord (Loeb et al., 1977).
82 Afferent feedback is crucial to modulate sensory-motor processing (Mandadi and Whelan,
83 2009; Mandadi et al., 2013; Sirois et al., 2013) and locomotor patterns (Hayes et al., 2009;
84 Brumley et al., 2017). Indeed, locomotor patterns induced by neurochemicals disappear when
85 afferent inputs are removed from a neonatal rat spinal cord preparations with limbs attached
86 (Acevedo and Diaz-Rios, 2013). On the other hand, protocols of electrical stimulation applied
87 to dorsal afferents elicit an epoch of locomotor-like cycles (Marchetti et al., 2001; Taccola,
88 2011; Dose and Taccola, 2016; Dose et al., 2016) and increase spontaneous activity of dorsal
89 horn networks when repeatedly supplied (Dingu et al., 2016).

90 In clinics, the continuous flow of afferent input determined by sessions of repetitive and
91 alternated limb movement facilitate the re-expression of locomotor patterns after a spinal
92 lesion, probably because of the consequent plastic changes occurring in spared spinal circuits
93 (Dietz and Fouad, 2014). Likewise, in preclinical models, increased expression of genes
94 involved in motoneuronal plasticity (Joseph et al., 2012; Keeler et al., 2012; Chopek et al.,
95 2015) and restored tuned balance between inhibitory and excitatory synaptic boutons to
96 motoneurons (Ichiyama et al., 2011) were observed with alternating and passive hindlimb
97 mobilization, as well as following activity-based interventions, such as passive cycling
98 (Chopek et al., 2014; Côté et al., 2014).

99 This evidence confirms that afferent input triggers locomotor-like cycles, but it remains
100 unclear how the afferent feedback evoked by the continuous mobilization of limbs modulates
101 the ongoing activity of dorsal sensory-related and ventral motor-related spinal networks. It
102 also needs to be determined whether a different duration of passive exercise can selectively
103 modulate distinct spinal networks and extend its functional effects on spinal circuits even
104 after session ending. Our hypothesis is that CPG-driven locomotor patterns are facilitated by
105 afferent input generated during and after alternating leg movements. This study explores how
106 the afferent input evoked by repetitive, passive alternating movements of hindlimbs
107 modulates patterns generated by spinal networks, and how exercise sessions of different
108 duration affect distinct spinal circuits.

109 We adopted an innovative robotic model that permits recording ventral and dorsal root (VR
110 and DR) activity during passive pedaling in a neonatal preparation of isolated spinal cord with
111 legs attached. Although immature, this preparation can extend understanding of neuromotor
112 system organization in humans, as well as of basic mechanisms of clinical rehabilitation,
113 since many “building blocks” of the mammalian spinal circuitry are already present at birth
114 (Getting, 1989; Stein, 1995; Nishimaru and Kudo, 2000). In neonatal isolated cords, rhythmic
115 activity of ventral locomotor networks arises as epochs of electrical discharges alternating
116 among homosegmental left and right VRs (Fictive Locomotion, FL; Juvin et al., 2007, Nistri
117 et al., 2010). On the other hand, activity of dorsal sensory circuits is probed with monitoring
118 spontaneous rhythmic antidromic discharges recorded synchronously among DRs (Vinay et
119 al., 1999).

120 The question addressed by the present paper consists in the key physiological mechanisms
121 driving spinal processing of afferent input, as elicited by passive limb mobilization. This issue
122 is at the base of numerous rehabilitative techniques, which are currently being improved by
123 exploiting passive and robotic walking for alleviating neuropathic pain and facilitating
124 recovery of function in people with spinal cord injury (Harkema et al., 2012; Hubli and Dietz,
125 2013; Dugan and Sagen, 2015).

126

127 **Experimental Procedures**

128 *In vitro preparations of neonatal rat spinal cord and nerves*

129 All procedures were approved by the International School for Advanced Studies (SISSA)
130 ethics committee and are in accordance with the guidelines of the National Institutes of Health
131 (NIH) and with the Italian Animal Welfare Act 24/3/2014 n. 26 implementing the European
132 Union directive on animal experimentation (2010/63/EU). Experiments were performed on
133 preparations of isolated thoraco-sacral (from T3-4 to *cauda equina*) spinal cord with
134 hindlimbs attached, obtained from neonatal Wistar rats at postnatal (P) days 0–4. All efforts
135 were made to minimize the number and suffering of animals used for experiments. Spinal
136 roots were bilaterally dissected from high thoracic spinal levels to the second lumbar segment
137 (L2) included, leaving all spinal segments below L2 (from L3 on) ventrally and dorsally
138 connected to the periphery. In a subset of experiments, a mechanical compression of the
139 hindpaw was performed to elicit the corresponding afferent feedback from lumbar DRs. All
140 preparations were placed in a recording chamber continuously superfused (5 mL/min) with
141 oxygenated (95% O₂ – 5% CO₂) Krebs solution containing (in mM): 113 NaCl, 4.5 KCl, 1
142 MgCl₂·7H₂O, 2 CaCl₂, 1 NaH₂PO₄, 25 NaHCO₃ and 11 glucose, pH 7.4.

143

144 *A new device to induce passive training*

145 We designed and created a novel device, named BIKE (Bipedal Induced Kinetic Exercise), to
146 induce passive training in the isolated spinal cord with legs attached (Fig. 1 A). The
147 preparation was placed in the recording chamber (maintained at room temperature, 23-25 °C)
148 using acrylic glue to attach the hindpaws to the pedals and to position the legs above the bath
149 (Fig. 1 B). In this position, pedal rotation produced a maximal knee excursions of
150 approximately 140 to 180 degrees (Fig. 1 C). BIKE was connected to a stabilized power
151 supply (K.E.R.T., Treviso, Italy), to allow an adaptable speed of rotation. The design of BIKE
152 carefully considered grounding and shielding from noise, by adopting a brushless DC electric
153 motor. Movement was set at an operative speed of 30-35 cycles/min (pedaling frequency =
154 0.5 Hz) to mimic the standard periodicity of a pharmacologically-induced locomotor-like
155 pattern by NMDA (5 μ M) and 5HT (10 μ M; Dose and Taccola, 2012; Taccola et al., 2012).
156 To verify that recordings remained stable even after a long maintenance of preparations in
157 experimental conditions, sham experiments were performed by keeping the spinal cord with
158 legs attached in Krebs solution with hindpaws firmly fixed to BIKE pedals while the device
159 was switched off. The preparation underwent the same stimulation and recording protocols
160 and at the same time points used on BIKE samples for testing spinal network activity (Fig. 1
161 A).

162

163 *Nerve recordings*

164 All recordings were taken after 40–60 min of steady state period to normalize the specimen
165 from any post-surgical depressions. In Fig. 1 A are summarized protocols of extracellular
166 recordings and stimulations. Using tight-fitting monopolar suction electrodes, simultaneous
167 DC-coupled recordings were extracellularly obtained from whole L2 ventral roots (VRs) right
168 (r) and left (l) and from the whole dorsal root (DR), either L1 or L2. Recordings of DR
169 potentials from L5 were performed *en passant* by applying a negative pressure through a
170 pipette close to the root surface. To isolate the sole contribution of the sensory input elicited
171 in the periphery by passive limb mobilization, in a sub group of preparations, all spinal nerves
172 were bilaterally transected, the spinal cord removed and distal stumps suctioned in glass
173 pipette electrodes connected to an AC-coupled amplifier. Afterwards, a pair of hooked needle
174 electrodes (Sei s.r.l., Padova, Italy) was used to record compound action potentials (CAPs)
175 from one sciatic nerve (exposed proximally to its trifurcation) and dorsal afferent nerves
176 following electrical stimulation of the territory of the hindpaw innervated by the sural nerve.

177 AC- and DC-coupled recordings were acquired with a differential amplifier (DP-304[®],
178 Warner Instruments, CT, USA; low-pass filter = 10 Hz, high-pass filter = 0.1 Hz, gain =
179 1000) at a sampling rate of 10 or 50 kHz, digitized (Digidata 1440[®], Molecular Devices
180 Corporation, Downingtown, PA, USA), visualized real time with the software Clampex 10.3[®]
181 (Molecular Devices Corporation, Downingtown, PA, USA) and stored on a PC for off-line
182 analysis. A bipolar suction electrode connected to a programmable stimulator (STG4002[®],
183 Multichannel Systems, Reutlingen, Germany) was used to deliver single or repeated electrical
184 pulses to a DR (either l or r T13 - L2). Intensity of stimulation was determined in terms of
185 threshold (Th), namely the lowest stimulus intensity capable of eliciting an appreciable
186 response from the homologous VR for determining DRVRPs and from the homosegmental
187 DRs for DRDRPs (Bracci et al., 1996a). Overall, the mean value of Th was $27.40 \pm 18.35 \mu\text{A}$.
188 Responses were evoked by delivering single rectangular pulses (duration = 0.1 ms; intensity =
189 $94.69 \pm 42.56 \mu\text{A}$, $3.15 \pm 0.67 \times \text{Th}$) every 50 seconds. Episodes of fictive locomotion (FL)
190 were induced by trains of electrical stimuli (120 rectangular pulses; frequency = 2 Hz;
191 duration = 0.1 ms) delivered every 3 minutes to a DR at suprathreshold intensity ($58.82 \pm$
192 $35.80 \mu\text{A}$, $2.07 \pm 0.58 \times \text{Th}$). Single rectangular pulses (duration = 5 ms) were delivered every
193 50 seconds to the territory of the hindpaw innervated by the sural nerve, using STG4002[®]
194 stimulator (Multichannel Systems, Reutlingen, Germany). Since the stimulating electrode
195 around the nerve was kept out of the electrolyte solution, high impedance was overcome by
196 applying an increased current (intensity = 16 mA; $4 \times \text{Th}$ for eliciting an orthodromic CAP).

197

198 *Parameters of spinal network activity*

199 Afferent volleys on DRs were quantified using Clampex 10.3[®] (Molecular Devices
200 Corporation, Downingtown, PA, USA) during off-line analysis. A template of incoming
201 events was generated for each dorsal afferent nerve and used for the selection of discharges
202 from the same nerve. Spontaneous activity was quantified in terms of power spectrum
203 magnitude and expressed as Root Mean Square (RMS; Deumens et al., 2013). Briefly, Fast
204 Fourier Transform (FFT) analysis decomposed fixed-length time windows (10, 20 or 90
205 minutes) into a number of discrete frequencies and their power distribution was measured
206 with Clampex 10.3[®] (Molecular Devices Corporation, Downingtown, PA, USA). Analysis
207 adopted a default rectangular windowing function, with data segments not overlapping,
208 window length set at the largest value fitting within the data segments to be processed and the
209 first spectral bin of the periodogram excluded from RMS measurements. The magnitude of
210 the resulting spectrum is the summed power of all rhythm frequencies. This statistical tool

211 quantifies any increase in frequency and/or amplitude of spontaneous activity, expressed as a
212 complex rhythm composed of multiple harmonics.

213 Ventral reflexes and electrically-evoked antidromic activity were assessed through series of
214 single electrical stimuli delivered to a DR in pre-BIKE control, during training and after 30-
215 and 90-minute BIKE sessions. At least 10 consecutive reflex responses were simultaneously
216 recorded from one DR (dorsal root - dorsal root potentials, DRDRPs) and from one VR
217 (dorsal root - ventral root potentials, DRVRPs; Kerkut and Bagust, 1995). Samples of equal
218 duration were collected at each different time point. For analysis, multiple sweeps were
219 averaged and the mean peak amplitude and area were quantified.

220 Alternating activity of the left and right VRs is indicative of a CPG-driven locomotor pattern
221 (Fictive Locomotion, FL; Juvin et al., 2007). A FL oscillation is defined as a period of
222 sustained membrane depolarizations remaining above a preset threshold (usually 5 times the
223 standard deviation of baseline noise) for more than 400 ms (Bracci et al., 1996b). To
224 determine the correlation between left and right VR activity, Cross-Correlation Function
225 (CCF) was computed using Clampfit 10.3[®] software (Molecular Devices Corporation, PA,
226 USA). A CCF > 0.5 at lag 0 correlation indicates that two roots are synchronous, while a CCF
227 < - 0.5 shows full alternation.

228

229 *Patch clamp recordings*

230 Sacrolumbar cords were completely isolated from the leg-attached preparation. The dorsal
231 surface of the cord was glued to an adjustable and articulated plastic support and bent at the
232 level of upper sacral segments in a perpendicular upright position, by means of a Sylgard[®]
233 184 silicone elastomer cube (Dow Corning Corporation, Auburn, MI, USA), with the caudal
234 cord facing upwards (Fig. 8 A). A horizontal section was cut at lumbar (L) 4-5 spinal
235 segments using a vibratome (Leica VT 1000 S, Leica Biosystems) to remove the caudal
236 lumbosacral segments. This upside-down configuration allows patch clamp recordings from
237 spinal motoneurons (Fig. 8 A), still keeping intact the segments where the locomotor
238 networks are mainly localized (Kjaerulff et al., 1994; Kremer and Lev-Tov, 1997; Cowley
239 and Schmidt, 1997). The entire procedure from the end of BIKE to onset of patch clamp
240 recordings requires at least 45 min. Patch clamp recordings in whole-cell configuration were
241 made on L4-L5 motoneurons from isolated spinal cords, either after training (30, 90 min) or
242 in experiments in which spinal cords were kept still in the BIKE recording chamber without
243 BIKE functioning, for the same time interval as the trained cords (sham). Note that
244 experimental protocols adopted in sham preparations were identical to BIKE-trained

245 preparations, with the exception that shams did not undergo any passive cycling. Recordings
246 were performed in both voltage clamp (VC) and current clamp (CC) modes. Up-right bent
247 spinal cords were continuously superfused with oxygenated Krebs solution (flow rate = 7
248 ml/min), illuminated by a far-red-emitting optical fiber (Scientifica Ltd, Uckfield, UK) and
249 visualized through two switchable objectives (4x and 40x; Olympus, Tokyo, Japan) of an
250 infrared video camera (Olympus U-TV1x-2, Tokyo, Japan) connected to the monitor through
251 a C-mount adapter (Olympus U-CMAD3, Tokyo, Japan). Firstly, motoneurons were visually
252 identified in the ventral horn (Rexed laminae VIII – IX) based on their morphology (21-25
253 μm diameter and one or two large processes; Fulton and Walton, 1986; Cifra et al., 2012) and
254 location (close to the VR, in an area corresponding to the Rexed's lamina IX; Molander et al.,
255 1984). Once patched, their functional identity was confirmed by the appearance of antidromic
256 action potentials in response to electrical stimulation of the corresponding VR. Recordings
257 were obtained using borosilicate pipettes with a mean resistance of $6.07 \pm 2.08 \text{ M}\Omega$ and filled
258 with a solution containing (in mM): 120 K gluconate, 20 KCl, 10 HEPES, 10 EGTA, 2
259 MgCl_2 , 2 Na_2ATP , adjusted to pH 7.3 with KOH (Fabbro et al., 2012). Series resistance
260 (lower than $18 \text{ M}\Omega$) was monitored throughout the experiment at specific time points and was
261 not compensated. Cells were discarded if series resistance was higher than $25 \text{ M}\Omega$ and if it
262 varied more than 20% of the initial value (Tartas et al., 2010; Bouhadfane et al., 2013). Liquid
263 junction potential in our experimental conditions was equal to 12.8 mV (Barry, 1994) and all
264 membrane potential values were corrected. Electrophysiological responses were amplified
265 using a differential amplifier (ELC-03XS Amplifier, npi electronic GmbH, Tamm, Germany),
266 digitized by Digidata 1440[®] (Molecular Devices Corporation, Downingtown, PA, USA) and
267 visualized in real time with Clampex 10.3[®] (Molecular Devices Corporation, Downingtown,
268 PA, USA). Data were acquired at a sampling rate of 10 kHz and subsequently analyzed off-
269 line.

270

271 *Parameters of motoneuronal activity*

272 Synaptic activity to lumbar motoneurons was recorded in VC mode, keeping cells clamped at
273 -60 mV. Spontaneous post-synaptic currents (sPSCs) were selected using templates and,
274 based on their decay time, classified as fast- ($\tau = 5.43 \pm 1.17 \text{ ms}$) and slow-decaying currents
275 ($\tau = 20.10 \pm 6.61 \text{ ms}$). Kinetic analysis was adopted to dissect out the following parameters:
276 current frequency (Hz), peak amplitude (pA), time of peak (ms), area (pA * ms), half-width
277 (ms), rise time (ms), rise slope (pA/ms), decay time (ms) and decay slope (pA/ms). Main
278 kinetic properties of fast and slow sPSCs were calculated with Clampex 10.3[®] (Molecular

279 Devices Corporation, Downingtown, PA, USA). In addition, discrimination between fast
280 glutamate-related currents and slow GABA/glycine-related currents was obtained by
281 performing patch clamp recordings at different holding potentials. Holding potential was first
282 equaled to the reversal potential for Cl⁻ to abolish GABA/glycine currents and to observe only
283 glutamatergic PSCs. Similarly, the holding potential was then matched with the reversal
284 potential for Na⁺/K⁺ cations to zero AMPA receptors contributions and to consider only
285 chloride currents. Resting membrane potential ($V_m = -82.90 \pm 7.19$ mV) of motoneurons was
286 determined in CC mode without injecting any holding current ($I = 0$ nA). Afterwards,
287 increasing steps of current were injected and membrane resistance (R_m) was calculated as the
288 slope of the current-voltage (I-V) curve, which was linear in the interval considered. All
289 membrane potentials were corrected for offset voltage (V_{off}), as obtained by raising the
290 electrode from the cell at the end of each recording.

291

292 *Statistical analysis*

293 Data are expressed as mean \pm SD. “n” indicates the number of preparations, while “n_{cells}”
294 represents the number of recorded motoneurons. Normality and equal variance tests were
295 used to determine appropriateness of parametric versus nonparametric comparisons. All
296 parametric values were analyzed using Student’s t-test (paired or unpaired) to compare two
297 groups of data, or with one-way repeated measures ANOVA for more than two groups.
298 Nonparametric comparisons were performed using Mann-Whitney rank sum test (unpaired)
299 and Wilcoxon signed rank test (paired) for two groups, and with Friedman repeated measures
300 ANOVA on ranks for multiple comparisons. Multiple comparisons were followed by a post
301 hoc test versus control (Bonferroni t-test). Statistical analysis was performed using
302 SigmaStat[®] 3.5 software (Systat Software Inc, San Jose, CA, USA). Results were considered
303 significant when $P < 0.05$.

304

305 **Results**

306 *BIKE generates real alternating hindlimb movement in vitro*

307 We created a robotic device, named BIKE (Fig. 1 B), to allow stable recordings during
308 passive hindlimb movement *in vitro*. Once a leg-attached isolated spinal cord preparation was
309 placed in the recording chamber and hindpaws were fixed to the pedals, BIKE passively and
310 alternatively propelled limbs (Fig. 1 C). BIKE was created with a unique low-noise design
311 that abolishes electrical interference, as confirmed by the lack of artifacts during BIKE
312 functioning (Fig. 1 D, E). In addition, no electrical nor mechanical interferences due to the

313 rotor engine or pedaling limb motion were detected by the probe electrode filled with Krebs
314 solution placed close to the recorded spinal roots (Fig. 1 E). Indeed, from the spectral analysis
315 of the background noise detected during BIKE, the rhythmic component of the main
316 frequency of pedaling (0.5 Hz) was absent (Fig. 1 F).

317 To confirm persistence of consistent baseline recordings, in exemplar sham experiments, VR
318 and DR traces were acquired and the spontaneous activity was calculated in 20-min bin
319 intervals (Fig. 2). Power spectrum magnitude, expressed as RMS, remained consistent for
320 more than three hours, confirming that the leg-attached isolated spinal cord provides stable
321 long-term baseline values of the spontaneous activity recorded from DRs (Fig. 2 C; $F_{(9, 18)} =$
322 0.849 , $P = 0.584$, one-way repeated measures ANOVA, $n = 3$) and VRs (Fig. 2 D; $F_{(9, 18)} =$
323 1.014 , $P = 0.465$, one-way repeated measures ANOVA, $n = 3$).

324

325 *BIKE evokes afferent input*

326 BIKE can thus be used for testing whether passive hindlimb movement *in vitro* evokes a
327 sensory feedback on the spinal cord. We faced this hypothesis using *en passant* AC-coupled
328 nerve recordings from the whole DRIL5 (left L5 DR) in the isolated spinal cord with
329 hindlimbs attached, during pre-BIKE control (90-minute), BIKE training and post-BIKE
330 resting phases (Fig. 3 A). Exemplar traces highlight an increase of events from 10659 during
331 pre-BIKE control to 12411 during BIKE, and the return to pre-BIKE control values with post-
332 BIKE rest (9057; Fig. 3 A). Pooled experiments show an average increase of discharges
333 during passive pedaling (138.11 ± 30.65 % of pre-BIKE control), an effect that waned in the
334 following post-BIKE resting phase (110.70 ± 36.39 % of pre-BIKE control; $\chi^2_{(2)} = 3.000$, $P =$
335 0.500 , Friedman repeated measures ANOVA on ranks). To characterize the pattern of afferent
336 discharges, Fast Fourier Transform (FFT) analysis was performed. During BIKE, a main
337 rhythmic frequency component at 0.5 Hz appeared, which was absent both during pre-BIKE
338 control (left) and after BIKE termination (right, Fig. 3 B). This rhythmic component at 0.5 Hz
339 was not an electrical interference produced by BIKE functioning, since it was not detected by
340 the probe electrode measuring bath polarization during pedaling (Fig. 1 E, F). Thus, during
341 BIKE, a rhythmic component is elicited from afferents, pairing the frequency of passive limb
342 movement. This rhythm had small amplitude and became appreciable only when magnified,
343 as it was otherwise covered by the baseline background noise (Fig. 3 C).

344 Next, we wanted to isolate the sole contribution of sensory input with respect to antidromic
345 discharges from dorsal horns. Thus, in a sub group of preparations (data not shown), we
346 bilaterally transected spinal roots and removed the cord from the bath, with dorsal nerves still

347 connected to hindlimbs. Then, the distal stump of DRL5 was suctioned in glass pipette
348 electrodes connected to an AC-coupled amplifier. In these experiments, the number of events
349 recorded during BIKE was more than double the pre-BIKE control values ($218.05 \pm 65.81 \%$,
350 $W = 21$, $P = 0.031$, Wilcoxon signed rank test, $n = 6$).

351 To exclude any bias due to a possible damage of afferent pathways, we defined a random
352 sample of preparations, where each hind paw was peripherally stimulated to record afferent
353 discharges from dorsal nerves (Fig 4). Orthodromic activity was recorded from DRIL4 during
354 mechanical compression of the left hindpaw (an almost eight-fold frequency increase with
355 respect to pre-compression control; see AC-coupled traces and raster plots in Fig. 4 A), as
356 highlighted by the magnification of selected events in Fig. 4 B. Moreover, in another subset of
357 experiments (Fig. 4 C), electrical stimulation of the skin innervated by the sural nerve evoked
358 responses from the sciatic nerve and, with a higher latency, from spinal afferent nerves L4 and
359 L5 (Fig. 4 D), demonstrating that afferent pathways still convey input from periphery to the
360 dorsal spinal cord.

361

362 *BIKE onset does not usually elicit FL per se, nor does it vary the number of oscillations of an*
363 *ongoing FL.*

364 To verify the effects of passive movement on rhythmic patterns generated by the locomotor
365 CPG, we investigated whether BIKE functioning elicits spontaneous locomotor-like
366 oscillations from VRs. In 85% of experiments (52/61), no FL was evoked during pedaling. In
367 the remaining preparations (9/61), only a brief episode of spontaneous left-right alternating
368 oscillations (0.55 ± 0.11 mV; 5 ± 3 cycles; data not shown) temporarily appeared when BIKE
369 was switched on. Likewise, during 90 mins of BIKE (Fig. 5 A), we derived in DC mode a
370 spontaneous tonic activity from VRs, with sporadic bursts provided of few superimposed
371 oscillations with poor phase coupling (Fig. 5 B, C), as confirmed by CCF analysis performed
372 on top of bursts (Fig. 5 D, E). In more details, the CCF at 0 lag for the first burst (B, D) shows
373 a positive peak (0.18) for synchronous superimposed oscillations. On the other hand, the
374 second event (C, E) at 0 lag depicts a negative CCF peak (-0.27), pointing out some
375 alternating activity, albeit below the cutoff value (-0.5) for a full VR alternation, caused by
376 the irregular rhythm. Spectral analysis of the spontaneous rhythmic activity (Fig. 5 A) at
377 different time intervals during a 90 min BIKE session indicated that BIKE does not affect the
378 spontaneous VR rhythmic pattern (Fig. 5 F; $\chi^2_{(6)} = 5.571$, $P = 0.473$, Friedman repeated
379 measures ANOVA on ranks, $n = 9$).

380 Although BIKE onset only rarely elicited episodes of FL on its own, we explored whether
381 BIKE still facilitates FL episodes evoked by electrical stimulation. We thus analyzed the
382 rhythmic, alternating activity of l and r VRs when applying a train of rectangular pulses (2
383 Hz, 120 to 180 stimuli, 1.92 ± 0.38 Th) to different DRs (from DRT13 to DRL2). During
384 pedaling, the addition of a train of electrical pulses did not change the number of FL
385 oscillations (108.93 ± 19.06 % of pre-BIKE control; $W = -1$, $P = 1.000$, Wilcoxon signed rank
386 test, $n = 5$) nor the cumulative depolarization peak (102.46 ± 3.83 % of pre-BIKE control; $t_{(4)}$
387 $= -1.508$, $P = 0.206$, paired t-test, $n = 5$). However, during pedaling, the amplitude of FL
388 oscillations consistently increased (104.44 ± 3.55 % of pre-BIKE control) without any
389 relation to either cycling phase or pulse occurrence. This mild modulatory action over the
390 electrically-evoked FL faded away after BIKE termination ($F_{(2, 8)} = 7.433$, $P = 0.015$, one-way
391 repeated measures ANOVA, $n = 5$).

392

393 *Different durations of BIKE application affect locomotor spinal circuits in a nonlinear*
394 *manner*

395 To test whether a protracted passive limb exercise could facilitate the locomotor pattern in the
396 post-BIKE resting phase, 2 Hz trains of pulses were delivered before and right-after BIKE
397 sessions of increasing duration to compare features of locomotor-like patterns.

398 Initially, we applied a 10-min session of BIKE to five samples, without observing any
399 significant increase in the number of oscillations (96.62 ± 37.83 of pre-BIKE control; $W = -4$,
400 $P = 0.625$, Wilcoxon signed rank test), cumulative depolarization peak (100.24 ± 5.39 % of
401 pre-BIKE control; $t_{(4)} = -0.402$, $P = 0.709$, paired t-test) or amplitude of FL oscillations
402 (100.55 ± 2.87 % of pre-BIKE control; $t_{(4)} = -0.569$, $P = 0.600$, paired t-test).

403 Thereafter, we considered prolonged BIKE sessions. In an exemplar experiment, episodes of
404 FL were electrically-elicited by DR stimulation before and after 30 minutes of training (see
405 DC-coupled traces in Fig. 6 A, B). In pre-BIKE control conditions, the electrical stimulation
406 protocol induced a cumulative depolarization with 8 locomotor-like oscillations (Fig. 6 A). At
407 the end of 30-min BIKE, the number of oscillations increased to 12 (Fig. 6 B). Pooled data
408 from nine independent samples showed a statistically significant increase in the number of
409 oscillations at the end of a 30-min BIKE session compared to pre-BIKE control (pre-BIKE
410 control: 11 ± 4 FL cycles; after 30 minutes BIKE: 14 ± 4 FL cycles; $W = 36$, $P = 0.008$,
411 Wilcoxon signed rank test). However, the number of oscillations diminished to initial values
412 20 min after ceasing BIKE training (Fig. 6 C, 11 ± 3 FL cycles; $\chi^2_{(2)} = 8.087$, $P = 0.012$,
413 Friedman repeated measures ANOVA on ranks, $n = 6$). Conversely, training did not affect the

414 amplitude of FL cycles (0.123 ± 0.088 mV in pre-BIKE control; 101.99 ± 25.82 % of pre-
415 BIKE control after 30-mins BIKE and 123.18 ± 46.28 % of pre-BIKE control after 20 min
416 post-BIKE rest; $H_{(2)} = 0.286$, $P = 0.867$, Kruskal-Wallis one-way ANOVA on ranks, $n = 9, 9,$
417 6) nor the peak of cumulative depolarization (0.538 ± 0.393 mV in pre-BIKE control; 111.89
418 ± 39.79 after 30-mins BIKE and 131.89 ± 43.31 % of pre-BIKE control after 20 min post-
419 BIKE rest; $H_{(2)} = 0.364$, $P = 0.833$, Kruskal-Wallis one-way ANOVA on ranks, $n = 9, 9, 6$).
420 Surprisingly, an extended training session of 90 minutes brought the number of FL cycles
421 back to pre-BIKE control levels ($W = -22$, $P = 0.148$, Wilcoxon signed rank test, $n = 8$) and
422 vanished the previous transitory FL facilitation induced by 30 min of BIKE (Fig. 6 D; $61.20 \pm$
423 23.38 % of 30 min BIKE; $U_{(8, 9)} = 8$, $P = 0.008$, Mann-Whitney rank sum test, $n = 9, 8$). Sham
424 experiments reported that the *in vitro* maintenance for 90 min did not affect number of
425 locomotor-like oscillations ($W = -1$, $P = 1.000$, Wilcoxon signed rank test, $n = 3$) nor
426 cumulative depolarization ($W = 2$, $P = 0.750$, Wilcoxon signed rank test, $n = 3$).

427

428 *A long session of BIKE depresses VR reflexes*

429 To explore whether the lack of FL facilitation after 90 min BIKE was due to a depression in
430 sensory-motor connections caused by long pedaling, we studied the sensory-motor reflex arc
431 through the delivery of single pulses at low intensity ($1 \times Th$; 30.53 ± 9.66 μA) to a DR (T13 or
432 L1), while recording potentials from the homologous VR. For a sample DC-coupled trace
433 (Fig. 7 A), a long BIKE session stably reduced the area of DRVRPs for up to one hour after
434 session end, but did not affect the peak of DRVRPs. Time course from many experiments
435 (Fig. 7 B) demonstrated that the area of DRVRPs progressively decreased over longer BIKE
436 durations, with a significant reduction at the end of session (90 min; $F_{(7, 91)} = 7.780$, $P \leq 0.001$,
437 one-way repeated measures ANOVA followed by post-hoc analysis with Bonferroni t-test
438 versus pre-BIKE control, $n = 14$). A subgroup of experiments, in which VR reflexes were not
439 affected by a single 30-minute BIKE session (peak: $F_{(4, 16)} = 0.655$, $P = 0.632$, one-way
440 repeated measures ANOVA, $n = 5$; area: $F_{(4, 16)} = 0.822$, $P = 0.530$, one-way repeated
441 measures ANOVA, $n = 5$), confirmed that BIKE sessions shorter than 90 min did not affect
442 the area of DRVRPs. Moreover, single pulses delivered during long sham experiments
443 confirmed that long recordings did not significantly deteriorate the area of DRVRPs (Fig. 7 C;
444 $P = 0.971$, one-way ANOVA, $n = 8, 6, 5, 6$).

445

446 *A long session of BIKE increases spontaneous post-synaptic currents and membrane* 447 *resistance of motoneurons*

448 To identify whether the depressing effect of long BIKE applications corresponds to potential
449 alterations in motoneuronal excitability, we performed whole-cell patch clamp recordings on
450 single antidromically-identified motoneurons. These measurements were performed on a
451 perpendicular upright arrangement of the isolated cord (Fig. 8 A) and lasted for up to five
452 hours after termination of 90-min of BIKE or sham experiments.

453 Frequency of currents recorded in VC mode from single motoneurons increased after training,
454 compared to sham experiments (Fig. 8 C, upper trace, and plot in D; $U_{(32, 33)} = 282.5$, $P =$
455 0.001 , Mann-Whitney rank sum test, $n_{\text{cells}} = 32, 33$, $n = 11, 14$), indicating an augmentation of
456 input converging onto the motoneuron. However, this input was not sufficient to generate a
457 spontaneous firing in CC mode, despite the presence of a more ragged baseline (Fig. 8 C,
458 bottom trace), in line with the reduced membrane resistance of motoneurons after training
459 (Fig. 8 E, F; $t_{(15)} = 2.182$, $P = 0.04$, t-test, $n_{\text{cells}} = 5, 12$, $n = 5, 8$).

460 After selecting single events, we obtained average traces for fast-decaying sPSCs (Fig. 8 G; τ
461 $= 5.43 \pm 1.17$ ms, $n_{\text{cells}} = 79$, $n = 33$) and slow-decaying sPSCs (Fig. 8 H; $\tau = 20.10 \pm 6.61$ ms,
462 $n_{\text{cells}} = 80$, $n = 33$), which indicate that BIKE increased frequency of both fast (Fig. 8 I; $U_{(32,$
463 $33)} = 361.5$, $P = 0.029$, Mann-Whitney rank sum test, $n_{\text{cells}} = 32, 33$, $n = 11, 14$) and slow
464 currents (Fig. 8 J; $U_{(32, 33)} = 357.5$, $P = 0.026$, Mann-Whitney rank sum test, $n_{\text{cells}} = 32, 33$, $n =$
465 $11, 14$). Moreover, kinetic analysis of currents revealed no difference between motoneurons
466 undergoing 90-minute training protocols vs. sham (data not shown).

467 Then, we selectively discriminated between fast glutamate-related currents and slow
468 GABA/glycine-related currents through patch clamp recordings at reversal potentials for Cl^-
469 and Na^+/K^+ cations. VC recordings from BIKE-trained and sham motoneurons at the two
470 different holding potentials indicated that frequency of both fast ($U_{(5, 10)} = 9$, $P = 0.05$, Mann-
471 Whitney rank sum test, $n_{\text{cells}} = 5, 10$, $n = 4, 6$) and slow currents ($U_{(5, 10)} = 9$, $P = 0.05$, Mann-
472 Whitney rank sum test, $n_{\text{cells}} = 5, 10$, $n = 4, 6$) increased with BIKE.

473 We also analyzed the effects of 30-mins of BIKE over sPSCs recorded from motoneurons.
474 Compared to sham experiments, input frequency to single motoneurons remained unchanged
475 (sPSCs in sham preparations: 1.87 ± 2.32 Hz, $n_{\text{cells}} = 17$, $n = 6$; sPSCs in BIKE preparations:
476 1.54 ± 1.33 Hz, $n_{\text{cells}} = 20$, $n = 9$; $U_{(17, 20)} = 156$, $P = 0.681$, Mann-Whitney rank sum test), as
477 did fast-decaying (fast sPSCs in sham preparations: 1.19 ± 2.06 Hz, $n_{\text{cells}} = 17$, $n = 6$; fast
478 sPSCs in BIKE preparations: 0.60 ± 0.89 Hz, $n_{\text{cells}} = 20$, $n = 9$; $U_{(17, 20)} = 162.5$, $P = 0.831$,
479 Mann-Whitney rank sum test) and slow-decaying sPSCs (slow sPSCs in sham preparations:
480 0.69 ± 0.73 Hz, $n_{\text{cells}} = 17$, $n = 6$; slow sPSCs in BIKE preparations: 0.94 ± 0.85 , $n_{\text{cells}} = 20$, n
481 $= 9$; $U_{(17, 20)} = 134.5$, $P = 0.286$, Mann-Whitney rank sum test). Likewise, membrane

482 resistance of single motoneurons was unaffected by 30-mins BIKE, as measured by
483 comparing a random sample of five cells from four trained preparations to a group of nine
484 cells from four sham experiments ($t_{(13)} = -0.771$, $P = 0.455$, t-test).

485

486 *Training modulates dorsal spinal networks*

487 To clarify whether BIKE could actually affect synaptic transmission in the dorsal cord, we
488 first recorded DRDRPs, both in pre-BIKE control conditions and after training. At the end of
489 30 minutes of BIKE, peaks of DRDRPs were unchanged (110.94 ± 26.18 % of pre-BIKE
490 control) and remained similar to baseline values for up to 1 hour of post-BIKE rest ($111.75 \pm$
491 27.46 % of pre-BIKE control; $\chi^2_{(4)} = 1.440$, $P = 0.837$, Friedman repeated measures ANOVA
492 on ranks, $n = 5$). When training was prolonged to 90 min, the peak of DRDRPs increased by
493 40% right after BIKE (Fig. 9 A) and the amplitude of responses remained higher than pre-
494 BIKE control, even after a long post-BIKE rest (time course in Fig. 9 B; $\chi^2_{(6)} = 14.657$, $P =$
495 0.023 , Friedman repeated measures ANOVA on ranks followed by Dunn's test versus control,
496 $n = 5$). However, to explore whether peaks of DRDRPs become higher after a long post-BIKE
497 rest, a pairwise statistical analysis was performed only among the data points collected at
498 post-BIKE rest in Fig. 9 B, showing no variation in DRDRPs at different time points during
499 post-BIKE rest ($\chi^2_{(5)} = 9.914$, $P = 0.078$, Friedman repeated measures ANOVA on ranks, $n =$
500 5). Moreover, spontaneous antidromic activity recorded from left L2 DR in DC mode during
501 training displays an increased frequency of antidromic discharges (Fig. 9 C). Pooled data
502 from many experiments point out that the power spectrum magnitude of spontaneous DR
503 activity significantly increased upon BIKE start (Fig. 9 D) and values of DR antidromic
504 discharges remained significantly higher than pre-BIKE control throughout training ($F_{(6, 18)} =$
505 14.865 , $P \leq 0.001$, one-way repeated measures ANOVA followed by post-hoc analysis with
506 Bonferroni t-test versus control, $n = 4$).

507

508 *Ninety minutes of passive mobilization long-lastingly increase spontaneous dorsal discharges*

509 To explore whether spinal circuit excitability is affected by longer BIKE sessions and can
510 persist even after the end of pedaling, spontaneous activity was continuously recorded in DC-
511 mode from cut L2 spinal roots before, during and at the end of a 90-min session, as well as for
512 over two hours of post-BIKE rest (Fig. 10).

513 During a 20-min pre-BIKE control period, VR bursts emerged (Fig. 10 A, left panels) while
514 measuring a simultaneous intense tonic activity from DRs (Fig. 10 A, left panels). Recordings
515 continued during the subsequent 90-min BIKE period (Fig. 10 A, middle panels) and showed

516 an increase in DR activity (in line with Fig. 9 C, D), while VR discharges remained
517 unaffected (compare with Fig. 5). Twenty minutes after cessation of BIKE, only DR activity
518 strongly augmented both in frequency and amplitude (Fig. 10 A, right panels). From the same
519 experiment, FFTs described the rhythmic activity of all the VRs and the single DR during
520 BIKE. No 0.5 Hz rhythmic component was detected, indicating the lack of any phase
521 relationship between pedaling pace and spontaneous VR activity (Fig. 10 B, top and middle
522 plots). On the contrary, antidromic DR activity was phase-related with pedaling, as confirmed
523 by the peak of discharge frequency observed closely around 0.5 Hz (Fig. 10 B, bottom plot),
524 which was absent from DR activity during pre-BIKE control and post-BIKE rest (not shown).
525 Power spectrum magnitude from many experiments confirmed that VR discharges were not
526 significantly affected by 90 min training (Fig. 10 C; $t_{(7)} = 2.119$, $P = 0.072$, paired t-test, $n =$
527 8), although the amplitude of events recorded from DRs was significantly higher than pre-
528 BIKE control (Fig 10 D; $t_{(7)} = -4.058$, $P = 0.005$, paired t-test, $n = 8$).
529 To identify the minimal protocol duration to elicit a significant increase in spontaneous dorsal
530 activity after training, 30 min-long BIKE sessions were applied in sequence while DR rhythm
531 magnitude was monitored at the end of each session (Fig. 10 E). Cumulative BIKE sessions
532 lasting less than 90 minutes did not change magnitude of dorsal discharges, while longer
533 sessions evoked a statistical variation in DR rhythmicity that persisted after turning off BIKE
534 (Fig. 10 F; $F_{(3, 9)} = 5.010$, $P = 0.026$, one-way repeated measures ANOVA followed by post-
535 hoc analysis with Bonferroni t-test versus control, $n = 4$).
536 In a subset of experiments, the activity recorded from DRs and VRs, expressed as RMS of the
537 power spectrum, was calculated in slots of 20 min for up to 140 minutes of post-BIKE rest.
538 VR spontaneous activity was not significantly affected by 90 min BIKE even in the long run
539 (see time course in Fig. 10 G, in grey; $F_{(7, 28)} = 2.102$, $P = 0.077$, one-way repeated measures
540 ANOVA, $n = 5$). Conversely, DR discharges remained significantly higher than pre-BIKE
541 control for the entire observation period (Fig. 10 G, in black; $F_{(7, 28)} = 5.726$, $P < 0.001$, one-
542 way repeated measures ANOVA followed by post-hoc analysis with Bonferroni t-test versus
543 pre-BIKE control, $n = 5$).

544

545 **Discussion**

546 In this study, we associated intra- and extra-cellular electrophysiological recordings with an
547 innovative robot that generates consistent afferent input to DRs. We proved that 30 minutes of
548 alternating passive limb mobilization increases the number of locomotor-like oscillations
549 induced by DR electrical stimulation, without affecting VR reflexes and DR potentials. This

550 facilitatory effect on the locomotor CPG was lost with longer training sessions, which also
551 reduced the area of VR reflexes and increased both frequency of currents recorded from
552 single motoneurons and antidromic discharges recorded from DRs.

553

554 *A novel robotic instrument to induce a real alternate movement of hindlimbs in vitro*

555 We invented a new robotic device, BIKE, for the standardized mobilization of limbs in an
556 isolated neonatal rat spinal cord preparation with legs attached. Our innovative model proved
557 suitable to trace an earlier modulation of spinal networks before, during and up to five hours
558 after the end of passive exercise. In the past decades, the modulatory effects of actual limb
559 movement on the *in vitro* CPG output have been studied using several experimental models
560 (Wheatley and Stein, 1992; Hayes et al., 2009). In particular, Hayes and collaborators (2009)
561 pharmacologically activated the CPG to induce real locomotion, and then studied how the
562 resulting limb movements modulated the ongoing neurochemically-driven CPG rhythmic
563 pattern. Our model, on the other hand, induces mere passive limb movement to study how a
564 session of passive mobilization can facilitate locomotor patterns. Our preparation allows to
565 study the role of afferent feedback evoked by dorsal spinal nerves during the sole limb
566 movement, which cannot be explored if the whole preparation is in contact with
567 neurochemicals. Indeed, neurochemicals would inevitably contaminate afferent volleys,
568 inducing antidromic rhythmic activity along spinal dorsal networks (Kremer and Lev-Tov,
569 1998) and increasing excitability of spinal ganglia (Lovinger and Weight, 1988; Cardenas et
570 al., 2001).

571 In this study, BIKE generated stereotyped rotations at the same average frequency as a
572 neurochemically-induced (NMDA+5HT) FL previously evoked from the same preparation.
573 Moreover, skin compression experiments demonstrated that afferent pathways still convey
574 input from the periphery to the dorsal spinal cord, even though oxygenation of hindlimb
575 tissues in our preparation was probably perturbed after dissection and for the entire
576 experimental protocol due to the absence of blood circulation.

577 Future studies can exploit BIKE's ability to variate exercise frequency during each pedaling
578 session to define the optimal intensity of training and assess how variations in pedaling speed
579 affect spinal networks. Moreover, versatility of BIKE also allows to assess the importance of
580 rhythmic, bilateral alternation vs. non-stereotyped movements to improve CPG functionality
581 as a result of input variability (Ziegler et al., 2010). These perspectives would provide an
582 important link to some current open questions about neurorehabilitation strategies in humans.

583

584 *Suggested origin of afferent input induced by BIKE*

585 Using *en passant* recordings from DRs during BIKE, we consistently observed afferent
586 volleys of a yet unclear origin. Both the activity of the locomotor CPG and the adaptation of
587 locomotion to the environment are facilitated by sensory stimulation and are mainly provided
588 by the sensory feedback from both load bearing structures and mechanoreceptors of the hip
589 (Dietz et al., 2002). In our model, cyclic pressure of the foot plant with each pedaling
590 movement recruits only a limited number of load receptors, as limbs are in an antigravity
591 upside-down position. Nevertheless, experiments with electrical skin stimulation indicate that
592 our model can still exploit cutaneous afferents. Although, cutaneous responses can also
593 facilitate locomotor activity in spinal and decerebrate preparations (Pearson and Rossignol,
594 1991; Hiebert and Pearson, 1999), input from cutaneous receptors physiologically modulates
595 the activity of lumbar locomotor circuits mostly by recruiting supraspinal systems, which are
596 absent in our preparation (Drew et al., 2004; McVea et al., 2007; Wong et al., 2018). As
597 shown by afferent volleys recorded in this study following mechanical compression of the
598 paw, input from nociceptive cutaneous afferents are present in our preparation and they might
599 be recruited during BIKE to further modulate the CPG (Mandadi and Whelan, 2009).
600 Pedaling evokes a hip joint range of motion that can generate a proprioceptive input, in turn
601 facilitating locomotion. However, proprioceptors are poor stretch transducers without any
602 alpha-gamma recruitment to shorten and/or increase intrafusal fiber stiffness, thus rendering
603 their contribution only marginal in our model.

604 It should be noted that the paucity of afferent input reflects the low amplitude of discharges
605 recorded in our study and might also be linked to immaturity of the preparation, which
606 reaches full development of afferent pathways only in the first three postnatal weeks
607 (Fitzgerald et al., 1994). Nevertheless, the same afferent feedback from the periphery to the
608 central spinal circuits present at birth is only weakly inhibited pre-synaptically. Therefore,
609 even a scarce peripheral feedback during early postnatal stages (Sonner and Ladle, 2013)
610 might contribute to optimally modulate the ontogeny of spinal circuits (Vinay et al., 2002).

611 Overall, BIKE increased afferent activity with a distinctive frequency spectrum, where the
612 rhythmic component of pedaling stands out from a highly variable background activity.
613 Interestingly, the combination of a baseline noisy input and a phasic component at the
614 frequency of stepping resembles the stimulating protocol that optimally activates the
615 locomotor CPG in the neonatal rat isolated spinal cord (Dose and Taccola, 2016).

616

617 *Mechanisms of BIKE in facilitating excitatory and inhibitory input, with different timing*

618 BIKE generated both excitatory and inhibitory effects. A short application of BIKE
619 transitorily increased the number of locomotor-like oscillations induced by trains of dorsal
620 stimuli. On the other hand, a longer application abolished this effect and reduced the area of
621 DRVRPs. At the same time, a longer application also potentiated antidromic discharges,
622 whether spontaneous or induced by dorsal stimulation, lasting for even several hours after the
623 end of training. The increased spontaneous DRPs and evoked DRDRPs parallel the decrease
624 in DRVRPs, likely due to deletion of input incoming from DR stimulation, determined by
625 spike occlusion at the level of sensory afferents. An additional explanation might reside in an
626 increased concentration of GABA, generating a two-stage effect: pre-synaptically
627 depolarizing primary afferents that originate antidromic DR discharges, and post-synaptically
628 hyperpolarizing post-synaptic targets, in turn inhibiting motor reflexes.

629 The analysis of current kinetics, intracellularly recorded from motoneurons after 90-min
630 BIKE, revealed an increased frequency of fast spontaneous currents, mainly attributed to
631 AMPA receptors (Hestrin, 1993; Wyllie et al., 1994; Galante et al., 2000), paired with
632 increased slow spontaneous currents, putatively ascribed to a GABAergic nature (Lewis and
633 Faber, 1996; Galante et al., 2000). The increased number of input directed to motoneurons is
634 consistent with an augmented release of vesicles from presynaptic terminals (Katz and Miledi,
635 1967). Conversely, peak amplitude of spontaneous PSCs remained unaltered, presumably
636 indicating that the probability of channel opening in motoneurons was unaffected (Katz and
637 Miledi, 1967). These results suggest that a prolonged training mainly affects pre-
638 motoneuronal networks rather than motoneuron membranes. Unfortunately, the longer time
639 frame needed to start patch clamp recordings did not allow us to investigate properties of
640 spontaneous currents right after the shorter BIKE sessions that transiently facilitated
641 locomotor networks. Therefore, we cannot exclude that some modifications in the biophysical
642 properties of motoneurons did transitorily take place during FL facilitation induced by short
643 BIKE sessions.

644 The most parsimonious explanation of the effects observed in this study is that excitatory and
645 inhibitory contributions were recruited with a different timing during BIKE. This hypothesis,
646 although not tested in this study, would predict an increase in DRVRPs over short BIKE
647 periods. However, FL facilitation seen for shorter training sessions might reflect an early
648 increase in spinal excitation, in line with the facilitation of weak electrical stimulation in the
649 presence of low concentrations of glutamatergic agents (Dose and Taccola, 2012). Moreover,
650 this study indicates that BIKE increases fast spontaneous PSCs attributed to AMPA
651 glutamatergic receptors.

652 Nevertheless, AMPA input on the motoneuron might be overwhelmed by longer sessions of
653 BIKE because of the appearance, or the progressive increase, of GABAergic post-synaptic
654 input (Fontana et al., 2001), which have been reported to depress FL (Cazalets et al., 1994;
655 Cowley and Schmidt, 1995). However, we could not test the selective pharmacological
656 antagonism of AMPA or GABA receptors, as these drugs would have interfered with the
657 expression of FL (Bracci et al., 1996b; Beato et al., 1997).

658 An alternative explanation suggests that potentiation of AMPA receptors (and the
659 corresponding facilitation of FL) is lost with a prolonged BIKE application because of the
660 desensitization of AMPA channels (Ballerini et al., 1995; Tsvetlynska et al., 2005).
661 Nonetheless, this hypothesis seems unlikely, since fast currents on the motoneuron are
662 augmented after 90-min BIKE. Moreover, in correspondence to longer BIKE sessions,
663 motoneuron membrane resistance was found to diminish, in line with previous reports on
664 trained adult rats (Beaumont et al., 2004).

665 Apart from changes in motoneurons, efficacy of BIKE relies on a more complex mechanism,
666 potentially affecting many other properties of the spinal cord. As a matter of fact, the state of
667 CPG interneurons is perturbed by afferent input (Burke, 1999), primary afferent
668 depolarization and other sensorimotor functions (Ménard et al., 2002; Hayes et al., 2012).
669 Overall, BIKE enhances spontaneous synaptic transmission within the spinal network, in line
670 with training affecting the proportion of inhibitory vs. excitatory boutons in rat motoneurons
671 (Ichiyama et al., 2011).

672

673 *Clinical perspectives*

674 We are aware that neonatal rodents are not the best suited model to understand the
675 mechanisms of passive pedaling in humans, adults in particular. Indeed, P0–P4 neonatal rats
676 cannot bear their own weight and descending and sensory input to motoneurons, as well as
677 their membrane properties, are still at an immature developing stage (Vinay et al., 2002;
678 Clarac et al., 2004). Nevertheless, these models share important basic characteristics with
679 adult animals and humans. In the present study, the transient facilitation of locomotor patterns
680 with a precise timing after passive cycling might suggest the existence of an optimal
681 therapeutic window for co-administering pharmacological agents and neurorehabilitation to
682 activate spinal locomotor circuits after SCI (NCT01621113, NCT01484184,
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684

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690

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692

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695

696 **References**

697

698 Acevedo JM, Díaz-Ríos M (2013) Removing sensory input disrupts spinal locomotor activity
699 in the early postnatal period. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol*
700 199:1105–1116.

701

702 Ballerini L, Bracci E, Nistri A (1995) Desensitization of AMPA receptors limits the
703 amplitude of EPSPs and the excitability of motoneurons of the rat isolated spinal cord. *Eur J*
704 *Neurosci* 7:1229–1234.

705

706 Barry PH (1994) JPCalc, a software package for calculating liquid junction potential
707 corrections in patch-clamp, intracellular, epithelial and bilayer measurements and for
708 correcting junction potential measurements. *J Neurosci Methods* 51:107–116.

709

710 Beato M, Bracci E, Nistri A (1997) Contribution of NMDA and non-NMDA glutamate
711 receptors to locomotor pattern generation in the neonatal rat spinal cord. *Proc Biol Sci*
712 264:877–884.

713

714 Beaumont E, Houlé JD, Peterson CA, Gardiner PF (2004) Passive exercise and fetal spinal
715 cord transplant both help to restore motoneuronal properties after spinal cord transection in
716 rats. *Muscle Nerve* 29:234–242.

717

718 Bouhadfane M, Tazerart S, Mogrich A, Vinay L, Brochard F (2013) Sodium-mediated plateau
719 potentials in lumbar motoneurons of neonatal rats. *J Neurosci* 33:15626–15641.

720

721 Bracci E, Ballerini L, Nistri A (1996a) Localization of rhythmogenic networks responsible for
722 spontaneous bursts induced by strychnine and bicuculline in the rat isolated spinal cord. *J*
723 *Neurosci* 16:7063–7076.

724

725 Bracci E, Ballerini L, Nistri A (1996b) Spontaneous rhythmic bursts induced by
726 pharmacological block of inhibition in lumbar motoneurons of the neonatal rat spinal cord. *J*
727 *Neurophysiol* 75:640–647.

728

729 Brumley MR, Guertin PA, Taccola G (2017) Multilevel analysis of locomotion in immature
730 preparations suggests innovative strategies to reactivate stepping after spinal cord injury. *Curr*
731 *Pharm Des* 23:1764–1777.
732

733 Burke RE (1999) The use of state-dependent modulation of spinal reflexes as a tool to
734 investigate the organization of spinal interneurons. *Exp Brain Res* 128:263–277.
735

736 Cardenas LM, Cardenas CG, Scroggs RS (2001) 5HT increases excitability of nociceptor-like
737 rat dorsal root ganglion neurons via cAMP-coupled TTX-resistant Na(+) channels. *J*
738 *Neurophysiol* 86:241–248.
739

740 Cazalets JR, Sqalli-Houssaini Y, Clarac F (1994) GABAergic inactivation of the central
741 pattern generators for locomotion in isolated neonatal rat spinal cord. *J Physiol* 474:173–181.
742

743 Chopek JW, MacDonell CW, Gardiner K, Gardiner PF (2014) Daily passive cycling
744 attenuates the hyperexcitability and restores the responsiveness of the extensor monosynaptic
745 reflex to quipazine in the chronic spinally transected rat. *J Neurotrauma* 31:1083–1087.
746

747 Chopek JW, Sheppard PC, Gardiner K, Gardiner PF (2015) Serotonin receptor and KCC2
748 gene expression in lumbar flexor and extensor motoneurons posttransection with and without
749 passive cycling. *J Neurophysiol* 113:1369–1376.
750

751 Cifra A, Mazzone GL, Nani F, Nistri A, Mladinic M (2012) Postnatal developmental profile
752 of neurons and glia in motor nuclei of the brainstem and spinal cord, and its comparison with
753 organotypic slice cultures. *Dev Neurobiol* 72:1140–1160.
754

755 Clarac F, Brocard F, Vinay L (2004) The maturation of locomotor networks. *Prog Brain Res*
756 143:57-66.
757

758 Côté MP, Gandhi S, Zambrotta M, Houlé JD (2014) Exercise modulates chloride homeostasis
759 after spinal cord injury. *J Neurosci* 34:8976–8987.
760

761 Cowley KC, Schmidt BJ (1995) Effects of inhibitory amino acid antagonists on reciprocal
762 inhibitory interactions during rhythmic motor activity in the in vitro neonatal rat spinal cord. *J*
763 *Neurophysiol* 74:1109–1117.
764

765 Cowley KC, Schmidt BJ (1997) Regional distribution of the locomotor pattern-generating
766 network in the neonatal rat spinal cord. *J Neurophysiol* 77:247–259.
767

768 Deumens R, Mazzone GL, Taccola G (2013) Early spread of hyperexcitability to caudal
769 dorsal horn networks after a chemically-induced lesion of the rat spinal cord in vitro.
770 *Neuroscience* 229:155–163.
771

772 Dietz V, Müller R, Colombo G (2002) Locomotor activity in spinal man: significance of
773 afferent input from joint and load receptors. *Brain* 125:2626–2634.
774

775 Dietz V, Fouad K (2014) Restoration of sensorimotor functions after spinal cord injury. *Brain*
776 137:654–667.
777

778 Dingu N, Deumens R, Taccola G (2016) Electrical stimulation able to trigger locomotor
779 spinal circuits also induces dorsal horn activity. *Neuromodulation* 19:38–46.
780

781 Dose F, Taccola G (2012) Coapplication of noisy patterned electrical stimuli and NMDA plus
782 serotonin facilitates fictive locomotion in the rat spinal cord. *J Neurophysiol* 108:2977–2990.
783

784 Dose F, Taccola G (2016) Two distinct stimulus frequencies delivered simultaneously at low
785 intensity generate robust locomotor patterns. *Neuromodulation* 19:563–575.
786

787 Dose F, Deumens R, Forget P, Taccola G (2016) Staggered multi-site low-frequency
788 electrostimulation effectively induces locomotor patterns in the isolated rat spinal cord. *Spinal*
789 *Cord* 54:93–101.
790

791 Drew T, Prentice S, Schepens B, (2004) Cortical and brainstem control of locomotion. *Prog.*
792 *Brain Res* 143:251–261.
793

794 Dugan EA, Sagen J (2015) An intensive locomotor training paradigm improves neuropathic
795 pain following spinal cord compression injury in rats. *J Neurotrauma* 32:622–632.
796

797 Fabbro A, Villari A, Laishram J, Scaini D, Toma FM, Turco A, et al (2012) Spinal cord
798 explants use carbon nanotube interfaces to enhance neurite outgrowth and to fortify synaptic
799 inputs. *ACS Nano* 6:2041–2055.
800

801 Fitzgerald M, Butcher T, Shortland P (1994) Developmental changes in the laminar
802 termination of A fibre cutaneous sensory afferents in the rat spinal cord dorsal horn. *J Comp*
803 *Neurol* 348:225–233.
804

805 Fontana G, Taccola G, Galante J, Salis S, Raiteri M (2001) AMPA-evoked acetylcholine
806 release from cultured spinal cord motoneurons and its inhibition by GABA and glycine.
807 *Neuroscience* 106:183–191.
808

809 Fulton BP, Walton K (1986) Electrophysiological properties of neonatal rat motoneurons
810 studied in vitro. *J Physiol* 370:651–678.
811

812 Galante M, Nistri A, Ballerini L (2000) Opposite changes in synaptic activity of organotypic
813 rat spinal cord cultures after chronic block of AMPA/kainate or glycine and GABAA
814 receptors. *J Physiol* 523:639–651.
815

816 Getting PA (1989) Emerging principles governing the operation of neural networks. *Annu*
817 *Rev Neurosci* 12:185–204.
818

819 Harkema SJ, Hillyer J, Schmidt-Read M, Ardolino E, Sisto SA, Behrman AL (2012)
820 Locomotor training: as a treatment of spinal cord injury and in the progression of neurologic
821 rehabilitation. *Arch Phys Med Rehabil* 93:1588–1597.
822

823 Hayes HB, Chang YH, Hochman S (2009) An in vitro spinal cord-hindlimb preparation for
824 studying behaviorally relevant rat locomotor function. *J Neurophysiol* 101:1114–1122.
825

826 Hayes HB, Chang YH, Hochman S (2012) Stance-phase force on the opposite limb dictates
827 swing-phase afferent presynaptic inhibition during locomotion. *J Neurophysiol* 107:3168–
828 3180.

829

830 Hestrin S (1993) Different glutamate receptor channels mediate fast excitatory synaptic
831 currents in inhibitory and excitatory cortical neurons. *Neuron* 11:1083–1091.

832

833 Hiebert GW, Pearson KG (1999) Contribution of sensory feedback to the generation of
834 extensor activity during walking in the decerebrate Cat. *J Neurophysiol* 81:758-770.

835

836 Hubli M, Dietz V (2013) The physiological basis of neurorehabilitation--locomotor training
837 after spinal cord injury. *J Neuroeng Rehabil* 10:5.

838

839 Ichiyama RM, Broman J, Roy RR, Zhong H, Edgerton VR, Havton LA (2011) Locomotor
840 training maintains normal inhibitory influence on both alpha- and gamma-motoneurons after
841 neonatal spinal cord transection. *J Neurosci* 31:26–33.

842

843 Joseph MS, Tillakaratne NJ, de Leon RD (2012) Treadmill training stimulates brain-derived
844 neurotrophic factor mRNA expression in motor neurons of the lumbar spinal cord in spinally
845 transected rats. *Neuroscience* 224:135–144.

846

847 Juvin L, Simmers J, Morin D (2007) Locomotor rhythmogenesis in the isolated rat spinal
848 cord: a phase- coupled set of symmetrical flexion extension oscillators. *J Physiol* 583:115–
849 128.

850

851 Katz B, Miledi M (1967) A study of synaptic transmission in the absence of nerve impulses. *J*
852 *Physiol* 192:407–436.

853

854 Keeler BE, Liu G, Siegfried RN, Zhukareva V, Murray M, Houllé JD (2012) Acute and
855 prolonged hindlimb exercise elicits different gene expression in motoneurons than sensory
856 neurons after spinal cord injury. *Brain Res* 1438:8–21.

857

858 Kerkut GA, Bagust J (1995) The isolated mammalian spinal cord. *Prog Neurobiol* 46:1–48.

859

860 Kiehn O, Kjaerulff O (1996) Spatiotemporal characteristics of 5-HT and dopamine-induced
861 rhythmic hindlimb activity in the in vitro neonatal rat. *J Neurophysiol* 75:1472–1482.
862

863 Kiehn O (2006) Locomotor circuits in the mammalian spinal cord. *Annu Rev Neurosci*
864 29:279–306.
865

866 Kjaerulff O, Barajon I, Kiehn O (1994) Sulphorhodamine-labelled cells in the neonatal rat
867 spinal cord following chemically induced locomotor activity in vitro. *J Physiol* 478:265–273.
868

869 Klein DA, Tresch MC (2010) Specificity of intramuscular activation during rhythms
870 produced by spinal patterning systems in the in vitro neonatal rat with hindlimb attached
871 preparation. *J Neurophysiol* 104:2158–2168.
872

873 Kremer E, Lev-Tov A (1997) Localization of the spinal network associated with generation of
874 hindlimb locomotion in the neonatal rat and organization of its transverse coupling system. *J*
875 *Neurophysiol* 77:1155–1170.
876

877 Kremer E, Lev-Tov A (1998) GABA-receptor-independent dorsal root afferents
878 depolarization in the neonatal rat spinal cord. *J Neurophysiol* 79:2581–2592.
879

880 Lewis CA, Faber DS (1996) Properties of spontaneous inhibitory synaptic currents in cultured
881 rat spinal cord and medullary neurons. *J Neurophysiol* 76:448–460.
882

883 Loeb GE, Bak MJ, Duysens J (1977) Long-term unit recording from somatosensory neurons
884 in the spinal ganglia of the freely walking cat. *Science* 197:1192–1194.
885

886 Lovinger DM, Weight FF (1988) Glutamate induces a depolarization of adult rat dorsal root
887 ganglion neurons that is mediated predominantly by NMDA receptors. *Neurosci Lett* 94:314–
888 320.
889

890 Mandadi S, Whelan PJ (2009) A new method to study sensory modulation of locomotor
891 networks by activation of thermosensitive cutaneous afferents using a hindlimb attached
892 spinal cord preparation. *J Neurosci Methods* 182:255–259.
893

894 Mandadi S, Hong P, Tran MA, Bráz JM, Colarusso P, Basbaum AI, Whelan PJ (2013)
895 Identification of multisegmental nociceptive afferents that modulate locomotor circuits in the
896 neonatal mouse spinal cord. *J Comp Neurol* 521:2870–2887.
897

898 Marchetti C, Beato M, Nistri A (2001) Alternating rhythmic activity induced by dorsal root
899 stimulation in the neonatal rat spinal cord in vitro. *J Physiol* 530:105–112.
900

901 McVea DA, Pearson KG (2007) Long-lasting, context-dependent modification of stepping in
902 the cat after repeated stumbling-corrective responses. *J Neurophysiol* 97:659–669.
903

904 Ménard A, Leblond H, Gossard JP (2002) Sensory integration in presynaptic inhibitory
905 pathways during fictive locomotion in the cat. *J Neurophysiol* 88:163–171.
906

907 Molander C, Xu Q, Grant G (1984) The cytoarchitectonic organization of the spinal cord in
908 the rat. I. The lower thoracic and lumbosacral cord. *J Comp Neurol* 230:133–141.
909

910 Nishimaru H, Kudo N (2000) Formation of the central pattern generator for locomotion in the
911 rat and mouse. *Brain Res Bull* 53:661–669.
912

913 Nistri A, Taccola G, Mladinic M, Margaryan G, Kuzhandaivel A. (2010) Deconstructing
914 locomotor networks with experimental injury to define their membership. *Ann N Y Acad Sci*
915 1198:242–251.
916

917 Pearson KG, Rossignol S (1991) Fictive motor patterns in chronic spinal cats. *J Neurophysiol*
918 66:1874–1887.
919

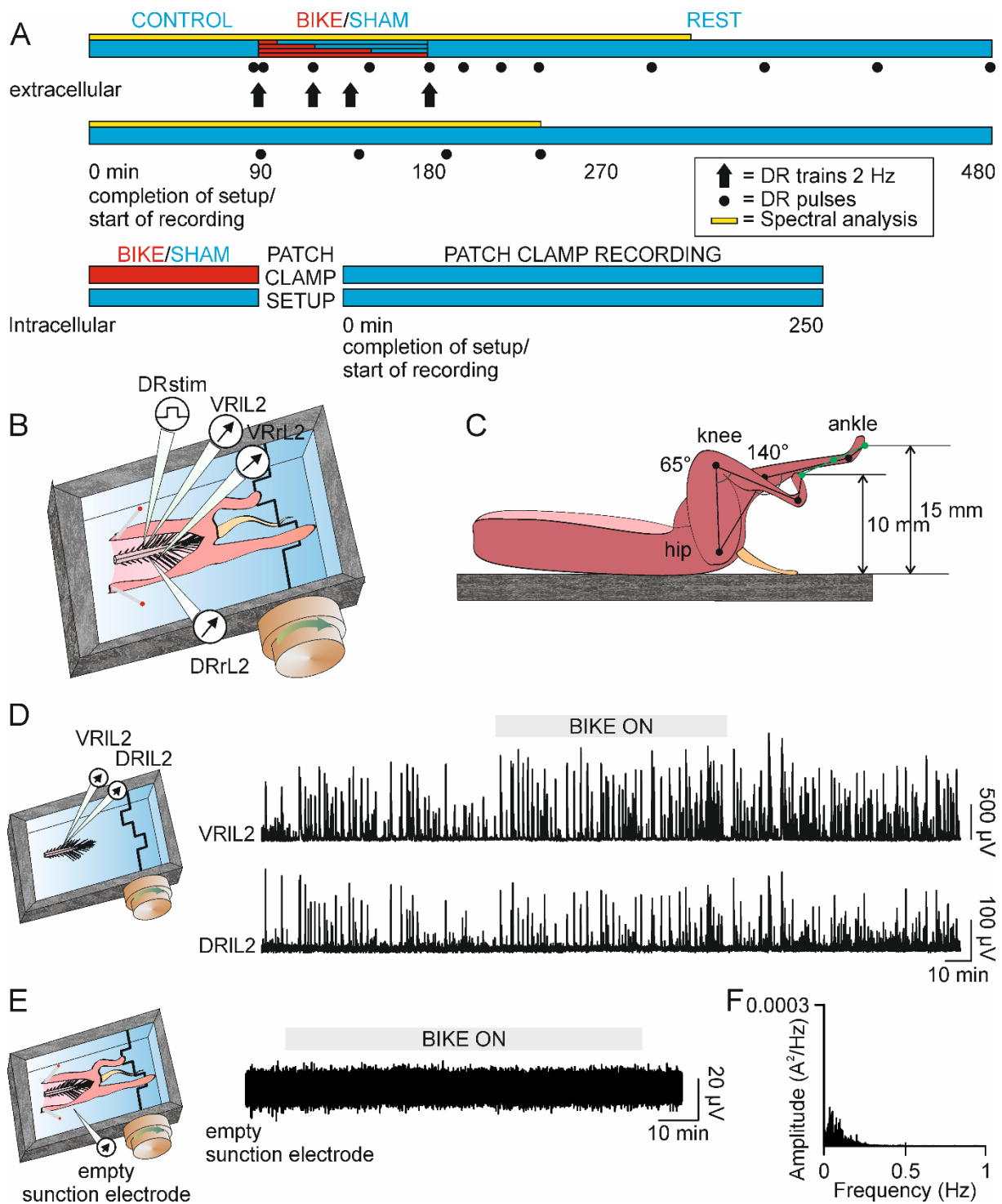
920 Sirois J, Frigon A, Gossard JP (2013) Independent control of presynaptic inhibition by
921 reticulospinal and sensory inputs at rest and during rhythmic activities in the cat. *J Neurosci*
922 33:8055–8067.
923

924 Sonner PM, Ladle DR (2013) Early postnatal development of GABAergic of Ia
925 proprioceptive afferent connections in mouse spinal cord. *J Neurophysiol* 109:2118–2128.
926

927 Stein RB (1995) Presynaptic inhibition in humans. *Prog Neurobiol* 47:533–544.

928
929 Taccola G (2011) The locomotor central pattern generator of the rat spinal cord in vitro is
930 optimally activated by noisy dorsal root waveforms. *J Neurophysiol* 106:872–884.
931
932 Taccola G, Olivieri D, D'Angelo G, Blackburn P, Secchia L, Ballanyi K (2012) A₁ adenosine
933 receptor modulation of chemically and electrically evoked lumbar locomotor network activity
934 in isolated newborn rat spinal cords. *Neuroscience* 222:191–204.
935
936 Tartas M, Morin F, Barrière G, Goillandeau M, Lacaille JC, Cazalets JR, Bertrand SS (2010)
937 Noradrenergic modulation of intrinsic and synaptic properties of lumbar motoneurons in the
938 neonatal rat spinal cord. *Front Neural Circuits* 4:4.
939
940 Tsvyetlynska NA, Hill RH, Grillner S (2005) Role of AMPA receptor desensitization and the
941 side effects of a DMSO vehicle on reticulospinal EPSPs and locomotor activity. *J*
942 *Neurophysiol* 94:3951–3960.
943
944 Vinay L, Brocard F, Fellippa-Marques S, Clarac F (1999) Antidromic discharges of dorsal
945 root afferents in the neonatal rat. *J Physiol Paris* 93:359–367.
946
947 Vinay L, Brocard F, Clarac F, Norreel JC, Pearlstein E, Pflieger JF (2002) Development of
948 posture and locomotion: an interplay of endogenously generated activities and neurotrophic
949 actions by descending pathways. *Brain Res Brain Res Rev* 40:118–129.
950
951 Wheatley M, Stein RB (1992) An in vitro preparation of the mudpuppy for simultaneous
952 intracellular and electromyographic recording during locomotion. *J Neurosci Methods*
953 42:129–137.
954
955 Wyllie DJ, Manabe T, Nicoll RA (1994) A rise in postsynaptic Ca²⁺ potentiates miniature
956 excitatory postsynaptic currents and AMPA responses in hippocampal neurons. *Neuron*
957 12:127–138.
958
959 Wong C, Wong G, Pearson KG, Lomber SG (2018) Memory-guided stumbling correction in
960 the hindlimb of quadrupeds relies on parietal area 5. *Cereb Cortex* 28:561–573.
961

962 Ziegler MD, Zhong H, Roy RR, Edgerton VR (2010) Why variability facilitates spinal
963 learning. *J Neurosci* 30:10720–10726.
964



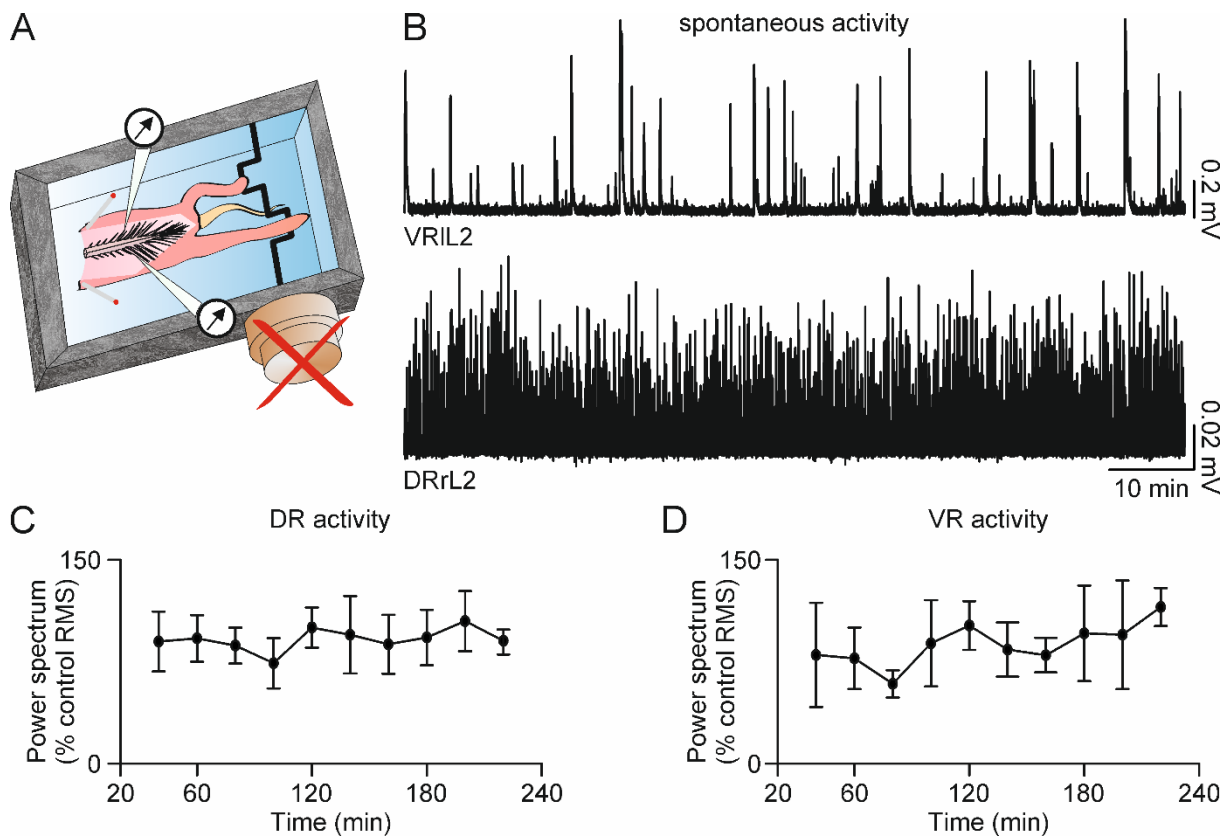
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967 **Figure 1. BIKE allows passive hindlimb movement while simultaneously recording from**
 968 **spinal roots.**

969 **A:** Bars summarize protocols for extracellular (top bars) and patch clamp (bottom bars)
 970 experiments. Time zero was arbitrarily assigned to the beginning of each recording. The
 971 extracellular protocol consisted in a pre-BIKE control, a session of passive pedaling driven by
 972 BIKE (10, 30, 60 or 90 minutes; in red), and a long post-BIKE rest. Throughout the

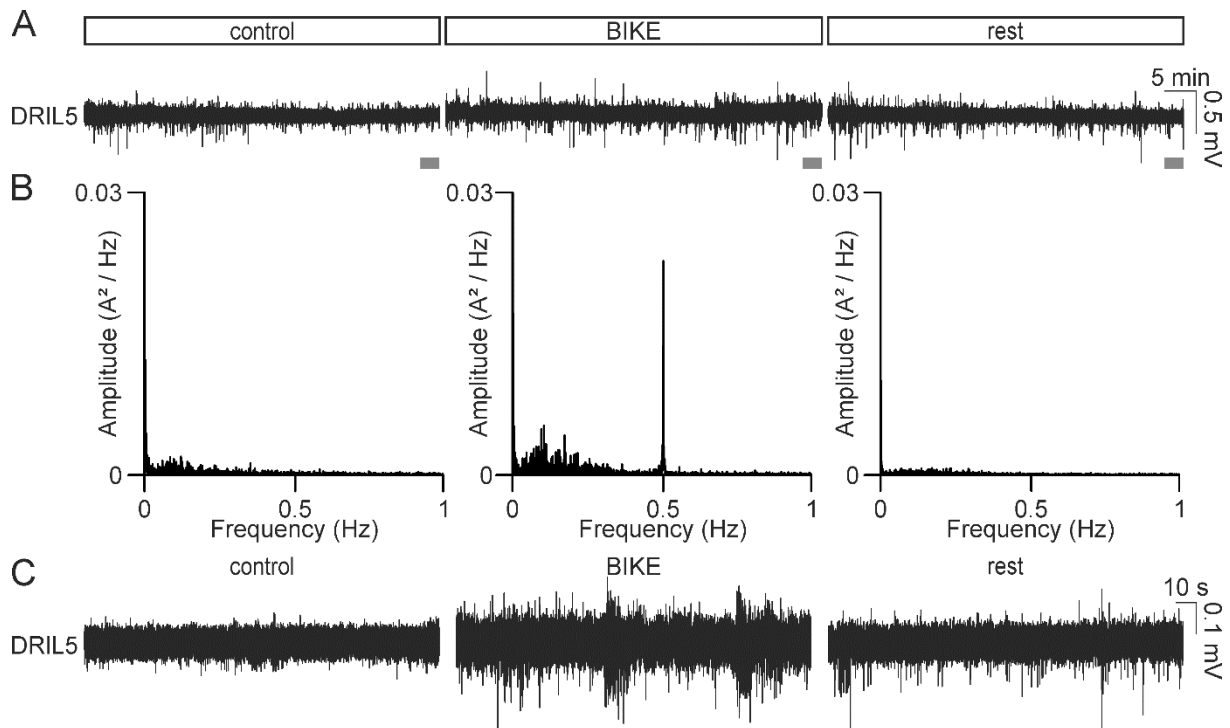
973 experiment, spontaneous activity (yellow bar), evoked activity (black dots) and electrically-
974 induced locomotor-like activity (black arrows) were continuously monitored. Sham
975 experiments were also carried out and recordings were performed at comparable time points
976 as BIKE preparations. As for patch clamp experiments, leg-attached preparations were first
977 trained with BIKE (30- or 90-minute sessions) or maintained still in the BIKE recording
978 chamber for an equivalent time interval. Then, each spinal cord was isolated and arranged for
979 patch clamp recordings from lumbar motoneurons. **B:** The cartoon shows the experimental
980 setup using a spinal cord with hindlimbs attached. Spinal roots were bilaterally dissected from
981 the high thoracic region down to the second lumbar segment (L2) included. Preparation was
982 maintained in a continuously superfused chamber, with hindpaws firmly fixed to the pedals of
983 BIKE above the chamber. Speed rotation was adjusted through a stabilized power supply at
984 around 30 cycles/min (0.5 Hz). Only forward movement was applied, as indicated by the
985 arrow. Simultaneous recordings were performed with suction glass electrodes from both right
986 (r) and left (l) L2 ventral roots (VRs) and from a single dorsal root (DR), either in the
987 presence or in the absence of DR stimulation. **C:** A lateral view of the leg-attached isolated
988 spinal cord on the BIKE device. The cartoon was modeled from goniometric measurements of
989 knee joints taken from P2 animals. Actual angles might slightly change with younger or older
990 subjects (P0, P4). **D:** Sample traces from VRIL2 and DRIL2 in an isolated spinal cord during
991 BIKE pedaling show that BIKE does not induce any electrical interference to perturb
992 electrophysiological recordings of spontaneous baseline activity. **E:** Background noise
993 recorded with a glass electrode placed close to the L5 spinal segment demonstrates that
994 passive hindlimb movement does not induce any baseline interference. **F:** The FFT analysis
995 reports spectral analysis for the trace in **D**. Note the absence of any rhythmic components
996 around the frequency of pedaling (0.5 Hz).
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Figure 2. Spontaneous activity from spinal roots of the leg-attached isolated spinal cord is consistent over a long *in vitro* maintenance.

A: In sham experiments, preparations were set in the recording chamber following all usual procedures, apart from switching on BIKE, as depicted by the red cross on the rotor. **B:** Sample traces represent long recordings of spontaneous activity from VRIL2 (on the top) and DRrL2 (on the bottom). **C, D:** Time courses show the magnitude of the power spectrum calculated by 20-min bins of activity for DR discharges (**C**) and VR activity (**D**).



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1010 **Figure 3. BIKE-induced passive training evokes afferent sensory feedback coupled with**
 1011 **pedaling.**

1012 **A:** Sample traces were recorded during a 90-min pre-BIKE control (control), BIKE and post-

1013 BIKE rest (rest), as depicted by the protocol bars on top. Activity was derived from DRIL5 by

1014 applying a negative pressure on its surface through a suction glass electrode (*en passant*

1015 recordings). **B:** The FFT analysis for traces in **A** isolates a main frequency component at 0.5

1016 Hz during BIKE (middle), which reflects the pedaling frequency. No components at 0.5 Hz

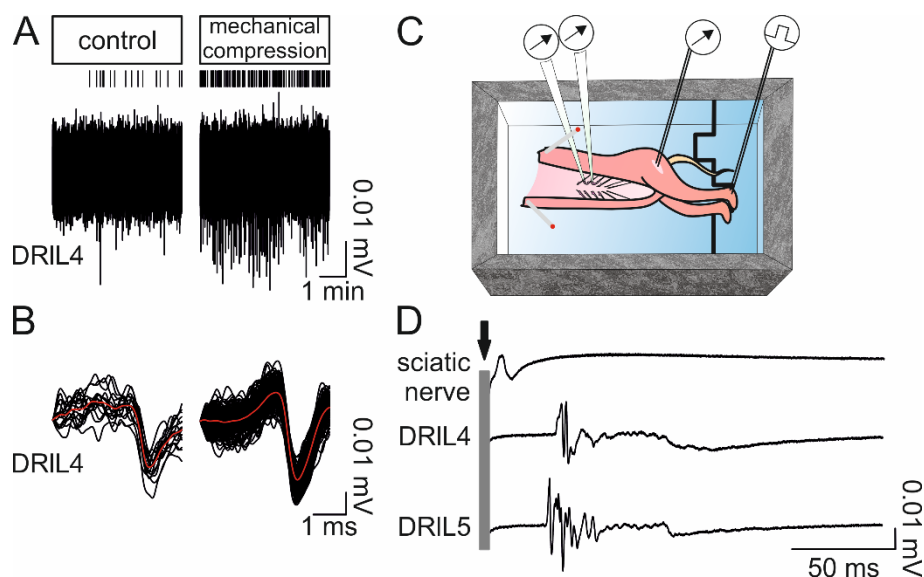
1017 could be detected either in pre-BIKE control (left) or at post-BIKE rest (right). **C:** Higher

1018 magnifications of traces in **A**, corresponding to grey rectangles, indicate the increase in

1019 afferent discharges during BIKE (middle) compared to pre-BIKE control (left) and post-BIKE

1020 rest (right).

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1024 **Figure 4. Afferent pathways to the spinal cord are recruited in our *in vitro* model.**

1025 **A:** Incoming discharges were recorded from a leg-attached preparation where the spinal cord

1026 was removed to allow recordings from the distal stump of DRIL4. Recordings were

1027 performed before and during a mechanical compression of the left hindpaw, as shown on top.

1028 Raster plots above traces highlight a greater incoming activity during peripheral mechanical

1029 compression of the left hindpaw. **B:** Identified events were superimposed and shown in black,

1030 while averaged traces were depicted in red. **C:** In a random group of experiments, leg-

1031 attached preparations deprived of the spinal cord were arranged in the BIKE recording

1032 chamber without any pedaling, as depicted by the cartoon. The left hindpaw was firmly fixed

1033 to the right pedal for single-pulse stimulation of the sural-innervated territory and for

1034 recording from the branch of the exposed sciatic nerve. Pairs of hooked needle electrodes

1035 were used for bipolar stimulation and recordings from the sciatic nerve. Moreover, monopolar

1036 recordings with suction glass electrodes were performed from the spinal stumps of DRIL4 and

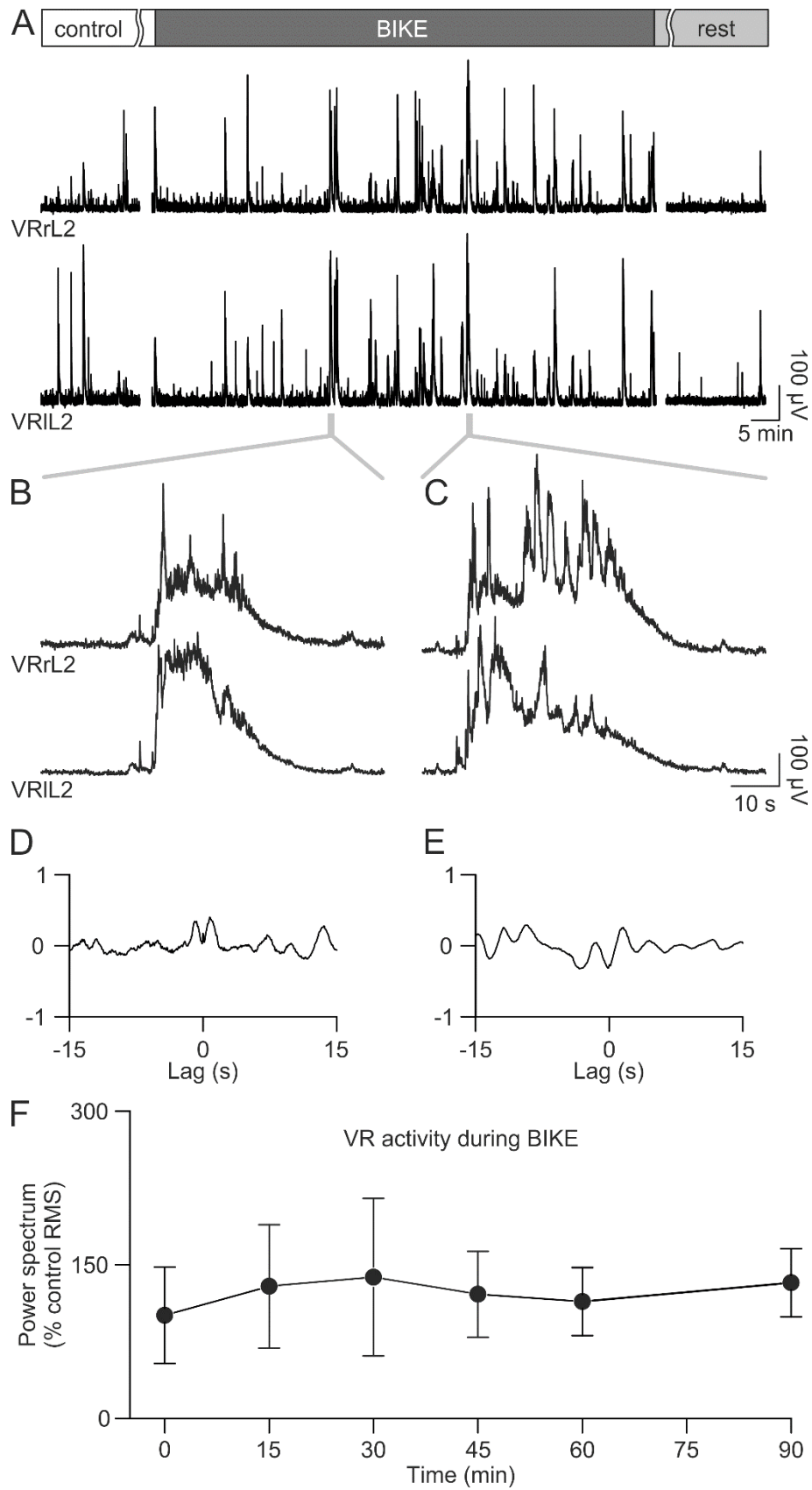
1037 DRIL5. **D:** Average traces of 500 sweeps are reported. Compound action potentials could be

1038 elicited from both the sciatic nerve and, with a higher latency, from spinal afferent nerves L4

1039 and L5. The grey rectangle on the left represents a 5-millisecond rectangular pulse applied to

1040 the sural territory on the left hindpaw.

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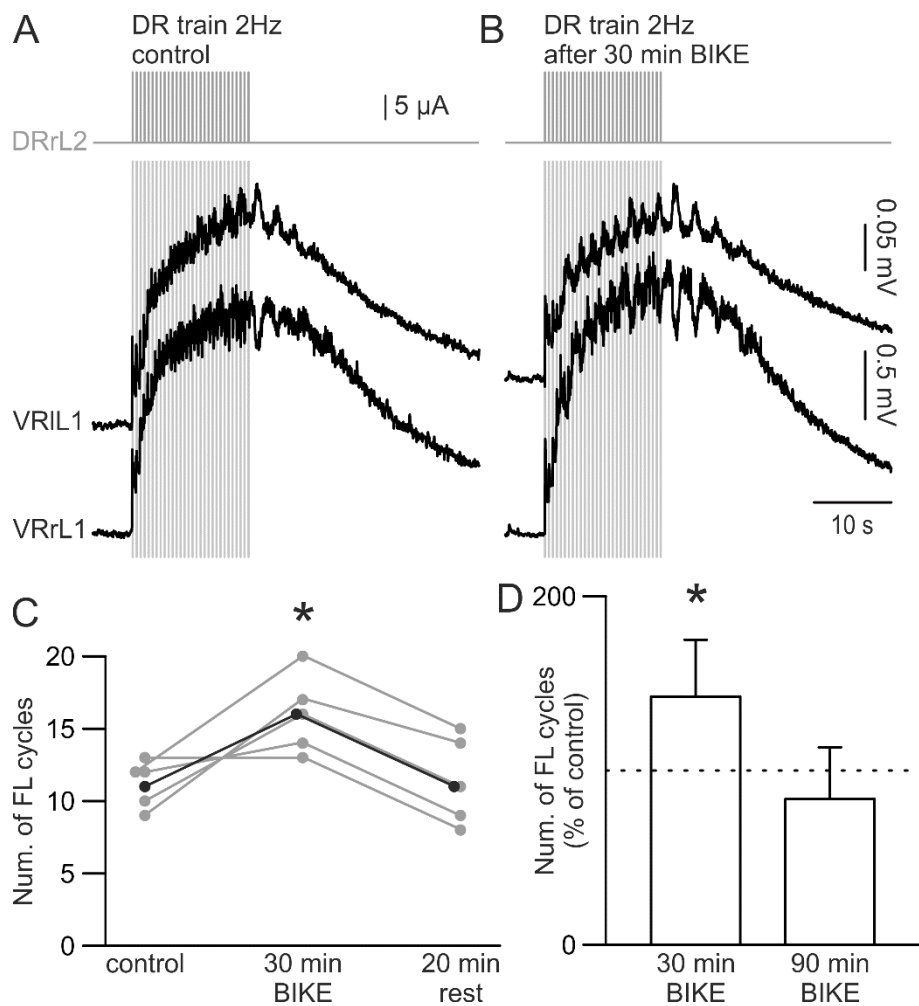
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Figure 5. Long application of BIKE does not vary spontaneous VR activity.

1045 **A:** Spontaneous activity was recorded from bilateral VRs L2 in pre-BIKE control (left),
1046 during a BIKE session (90 min; middle) and at the end of training (right). Traces are
1047 interrupted in correspondence to the artifacts from single or repetitive pulse stimulation (20
1048 min). **B, C:** Magnifications of two bilateral bursts from VRs corresponding to the grey
1049 rectangles in **A**. **D, E:** Cross-correlograms between homosegmental L2s report poor phase
1050 coupling between a pair of VRs. **F:** The time course points out that magnitude of the power
1051 spectrum for VR spontaneous activity during BIKE was not significantly different from pre-
1052 BIKE control values.
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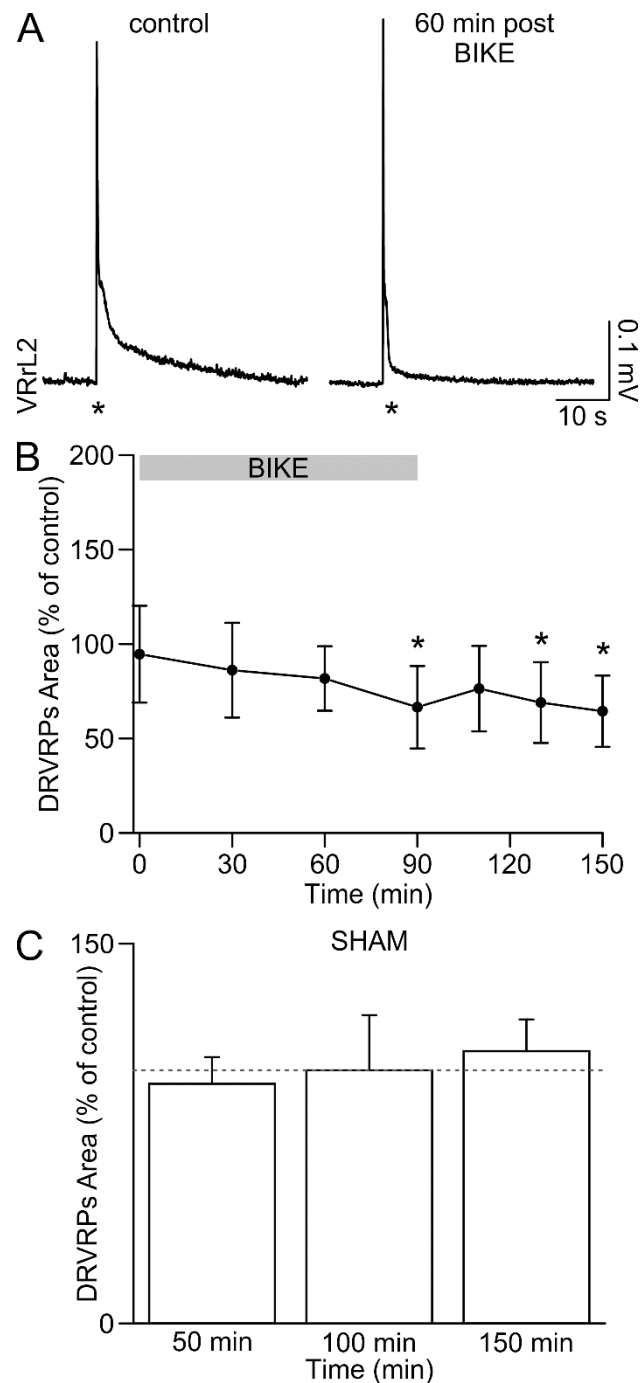


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1056 **Figure 6. A 30-minute BIKE session transiently facilitates electrically-evoked FL.**

1057 **A:** Before BIKE, a train of stereotyped electrical stimuli (30 rectangular pulses; pulse duration
1058 = 0.1 ms; intensity = 45 μ A, 3 x Th; frequency = 2 Hz) was delivered to DRrL2 (upper panel),
1059 inducing a characteristic episode of FL consisting in a cumulative depolarization with 8
1060 superimposed alternating cycles recorded from homosegmental L1 VRs. **B:** At the end of a
1061 30-minute BIKE session, the same train of pulses induced a higher number of locomotor-like
1062 oscillations. **C:** Plot summarizes, for six experiments, the time course related to the number of
1063 FL cycles induced by a train of stimuli, before BIKE, right after switching off BIKE (at the
1064 end of a 30-minute session) and after 20 minutes of post-BIKE rest. BIKE transiently
1065 augmented the number of FL cycles, which returned to pre-BIKE control values 20 minutes
1066 after the end of training (*; P = 0.012). Note that grey dots represent raw data and black dots
1067 indicate mean values. **D:** Histogram compares the average increase in the number of FL
1068 cycles induced by DR trains in preparations subjected to 30 or 90 min BIKE. The facilitatory

1069 effect mediated by 30 minutes BIKE was lost if training was prolonged to 90 minutes (*; P =
1070 0.008).

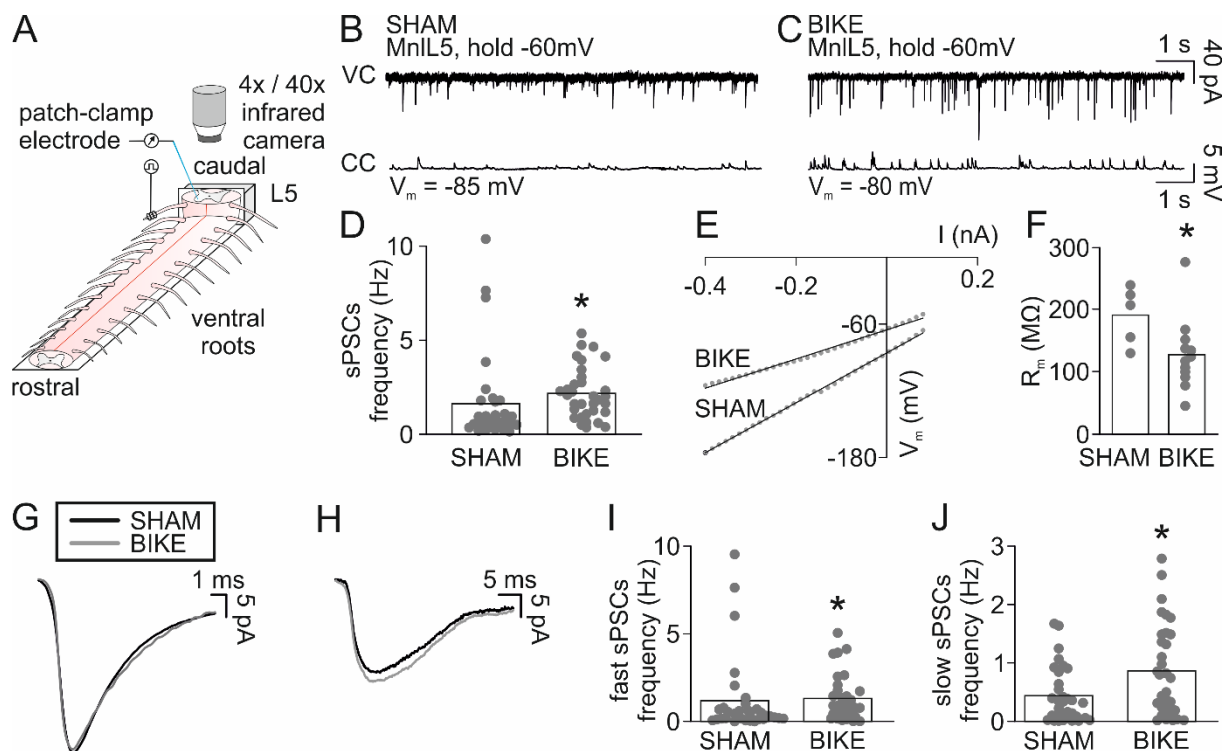


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1073 **Figure 7. Only longer sessions of BIKE reduce the area of DRVRPs.**

1074 **A:** A spinal reflex (averaged traces from 10 sweeps) is induced by a single pulse (22 μ A, Th,
1075 100 μ s) delivered to DRIT13 at the time indicated by the stars. In pre-BIKE control conditions
1076 (left), responses present an early peak followed by a slow decaying repolarization. One hour
1077 after a BIKE training session of 90 mins (right), the same pulse elicited a response of smaller
1078 area, while the peak remained unaffected. **B:** The time course plot summarizes mean values

1079 collected from 14 experiments, showing a progressive reduction in the DRVRP area by
1080 prolonging the duration of the BIKE session. After 90 min BIKE, a significant reduction of
1081 the reflex response is reached and maintained for at least the following hour (*; $P = <0.001$).
1082 **C:** Bars report the area of DRVRPs in sham experiments (without any actual pedaling), at
1083 different time intervals. DRVRPs were monitored up to 150 min and they remained
1084 unchanged.
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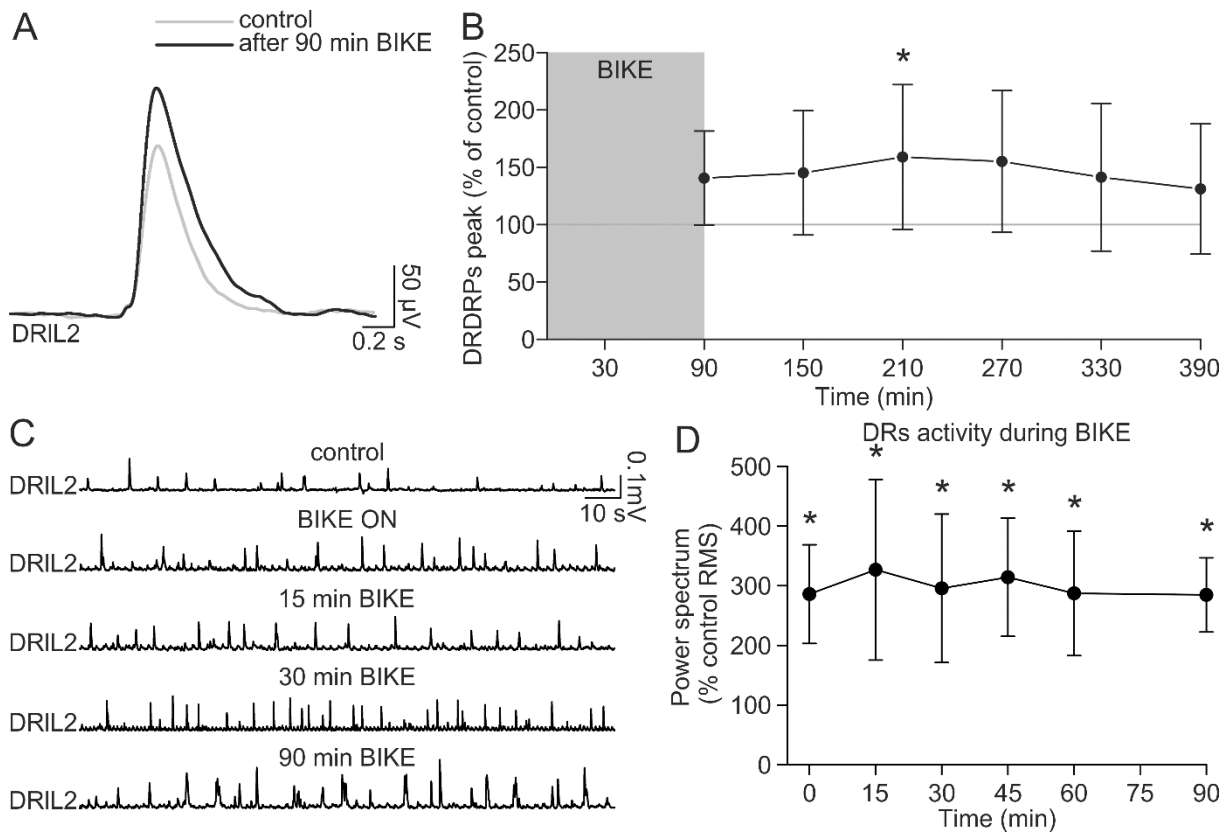


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1088 **Figure 8. 90-minutes of BIKE affect synaptic transmission and membrane resistance of**
1089 **lumbar motoneurons.**

1090 **A:** Cords were isolated from leg-attached preparations at the end of BIKE training or sham
1091 experiments. The dorsal surface was glued on a plastic strip and bent in an upright position
1092 against a sylgard cube. In this configuration, L5 spinal segment faces upwards and
1093 motoneurons are visualized with an infrared camera for patch clamp recordings. L5 VR is
1094 antidromically stimulated using bipolar suction electrodes to functionally identify
1095 ipsilaterally-patched motoneurons. **B:** Whole-cell patch clamp recordings were performed in
1096 voltage clamp (VC; top trace) and current clamp (CC; bottom trace) modes from the same LL5
1097 motoneuron in a sham preparation. Holding potential in VC mode is -60 mV, while cell
1098 membrane potential (V_m) in CC mode, without injecting any holding current, is -85 mV. **C:**
1099 Recordings were carried out in VC mode (top) and CC mode (bottom) from the same LL5
1100 motoneuron after 90 minutes of BIKE. Holding potential in VC mode was -60 mV, while V_m
1101 in CC mode was -80 mV. Recordings from motoneurons in **B** and **C** were obtained from an
1102 equivalent period of time after isolation of the cord from the leg-attached preparation. Note
1103 the higher number of sPSCs (top trace in **C**) with respect to sham (top trace in **B**), as
1104 confirmed by the plot in **D**, reporting a significant increase in sPSCs frequency after BIKE-
1105 training (*; $P = 0.001$). **E:** In the graph are shown the I-V curves for sample motoneurons
1106 from BIKE and sham preparations. **F:** The plot indicates a significantly lower membrane

1107 resistance (R_m) in BIKE-trained motoneurons with respect to sham (*; $P = 0.04$). **G** and **H**:
1108 Superimposed averaged traces represent fast sPSCs (**G**) and slow sPSCs (**H**) from a pair of
1109 sample motoneurons in sham (black lines) and BIKE-trained preparations (grey lines). **I** and
1110 **J**: Plots indicate that 90 minutes of BIKE increase frequency of fast sPCSs (**I**; *, $P = 0.029$),
1111 and slow sPCSs (**J**; *, $P = 0.026$), compared to sham experiments. In plots, grey dots
1112 represent raw data, bars indicate mean values.
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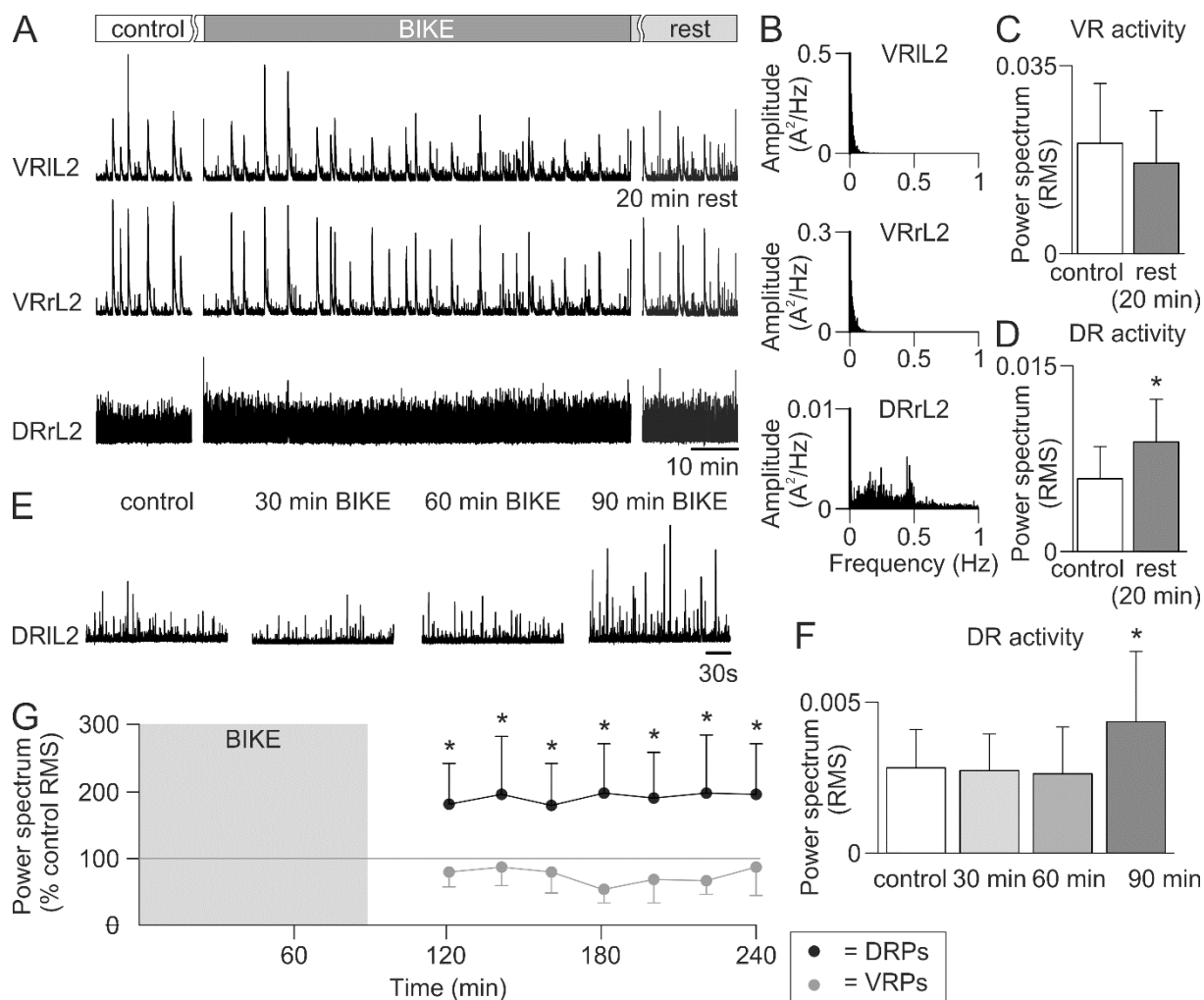
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1116 **Figure 9. A 90-minute BIKE session stably increases DR reflexes and spontaneous**
 1117 **discharges during training.**

1118 **A:** Averaged traces (mean of 5 sweeps) of DRDRPs were recorded from DRIL2 in response
 1119 to electrical pulses applied to DRrL1 (rectangular pulses; duration = 0.1 ms; intensity = 80
 1120 μA , 4 x Th; frequency = 0.02 Hz) in pre-BIKE control (grey trace) and after a 90-minute
 1121 BIKE session (black trace). **B:** The time course of DRDRP peaks recorded for up to five
 1122 hours after BIKE (grey rectangle) highlights a significant increase in DRDRPs amplitude at
 1123 two hours after training (*; $P = 0.023$). **C:** Spontaneous activity was recorded from DRIL2 in
 1124 pre-BIKE control (top trace) and at different time points during BIKE training. **D:** The time
 1125 course points out that magnitude of the power spectrum for spontaneous antidromic DR
 1126 discharges was significantly increased as soon as BIKE was switched on and stably persisted
 1127 throughout training duration (*; $P \leq 0.001$).

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1131 **Figure 10. At least 90 minutes of BIKE are needed to increase spontaneous dorsal**
 1132 **activity, which lasts throughout the following resting period.**

1133 **A:** As reported in the top bar, spontaneous activity was recorded from homosegmental L2
 1134 VRs and from DRrL2 in pre-BIKE control (left traces), during a 90-minute BIKE session
 1135 (middle traces) and for the first 20 minutes of post-BIKE rest (right traces). Note that, during
 1136 BIKE, VR activity did not change (middle, top traces; see also Fig. 5), while DR rhythm
 1137 increased (middle, bottom trace; see also Fig. 9 C-D). Dorsal activity remained higher than
 1138 pre-BIKE control also at the end of training (right, bottom trace). **B:** During BIKE
 1139 functioning, power spectra of activity from bilateral VRs L2 (above panels) reveal the
 1140 absence of any frequency component at 0.5 Hz, while rhythmic activity coupled with pedaling
 1141 can be detected from DRrL2 (bottom panel). **C, D:** Histograms summarize average data of
 1142 VR (**C**) and DR (**D**; *, $t_{(7)} = -4.058$, $P = 0.005$) spontaneous discharges recorded after 20
 1143 minutes post-BIKE rest. **E:** Three subsequent sessions of BIKE (30 minutes each) were
 1144 cumulatively applied, meanwhile spontaneous dorsal activity was recorded from DRIL2 at the

1145 end of each session. Please note that the calibration bar on the right is the same for all traces
1146 in **E**. **F**: Bars show that only three consecutive 30-minute sessions of BIKE (for a total
1147 training period of 90 minutes) significantly increased DR rhythm magnitude (expressed as
1148 root mean square; RMS; *, $P = 0.026$), while a lower training duration was ineffective. **G**:
1149 The time course points out that after a 90-minute BIKE training (grey rectangle), DR
1150 spontaneous activity (black dots) remained higher than pre-BIKE control for at least two
1151 hours of post-BIKE rest (*; $P \leq 0.001$); on the other hand, VR spontaneous activity (grey
1152 dots) was unaffected by training. Spontaneous activity was assessed in 20-min bins, starting
1153 10 minutes after the end of training.