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# 1 Conservation of locomotion-induced oculomotor 2 activity through evolution in mammals

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4 *Filipa França de Barros*<sup>1</sup>, *Julien Bacqué-Cazenave*<sup>2</sup>, *Coralie Taillebuis*<sup>1,2</sup>, *Gilles Courtand*<sup>2</sup>,  
5 *Marin Manuel*<sup>3,4</sup>, *Hélène Bras*<sup>5</sup>, *Michele Tagliabue*<sup>1</sup>, *Denis Combes*<sup>2</sup>, *François M Lambert*<sup>2, 6,\*</sup>  
6 , *Mathieu Beraneck*<sup>1, 6, 7,\*</sup>

7 1-Université de Paris, CNRS UMR 8002, INCC - Integrative Neuroscience and Cognition Center, F-  
8 75006, Paris, France.

9 2-Institut des Neurosciences Cognitives et Intégratives d'Aquitaine, INCIA CNRS UMR 5287, 33076  
10 Université de Bordeaux, Bordeaux, France

11 3-Université de Paris, CNRS UMR 8003, SPPIN - Saints-Pères Paris Institute for the Neurosciences,  
12 F-75006 Paris, France.

13 4-Department of Biomedical and Pharmaceutical Sciences and George and Anne Ryan Institute for  
14 Neuroscience, University of Rhode Island, Kingston, RI

15 5-Institut de Neurosciences de la Timone, UMR 7289 CNRS-AMU, 13385 Marseille, France.

16 6- Equal contribution

17 7- Lead contact

## 18 \* Corresponding authors

19 Dr. M. Beraneck. CNRS UMR 8002, Université de Paris, 45 rue des St-Pères, Paris 75270,  
20 France. Email: mathieu.beraneck@u-paris.fr

21 Dr. FM Lambert CNRS UMR 5287, Université de Bordeaux, 146 rue Léo Saignat, Bordeaux  
22 33076, France. Email: francois.lambert@u-bordeaux.fr

23

## 24 Summary

25 Efference copies are neural replicas of motor outputs used to anticipate the sensory  
26 consequences of a self-generated motor action or to coordinate neural networks involved in  
27 distinct motor behaviors<sup>1</sup>. An established example of this motor-to-motor coupling is the  
28 efference copy of the propulsive motor command, that supplements classical visuo-vestibular  
29 reflexes to ensure gaze stabilization during amphibian larval locomotion<sup>2</sup>. Such feedforward  
30 replica of spinal pattern-generating circuits, produces a spino-extraocular motor coupled  
31 activity that evokes eye movements, spatio-temporally coordinated to tail undulation  
32 independently of any sensory signal<sup>3,4</sup>. Exploiting the developmental stages of the frog<sup>1</sup>,  
33 studies in metamorphosing *Xenopus* demonstrated the persistence of this spino-extraocular  
34 motor command in adults, and its developmental adaptation to tetrapodal locomotion<sup>5,6</sup>. Here,  
35 we demonstrate for the first time the existence of a comparable locomotor-to-ocular motor  
36 coupling in the mouse. In neonates, *ex vivo* nerve recordings of brainstem-spinal cord  
37 preparations reveal a spino-extraocular motor coupled activity similar to the one described in  
38 *Xenopus*. In adult mice, trans-synaptic rabies virus injections in lateral rectus eye muscle label  
39 cervical spinal cord neurons closely connected to abducens motor neurons. Finally, treadmill-  
40 elicited locomotion in decerebrated preparations<sup>7</sup> evokes rhythmic eye movements in  
41 synchrony with the limb gait pattern. Overall, our data are evidence for the conservation of  
42 locomotor-induced eye movements in vertebrate lineages. Thus, in mammals as in  
43 amphibians, CPG-efference copy feedforward signals might interact with sensory feedback to  
44 ensure efficient gaze control during locomotion.

## 45 Results

### 46 **Fictive locomotor activity induces ex vivo spino-extraocular motor coupling in** 47 **neonates in absence of sensory inputs**

48 The ability of locomotor CPG rhythmic activity to elicit a spino-extraocular motor coupling was  
49 evaluated on isolated *ex vivo* brainstem-spinal cord preparations of neonatal mice (Figure 1A).  
50 Electrical stimulation of either the first sacral dorsal root (S1Dr, Figure 1A) or the 8th cervical  
51 dorsal root (C8Dr, Figure S1A-B) evoked episodes of fictive locomotion with the typical  
52 coordination pattern between cervical and lumbar ventral roots (Vr) rhythmic activity (Vr;  
53 Figure 1A), as previously reported<sup>8,9</sup>. In accordance with this typical locomotor pattern, the  
54 example shown in Figure 1A demonstrates a paired bursting discharge between the  
55 homolateral C8 (light green in Figure 1A, bottom panel in B) and L2 (orange in Figure 1A, B  
56 bottom panel) Vr<sup>8</sup>. In most cases (66%, Figure 1C) this rhythmic cervico-lumbar coordinated  
57 locomotor activity was coupled to a bursting activity of the abducens motor nerves (Figure 1A-  
58 B) within the same frequency range (0.5-1.5Hz; Figure 1D, E) and a latency of ~62ms to C8  
59 Vr burst and ~80ms to L2 Vr burst, respectively (Figure 1F), compatible with a monosynaptic  
60 delay<sup>10-12</sup>. Inversely, non-coordinated spinal activity failed to evoke a coupled rhythmic  
61 discharge in the abducens nerves (Figure 1C). Locomotor-related bursts of an abducens nerve  
62 were synchronized to the ipsilateral C8-L2 Vr discharge (Figure 1B, bottom panel and Figure  
63 G), and alternated with the contralateral abducens discharge (Figure 1B, top panel).

64 Coordinated fictive locomotor episodes evoked by NMDA/5HT spinal immersion also  
65 produced a well correlated rhythmic activity in the abducens motor nerves (Figure S1C-F) in  
66 a lower frequency range (0.15-0.25Hz; Figure S1E) and with a preserved phase relationship  
67 relative to the Vr burst discharge (Figure S1F). In addition, frequency analysis of abducens  
68 discharge in response to S1Dr stimulation (Figure S1G-I) demonstrated that the spino-  
69 extraocular coupling was not induced by proprioceptive feedback inputs. Since all these  
70 brainstem-spinal preparations lacked visuo-vestibular sensory inputs as well as cortical,  
71 mesencephalic, and cerebellar inputs (see methods), the spino-extraocular motor coupling  
72 was produced by a locomotor efference copy signal, originating from the spinal motor CPGs.

73 **Spino-extraocular motor coupling depends on the integrity of the cervical, and**  
74 **not lumbar, spinal cord**

75 To determine the respective roles of the cervical and lumbar CPG networks in the origin of  
76 locomotor-induced spino-extraocular motor coupling, following the experimental approach  
77 previously used in xenopus<sup>3</sup>, cervical and lumbar regions were selectively isolated in  
78 brainstem-spinal cord preparations using spinal split-baths (Figure 2A). When synaptic  
79 transmission in the cervical cord was blocked with calcium-free aCSF<sup>9,12</sup> (Figure 2A, left),  
80 electrical stimulation of the S1Dr evoked typical alternated fictive locomotor activity in the  
81 lumbar Vr (Figure 2B bottom red traces, Figure 2D upper panel). However, it failed to evoke  
82 correlated fictive locomotor activity in the cervical Vr or rhythmic discharge in the abducens  
83 motor nerves (Figure 2B upper blue and green traces, Figure 2D, lower panel). This  
84 experiment demonstrates the instrumental role of the activity of the cervical motor networks in  
85 the genesis of the spino-extraocular motor coupling.

86 Next, we tested whether the cervical cord acted as a neuronal relay, or whether it was the  
87 origin of the spino-extraocular motor coupling. Locomotor-induced rhythmic discharge of  
88 abducens motor nerves was recorded in response to electrical stimulation of either S1Dr or  
89 C8Dr, in isolated *ex vivo* preparations exposed to a complete mid-thoracic section (Figure 2E).  
90 After the thoracic section, S1Dr stimulation failed to produce any spino-extraocular motor  
91 coupling (Figure 2F) whereas C8Dr electrical stimulation evoked locomotor-induced rhythmic  
92 discharge of the abducens motor nerves (Figure 2G). Even though the cervical CPG-related  
93 abducens rhythmic activity was recorded at relatively low frequency (Figure 2H, 0.2-0.35Hz,  
94 orange circles), it remained in synchrony with C8Vr bursting discharge (Figure 2I and 2J).  
95 Overall, these results show that, while the activation of the lumbar CPG allowed for a more  
96 robust coupling, the locomotor-related activity of the abducens motor nerves specifically  
97 originated from the cervical CPG.

98 **Cervical neuronal relay implicated in the spino-extraocular coupling**

99 To determine the cervical neuronal relay involved in the spino-extraocular coupling (Figures 1  
100 and 2), transsynaptic retrograde tracing of neuronal populations was performed by rabies virus  
101 (RV) injection in the lateral rectus muscle (LRM) of adult mice (Figure 3A). 55h after RV

102 infection of motoneurons innervating the LRM (Figure 3A and 3B; see methods), several  
103 neuronal populations closely related to RV<sup>+</sup> abducens motoneurons (visualized by ChAT  
104 immunolabeling, Figure 3B-D) were labelled. As expected, based on the vestibulo-ocular  
105 reflex circuitry<sup>13,14</sup>, RV<sup>+</sup> neurons were found in the vestibular nuclei (VN, Figure 3C) and  
106 contralateral oculomotor nucleus (contra nIII, Figure 3D). Moreover, RV<sup>+</sup> neurons were  
107 observed bilaterally throughout the cervical spinal cord (Figure 3E-F) in all preparations (n =  
108 3; Figure 3G-I). The average RV infection ratio was of 1 abducens motoneuron contacted by  
109 6 cervical neurons (Figure 3J). RV<sup>+</sup> cervical neurons were mainly located in the spinal ventral  
110 horn or around the central canal (Figure 3E-G and K). Notably, at this 55h labeling time  
111 window, no RV<sup>+</sup> neurons were observed caudally from the C8 segment. An extended infection  
112 time of 70h, which labelled extended network (Figure S3A) as e.g. cerebellum (Figure S3C)  
113 revealed spinal neuronal populations polysynaptically connected with abducens motoneurons  
114 (Fig S3B), scattered throughout the gray matter as well as beyond the cervical region (Figure  
115 S3D-G). The presence of RV<sup>+</sup> neurons in the dorsal horn (Figure S3E) suggests that  
116 brainstem-projecting spinal cord interneurons not only receive inputs from CPG but also  
117 putatively from sensory networks (Figure S3A). With this infection time (70h), rare RV<sup>+</sup> INs  
118 were detected in the rostral part of the lumbar spinal cord (L1-L2, n = 1 mouse, data not  
119 shown), whereas no RV<sup>+</sup> lumbar neurons were visible in lumbar spinal cord after 55h of  
120 infection (n = 3 mice). Overall, the use of RV trans-synaptic injections revealed a spinal-  
121 brainstem pathway likely to support the functional coupling of abducens motor nerve discharge  
122 with the rhythmic activity evoked by the cervical-CPG during locomotion in mice.

### 123 **Coupling between forelimb and eye movements in decerebrated preparations**

124 To determine if locomotor-induced eye movements occur *in vivo* in the absence of visual and  
125 vestibular sensory signals, eye movements were recorded in the dark during head-fixed  
126 treadmill locomotion in decerebrated adult mice (Figure 4A and Figure S4A). Such  
127 preparations enable the study of the spinal cord output and sensorimotor integration in the  
128 absence of descending cortical motor commands<sup>7</sup>. Figure 4A displays a representative  
129 example of the combined occurrence of locomotion and eye movements. Binocular nystagmic-  
130 like eye movements were observed during treadmill-evoked bouts of 10-40s sustained  
131 locomotion, after a >2hz locomotion was reached (Figure 4A, area in between the green  
132 arrows). This locomotor-induced oculomotor behavior consisted of an alternation of short

133 ocular movements (quick-phase like) in one direction, followed by longer eye movements  
134 (slow phase-like) of comparable amplitude in the opposite direction (Figure 4A-C; additional  
135 examples in Figure S4B). Eye movements were mostly restricted to the horizontal plane  
136 (compare vertical, in light colors, and horizontal components, in dark colors, in Figure 4B and  
137 4C) and their orientation (Figure 4D) remained homogeneous throughout the recordings. Left  
138 and right eye movements were always conjugated, with similar directionality for both eyes,  
139 and appeared well coordinated (see quick and slow eye movement phases on mean cycles of  
140 panel C; horizontal binocular coordination on oculogram in Figure 4E). To determine the  
141 temporal relationship between locomotor and ocular movements, the occurrence of the limbs  
142 (left stance onset) and eye events (quick phase-like peak velocity) during this sequence were  
143 compared. The frequency of the oculomotor cycles increased with the frequency of the  
144 locomotor cycles (Figure 4F). Moreover, eye and forelimb movements were generally  
145 synchronized (Figure 4G). All preparations (n=5) shared two main features: first, consistent  
146 eye movements only occurred once a locomotion frequency above 1Hz was attained (Figure  
147 4H, I)<sup>15</sup>. Second, the quantification of the event occurrence, comparing eye, forelimb and  
148 hindlimb movements, showed a match between eye and limb movements in about 80% of the  
149 cycles (Figure 4I, left bars), and an eye mismatch (limb movement, no eye movement; Figure  
150 4I, middle bars) in about 20% of the cycles. Notably, locomotor-induced eye movements never  
151 occurred in the absence of forelimb movements (Figure 4I, right bars). Analysis of sequences  
152 with a ratio of forelimb/eye events near unity (see methods) revealed that the frequency of the  
153 eye movement was closely correlated with the frequency of the forelimb (Figure 4J, left panel),  
154 while locomotor and oculomotor cycles were well synchronized (Figure 4J, right panel). Left  
155 and right eye movements were tightly correlated and synchronized ( $r^2 = 0.98$ ; Figure 4K; phase  
156 in right panel). In all recordings, eye movements consisted of nystagmic-like movements  
157 composed of alternated quick ( $0.066 \pm 0.022$  s) and slow ( $0.223 \pm 0.095$  s) duration phases  
158 (Figure 4L, left panel) lasting 20% and 80% of the oculomotor cycles, respectively (Figure 4L,  
159 right panel). Both components had similar amplitudes in range 1-8° (Figure 4M, quick phases  
160 - black, slow phases - gray, and Figure S4C), leading to different velocities (amplitude/velocity  
161 relation on Panel M; slopes of 20.27 and 2.09 for short and long duration eye movements,  
162 respectively; Figure S4C, right panel). Overall, eye movements were observed during >80%  
163 of trot-like locomotor cycles and closely matched forelimb movements. Thus, the results  
164 obtained in decerebrated preparations suggest the presence of a spino-extraocular motor

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165 coupling based on an efference copy from the locomotor cervical CPG in adult mice, as  
166 described in newborn mice (Figures 1 and 2). Additionally, they demonstrate that the  
167 persistence of this functional short-latency pathway in adults (Figure 3) enables a tight  
168 coupling between forelimb locomotor movements and eye movements during sustained high  
169 dynamic (>2Hz) locomotion (Figure 4).

170



## 171 Discussion

### 172 Conservation of locomotor-induced spino-extraocular motor coupling through 173 vertebrate evolution

174 Locomotor-induced spino-extraocular motor coupling was initially described in the *Xenopus*  
175 frog<sup>2–6</sup>. During swimming, both larvae and adult frogs exhibited a CPG efference copy-based  
176 extraocular bursting activity tightly coupled to the spinal motor rhythmic discharge. This spino-  
177 extraocular motor coordination adapted through metamorphosis to produce appropriate  
178 horizontal eye movements that counteracted either tail (larva) or limb (adults) movements  
179 during strong propulsive swimming<sup>4–6</sup>. The efference copy mechanism was supported by an  
180 ascending spinal-brainstem neural pathway connecting spinal cord neurons to extraocular  
181 motoneurons, crossed in larvae and bilateral in adult *xenopus*<sup>3,4,5</sup>. Using newborn and adult  
182 mice preparations, the present study reports a locomotor-induced spino-extraocular motor  
183 functional coupling that exhibits common features with the one found in frogs. First, in both  
184 species this coupling is supported by a spino-extraocular pathway that persists throughout  
185 ontogeny. Second, the bilateral distribution of cervical neurons revealed in mice by RV tracing  
186 suggests a comparable organization of the spinal-brainstem pathway between adult frog and  
187 mouse. Third, the locomotor-induced ocular signal, recorded either *ex vivo* or in decerebrated  
188 mice, was only observed during sustained and vigorous locomotor activity, corresponding to  
189 running behavior, a terrestrial equivalent for propulsive undulatory swimming. *Ergo*, our  
190 findings, combined with previous studies in *Xenopus*, strongly support the hypothesis that the  
191 CPG efference copy-based ocular signal is a conserved mechanism from amphibians to  
192 mammals, and putatively in other vertebrates. This locomotor-induced ocular behavior is in  
193 accordance with the absolute necessity to stabilize the visual field during motion, by  
194 performing rapid counteracting eye adjustments in all vertebrates, including humans<sup>16</sup>. There  
195 is considerable behavioral evidence for the contribution of locomotor-related spinal signals  
196 during gaze stabilization in several mammalian species. In cats<sup>17,18</sup> and in humans<sup>19,20</sup>,  
197 locomotor signals are integrated to favor vertical eye movements by projecting gaze towards  
198 the direction of locomotion and canceling VOR during walking or running on a treadmill. In  
199 monkeys performing circular running in the dark, compensatory horizontal eye movements are  
200 favored by non-vestibular velocity signals<sup>21</sup>. Here, we report new evidence demonstrating the

201 existence of the spino-extraocular motor command in mice that could constitute a neural  
202 mechanism sustaining the coupling of locomotion to eye movements, as suspected in other  
203 mammalian species<sup>20</sup>.

#### 204 **Functional considerations regarding the coupling of locomotion and eye movements in** 205 **mice**

206 In all vertebrates, gaze control largely results from the sensory-motor transformations which  
207 primarily depend on the integration of visual and vestibular inputs (optokinetic and vestibulo-  
208 ocular reflex)<sup>22</sup>. Interestingly, no locomotor-induced ocular movements were observed in  
209 head-fixed alert mice during sustained treadmill locomotion in the dark (Figure S4D),  
210 suggesting a sensory-dependent supraspinal control of the CPG efference copy signal in  
211 natural conditions, which is suppressed in decerebrated mice. Recent studies in mice reported  
212 that the vestibulo-ocular reflex accounts for a dominant part of the conjugated gaze  
213 stabilization observed during head-free locomotion<sup>23–25</sup>. VOR-based eye movements were  
214 found to reach higher dynamics, compensating naturalistic head movements interspersed with  
215 non-conjugated (vergence) and shifting movements relocating gaze during directed head  
216 turn<sup>25,26</sup>. The participation of the spino-extraocular motor coordination in the different repertoire  
217 of eye movements during natural locomotion remains to be determined. Nonetheless, our data  
218 provide clues regarding its physiological characteristics. First, regarding the pathway involved,  
219 viral tracing experiment revealed brainstem-projecting neurons in the motor ventral horn of the  
220 cervical spinal cord, which likely convey the locomotor efference copy signal to ocular motor  
221 neurons. Additionally, an extended time window demonstrated, in one preparation, connected  
222 neurons in the sensory dorsal horn, suggesting the potential integration of sensory and motor  
223 information by the spino-extraocular circuitry, which will require further confirmation. The  
224 restriction of infected neurons in the cervical segments at both short and extended time  
225 window, and tight coupling between forelimb and eye movements suggest that this coupling  
226 could produce ocular adjustments in response to the movements of the rostral part of the body  
227 (forelimb, shoulder girdle, neck, head). Second, regarding the necessary conditions for the  
228 coupling to occur, in the in vitro neonatal preparation the spino-extraocular coupling was  
229 consistently observed at frequencies <2Hz. However, in the adult decerebrated preparation,  
230 eye movements were observed when sustained locomotion reached frequency >2Hz. This  
231 disparity likely reflects differences in the age and/or preparation. Nevertheless, we cannot

232 exclude that the efference copy would already contribute to gaze control at subthreshold  
233 levels, during low dynamic locomotion which in decerebrated mice did not produce a tight  
234 coupling between forelimb and eye movements. This observation suggests that the spinal  
235 circuits driving the forelimb and ocular motor neurons are independent. However, both might  
236 be controlled by a common motor network whose activity would determine the efficacy of the  
237 coupling between forelimb and eye movements when high-dynamic locomotion is produced,  
238 as previously suggested<sup>1</sup>. Third, concerning the functional role of the efference copy, because  
239 the eye movements observed were restricted to the horizontal plane, this mechanism could  
240 serve as an additional signal complementing the VOR to stabilize gaze during horizontal  
241 whole-body turn or lateral deviations of the head. Alternatively, and as proposed for vertical  
242 VOR in humans, it could serve as a feedforward signal to anticipate the horizontal perturbation  
243 of gaze provoked during quadrupedal running. In sum, data reported here suggests that spino-  
244 extraocular motor coupling might, in the mouse, complement the visuo-vestibular based  
245 reflexes, accounting for the biomechanical coupling between the rostral part of the body and  
246 horizontal eye movements during high-dynamic locomotor patterns. All in all, the comparison  
247 of the mouse and the xenopus concerning the biomechanical constraints, type of locomotion,  
248 and changes during development suggests an adaptation of the CPG efference copy-based  
249 mechanism through the vertebrate lineages in relation with changes in sensory capacities and  
250 locomotor repertoire during evolution.

251

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### 266 **Author contribution**

267 Conceptualization: FFB; FML, DC, MB. Methodology: FFB; MM, JBC, MT, HB, FML, MB.  
268 Software: JBC, MM, MT. Formal analysis: FFB; JBC, MT, CT; MM, FML, MB. Investigation:  
269 FFB; CT, MM, HB. Writing original manuscript: FFB; JBC, FML, MB. Writing review and  
270 editing: FFB; MM, JBC, MT, HB, FML, MB. Visualization: FFB; HB; FML; MB. Supervision:  
271 HB; FML; MB. Project administration: DC, FML, MB. Funding acquisition: DC, FML; MB.

### 272 **Declaration of interests**

273 The authors declare no competing interests.

274

## 275 Figure Legends

276

277 **Figure 1. Fictive locomotor activity evokes spino-extraocular motor coupling in**  
278 **neonatal brainstem-spinal cord isolated preparations.**

279 **(A)** Left side, schematic of the neonatal mouse brainstem and spinal cord preparation with  
280 stimulated and recorded nerve roots. In all experiments C5, C8, L2 and L5 Vr were targeted,  
281 as main flexor (C5, L2) and extensor (C8, L5) motor nerve of fore- and hindlimbs<sup>8,9</sup>,  
282 respectively. Preparations with recording of at least 3 of these 4 ventral roots were retained  
283 for analysis. Right side, Extracellular nerve recordings (raw traces in light gray, integrated  
284 traces in color) of the left (le, light blue) and right (ri, dark blue) abducens nerves (Abd.), the  
285 left and right 8th cervical roots (le C8, light green; ri C8, dark green) and the right 2nd lumbar  
286 ventral root (le L2, orange). Discharges recorded during an episode of fictive locomotion  
287 evoked by the electrical stimulation of the S1 dorsal root (stim. S1Dr) with a 4Hz pulse train  
288 (black vertical bars).

289

290 **(B)** Average cyclic modulation of the discharge activity (integrated trace) from the motor  
291 nerves shown in (A) over 10 consecutive fictive locomotor cycles. The right C8 trace (dark  
292 green) was used as the reference to determine locomotor cycles.

293

294 **(C)** Percentage of total (C8: 7 mice, 19 sequences; L2: 8 mice, 13 sequences) preparations  
295 with a coordinated fictive locomotor pattern coupled with a rhythmic abducens discharge  
296 (coord./rhyth, black histogram; 66%), with an absence of coordinated fictive locomotor pattern  
297 and an absence of rhythmic abducens discharge (non coord./non rhyth, dark gray histogram;  
298 33%), with a coordinated fictive locomotor pattern and an absence of rhythmic abducens  
299 discharge (coord./non rhyth, light gray histogram; 1%), with an absence of coordinated fictive  
300 locomotor pattern and a rhythmic Abducens discharge (non coord./rhyth; 0%).

301

302 **(D)** Linear correlation in bursting frequencies between the left abducens (Abd) nerve discharge  
303 and the left C8 (green) (Abd vs C8,  $n = 7$ ,  $R = 0.9567$ , Pearson test,  $p < 0.0001$ ;  $r^2 = 0.9153$ ),  
304 or left L2 (orange) (Abd vs L2,  $n = 8$ ,  $R = 0.9696$ , Spearman test,  $p < 0.0001$ ;  $r^2 = 0.9400$ ) spinal  
305 ventral roots.

306

307 **(E-F)** Individual (empty circles) and mean (bars)  $\pm$ SEM of the frequency linear regression (**E**,  
308  $r^2$ ) and the latency (**F**, milliseconds, ms) between firing bursts of the abducens nerve (Abd)  
309 and spinal ventral root (sp. Vr) discharges (Abd vs C8Vr in green, mean  $r^2 = 0.75 \pm 0.06$ , mean  
310 latency =  $61.7 \pm 7.2$ ms; Abd vs L2Vr in orange, mean  $r^2 = 0.82 \pm 0.06$ , mean latency =  $79.6 \pm$   
311  $9.7$ ms) for each preparation, independently of the frequency.

312

313 **(G)** Circular plots showing the phase relationships between the firing discharge burst in the  
314 left abducens nerve and ipsilateral C8 ( $\mu = 357.359^\circ \pm 2.213$ ;  $r = 0.936$ ;  $n = 7$ ) and L2 ( $\mu =$   
315  $354.288^\circ \pm 1.467$ ;  $r = 0.97$ ,  $n = 8$ ) and contralateral L5 ( $\mu = 198^\circ \pm 5.04$ ;  $r = 0.868$ ,  $n = 3$ ) Sp.  
316 Vr. In this and all polar plots, the width of the wedges is 0.05.

317

318 Supplemental data related to figure 1 is available as Figure S1.

319

320

321 **Figure 2. The efference copy signaling responsible for spino-extraocular motor**  
322 **coupling originates in cervical locomotor CPG.**

323 **(A)** Left side, schematic of the neonatal mouse brainstem and spinal cord preparation with the  
324 recorded nerve root and the calcium free ( $0Ca^{2+}$ ) aCSF split bath on the cervical cord.  
325 Extracellular nerve recordings (raw traces in light gray, integrated traces in color) of the right  
326 (ri, dark blue) abducens nerves (Abd.), the right 8th cervical roots (ri C8, dark green) and the  
327 right and left 5th lumbar ventral roots (ri L5, dark red; le L5, light red) discharges during an  
328 episode of fictive locomotion evoked by the electrical stimulation of the S1 dorsal root (stim.  
329 S1Dr) with a 4Hz pulse train (black vertical bars). In the control condition (**A**), during bath  
330 application of  $0Ca^{2+}$  aCSF (**B**) restricted to the cervical spinal cord and after washout (**C**).

331 **(D)** Averaged cyclic modulation of the discharge activity (integrated trace) from the motor  
332 nerves shown in A (top panel, control condition) and B (bottom,  $0Ca^{2+}$ ) over 10 consecutive  
333 locomotor cycles. The right L5 trace was used as the reference to determine locomotor cycles.

334 **(E)** Left side, schematic of the neonatal mouse brainstem and spinal cord preparation with the  
335 recorded nerve branches during the mid-thoracic section experiments. Extracellular nerve  
336 recordings (raw traces in light gray, integrated traces in color) of the right (ri, dark blue) and

337 left (le, light blue) abducens nerves (Abd.), the right 8th cervical roots (ri C8, dark green) and  
338 the right 5th lumbar ventral root (ri L5, dark red) discharges during an episode of fictive  
339 locomotion evoked by the electrical stimulation of the S1 dorsal root (stim. S1Dr) with a 4Hz  
340 pulse train (black rectangle). Responses for control (**E**), after mid-thoracic complete  
341 transection during an episode of fictive locomotion evoked by the electrical stimulation of the  
342 S1 dorsal root (**F**, stim. S1Dr) and after mid-thoracic section during an episode of fictive  
343 locomotion evoked by the electrical stimulation of the C8 dorsal root (**G**, stim. C8Dr) with a  
344 4Hz pulse train.

345 **(H)** Linear correlation in bursting frequencies between Abducens (Abd) nerve discharge and  
346 ipsi C8 Vr discharge in response to stimulation of the S1Dr during control conditions (black  
347 empty circles,  $R = 0.9817$ , Pearson test,  $p < 0.0001$ ,  $n = 3$ ;  $r^2 = 0.9637$ ), and after the mid-  
348 thoracic section (orange filled circles) ( $R = 0.9983$ , Pearson test,  $p < 0.001$ ,  $n = 3$ ;  $r^2 = 0.9966$ ).

349 **(I)** Polar plots of the phase relationships between the abducens and C8Vr before (control, left)  
350 abd C8 ipsi before ( $\mu = 345.161^{\circ} \pm 2.546$ ;  $r = 0.974$ ) abd C8 contra before ( $\mu = 162.803 \pm 4$   
351  $.285$ ;  $r = 0.943$ ) and after mid-thoracic section (after cut, right) abd C8 ipsi after cut ( $\mu = 353$   
352  $.912 \pm 2,463$ ;  $r = 0.999$ ), abd C8 contra after cut ( $\mu = 177.873 \pm 4.909$ ;  $r = 0.939$ ) ( $n = 3$  mice).  
353 The phase relationship is conserved after the cut.

354 **(J)** Mean absolute latency time between the activity in the C8 Vr and the abducens nerve in  
355 control ( $52.96 \pm 4.51$ ms) and after cut ( $66.49 \pm 5.96$ ms). There was no significant difference in  
356 the delay observed before and after the mid-thoracic cut (t-test,  $p = 0.1038$ ,  $n = 3$ ).

357

358

### 359 **Figure 3. Anatomy of the spino-extraocular pathway**

360 **(A)** Depiction of the rabies virus (RV) injection protocol. RV injections were performed in the  
361 lateral rectus (Lat. rectus) muscle of the left eye. After 55h of RV infection, labeled neurons  
362 (red asterisks) were found in the abducens (Abd) motor nuclei, and in the closely connected  
363 structures. PC, Purkinje cells; VN, vestibular nucleus neuron; EC, efferent copy spinal neuron.

364 **(B)** Representation (top panel) of the location (red square) of the RV<sup>+</sup> neurons in the abducens  
365 nucleus (nVI) and its landmarks: geniculum of the facial nerve (g7) and the 4th ventricle IVth  
366 v). The bottom panel shows a fluorescence microscopy image (20x) of an RV<sup>+</sup>/ChAT<sup>+</sup> neuron  
367 (RV, red) and abducens motoneurons (Abd. MN, ChAT, green) 55h after the infection.

368 **(C)** Representation (top panel) of the location (red square) of the RV<sup>+</sup> neurons in the medial  
369 vestibular nucleus (MVN) 55h after the infection. The fluorescence microscopy image (bottom  
370 panel, 20x) shows RV<sup>+</sup> but ChAT<sup>-</sup> (left insets) vestibular nucleus (VN) neurons.

371 **(D)** Confocal microscopy image (20x magnification) of a brainstem sagittal slice of the  
372 oculomotor nucleus (nIII) showing RV<sup>+</sup> (red) but ChAT<sup>-</sup> (green) interneurons (INs; white  
373 arrowheads in e1 and e2,) contralateral to the inoculation side, while MNs (ChAT<sup>+</sup>) are not  
374 RV-infected.

375 **(E)** Fluorescence microscopy reconstructed image (5x magnification) of a cervical cross  
376 section showing RV<sup>+</sup> neurons in the ventral horn (white arrowheads and **(E1)**) as well as  
377 ChAT<sup>+</sup> neurons (**(E2)**). c.c, central canal; DR, dorsal root.

378 **(F)** Example (20x magnification) of a RV<sup>+</sup> neuron located near the central canal (c.c), not  
379 ChAT<sup>+</sup> (F, left bottom inset).

380 **(G)** Location of the RV<sup>+</sup> neurons after 55h of infection. RV<sup>+</sup> neurons were represented by  
381 colored dots in the different segments of the cervical spinal cord for each preparation (prep #1  
382 cyan, prep#2 purple, prep#3 blue). Ipsi: cervical spinal cord side ipsilateral to the RV injected  
383 in the left lateral rectus muscle.

384 **(H)** Plot of the number of RV<sup>+</sup> neurons (nb of neurons, x axis) in ipsilateral and contralateral  
385 sides and location along (distance, millimeters, y axis) the rostro-caudal cervical (C1-C8)  
386 segments for each preparation (prep #1 cyan, prep#2 purple, prep#3 blue).

387 **(I)** Number of RV<sup>+</sup> motoneurons (MN, x axis) in the abducens motor nucleus and  
388 corresponding number of RV<sup>+</sup> brainstem-projecting cervical neurons (Brainstem-projecting)  
389 for each preparation (prep #1 cyan, prep#2 purple, prep#3 blue).



390 **(J)** Mean +/- SD (black circle,  $6.83 \pm 3.33$ ) of the infection ratio indicating the average of RV<sup>+</sup>  
391 brainstem-projecting cervical neurons labelled *per* RV<sup>+</sup> abducens motoneuron infected.

392 **(K)** Scheme of the spatial distribution of all the RV<sup>+</sup> brainstem-projecting cervical neurons,  
393 shown in (G), *per* lamina of the cervical spinal cord. Gray lines represent the superimposed  
394 limits of the gray matter from panel G, purple outlines represent the limits of the laminae on a  
395 typical cervical spinal cross-section.

396 Supplemental data related to figure 3 is available as Figure S3.

397

398

#### 399 **Figure 4. Locomotion-induced eye movements in adult mice**

400 **(A-E)** Example of the eye movements observed during an episode of treadmill-induced  
401 locomotion. **(A)** Left (le Eye position, blue trace) and right (ri Eye position, red trace) horizontal  
402 movements are observed after the onset of locomotion. Rectangles represent the stance  
403 phases of the left forelimb (dark green) and left hindlimb (light green); gaps between  
404 rectangles represent swing phases; green arrows contain a bout of continuous locomotion  
405 with instantaneous frequency above 2Hz). Upper right inset, depiction of the set up used for  
406 recording eye movements in decerebrated mice. A video oculography camera recorded the  
407 movement of each eye and an infra-red camera tracked the movement of the limbs while mice  
408 ran on a motorized treadmill. **(B)** Segment of the recording (dotted rectangle, panel A) showing  
409 the horizontal (dark blue and red traces) and vertical (light blue and light red traces)  
410 components of the eye movements and the corresponding locomotor cycles of the left fore-  
411 and hindlimbs. **(C)** Average modulation (over 16 consecutive cycles) of the eye movements  
412 and limb stance phases shown in panel B. For horizontal ocular traces, right is up. **(D)**  
413 Oculogram showing the stable orientation of the left eye movement during the segment in  
414 panel B. The reported angle of  $16.1^\circ$  is expressed relative to the horizontal plane with head in  
415 a stereotaxic position (Hor.pos. vs Ver.pos,  $R = -0.8699$ , Spearman test,  $p = 0.031$ ;  $r^2 = 0.75$ ).  
416 **(E)** Right eye vs left eye horizontal components (le+ri eyes) (riHor.pos. vs leHor.pos,  $R =$

417 0.7152, Spearman test,  $p = 0,47$ ;  $r^2 = 0.51$ ), showing comparable amplitude and synchronized  
418 movements.

419 **(F-G)** Analysis of the eye movements in panel **(A)**. **(F)** Relation between the instantaneous  
420 frequency of the forelimb (dark green dots) or the hindlimb (light green dots), and the left  
421 horizontal eye movements (Right eye vs left forelimb,  $R = 0.7248$ , Spearman test,  $p < 0.001$ ;  
422  $r^2 = 0.5253$ ; Right eye vs left hindlimb,  $R = 0.3195$ , Spearman test,  $p = 0.0852$ ;  $r^2 = 0.1021$ ). **(G)**  
423 Polar plot of the phase coupling between the peak velocity of the left eye quick phase and  
424 forelimb ( $\mu = 18.272^{\circ} \pm 8,61$ ;  $r = 0.718$ ).

425 **(H-J)** Relation between the eye and limb movements. **(H)** Cumulative distribution of the  
426 locomotor frequency measured from the left forelimb (le fore.) and left hindlimb (le hind.)  
427 movements (beginning of stance phase). Rhythmic eye movements were observed when  
428 locomotion frequency reached 2-4Hz, corresponding to trot-like gait (gray interval,  $n=5$  mice).  
429 **(I)** Proportions of the locomotor cycle showing eye and/or limb movements (left forelimb- dark  
430 green; left hindlimb- light green). **(J)** Correlation (left panel; Forelimb vs eye instantaneous  
431 frequency,  $R = 0.6569$ , Pearson test,  $p < 0.0001$ ;  $r^2 = 0.4315$ ) and temporal relationship (right  
432 panel;  $\mu = 358.763^{\circ} \pm 16.106$ ;  $r = 0.777$ ) between the instantaneous frequency of the eye (peak  
433 velocity of quick phase) and forelimb movements (beginning of stance phase).

434 **(K-M)** Quantification of eye movements during locomotion. **(K)** Instantaneous frequency of the  
435 left and right eye movements. The degree of conjugation of the eye movements is reflected in  
436 the high linear correlation (left panel; left vs right eye instantaneous frequency,  $R = 0,9873$ ,  
437 Pearson test,  $p < 0.0001$ ;  $r^2 = 0.9748$ ) between left and right eye frequency and the synchrony  
438 between them (right panel; polar plot;  $\mu = 355.641^{\circ} \pm 7.53$ ;  $r = 0.991$ ). **(L)** Distribution of the  
439 duration of eye movements. The short phases of the eye movements only last up to 200  
440 milliseconds and are mainly distributed around 100 milliseconds whereas, the long phase of  
441 the eye movements is scattered and distributed up to 400 milliseconds. Right panel shows the  
442 relative duration of short and long eye movements during each cycle. Short eye movements  
443 (dark gray) represent 20% of the cycle; long eye movements (light gray) 80% of the cycles.  
444 **(M)** Main sequence of the eye movements showing the amplitude-velocity relationship of short  
445 (dark gray; Amplitude vs velocity of short component of eye movements,  $R = 0.9169$ ,  
446 Spearman test,  $p = 0.00366$ ;  $r^2 = 0.8395$ ) and long (light gray; Amplitude vs velocity of long

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447 component of eye movements,  $R = 0.5412$ , Spearman test,  $p < 0.00001$ ;  $r^2 = 0.2929$ )

448 components of the eye movements.

449 Supplemental data related to figure 4 is available as Figure S4.

450

451 **STAR Methods**

452 **Resource availability**

453 *Lead contact*

454 Further information and requests for resources should be directed to M. Beraneck  
455 (mathieu.beraneck@univ-paris.fr).

456 *Materials availability*

457 This study did not generate any new materials or reagents.

458 *Data and code availability*

459 The datasets generated during this study are available at “Dataset\_Barros\_2021”, Mendeley  
460 Data, V1, doi: 10.17632/h8nbswgf95.1

461 **Experimental model and subject details**

462 Animals were used in accordance with the European Communities Council Directive  
463 2010/63/EU. All efforts were made to minimize suffering and reduce the number of animals  
464 included in the study. All protocols were performed on C57/BL6J male mice. *Ex vivo*  
465 experiments were performed on neonate (2-3 days old; n = 23) following procedures  
466 specifically approved by the ethical committee for animal research of the University of  
467 Bordeaux. Experiments on adults (8–14 weeks) were approved by the ethical committee for  
468 animal research of the University of Aix-Marseille (n = 6) and of the university of Paris (n =  
469 29).

470 **Ex vivo brainstem-spinal cord preparations**

471 The dissection protocol followed as previously detailed by Kasumacic<sup>10</sup>. Briefly, mice were  
472 deeply anesthetized by 4% isoflurane inhalation until the loss of the nociceptive reflexes. After  
473 being placed in Sylgard-coated Petri dish, they were decerebrated and eviscerated while  
474 submerged in an ice-cold (1°-5°C) oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) artificial cerebrospinal  
475 fluid (aCSF) (in mM: 128 NaCl, 4.5 KCl, 2.5 CaCl<sub>2</sub>.2H<sub>2</sub>O, 1.0 MgSO<sub>4</sub>.7H<sub>2</sub>O, 1.2

476 NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 5 Hepes, 25 NaHCO<sub>3</sub> and 11 D-glucose). In a dorsal view, a craniotomy was  
477 performed to expose and remove the cerebellum, and the abducens nerves were carefully cut  
478 so that they could be recorded. With a ventral approach, a full laminectomy was done to  
479 expose the spinal cord and all the dorsal and ventral roots of both cervical sides were cut so  
480 that the final brainstem-spinal cord preparation could be extracted. After removing the  
481 meninges, the preparation was fixed with the ventral side up and fixed to the Sylgard-coated  
482 dish with insect pins and bathed in oxygenated aCSF fluid at room temperature.

### 483 **Fictive locomotion protocols and recordings**

484 Isolated brainstem-spinal cord preparations had the distal ends of abducens nerves as well  
485 as bilateral spinal dorsal motor roots (sacral, lumbar or cervical) simultaneously recorded  
486 (Model 1700 AC amplifiers, A-M Systems, Carlsborg, US) with fire-polished borosilicate glass  
487 suction recording electrodes filled with aCSF solution. In all experiments C5, C8, L2 and L5  
488 Vr were targeted, as main flexor (C5, L2) and extensor (C8, L5) motor nerve of fore- and  
489 hindlimbs<sup>8,9</sup>, respectively. Preparations with recording of at least 3 of these 4 ventral roots  
490 were retained for analysis. The electrodes were connected to extracellular amplifiers and the  
491 analog signals recorded by an analog/digital interface (CED 1401; Cambridge Electronic  
492 Design, Cambridge, UK). Signals were amplified (x10000) and digitized at 10 KHz (CED 1401;  
493 Cambridge Electronic Design, Cambridge, UK), displayed and stored on computer with  
494 acquisition software (Spike 2, Cambridge Electronic Design) and finally analyzed off-line with  
495 customized scripts. Fictive locomotion was achieved in brainstem-spinal cord preparations  
496 either through electrical or pharmacological stimulation.

497 For electrical stimulation, the electrodes suctioning the dorsal roots were connected to a pulse  
498 generator (Grass stimulator); this allowed the delivery of pulse trains (4Hz for 10s, inter-  
499 stimulus interval of 0.25s and at least 5 minutes between pulse trains). The described pulse  
500 trains were delivered at the lumbar or at the cervical levels while the abducens nerves were  
501 recorded.

502 To obtain fictive locomotion through pharmacological stimulation, the *ex vivo* brainstem-spinal  
503 cord preparations (n = 2 mice) were bathed in an aCSF infused with glutamatergic receptor  
504 agonists; namely NMDA (7,5 μM) and serotonin (5-HT; 15 μM).

505 In a subset of experiments (n = 6 mice), the motor coupling was disrupted using a split-bath  
506 configuration that allowed reversibly isolating the brainstem from the spinal cord. Isolation was  
507 obtained by partitioning the recording chamber with two custom-made plastic walls that  
508 traversed the preparation cervical and lumbar regions with spinal split-baths. The cervical  
509 spine was immersed in an aCSF 0 Ca<sup>2+</sup> solution, in order to completely block transmission at  
510 that level, while lumbar root stimulation was performed. This calcium free ACSF perfusion was  
511 shown to block local synaptic transmission, but not axons *en passage*<sup>9,12</sup>. In a subset of  
512 experiments (n= 7 mice), a complete section was made at the thoracic spinal cord in order to  
513 disconnect the lumbar locomotor network from the cervical locomotor network. In these  
514 preparations cervical and lumbar networks were then stimulated independently from each  
515 other.

#### 516 **Methods for tracing experiments**

517 Rabies virus (RV) manipulation was done by vaccinated experimenters in a Biosafety level 2  
518 facility. The virus (Challenge Virus Strain, CVS) was produced and concentrated as previously  
519 described<sup>27</sup>. Aliquots of the virus were stocked at -80°C and thawed before use. Mice were  
520 anesthetized by an intraperitoneal injection of ketamine (Imalgene, 60 mg/kg) and xylazine  
521 (Rompun, 10 mg/kg) and deep anesthesia was confirmed by lack of response to interdigital  
522 pinching. The left extraocular muscles were exposed through an incision on the skin covering  
523 the mouse's eye, surrounding fatty tissue was removed and absorbable hemostatic gelatin  
524 sponges were used to curtail bleeding (Spongostan, Ethicon). After its identification, the lateral  
525 rectus muscle (LR) was isolated using small hooks and an injector cannula (gauge 33), linked  
526 to a 10µL Hamilton syringe, was inserted. 1µL of the RV was then slowly injected and the  
527 needle was left in place for an extra minute to prevent leaking<sup>28,29</sup>. Finally, the skin was closed  
528 using non-absorbable monofilament suture and mice were put back on their cages under a  
529 red light for post-surgery care. As in our previous studies using RV as retrograde  
530 transneuronal tracer to characterize synaptically connected networks in rodents after  
531 peripheral inoculations<sup>10,11,28–30</sup>, we determined the time windows required to infect neurons  
532 connected monosynaptically or polysynaptically to abducens motoneurons, based on  
533 anatomical data. A 70h window displayed RV<sup>+</sup> neurons in the cerebellum (Purkinje cells;  
534 Figure S3C), but an absence of RV<sup>+</sup> neurons in the pontine nuclei (directly projecting to  
535 Purkinje cells, data not shown), compatible with disynaptic connections to that delay. When

536 the inoculation time was lowered to 55h the RV + interneurons were restricted to the brainstem  
537 (including INs in the medial vestibular nucleus (MVN, Figure 3C, Baker et al., 1969),  
538 contralateral oculomotor nucleus (3N, Figure 3D)<sup>14</sup>, prepositus nucleus (Pr)<sup>31</sup>,  
539 reticulotegmental nucleus of the pons<sup>32</sup> and cervical spinal cord, with no infection in the  
540 cerebellum, suggesting monosynaptic connections to that delay. In case of infection of MNs  
541 in other oculomotor nuclei than the abducens, resulting from leakage during inoculation of the  
542 virus, the animal was excluded.

### 543 **Immunochemistry**

544 After 55 or 70h of RV infection, animals were deeply anesthetized with ketamine (Imalgene,  
545 60 mg/kg) and xylazine (Rompun, 10 mg/kg) and perfused intracardially with 20mL of 0.1M  
546 phosphate buffer saline (PBS) followed by 20mL of 4% paraformaldehyde (PFA). Immediately  
547 after, the brain was dissected coronally from the midbrain to the medulla oblongata and the  
548 spinal cord was removed in a single block. Tissue fixation was let for 4h at 4°C in 4% PFA,  
549 subsequently cryoprotected in 20% sucrose during at least 24h and freeze embedded at -  
550 80°C in Tissue Tek (Sakura, Zoeterwoude, The Netherlands). Transverse sections (30 µm  
551 thick) of the brainstem and cervical and lumbar spinal cord were serially cut in cryostat  
552 (Microm, Heidelberg, Germany), and two alternate set of sections were collected<sup>29</sup>. One set  
553 was placed into 48 well plates filled with cryoprotection liquid, and kept at -20°C, and the other  
554 set was mounted on poly-L-lysine-coated slides. These latter sections were first incubated in  
555 blocking solution (PBS, Triton X- 100, 0,2% bovine-serum albumin and normal donkey serum),  
556 to avoid nonspecific binding for 1h at room temperature and then left at 4°C for 48h with the  
557 anti-RV phosphoprotein<sup>29</sup> and anti-choline acetyltransferase (ChAT) diluted 1:100 in the  
558 blocking solution. After being rinsed thrice in PBS, sections were incubated at room  
559 temperature with the secondary antibodies Alexa546 goat anti-mouse (Thermo Fisher  
560 Scientific) and Alexa488 donkey anti-goat (Thermo Fisher Scientific) at a 1:200 dilution for 2h.  
561 Finally, sections were rinsed two times with PBS and once with tap water before being  
562 mounted (Immu-Mount, Fisher Scientific) under coverslips.

563 To analyze the distribution of RV<sup>+</sup> neurons, the obtained sections were visualized under an  
564 upright light microscope (Axioscope, Carl Zeiss, Germany) equipped with a camera lucida. A  
565 CCD camera (AxioCam MR3, Carl Zeiss) adapted on this microscope, or the use of a laser

566 scanning confocal microscopy (LSM ZEISS 510 Meta) allowed to photograph areas of interest  
567 at various magnifications. These images were afterwards treated in ZEN (Carl Zeiss) imaging  
568 software where brainstem RV<sup>+</sup>/ChAT<sup>+</sup> motoneurons and spinal cord RV<sup>+</sup> neurons were  
569 counted on each section and plotted from one out of two (60µm apart). Only neurons with  
570 visibly infected nuclei, determined by the presence of Negri bodies, were considered. The  
571 success of the viral infection was verified by the presence of double-positive RV<sup>+</sup> and ChAT<sup>+</sup>  
572 neurons on the abducens nucleus (Figure 3B). Abducens motoneurons simultaneously RV<sup>+</sup>  
573 and ChAT<sup>+</sup> were found in all mice (n = 3), ipsilateral to the injection site (left lateral rectus  
574 muscle), while motoneurons innervating other eye muscles (Figure 3B) were never marked,  
575 confirming the specificity of the infection.

#### 576 **Procedure for decerebration**

577 Procedures for decerebration were previously described by Meehan and colleagues<sup>7</sup>. Briefly,  
578 mice were deeply anesthetized with isoflurane (2–3% in 100% O<sub>2</sub>) in an induction chamber,  
579 and then transferred to a heating pad on the surgery table with a nose cone to maintain  
580 anesthesia (1.5–2% in 100% O<sub>2</sub>). A rectal thermometer (Physiosuite, Kent Scientific) was  
581 used to monitor body temperature throughout the surgery as well as a pulse oximeter  
582 (MouseSTAT, Kent Scientific) to monitor O<sub>2</sub> saturation. Mice were then artificially ventilated  
583 via tracheostomy by blunt dissection; first the sternohyoid muscle was exposed, then the upper  
584 half of the fibrous membrane between two cartilages was cut and finally, the air delivery mode  
585 was quickly switched to ventilator mode (SomnoSuite, Kent Scientific) and a plastic  
586 endotracheal tube was inserted into the trachea. After confirming that the pCO<sub>2</sub> levels were  
587 acceptable (>2.5%), the isoflurane was lowered to 1–1.2%. To avoid excessive bleeding during  
588 the decerebration, the left and right carotid arteries were bluntly isolated, ligatured and  
589 cauterized using an electric cauterizer (Change-a-tip cauteries, Bovie). The initial midline  
590 incision was sutured and, to avoid dehydration, 0.3 ml of sterile lactated Ringer's solution  
591 (Braun Medical) were injected subcutaneously. The mouse was placed in a stereotaxic frame  
592 and a superficial cut was made across the skin above the midline of the skull. Using a micro-  
593 drill (Foredom, David Kopf Instruments), the skull was scored, and the parietal bones removed.  
594 Decerebration was performed at the confluence of the sagittal and transverse sinus,  
595 perpendicular to the midline and at a 40±5° angle (Figure S4A). A surgical scalpel (no.10  
596 blade; Fine Science Tools) was used to perform a swift and gentle slicing motion. The



597 structures rostral to the incision were fully removed to obtain a premammillary and  
598 precollicular decerebration. Finally, a subcutaneous injection of Ringer's lactate (0.3 ml) was  
599 applied, and the isoflurane gas anesthesia was decreased to zero. Complete anesthesia  
600 withdrawal and muscular tonus occurred 10-20 minutes after isoflurane removal; PCO<sub>2</sub> levels  
601 rose consequently.

### 602 **Videoculography and locomotion recordings and analysis**

603 After the decerebration, mice were tightly secured using ear bars and a custom-made  
604 mouthpiece onto the motorized treadmill (Panlab, Harvard Apparatus). All recordings were  
605 performed in complete darkness. The locomotion was recorded using an infrared video  
606 camera (100Hz; Grasshopper3, FLIR Systems, Inc) fixed perpendicular to the left side of the  
607 body. To perform binocular video-oculography, the eyes of the mouse were illuminated with  
608 infrared emitters attached to 2 CCD cameras (120Hz; ISCAN, Burlington, MA) that were  
609 placed symmetrically on each side of the treadmill. Special care was provided to prevent eye  
610 dryness by regularly re-applying ophthalmic gel, to ensure that at least one eye could be  
611 tracked online. Eye video signals were processed online (ETL-200, ISCAN, Burlington, MA),  
612 sampled at 1kHz (CED 1401; Cambridge Electronic Design, Cambridge, UK) and recorded  
613 with Spike2 software. Online tracking of the eye movements with set-up fixed cameras allowed  
614 verifying the absence of head movements during the recordings. The video-oculography and  
615 locomotion cameras were synchronized using trigger signals generated using spike2 software.  
616 Locomotor cycles were tracked from the acquired videos using a markerless pose estimation  
617 (DeepLabCut)<sup>33</sup>. Eye movements and locomotion were analyzed offline using DataView  
618 software (W.J. Heitler, University of St.Andrews Scotland) at the Animotion collaborative core  
619 facility of the INCIA laboratory (CNRS UMR5287, Université de Bordeaux, [http://www.incia.u-  
620 bordeaux1.fr/spip.php?rubrique193](http://www.incia.u-bordeaux1.fr/spip.php?rubrique193)).

621 Overall, locomotion and eye movements were observed on a total of n = 26 preparations. Due  
622 to the experimental difficulties associated with the decerebrated mice, it was challenging to  
623 obtain preparations that would generate sustained, rapid, and well-coordinated locomotion.  
624 Additionally, the heavy surgery (long anesthesia; dehydration)<sup>34</sup> also impacted the possibility  
625 to track the pupil. However, for the preparations that fulfilled these quality criteria (n=6), all  
626 demonstrated the spino-extraocular coupling.

627 For locomotor analysis, the stance and swing phases of left forelimbs and hindlimbs, were  
628 defined based on the X, Y values issued from DeepLabCut pose estimation. Locomotor cycles  
629 were defined using the beginning of the stance phase of the left forelimb. The instantaneous  
630 frequency of locomotor cycles was calculated. Only sequences which exhibited bouts of  
631 sustained locomotion (>10 locomotor cycles) at frequency >1Hz were retained for analysis (n  
632 = 6). For oculomotor analysis, the position traces imported in DataView were integrated to  
633 obtain the velocity of the eye. The peak velocity of the alternated fast and slow phases of eye  
634 movements were detected using local peak detection. To analyze the coupling between limb  
635 and eye movements, beginning of locomotor cycles were defined as the onset of stance phase  
636 of the left forelimb. Oculomotor events were defined as the peak velocity of the quick phases.  
637 For each bout of locomotion, ratio of eye/limb events was calculated and sequences with a  
638 ratio in range 0.8-1.2 were retained. Analysis was then performed to compare the occurrence  
639 of both events, the instantaneous limb/eye frequency, and for each locomotor cycle, the  
640 correlation and temporal relation of limb (stance onset) and oculomotor (peak velocity of quick  
641 phase) events.

#### 642 **Quantification and statistical analysis**

643 A table featuring all the numerical values obtained from the statistical tests performed is  
644 available as Table S1. Prism (GraphPad) was used for statistical analysis.

645 For paired t-tests normality was first evaluated using the D'Agostino & Pearson normality test  
646 and Shapiro-Wilk in the case of smaller unpaired two-tailed samples. When testing locomotion  
647 frequency and latency as well as the integrated electrophysiological signals, differences  
648 between two results were obtained using the unpaired two-tailed Mann–Whitney *U*-test and  
649 Kolmogorov–Smirnov test to compare distributions. To compare several values, the non-  
650 parametric Kruskal–Wallis test was processed with a Dunn's multiple comparisons test. To  
651 evaluate the correlations between the frequencies of discharge of the locomotor nerves and  
652 the extraocular nerves, regression tests (R) and linear regression ( $r^2$ ) were performed.  
653 Regression results were expressed as (R,  $r^2$ ). Circular data was analyzed with Oriana 4.02  
654 (Kovach Computing Services, UK) and the phase and strength of coupling were indicated by  
655 their mean vector ( $\mu$ ) and its length ( $r$ ), respectively for non-uniform distributions values (tested  
656 with the Rayleigh's uniformity test,  $p$ ).

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657 The results are expressed as the mean  $\pm$  SEM and  $p$  values threshold determined as  $*p <$   
658 0.05;  $*p < 0.01$ ;  $**p < 0.001$ ;  $***p < 0.0001$ ; ns: non-significant.

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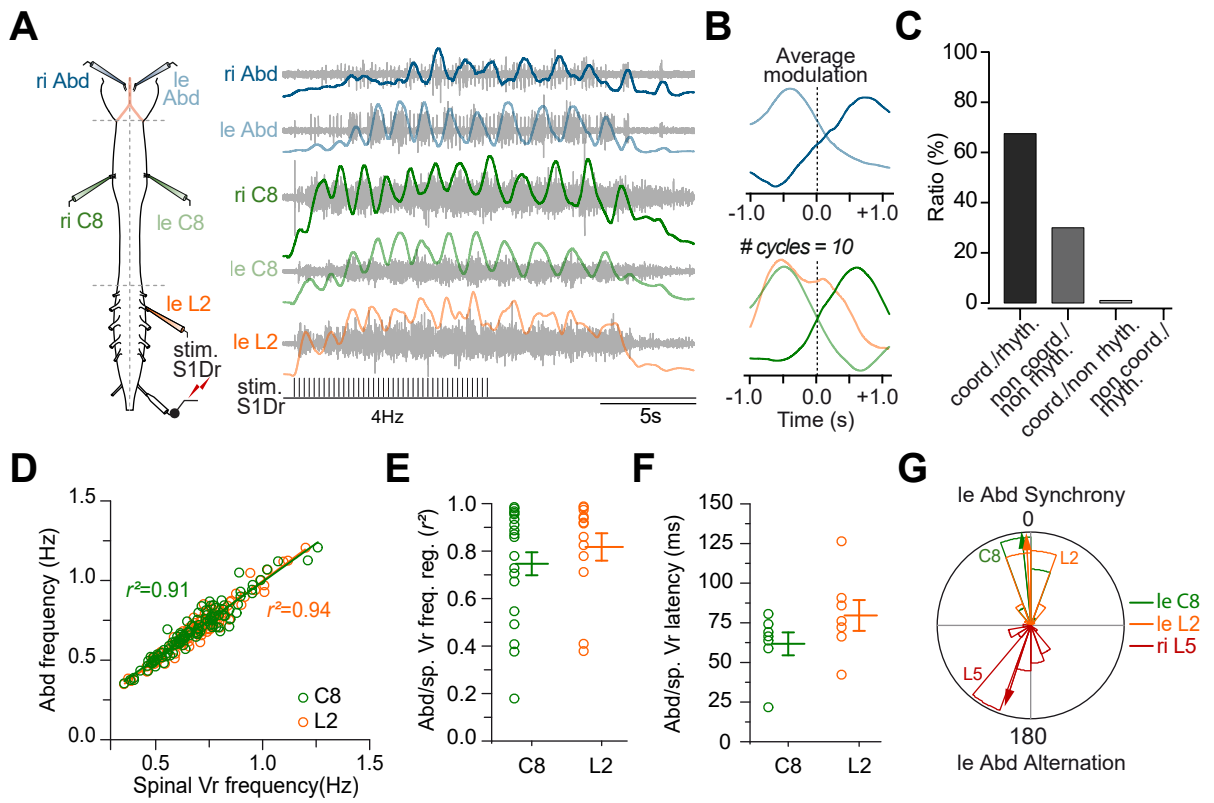
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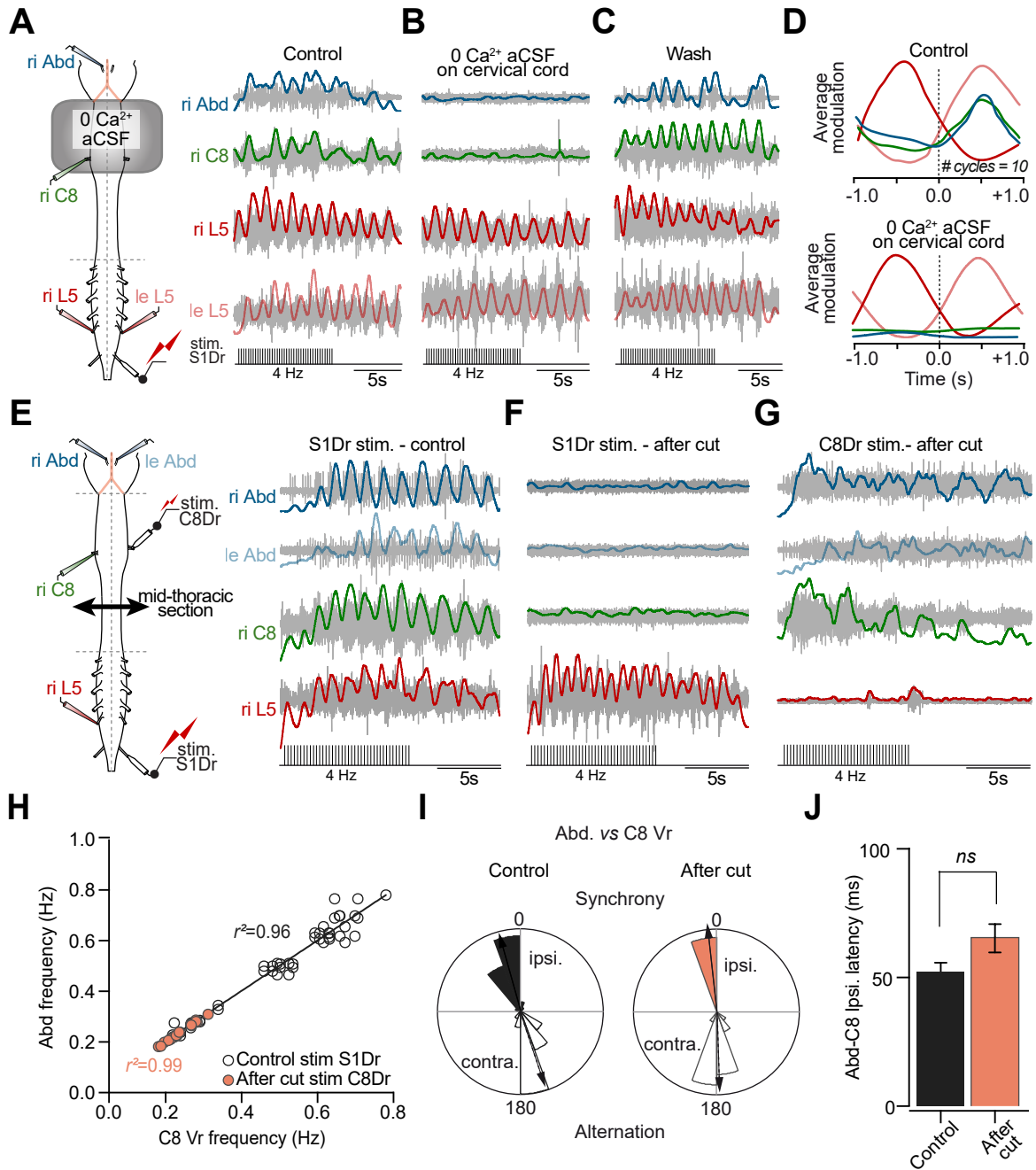
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Figure 1

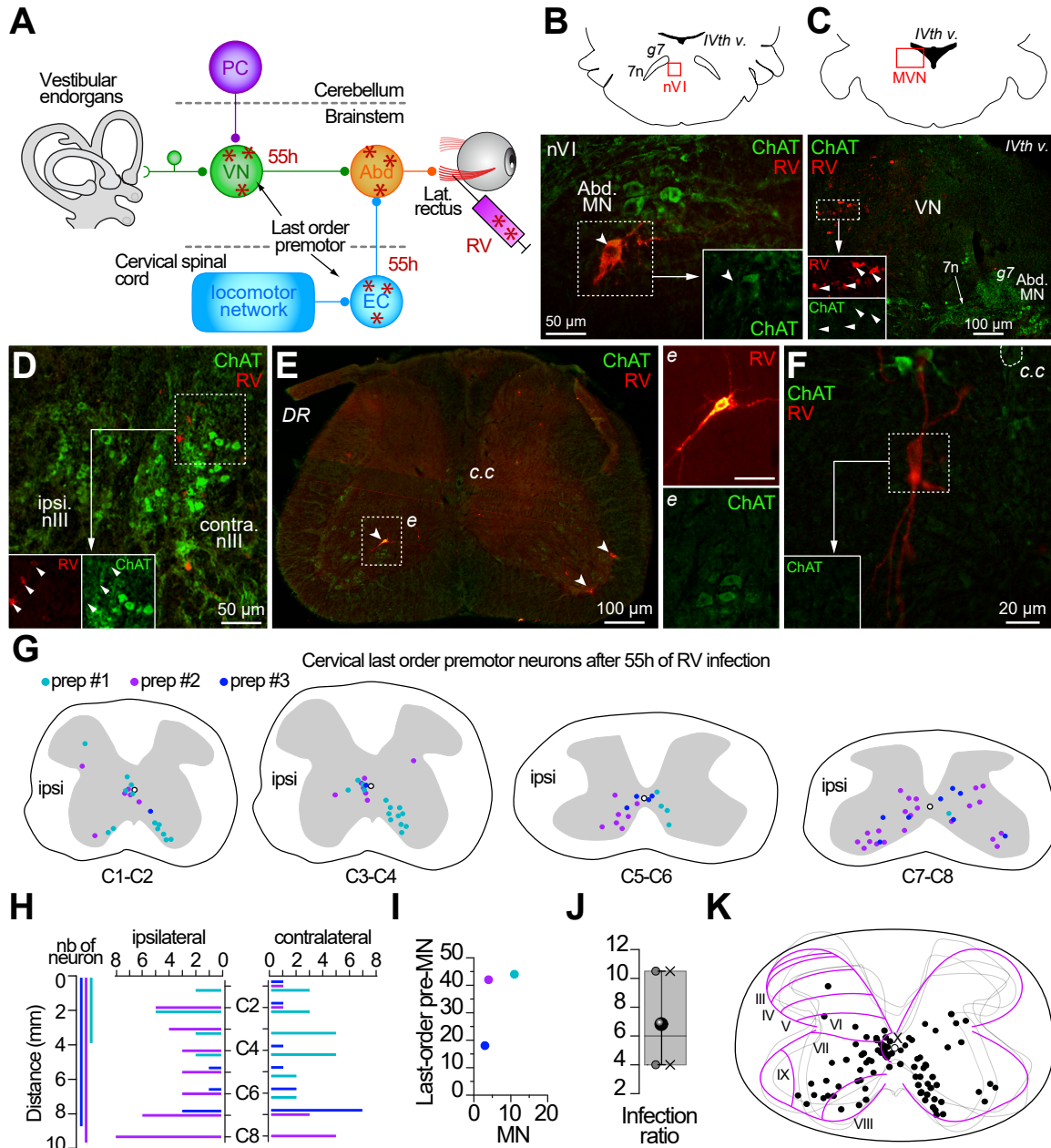


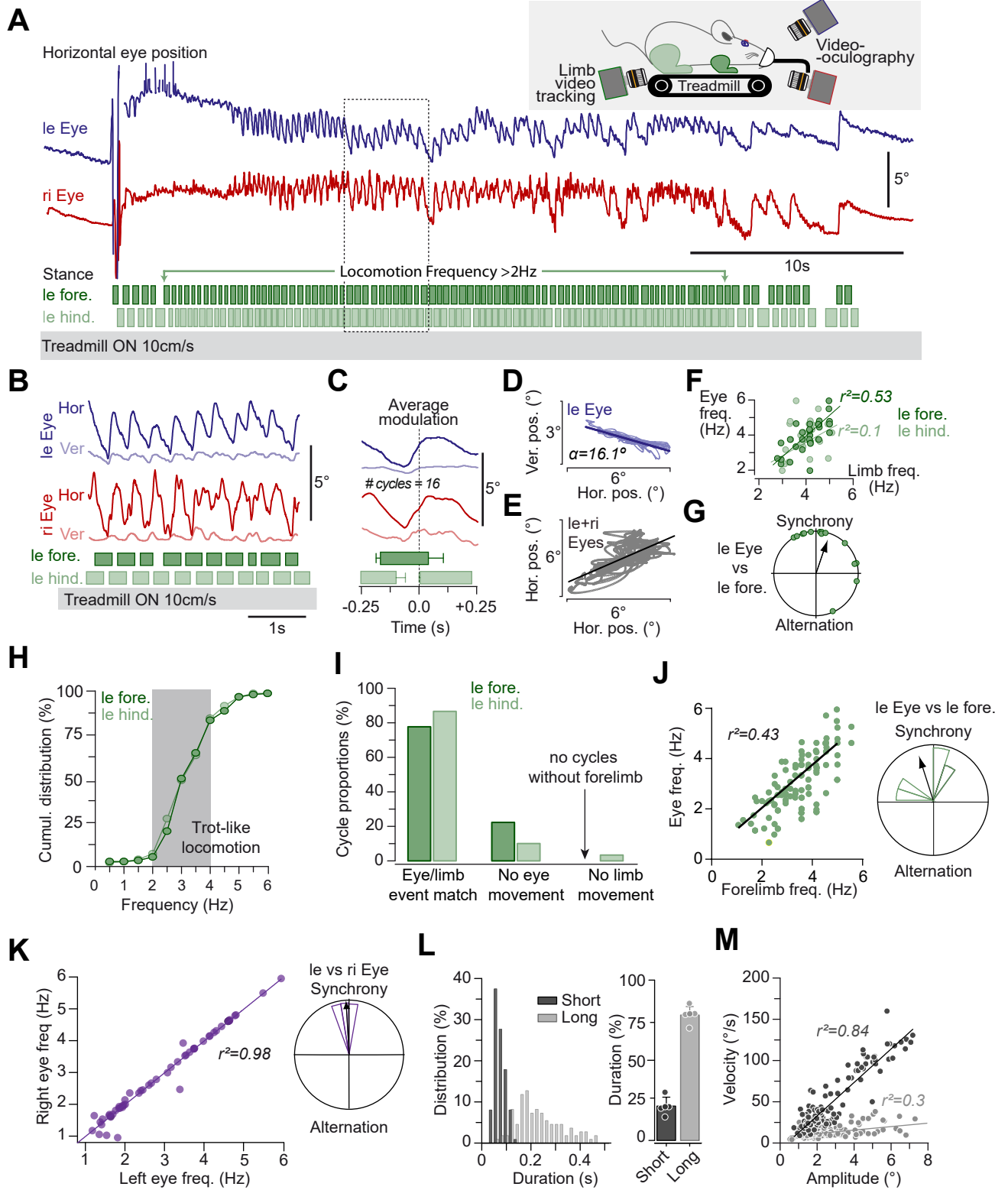
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Figure 2

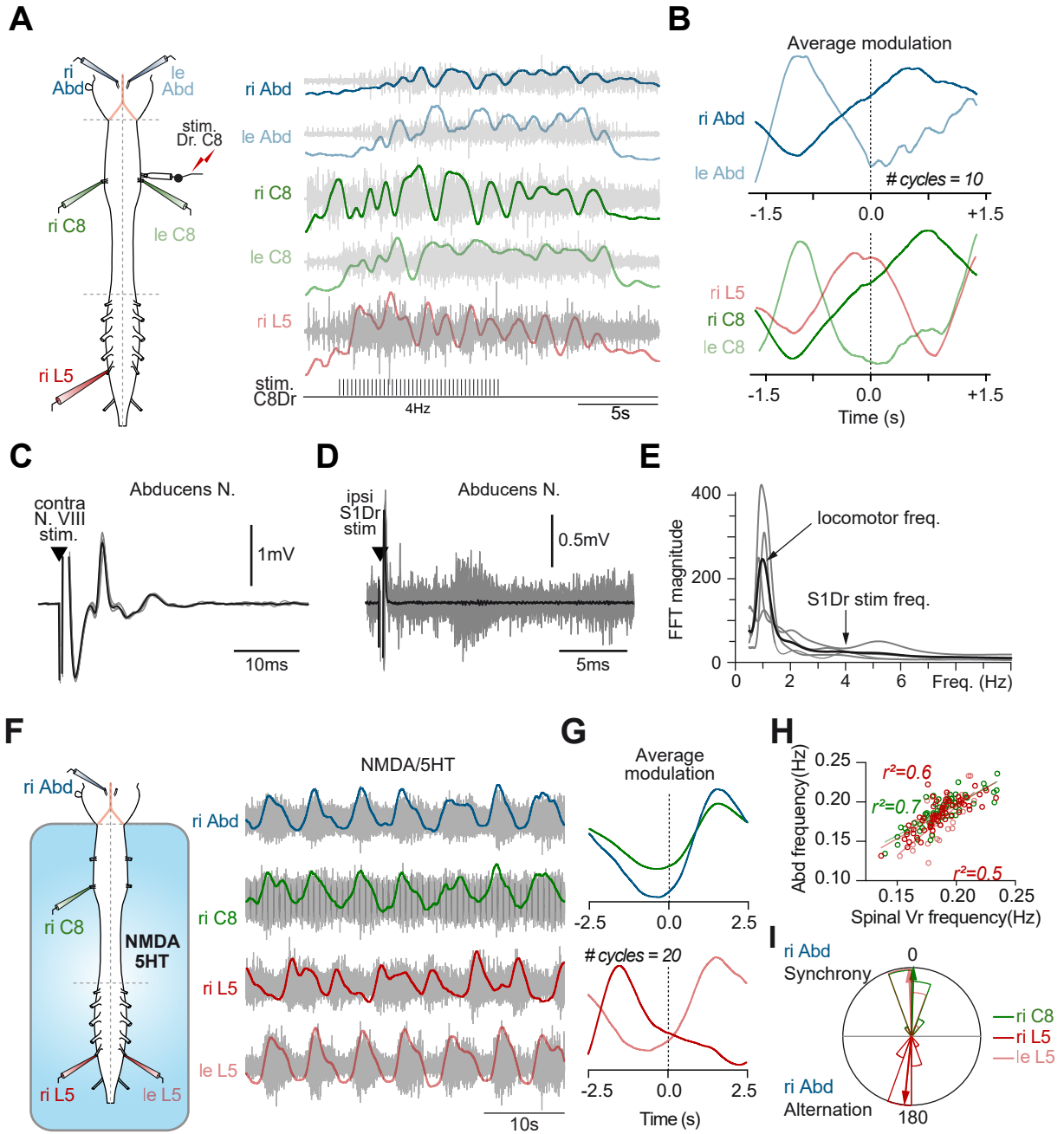




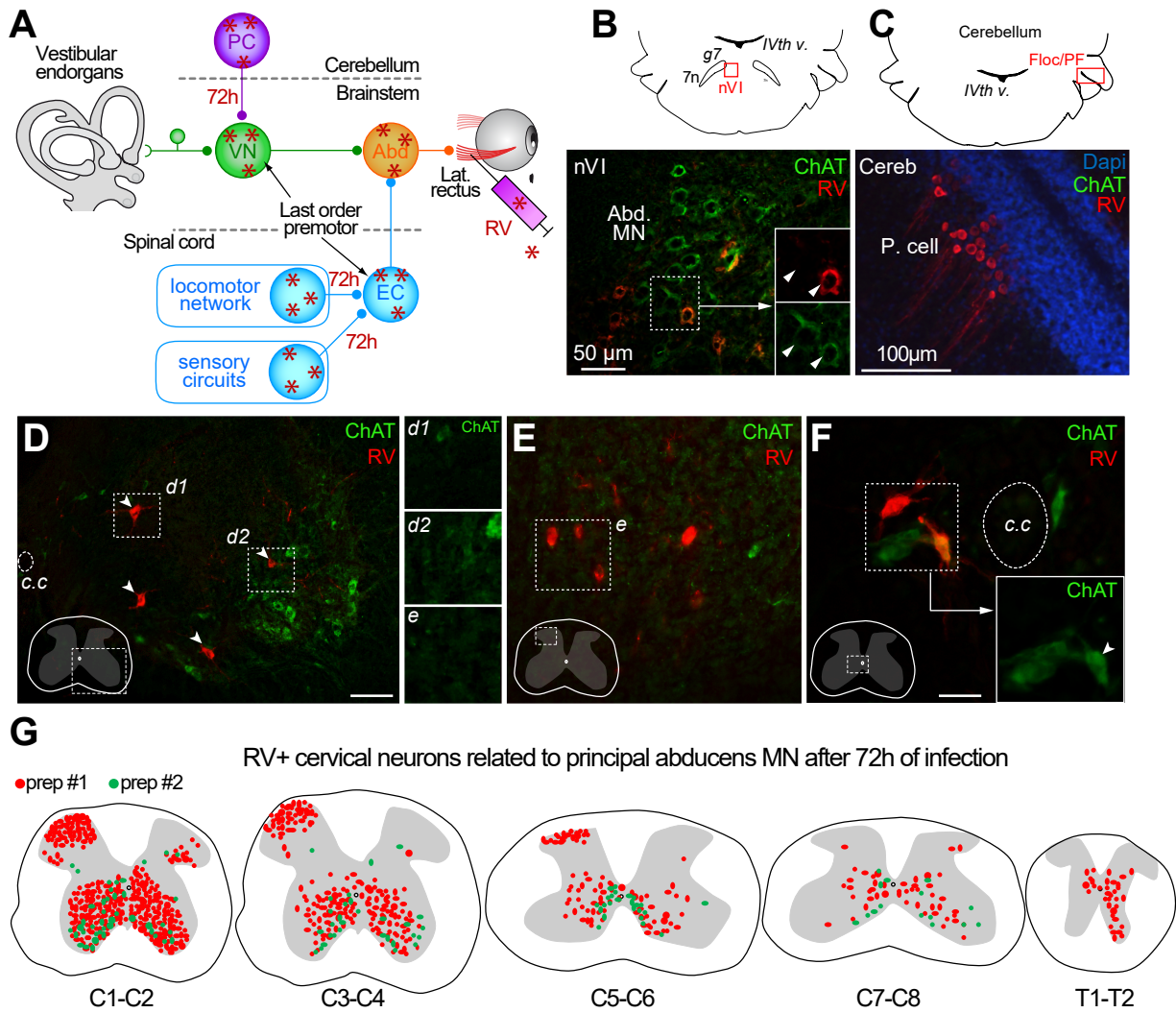
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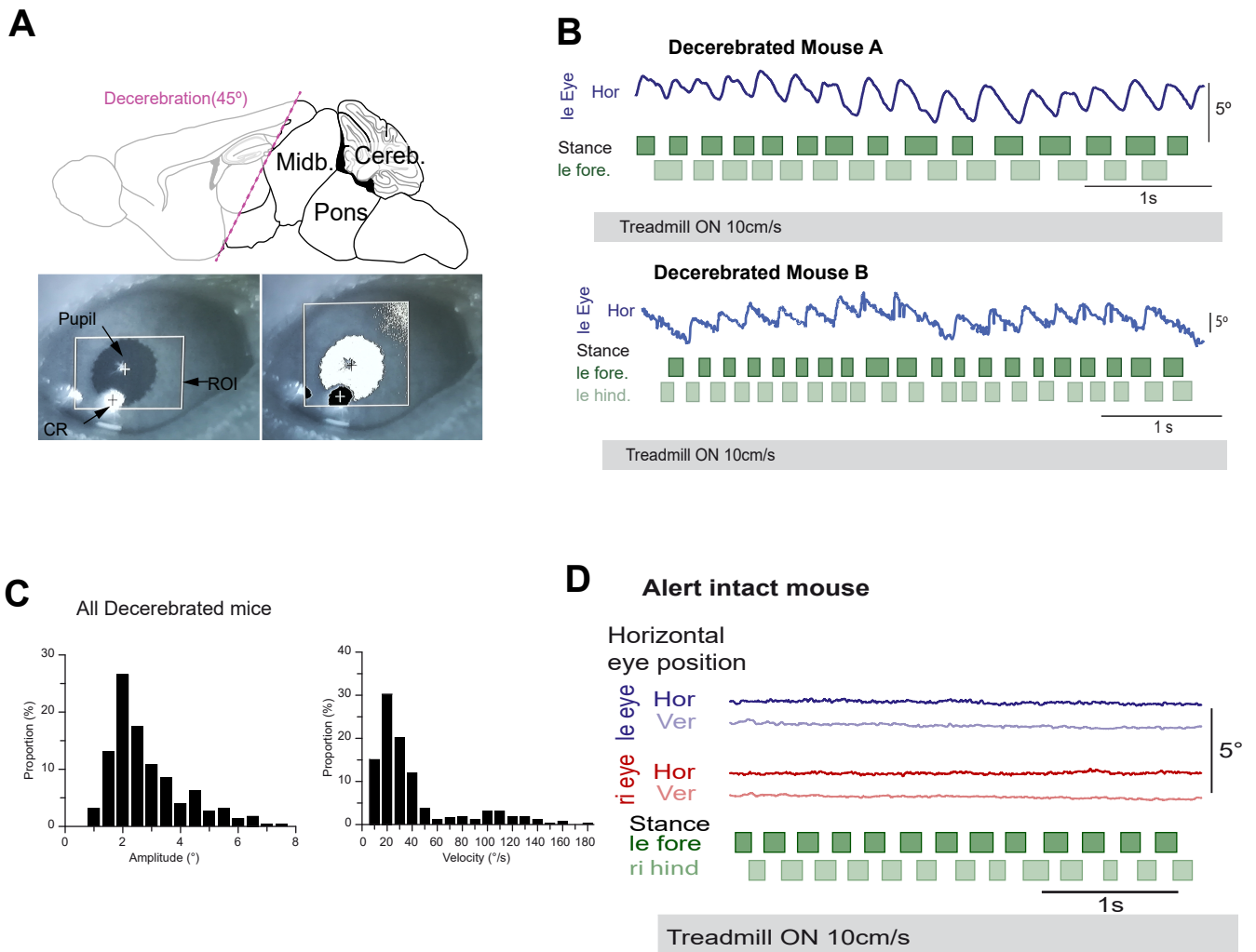






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 Figure S3





**Supplementary Figure 1. Fictive locomotor episodes evoked by electrical and NMDA/5HT produce a rhythmic activity in the abducens motor nerves, irrespective of proprioceptive feedback inputs. Related to Figure 1 and 2.**

**(A)** Fictive locomotion episodes evoked by cervical CPG stimulation. Left side, schematic of the neonatal mouse brainstem and spinal cord preparation with stimulated and recorded nerve branches. Right side, extracellular nerve recordings (raw traces in light gray, integrated traces in color) of the left (le, light blue) and right (ri, dark blue) abducens nerves (Abd.), the left and right 8th cervical roots (le C8, light green; ri C8, dark green) and the right 5th lumbar ventral root (ri L5, red). Discharges recorded during an episode of fictive locomotion evoked by the electrical stimulation of the C8 dorsal root (stim. C8Dr) with a 4Hz pulse train (bottom line).

**(B)** Average cyclic modulation of the discharge activity (integrated trace) from the motor nerves shown in (A) over 10 consecutive fictive locomotor cycles. The ri C8 trace (dark green) was used as the reference to determine locomotor cycles

**(C)** Left side, schematic of the neonatal mouse brainstem and spinal cord preparation with recorded nerve roots and NMDA/5HT split bath on the spinal cord. Right side, extracellular nerve recordings (raw traces in light gray, integrated traces in color) of the right (ri, dark blue) abducens nerves (Abd.), the right 8th cervical roots (ri C8, dark green) and the right and left 5th lumbar ventral root (ri L5, dark red; le L5, light red) discharges during an episode of fictive locomotion evoked by bath application of 5HT (15  $\mu$ M) and NMDA (7.5  $\mu$ M) restricted to the spinal cord.

**(D)** Averaged cyclic modulation of the discharge activity (integrated trace) of the motor nerves shown in panel H over 20 consecutive locomotor cycles. The right C8 trace (green) was used as the reference to determine locomotor cycles.

**(E)** Linear correlation in bursting frequencies between the abducens (Abd) nerve discharge and ipsilateral C8 (green) (ri Abd vs ri C8,  $n = 2$ ,  $R = 0.8187$ , Spearman test,  $p < 0.0001$ ;  $r^2 = 0.6702$ ), ipsilateral L5 (ri L5, red) (Abd vs L5,  $n = 2$ ,  $R = 0.7879$ , Spearman test,  $p < 0.0001$ ;  $r^2 = 0.6208$ ) and, contralateral L5 (le L5, pink) (Abd vs L5,  $n = 2$ ,  $R = 0.7045$ , Spearman test,  $p < 0.0001$ ;  $r^2 = 0.4964$ ) spinal ventral roots (Vr) discharges from the sequence shown in H.

**(F)** Circular plots showing the phase relationships between the discharge burst in the right abducens nerve and ipsilateral C8 ( $\mu = 1.432^{\circ} \pm 2.52$ ;  $r = 0.949$ ), ipsilateral L5 ( $\mu = 357.97^{\circ} \pm 1.826$ ;  $r = 0.9712$ ) and contralateral L5 ( $\mu = 185.835^{\circ} \pm 2.273$ ;  $r = 0.954$ ) spinal ventral roots ( $n = 2$  mice). The right C8 and the left L5 were in synchrony with the abducens nerve discharge while the right L5 was in alternation.

**(G)** VIIIth nerve-evoked (black arrowhead) abducens motor responses (individual traces in gray; mean in black) occur with a typical disynaptic latency compatible with the activation of the direct VOR pathway.

**(H)** Superimposed abducens discharge activity (gray traces) in response to electrical pulse applied on S1Dr (black arrowheads, as in figure 1A) and average activity (black trace). No reproducible dorsal root sensory-related response was observed following the S1Dr stimulation.

**(I)** Frequency periodograms obtained from FFT (Fast Fourier Transform) analysis of the abducens nerve discharge in response to S1Dr electrical stimulation evoking fictive locomotion episodes (Figure 1A and panel D) (gray traces represent a single locomotor sequence; the black trace is the average from the 4 gray traces). Periodograms revealed only one magnitude peak at the locomotor frequency (locomotor freq., about 1Hz) and did not show any peak at the S1Dr stimulation frequency (S1Dr freq., 4Hz), demonstrating the absence of spinal afference-evoked direct response in the abducens discharge.

**Supplementary Figure 3. Extended connections of the spino-extraocular pathway 70h after a rabies virus inoculation. Related to Figure 3.**

**(A)** Depiction of the RV labeling obtained 70h after RV inoculation in the lateral rectus muscle. RV<sup>+</sup> neurons are represented with red asterisks. Abd, abducens motor nuclei; PC, Purkinje cells; VN, Vestibular nucleus neurons; EC, efferent copy spinal neuron.

**(B)** Representation (top panel) of the location (red square) of the RV<sup>+</sup> neurons in the abducens motor nucleus (nVI) and its landmarks: geniculum of the facial nerve (g7, 7n) and 4th ventricle (IVth v.). The bottom panel shows a fluorescence microscopy image (20x) of a RV<sup>+</sup>/ChAT<sup>+</sup> Abducens motoneuron (Abd. MN; RV, red; ChAT, green) 70h after the RV inoculation.

**(C)** Representation (top panel) of the cerebellar location (red box, Floc/PF: Flocculus/Paraflocculus) of the RV<sup>+</sup> Purkinje cells (P. cell) shown in the fluorescence microscopy image (bottom panel, 20x magnification) 70h after the RV infection.

**(D, E, F)** Fluorescence microscopy images of cervical spinal cord sections showing the ventral **(D)** (10x magnification), dorsal **(E)** horns (10x magnification) and around the central canal (c.c) **(F)** (20x magnification) location of RV<sup>+</sup> neurons. The RV<sup>+</sup> spinal cord neurons found in the ventral **(D1, D2)** insets) and dorsal **(E1)** horns were not ChAT<sup>+</sup>. However, some neurons found around the central canal **(F1)** were simultaneously RV<sup>+</sup> and ChAT<sup>+</sup>.

**(G)** Scheme of the location of the RV<sup>+</sup> neurons found in the different cervical (C1-C8) and thoracic (T1, T2) segments following the 70h protocol. Red and green dots correspond to 2 different preparations (prep#1 red; prep #2 green). Variability in the labelling results from well-described methodological points<sup>S1-S4</sup>. Labeled neurons were found with a clear rostro-caudal gradient, in the ipsilateral and contralateral ventral horn, and in the ipsilateral dorsal horn of rostral cervical segments.



**Supplementary Figure 4. Eye movements during head-fixed treadmill locomotion in decerebrated and intact adult mice. Related to Figure 4.**

(A) Upper panel: scheme of the decerebration performed in adult mice. The 45° decerebration angle ensured a premammillary and precollicular decerebration; the structures outlined in black were preserved while the ones in gray were removed. Lower panel: (A1) picture of the right eye during a videoculography recording (see inset in Figure 4A) with region of interest (ROI) tracked; the pupil and corneal reflection (CR). (A2) shows the same eye with the detection threshold applied.

(B) Left eye movement traces and locomotor cycles of two additional decerebrated mice (A and B) running on a treadmill at 10 cm/s. As exemplified in Figure 4A-B, the horizontal (le Eye Hor, dark blue traces) and vertical (le Eye Ver, light blue traces) components of the eye movements are also shown for these mice. Rectangles represent the stance phases of the left forelimb (dark green) and left hindlimb (light green); gaps between rectangles represent swing phases.

(C) Distribution of the amplitude and velocity of the horizontal eye movements generated during treadmill locomotion in all decerebrated preparations (n= 5 mice).

(D) Eye movement traces and locomotor cycles of an intact mouse (head-fixed but not decerebrated) running on a treadmill at 10 cm/s in the dark. No horizontal (Leye; dark blue trace: horizontal, light blue trace: vertical) or vertical movements are observed after the onset of locomotion (dark green- left forelimb; light green- left hindlimb stance phases)

Figure #	Statistics									Data samples
	Panel	Data structure	Type of test	p value	R	r2	Mean	SEM	Length of mean vector	
1	D (L2. orange)	Correlation	Pearson correlation significance test	<0.0001	0.9696	0.94	N/A	N/A	N/A	C8: 7 mice, 19 sequences; L2: 8 mice, 13 sequences
1	D (C8. green)	Correlation	Pearson correlation significance test	<0.0001	0.9567	0.9158	N/A	N/A	N/A	
1	E (L2. orange)	Mean	Mean; Standard error of the mean	N/A	N/A	N/A	0.8175	0.05716	N/A	
1	E (C8. green)	Mean	Mean; Standard error of the mean	N/A	N/A	N/A	0.7468	0.05552	N/A	
1	F (L2. orange)	Mean	Mean; Standard error of the mean	N/A	N/A	N/A	N/A	N/A	N/A	
1	F (C8. green)	Mean	Mean; Standard error of the mean	N/A	N/A	N/A	N/A	N/A	N/A	
1	G (L2. orange)	Polar plot	Mean Vector; length of mean vector	N/A	N/A	N/A	354.288°	1.467*	0.936	
1	G (C8. green)	Polar plot	Mean Vector; length of mean vector	N/A	N/A	N/A	357.359°	2.213*	0.97	
1	G (L5. red)	Polar plot	Mean Vector; length of mean vector	N/A	N/A	N/A	198°	5.04	0.868	
S1	H (C8. green)	Correlation	Pearson correlation significance test	<0.0001	0.8187	0.6702	N/A	N/A	N/A	
S1	H (le L5. orange)	Correlation	Pearson correlation significance test	<0.0001	0.7045	0.4964	N/A	N/A	N/A	
S1	H (ri L5. red)	Correlation	Pearson correlation significance test	<0.0001	0.7879	0.6208	N/A	N/A	N/A	
S1	I (le L5. orange)	Polar plot	Mean Vector; length of mean vector	N/A	N/A	N/A	357.97°	1.826°	0.971	
S1	I (ri C8. green)	Polar plot	Mean Vector; length of mean vector	N/A	N/A	N/A	1.432°	2.434°	0.949	
S1	I (ri L5. red)	Polar plot	Mean Vector; length of mean vector	N/A	N/A	N/A	185.835°	2.273°	0.954	
2	A, B, C, D	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	6 mice. control: 11 sequences; 0 Ca2+: 13 sequences; washout: 13 sequences
2	E, F, G	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	7 mice. control lumbar: 11 sequences; control cervical: 13 sequences; cervical after-cut: 9 sequences; lumbar after-cut: 6 sequences
2	H (orange dots)	Correlation	Pearson correlation significance test	0.0001	0.9983	0.9966	N/A	N/A	N/A	3 mice. control lumbar: 6 sequences; control cervical: 7 sequences; after-cut lumbar: 3 sequences; after-cut cervical: 3 sequences
2	H (grey circles)	Correlation	Pearson correlation significance test	0.0001	0.9817	0.9637	N/A	N/A	N/A	
2	I (control. ipsi.black)	Distribution of mean angle values	Mean Vector; length of mean vector	N/A	N/A	N/A	345.161	2.546	0.974	
2	I (control. contra. empty)	Distribution of mean angle values	Mean Vector; length of mean vector	N/A	N/A	N/A	162.803	4.285	0.943	
2	I (after cut. ipsi. orange)	Distribution of mean angle values	Mean Vector; length of mean vector	N/A	N/A	N/A	353.912	2.463	0.999	
2	I (after cut. contra. empty)	Distribution of mean angle values	Mean Vector; length of mean vector	N/A	N/A	N/A	177.873	4.909	0.939	
2	J (before cut)	Normal distribution	Paired t-test	0.1038	N/A	N/A	52.97	4.51	N/A	
2	J (after cut)	Normal distribution	Paired t-test	0.1038	N/A	N/A	66.49	5.96	N/A	
3	J	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	3 mice
4	D	Correlation	Pearson correlation significance test	0.031	-0.8699	0.75	N/A	N/A	N/A	1 mouse
4	E	Correlation	Pearson correlation significance test	0.47	0.7152	0.51	N/A	N/A	N/A	
4	F (light green. forelimb)	Correlation	Pearson correlation significance test	<0.0001	0.7248	0.5253	N/A	N/A	N/A	
4	F (dark green. hindlimb)	Correlation	Pearson correlation significance test	0.0852	0.3195	0.1021	N/A	N/A	N/A	
4	G	Polar plot	Mean Vector (μ); length of mean vector	N/A	N/A	N/A	18.272	8.61	0.718	
4	J	Correlation	Pearson correlation significance test	<0.0001	0.6569	0.4315	N/A	N/A	N/A	
4	J	Polar plot	Mean Vector (μ); length of mean vector	N/A	N/A	N/A	342.719	28.957	0.727	
4	K (left panel)	Correlation	Pearson correlation significance test	<0.0001	0.9873	0.9748	N/A	N/A	N/A	
4	K (right panel)	Polar plot	Mean Vector (μ); length of mean vector	N/A	N/A	N/A	355.641	7.53	0.991	
4	M (dark gray)	Correlation	Pearson correlation significance test	0.00366	0.9169	0.8395	N/A	N/A	N/A	
4	M (light gray)	Correlation	Pearson correlation significance test	<0.00001	0.5412	0.2929	N/A	N/A	N/A	

**Supplementary Table 1. Statistical results and data samples. Related to Figure 1, 2, 3, 4 and Supplementary Figure 1.**

Statistical table with data samples for each figure. SEM, standard error of the mean; N/A, non-applicable.

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