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# 1     **Functional contribution of astrocytic Kir4.1 channels** 2                   **to spasticity after spinal cord injury**

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14  
15    **Running Title:** Restoring astrocytic Kir4.1 function decreases spasticity after spinal  
16    cord injury

## 17 18 19 20 21 22 23 24 25 26 27 28    **Highlights**

- 29       • Lumbar astrocytes adopt a reactive phenotype following a thoracic SCI
- 30       • NBCe1-mediated acidosis in astrocytes disrupts Kir4.1 function post-SCI.
- 31       • Impaired K<sup>+</sup> uptake leads to motoneuron hyperexcitability post-SCI.
- 32       • Enhanced astroglial Kir4.1 function reduces spastic-like symptoms in SCI mice.

## 34 **Summary**

35 Spasticity, a prevalent motor issue characterized by network hyperexcitability, causes  
36 pain and discomfort, with existing treatments offering limited relief. While past research  
37 has focused on neuronal factors, the role of astrocytes in spasticity has been  
38 overlooked. This study explores the potential of restoring astrocytic potassium ( $K^+$ )  
39 uptake to reduce spasticity following SCI. Astrocytes buffer extracellular  $K^+$  via Kir4.1  
40 channels, preventing neuronal hyperexcitability. Following spinal cord injury (SCI),  
41 Kir4.1 levels decrease at the injury site, though the consequences and mechanisms of  
42 this reduction within the motor output area have not been investigated. Utilizing  
43 advanced techniques, we demonstrate that lumbar astrocytes in a juvenile thoracic  
44 SCI mouse model switch to reactive phenotype, displaying morpho-functional and pro-  
45 inflammatory changes. These astrocytes also experience NBCe1-mediated  
46 intracellular acidosis, leading to Kir4.1 dysfunction and impaired  $K^+$  uptake. Enhancing  
47 Kir4.1 function reduces spasticity in SCI mice, revealing new therapeutic targets for  
48 neurological diseases associated with neuronal hyperexcitability.

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## 52 **Keywords**

53 spinal cord injury – spasticity – reactive astrocyte – Kir4.1 channels – astrocytic  
54 acidosis – NBCe1 transporter – astrocyte targeting therapy

## 55 Introduction

56 Traumatic spinal cord injury (SCI) is characterized by an acute mechanical injury,  
57 which drastically interrupts the sensory and motor tracts, leading to a wide spectrum  
58 of chronic dysfunctions, in particular spasticity affecting ~75% of the SCI patients<sup>1</sup>.  
59 Spasticity is characterized by a velocity-dependant increase in the tonic stretch reflex  
60 and spasms. It impairs the life quality of SCI patients by decreasing mobility and  
61 causing pain, sleep disturbances and depression<sup>2</sup>. Therapeutic approaches for  
62 spasticity are currently giving unsatisfactory results. Neurorehabilitation moderately  
63 attenuates the spastic symptoms<sup>3</sup>, this is why the main clinical strategy is focused on  
64 pharmacology. However, anti-spastic medications display limited benefits relative to  
65 numerous side effects. A better understanding of the pathophysiological mechanisms  
66 of spasticity is needed to propose a more appropriate therapeutic strategy.

67 Previous works including our own, revealed that spasticity after SCI is associated with  
68 a neuronal dysfunction resulting from an excitatory/inhibitory imbalance of both (i) pre-  
69 motor neurons<sup>4-6</sup> and (ii) motoneurons (MNs) innervating the hindlimb muscles<sup>7-13</sup>. A  
70 switch in neuronal excitability occurs at lumbar levels where MNs switch from  
71 hypoexcitability during the initial 'spinal shock' to hyperexcitability a few weeks or  
72 months after SCI in adults<sup>14</sup>. In our newborn SCI model, we showed that neuronal  
73 hyperexcitability occurs within few days post-SCI after an initial short-term  
74 hypoexcitability<sup>10,12</sup>. In this model, neuronal hyperexcitability has been associated with  
75 an increase in persistent sodium induced by a calpain-driven cleavage of voltage-  
76 dependent sodium channels Nav1.6<sup>11</sup>. Besides this increase in neuronal excitability,  
77 we previously observed a neuronal disinhibition in MNs resulting from a decrease of  
78 KCC2 function, a cation-chloride cotransporter involved in the intracellular chloride



79 homeostasis<sup>8,10</sup>. When KCC2 is reduced, the inhibitory neurotransmitters GABA and  
80 glycine contribute to hyperexcitability by evoking depolarization instead of  
81 hyperpolarization<sup>10</sup>. So far, most of the mechanisms proposed to explain spasticity in  
82 adult or juvenile SCI models focused on intrinsic neuronal elements. To determine the  
83 role of astrocytes, a subtype of glial cells<sup>15</sup>, in modulating the excitability of large-size  
84 MNs that innervate the fast-fatigable muscles, we chose a juvenile SCI model over an  
85 adult one. Indeed, recording these large-size MNs in adult lumbar spinal cord slices is  
86 extremely challenging largely due to (i) their extensive dendritic arborization<sup>16</sup> and (ii)  
87 their high metabolic demands, which makes them vulnerable to ischemia<sup>17</sup>, a common  
88 issue during *ex vivo* slicing. To overcome these limitations, we and others, conduct  
89 studies on spinal lumbar cord slices prepared from animals within the first 2 weeks  
90 after birth<sup>11,12,18–20</sup>.

91 In response to neuronal activity, astrocytes prevent neuronal hyperexcitability by  
92 buffering the increase in extracellular potassium concentration ( $[K^+]_o$ ) mainly through  
93 the inwardly rectifying  $K^+$  channel, Kir4.1<sup>21,22</sup>. Recent evidences also indicate that  
94 astrocytes switching to a reactive phenotype contribute to the pathophysiology of  
95 neurodegenerative diseases and traumatic CNS injury<sup>23</sup>. Reactive astrocytes are  
96 defined as astrocytes that undergo morphological, molecular, and functional changes  
97 in response to pathological situations in surrounding tissue<sup>23,24</sup>. Among the molecular  
98 changes, a decrease in the expression of Kir4.1 channels has been observed in  
99 various neurological disorders<sup>25</sup>, including SCI in the area of the injury site<sup>26</sup>. However,  
100 the functional consequences of such a decrease within the motor output region  
101 (specifically the lumbar region where MNs are located) have not been investigated,  
102 especially in the chronic phase when spasticity emerges. Understanding the glial

103 pathophysiological processes underlying this neuronal hyperexcitability might offer  
104 new therapeutic strategy for spasticity.

105 Here we investigated whether restoring astrocyte K<sup>+</sup> uptake alleviates spasticity after  
106 SCI. We demonstrate that, following thoracic SCI, lumbar astrocytes switch to a  
107 reactive phenotype exhibiting morpho-functional changes and inflammatory markers.  
108 These astrocytes also display prominent NBCe1-mediated intracellular acidosis  
109 leading to dysfunction of the inwardly-rectifying Kir4.1 channels, which ultimately  
110 results in impaired K<sup>+</sup> uptake, and MN hyperexcitability. Importantly, we demonstrate  
111 that enhancing Kir4.1 function alleviates spastic-like symptoms in SCI mice.

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## 121 Results

### 122 Morphological changes of astrocytes and inflammatory marker expression in 123 lumbar region after SCI

124 To determine whether astrocytic reactivity occurs in the lumbar spinal cord during the  
125 onset of spasticity following thoracic SCI, we performed immunohistofluorescence  
126 against GFAP in Aldh1L1-eGFP mice<sup>27</sup>, in which astrocytes express GFP (Figure 1A).  
127 This revealed significant astrocytic hypertrophy and a widespread increase in GFAP  
128 fluorescence intensity in the gray matter of the lumbar ventral horn in SCI mice (Figures  
129 1A-1B, Figure S1A-B). Using Sholl analysis based on GFAP labelling, we showed that  
130 lumbar GFP+ astrocytes from SCI mice display longer and more branched processes,  
131 characterized by an increased ramification index (Figure 1C), and occupy a larger  
132 space than their control counterparts (Figure 1D). These results suggest the  
133 emergence of astrocyte reactivity following SCI. Given that GFAP expression and  
134 morphological changes are not conclusive indicators of reactive astrocytes<sup>23</sup>, we  
135 focused on additional pro-inflammatory biomarkers, namely the complement  
136 component 3d (C3d) and the transcription factor Signal Transducer and Activator of  
137 Transcription 3 (STAT-3), both well-documented for their elevated levels in reactive  
138 astrocytes within cortical regions impacted by neurological disorders<sup>28-30</sup>. We found a  
139 notable rise in the proportion of Aldh1L1(+) astrocytes co-expressing the pro-  
140 inflammatory marker C3d, as shown by immunohistochemistry (Figures S1C-D). Active  
141 phosphorylated STAT3 could not be detected by immunohistochemistry in our SCI  
142 model. We used STAT3 nuclear localization as an indicator of pathway activation<sup>29,30</sup>,  
143 and observed a significant increase of astrocytic nuclear STAT3 translocation in SCI  
144 mice compared to controls (Figures S1E-F). Collectively, these findings reveal that

145 thoracic SCI triggers morphological changes in astrocytes and inflammatory marker  
146 upregulation in the vicinity of lumbar ventro-lateral  $\alpha$ -MNs.

### 147 **Functional alterations of astrocytes surrounding MNs after SCI**

148 To evaluate the functional deficits associated with morphological alterations in  
149 astrocytes post-SCI, we conducted patch-clamp electrophysiology on GFP(+)   
150 astrocytes in acute lumbar slices from Aldh1L1-eGFP mice (Figure 2A). Our results  
151 revealed that GFP(+) astrocytes from SCI mice exhibit altered electrophysiological  
152 properties characterized by a depolarized resting membrane potential (RMP), an  
153 increased input resistance, and a decreased conductance (Figure 2B), leading to lower  
154 macroscopic currents (Figures 2C-2E) compared to controls. In addition, to better  
155 characterize these functional alterations at a cell population level, we performed two-  
156 photon imaging of astrocytic intracellular  $\text{Ca}^{2+}$  in the lumbar ventro-lateral horn (Figure  
157 2F). Control and SCI mice were injected at birth with adeno-associated virus serotype  
158 5 (AAV5) designed to express GCaMP6f in all astrocytic compartments (Figure 2G).  
159 We observed an increased proportion of astrocytes displaying spontaneous  $\text{Ca}^{2+}$   
160 transients in the processes (+22%) and soma (+32%) post-SCI (Figures 2H and 2I;  
161 Figures S2A and S2B). Further analysis revealed a significant increase in the  
162 amplitude of spontaneous  $\text{Ca}^{2+}$  transients in processes and microdomains, with no  
163 change in the soma of SCI mice (Figures 2J-K; Figures S2C and S2F-H). While the  
164 duration and frequency of the spontaneous  $\text{Ca}^{2+}$  transients in soma and processes  
165 remained unchanged post-SCI, the duration decreased and the frequency increased  
166 in microdomains (Figures 2J-K; Figures S2C and S2F-H). Overall, these findings  
167 support the view that reactive astrocytes surrounding MNs undergo specific functional  
168 alterations, characterized by altered membrane properties and increased spontaneous  
169  $\text{Ca}^{2+}$  activity, which may contribute to the hyperexcitability of MNs following SCI.

170 **Glutamate-evoked Ca<sup>2+</sup> transients are enhanced in astrocytes and MNs after SCI**

171 Spasticity resulting from SCI is partly attributed to MNs hyperexcitability<sup>4,8,10,11</sup>. Given  
172 that reactive astrocytes are known to modulate neuronal excitability<sup>31,32</sup>, we  
173 investigated the SCI-induced changes in astrocyte-neuron signaling. To address this,  
174 we conducted simultaneous two-photon Ca<sup>2+</sup> imaging of astrocytes and neurons in the  
175 ventro-lateral lumbar region. At birth, wild-type mice were injected with AAV5 to  
176 express GCaMP6f in astrocytes (Figure 3A) and AAV9 to express jRGECO1a in  
177 neurons (Figure 3B). Glutamate was bath applied to trigger widespread increases in  
178 neuronal activity. Following SCI, we observed significantly elevated Ca<sup>2+</sup> transients in  
179 astrocytic processes (+214%), soma (+166%) and microdomains (+139%) in response  
180 to glutamate exposure, compared to control conditions (Figures 3C-E, Figures S2D-E  
181 and S2I-J). Additionally, large neurons in the ventro-lateral region, likely  $\alpha$ -MNs,  
182 exhibited an amplified glutamate-induced Ca<sup>2+</sup> signals (+34%) (Figures 3F-3H). This  
183 pronounced Ca<sup>2+</sup> activity spread progressively throughout both astrocytic and neuronal  
184 networks (Figures 3I-J). Notably, we observed that the number of co-active astrocytes  
185 is transiently increased upon glutamate exposure (+44% and +74% at 10 and 30s,  
186 respectively) in SCI mice compared to controls (Figure 3J). Altogether, these findings  
187 suggest that SCI amplifies astrocyte-neuron Ca<sup>2+</sup> signaling in the lumbar motor  
188 network.

189 **Glutamate-evoked astrocytic acidosis is enhanced after SCI**

190 In response to increased neuronal activity, astrocytes express a high level of  
191 electrogenic sodium (Na<sup>+</sup>) – bicarbonate (HCO<sub>3</sub><sup>-</sup>) cotransporter 1 (NBCe1, *Slc4a4*) to  
192 maintain brain extracellular pH (pHe) homeostasis<sup>33</sup>. NBCe1 is a high affinity carrier  
193 primarily responsible for moving HCO<sub>3</sub><sup>-</sup> across the astroglial membrane, and its  
194 activity is modulated by intracellular Ca<sup>2+</sup> rise<sup>34,35</sup>. Given our observation of increased

195  $\text{Ca}^{2+}$  signals in astrocytes post-SCI, we investigated whether NBCe1 expression and  
196 function in spinal astrocytes are affected by SCI. We found a slight but significant  
197 upregulation of membrane-bound NBCe1 in astrocytes after SCI (Figures 4A and 4B).  
198 Consistent with our previous experiment, we activated the neuronal network with  
199 glutamate. Using two-photon imaging with the pH-sensitive probe BCECF during  
200 glutamate exposure, we monitored pH shifts in Td-tomato (+) astrocytes in the ventro-  
201 lateral region of lumbar slices (Figure 4C). Confirming BCECF pH-sensitivity (Figures  
202 S3A-C), astrocytes loaded with BCECF exhibited either alkalinization or acidification  
203 in response to glutamate (Figures 4D and 4E). We found a significant increase (~20%)  
204 in acidic responses in astrocytes post-SCI compared to control (CTL) mice (Figure 4F).  
205 Detailed analysis showed a pronounced glutamate-induced functional acidification of  
206 astrocytes post-SCI, attenuated by NBCe1 blockade (Figures 4G and 4H). These data  
207 suggest that reactive astrocytes exhibit increased NBCe1 expression and undergo  
208 prominent intracellular acidosis during sustained neuronal activity post-SCI.

### 209 **NBCe1-mediated prominent acidosis in astrocytes decreases Kir4.1 function** 210 **after SCI**

211 Intracellular pH modulates various cellular functions, including the weakly inwardly  
212 rectifying  $\text{K}^+$  (Kir4.1) channel activity<sup>36,37</sup>. Given the altered astrocytic pH regulation  
213 post-SCI, we investigated its impact on Kir4.1 functionality in reactive astrocytes.  
214 Immunohistochemistry confirmed the presence of Kir4.1 channels in astrocytes  
215 enwrapping ventro-lateral MNs in the lumbar spinal segments (L4-L5) (Figure 5A). In  
216 parallel, we assessed their pH sensitivity in our preparation. Patch-clamp recordings  
217 of GFP(+) astrocytes in the ventro-lateral lumbar slices of Aldh1L1-eGFP mice  
218 revealed a barium ( $\text{Ba}^{2+}$ , 100 $\mu\text{M}$ ) -sensitive current indicative of Kir4.1 function, which  
219 was significantly reduced under intracellular acidic conditions or post-SCI (Figures 5B-

220 E). Interestingly, blockade of NBCe1 with S0859 (50 $\mu$ M) restored Kir4.1 conductance,  
221 highlighting the acidosis' detrimental effect on Kir4.1 function (Figures 5C-E). Then, we  
222 challenged GFP(+) astrocytes with high extracellular K<sup>+</sup> puff application (Figure 5F) to  
223 analyze K<sup>+</sup> inward current dynamics. We first confirmed that blockade of Kir4.1  
224 channels with Ba<sup>2+</sup> (100 $\mu$ M) significantly decreases the astrocytic K<sup>+</sup>-mediated inward  
225 current (Figures S4A-C). Additionally, the application of a K<sup>+</sup> puff (12 mM) induced an  
226 inward K<sup>+</sup> current, which was reduced in SCI astrocytes (Figures 5G and 5H). This  
227 decrease of the pH-sensitive current was restored by blocking NBCe1 . These results  
228 suggest that the NBCe1-mediated acidosis impairs K<sup>+</sup> uptake by decreasing Kir4.1  
229 function in reactive astrocytes post-SCI.

### 230 **Astrocytic Kir4.1 dysfunction leads to impaired K<sup>+</sup> homeostasis post-SCI**

231 MN hyperexcitability occurs within a few days post-SCI in a newborn SCI model<sup>10,12</sup>. It  
232 has been shown that an effective regulation of extracellular K<sup>+</sup> concentration ([K<sup>+</sup>]<sub>o</sub>) is  
233 essential for limiting network hyperexcitability<sup>38</sup>, with astrocytes primarily maintaining  
234 K<sup>+</sup> homeostasis through the activity of Kir4.1 channels<sup>21,25</sup>. Given that SCI induces  
235 Kir4.1 dysfunction, we investigated whether extracellular K<sup>+</sup> homeostasis was impaired  
236 in lumbar slices of SCI mice. To do so, we used K<sup>+</sup>-sensitive microelectrodes to  
237 measure glutamate-induced changes in extracellular K<sup>+</sup> concentration in control and  
238 SCI lumbar slices, specifically in the ventro-lateral region where MNs are located  
239 (Figure 6A). The [K<sup>+</sup>]<sub>o</sub> transients evoked by glutamate puff (Figure 6B) showed a larger  
240 peak amplitude and area in SCI mice compared to controls while their rise slope and  
241 decay time were identical in both groups (Figure 6C). These findings indicate impaired  
242 glutamate-evoked K<sup>+</sup> homeostasis in the lumbar cord post-SCI. This prompted us to  
243 explore the impact of increased K<sup>+</sup> on the properties of large lumbar  $\alpha$ -MNs in control  
244 mice (Figure 6D). We found that the rise in [K<sup>+</sup>]<sub>o</sub> that we observed in SCI condition,

245 enhanced MNs excitability by depolarizing their resting membrane potential (RMP)  
246 (Figure 6E) and decreasing their rheobase (Figures 6F), leading to a gain increase  
247 (Figure 6G). Additionally, we observed that the rise in  $[K^+]_o$  decreased the peak  
248 amplitude of the slow inactivating outward  $K^+$  currents, primarily mediated by the Kv1.2  
249 channels, which may contribute to the amplification of the motor output<sup>18</sup>. Altogether,  
250 these findings demonstrate that SCI impairs  $K^+$  homeostasis in the lumbar cord, which  
251 in turn enhances MN excitability.

### 252 **Restoration of astrocytic Kir4.1 function reduces spasticity post-SCI**

253 To evaluate whether restoring Kir4.1 function in astrocytes could alleviate spastic-like  
254 symptoms post-SCI, we performed EMG recordings of the *gastrocnemius* muscle in  
255 wild-type SCI mice (Figure 7A), injected at birth with AAV9 carrying GFP-tagged Kir4.1  
256 (Kir4.1-GFP) or GFP-tagged Cre (Control-GFP) under the astrocytic promoter,  
257 *gfaABC1D* or *GFAP*, respectively. First, we confirmed that Kir4.1-GFP transduction  
258 increases Kir4.1 expression in the lumbar spinal cord (Figures S5A-D), while no Kir4.1-  
259 GFP transduction occurs in the supraspinal structures involved in motor control (Figure  
260 S5E). Then, we observed a significant reduction in spontaneous muscle spasm  
261 frequency from P15 in Kir4.1-GFP-treated SCI mice, although spasm duration  
262 remained unchanged (Figures 7C ; Figures S6A and S6B). Using sensory-evoked  
263 stimulation, we found a reduced duration of triggered spasms from P10 in Kir4.1-GFP-  
264 treated SCI mice (Figures 7D and 7E). Complementary DeepLabCut analysis<sup>39</sup>  
265 (Figures 7F; Figures S6C and S6D) demonstrated several additional differences  
266 between the Kir4.1-GFP-treated SCI mice and their control counterparts: (i) a decrease  
267 in spontaneous hindlimb motion frequency and acceleration from P10 and P20,  
268 respectively (Figure 7G), (ii) a significant decrease in duration of the hindlimb  
269 hyperextension from P10 in Kir4.1-GFP-treated SCI mice (Figure 7H), confirming our



270 EMG recordings. Importantly, the tail pinch stimulation was consistent across both  
271 conditions (Figures S6F and S6G), and no change in ankle angle variation in response  
272 to tail pinch was observed in both conditions (Figure 7H; Figure S6E). These *in vivo*  
273 results suggest that restoring Kir4.1 function in astrocytes decreases spastic-like  
274 symptoms, suggesting Kir4.1 as a therapeutic target for neurological disorders  
275 characterized by neuronal hyperexcitability.

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## 290 **Discussion**

291 Our study reveals novel insights into neuro-glial interactions within spinal motor circuits  
292 and demonstrate that improving Kir4.1 function in astrocytes reduces spasticity in SCI  
293 mice. Employing a multifaceted methodology that includes advanced imaging  
294 techniques, electrophysiology, immunohistochemistry, and genetic tools, we explored  
295 the effects of enhancing astrocytic K<sup>+</sup> uptake in alleviating spasticity. We found that  
296 thoracic SCI prompts lumbar astrocytes to adopt a reactive phenotype characterized  
297 by significant morphological and functional alterations, as well as the upregulation of  
298 inflammatory markers. These reactive astrocytes exhibit increased intracellular  
299 acidosis mediated by NBCe1, disrupting the function of Kir4.1 channels and leading to  
300 compromised K<sup>+</sup> absorption which heightened MN excitability.

### 301 **Morphological and inflammatory changes from lumbar astrocytes following** 302 **thoracic SCI**

303 Reactive astrocytes undergo morphological, molecular and functional remodelling in  
304 response to various CNS injuries in surrounding tissue<sup>23,24,40</sup>. Reactive astrocytes at  
305 the glial scar protect tissue and preserve function after SCI by restricting the spread of  
306 inflammatory cells<sup>41,42</sup>. However, the occurrence and nature of astrocyte reactivity in  
307 regions distal to the injury site post-SCI remain poorly understood. Here, we reveal that  
308 thoracic SCI induces significant astrocyte reactivity within the lumbar region, evidenced  
309 by hypertrophied and increased complexity of astrocytic processes. We also report an  
310 overall rise in GFAP intensity within the ventral horn's gray matter, without modification  
311 of the astrocyte/motoneuron ratio, thus underscoring the pervasive astrocytic presence  
312 around  $\alpha$ -MNs as observed in amyotrophic lateral sclerosis<sup>43</sup> or spinal muscular  
313 atrophy<sup>44</sup> mouse models. In our study, we also observed a modest but significant

314 increase in the pro-inflammatory markers (C3d, STAT3) in lumbar astrocytes following  
315 SCI. Previous studies focusing on the spinal motor networks, indicated that pro-  
316 inflammatory cytokines from microglia, such as IL-1 $\alpha$ , IL-6, TNF- $\alpha$ , and C1q, may drive  
317 the enhanced astrocytic expression of C3<sup>28,43</sup> and pSTAT3 signaling<sup>45,46</sup>. Moreover,  
318 the persistent activation of astrocytic STAT3 has been shown to depend on increased  
319 Ca<sup>2+</sup> signaling in a mouse model of chronic inflammatory itch<sup>47</sup>. These findings further  
320 underscore the significance of exploring the astrocytic Ca<sup>2+</sup> dynamics after SCI.

### 321 **Functional changes from lumbar astrocytes following thoracic SCI**

322 In conjunction with morphological evaluations, we investigated the functional plasticity  
323 of lumbar astrocytes post-SCI. Reactive astrocytes demonstrate varied Ca<sup>2+</sup> dynamics  
324 across different pathological models, stages (acute vs. chronic), and anatomical  
325 regions<sup>40</sup>. Generally, these cells show dysregulated Ca<sup>2+</sup> signaling, predominantly  
326 characterized by heightened Ca<sup>2+</sup> activity<sup>48-50</sup>, though reductions in Ca<sup>2+</sup> signaling  
327 have also been reported<sup>51,52</sup>. Our two-photon Ca<sup>2+</sup> imaging data indicate significant  
328 alterations in Ca<sup>2+</sup> signaling in lumbar astrocytes from SCI mice. Specifically, we  
329 observed an increase in both (i) the proportion of spontaneous active astrocytic Ca<sup>2+</sup>  
330 transients and (ii) the glutamate-evoked Ca<sup>2+</sup> activity in astrocytic soma, processes,  
331 and microdomains. Ca<sup>2+</sup> signaling is crucial as it impacts numerous cellular processes,  
332 including the modulation of neurotransmitter release<sup>53</sup> and K<sup>+</sup> buffering capacity<sup>54,55</sup>.  
333 Moreover, simultaneous Ca<sup>2+</sup> imaging from both neurons and astrocytes using dual-  
334 color genetically encoded calcium indicators (GECIs) revealed that astrocyte-neuron  
335 Ca<sup>2+</sup> signalling is enhanced after SCI. While further studies are needed to elucidate the  
336 underlying mechanisms, these findings suggest that the increased and widespread  
337 Ca<sup>2+</sup> signalling in astrocytes may fuel neuronal hyperexcitability and subsequent  
338 spasticity following SCI. Supporting this notion, a proconvulsive potential of astrocyte

339  $\text{Ca}^{2+}$  signals in promoting the spread of epileptiform activity within the hippocampal  
340 networks has been demonstrated<sup>56</sup>.

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### 342 **pH alterations in lumbar astrocytes post-SCI**

343 Alterations in brain pH are recognized as critical indicators of various pathological  
344 states<sup>57</sup>, with astrocytes playing a key role in maintaining an intricate pH balance  
345 between the intra and extracellular environments<sup>15</sup>. In our study, we identified two  
346 distinct astrocytic populations in control mice that responded to glutamate in divergent  
347 ways affecting intracellular pH: the majority (~70%) exhibited intracellular acidification,  
348 likely for maintaining the extracellular pH homeostasis during sustained neuronal  
349 activity through activity-dependent  $\text{HCO}_3^-$  efflux<sup>33</sup>, whereas the minority (~30%)  
350 showed intracellular alkalinization, possibly facilitated by  $\text{K}^+$ -stimulated NBCE1 activity  
351 that supports energy metabolism during astrocytic glycolysis<sup>58</sup>. Following SCI, our two-  
352 photon imaging data indicated a rise in the proportion of acidifying astrocytes to 92%,  
353 with an increase in the amplitude of acidifying  $\text{pHi}$  responses. These changes could be  
354 attributed to a pSTAT3-dependent increase in NBCE1 expression<sup>59</sup> and a shift in  
355 NBCE1 activity towards its outward mode triggered by enhanced astrocytic  $\text{Ca}^{2+}$   
356 signaling<sup>34,35</sup>. Additionally, chronic hypoxia beneath the injury site, due to compromised  
357 capillary blood flow<sup>60</sup>, may further drive NBCE1-induced  $\text{Ca}^{2+}$  transients in astrocytes,  
358 thereby exacerbating intracellular pH acidification<sup>35,61,62</sup>. Additional research is needed  
359 to explore the roles of other factors (i.e. NCX, AE, ...) in this intensified astrocytic pH  
360 acidification<sup>57,63</sup>.

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363 **Acidosis-mediated dysfunction of Kir4.1 leading to increased extracellular K<sup>+</sup>**  
364 **post-SCI**

365 Effective extracellular potassium (K<sup>+</sup>) removal is essential for maintaining brain  
366 homeostasis and limiting network hyperexcitability, as impairments in K<sup>+</sup> clearance are  
367 associated with various pathological states<sup>38,64–66</sup>. The maintenance of K<sup>+</sup> homeostasis  
368 is predominantly performed by astrocytes, primarily through the activity of the weakly  
369 inwardly rectifying Kir4.1 K<sup>+</sup> channels<sup>21,25</sup>. Given the pH sensitivity of Kir4.1 to  
370 acidosis<sup>36,37</sup>, we confirmed that NBCe1-mediated pH acidification impairs Kir4.1  
371 channel function post-SCI, thereby compromising the reactive astrocytes' capacity to  
372 buffer extracellular K<sup>+</sup>. This dysfunction is exacerbated by acidosis but can be reversed  
373 by NBCe1 blockade, emphasizing the detrimental impact of acidosis on Kir4.1 activity  
374 and resulting K<sup>+</sup> imbalance post-SCI. We previously demonstrated that SCI displays  
375 early and chronic MN hyperexcitability<sup>10,12</sup>, which occurs alongside with the astrocytic  
376 Kir4.1 deficits observed in this study. Additionally, we observed that increase in  
377 extracellular K<sup>+</sup> reduces the delayed rectifying K<sup>+</sup> current, Kv1.2, potentially amplifying  
378 motor output<sup>18</sup>. However, unlike in the hippocampus<sup>67</sup>, the increase in K<sup>+</sup> did not affect  
379 the persistent sodium current (INaP) that is typically amplified post-SCI<sup>11</sup>. This  
380 discrepancy may be attributed to the moderate (~3mM) rise in K<sup>+</sup> in response to  
381 glutamate post-SCI, aligning with the sensory-evoked K<sup>+</sup> rise observed in striatal and  
382 hippocampal networks under pathological conditions<sup>65,66</sup>. Collectively, these findings  
383 underscore the importance of correcting K<sup>+</sup> dysregulation to decrease MN  
384 hyperexcitability and thus potentially reduce spasticity post-SCI.

385 **Kir4.1 gain of function for decreasing spasticity post-SCI**

386 The observation that impaired K<sup>+</sup> homeostasis leads to MN hyperexcitability post-SCI  
387 raise the question of whether increasing astrocytic Kir4.1 function could decrease

388 spasticity. Previous studies have shown that a selective restoration of Kir4.1 function  
389 in astrocytes reverts Huntington disease-like symptoms<sup>51,65</sup>, can relieve chronic  
390 hyperalgesia in neuropathic pain<sup>68</sup> and improves cognitive and social performance in  
391 a Fragile X syndrome mouse model<sup>66</sup>. Spinal astrocytes might thus play a key role in  
392 modulating motor troubles after SCI as: (i) they are present in high proportion in the  
393 ventral horn<sup>69</sup>, (ii) they control pathological tremor by tuning excitatory synaptic  
394 transmission through the release of purines<sup>19</sup>, and (iii) the Rac1 activity in astrocytes  
395 can contribute to hyperreflexia following SCI<sup>70</sup>. Our viral approach specifically targeted  
396 Kir4.1 channels, which are highly expressed in astrocytes around large  $\alpha$ -MNs<sup>22</sup>.  
397  $\alpha$ -MNs have the capacity to display a sustained firing pattern<sup>71,72</sup>, which is enhanced  
398 post-SCI<sup>4,7,12</sup>. By enhancing Kir4.1 function in spinal astrocytes, we observed a  
399 significant alleviation of spastic-like symptoms in SCI mice. The reduction in muscle  
400 spasm frequency and duration in treated mice highlights the functional importance of  
401 Kir4.1 channels in modulating neuronal excitability and controlling spasticity post-SCI.

## 402 **Limitations of the Study**

403 Our study provides new biological insights onto neuroglial crosstalk in the spinal motor  
404 network and broaden the spectrum of therapeutic targets for restoring compromised  
405 neuronal excitability in neurological diseases involving altered  $K^+$  homeostasis.  
406 However, some limitations warrant consideration. Primarily, by focusing on Kir4.1, we  
407 may have overlooked the potential contributions of other ion channels or molecular  
408 components, notably  $Na^+K^+$  ATPase pump, which could play a pivotal role in regulating  
409  $K^+$  homeostasis<sup>73</sup>. Additionally, in a translational point of view, the juvenile SCI model  
410 may not accurately mirror the complexities of adult SCI environment. Moreover,  
411 considering the diverse forms of spasticity in humans and the various models used in

412 mice<sup>74</sup>, it will be important in future work to establish whether our findings extend to the  
413 other forms and models of spasticity, such as those induced by SCI, stroke or  
414 neurodegenerative diseases like Parkinson's disease or multiple sclerosis. Our results  
415 also highlight the significance of astrocyte-selective AAV gene therapy in biomedical  
416 research. However, AAV technologies are still in their infancy regarding clinical trials  
417 aimed at treating CNS disorders, and optimizing delivery methods to enhance both  
418 safety and efficacy<sup>75</sup> are necessary.

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## 429 **Figure Legends**

430 **Figure 1. Lumbar astrocytes display morphological changes nearby**  
431 **motoneurons (MNs) after thoracic spinal cord injury (SCI).** (A) Confocal images  
432 showing endogenous GFP (top) and immunofluorescent staining with GFAP (middle),  
433 and Hoechst (bottom) in the ventro-lateral lumbar cord from control (CTL) Aldh1l1-  
434 eGFP (left) or SCI Aldh1L1-eGFP (right) mice. Images at different magnifications (left  
435 to right) depict the ventral section (scale bar: 120  $\mu$ m), an intermediate view of the  
436 motoneuron (MN) pool (scale bar: 40  $\mu$ m), and a high magnification of a single  
437 astrocyte near a MN (scale bar: 10  $\mu$ m). (B) 3D reconstruction (using GFAP signal) of  
438 a single astrocyte (purple) from A (left : CTL mouse, right : SCI mouse) near MNs,  
439 showing only nuclei of the astrocyte and adjacent MNs (scale bar: 10  $\mu$ m). (C-D)  
440 Quantification of key morphological parameters of GFAP (+) astrocytes from Aldh1L1-  
441 eGFP (+) mice, including primary process length, sum of intercepts, and ramification  
442 index (C), and the projected area from a single astrocyte (D), in control (black) and SCI  
443 (red) mice (CTL: n = 4 mice, n = 20 slices, n = 76 astrocytes; SCI: n = 4 mice, n = 20  
444 slices, n = 76 astrocytes). Data: mean  $\pm$  S.E.M. \*\*\*P < 0.001, Mann-Whitney test for E.  
445 See also Figures S1.

446 **Figure 2. Lumbar astrocytes exhibit functional alterations after SCI.** (A) Bright  
447 field image of a lumbar slice (L4) from an Aldh1L1-eGFP mouse under IR-DIC. Inset:  
448 High magnification of the outlined region overlaid with GFP signal. White arrows: GFP+  
449 astrocytes. The recording pipette is black, filled in gray. Scale bar, 30  $\mu$ m. (B) Resting  
450 membrane potential (RMP), input resistance, and mean conductance at -140mV of  
451 GFP+ astrocytes from control (CTL, black) and SCI (red) mice (CTL: n=7 mice, 61  
452 astrocytes; SCI: n=7 mice, 60 astrocytes for RMP; CTL: n=5 mice, 40 astrocytes; SCI:



453 n=5 mice, 25 astrocytes for resistance and conductance). **(C)** Macroscopic currents of  
454 GFP+ astrocytes clamped at RMP in response to voltage steps (-140 to +40mV) from  
455 CTL (black) and SCI (red) mice. **(D)** Quantification of macroscopic currents as a  
456 function of voltage steps. **(E)** Peak amplitude of macroscopic current at -140mV (CTL:  
457 n=5 mice, 23 astrocytes; SCI: n=5 mice, 25 astrocytes). **(F)** Experimental design for  
458 viral infection at birth followed by Ca<sup>2+</sup> imaging (GCaMP6f) two to three weeks later.  
459 **(G)** Astrocyte segmentation for sub-cellular compartment analysis (scale bar: 10 μm).  
460 **(H)** Heatmaps of spontaneous astrocytic processes ROIs transients (CTL: n=182  
461 ROIs, 7 mice; SCI: n=235 ROIs, 5 mice). Color code: fluorescence changes ( $\Delta F/F$ )  
462 (scale bar: 30 s). **(I)** Percentage of active versus silent astrocytic processes (CTL:  
463 n=182 ROIs, 7 mice; SCI: n=235 ROIs, 5 mice). **(J)** Examples of spontaneous  
464 GCaMP6f Ca<sup>2+</sup> transients in astrocyte processes from CTL (left) and SCI (right) mice.  
465 **(K)** Quantification of the spontaneous GCaMP6f Ca<sup>2+</sup> transients amplitude, duration,  
466 and frequency in astrocytic processes (CTL: n=83 ROIs, 7 mice; SCI: n=153 ROIs, 5  
467 mice). Data: mean  $\pm$  S.E.M. ns: not significant, \*\*P < 0.01, \*\*\*P < 0.001, Mann-Whitney  
468 test for B, E, and K. Fisher's test for I. See also Figure S2.

469

470 **Figure 3. Simultaneous two-photon Ca<sup>2+</sup> imaging of astrocytic and neuronal**  
471 **signals reveals increased glutamate-evoked Ca<sup>2+</sup> transients after SCI. (A-B)**

472 Fluorescence images of the ventro-lateral lumbar slice showing astrocytic GCaMP6f  
473 (A) and neuronal jRGECO1a (B) Ca<sup>2+</sup> signals from CTL (top left) and SCI (bottom left)  
474 mice. Pseudocolor images display Ca<sup>2+</sup> transients before (middle) and after (right) L-  
475 glutamate (1mM) bath application in CTL (top) and SCI (bottom) mice. Color changes  
476 indicate pixel intensity variations. **(C)** Heatmaps of glutamate-evoked GCaMP6f Ca<sup>2+</sup>  
477 transients in astrocytic processes from CTL (left) and SCI (right) mice, with color

478 representing fluorescence changes ( $\Delta F/F$ ). Scale bar: 30 s. **(D)** Examples of  
479 glutamate-evoked GCaMP6f  $Ca^{2+}$  transients in astrocyte processes from CTL (left) and  
480 SCI (right) mice. **(E)** Quantification of astrocytic area under the curve (AUC) of  
481 glutamate-evoked GCaMP6f  $Ca^{2+}$  transients in CTL (black) and SCI (red) mice (CTL:  
482  $n=121$  ROIs, 7 mice; SCI:  $n=95$  ROIs, 5 mice). **(F)** Heatmaps of glutamate-evoked  
483 jRGECO1a  $Ca^{2+}$  signals in neuronal soma from CTL (left) and SCI (right) mice, with  
484 color representing fluorescence changes ( $\Delta F/F$ ). Scale bar: 30 s. **(G)** Examples of  
485 glutamate-evoked jRGECO1a  $Ca^{2+}$  signals in neuronal soma from CTL (left) and SCI  
486 (right) mice. **(H)** Quantification of neuronal AUC of glutamate-evoked jRGECO1a  $Ca^{2+}$   
487 transients in neuronal soma from CTL (black) and SCI (red) mice (CTL:  $n=54$  ROIs, 6  
488 mice; SCI:  $n=57$  ROIs, 5 mice). **(I)** Quantification of the co-active neurons (%) in  
489 function of the time upon L-glutamate exposure (CTL:  $n= 6$  mice; SCI:  $n= 5$  mice). **(J)**  
490 Quantification of the co-active astrocyte processes (%) in function of the time upon L-  
491 glutamate exposure (CTL:  $n= 7$  mice; SCI:  $n= 5$  mice). Data: mean  $\pm$  S.E.M. \*\* $P < 0.01$ ,  
492 \*\*\* $P < 0.001$ , Mann-Whitney test for E and H. Two-way ANOVA with Sidak's multiple  
493 comparison test for I and J. See also Figure S2.

494

495 **Figure 4. Astrocytes display increased NBCe1 levels and undergo prominent**  
496 **glutamate-evoked acidosis after SCI. (A)** NBCe1 pseudo-gel images from capillary  
497 western blot of lumbar segment from CTL (left, black) and SCI (right, red) mice. **(B)**  
498 Group mean quantification of the NBCe1 band (~140 kDa) normalized to CTL group  
499 (CTL:  $n=4$  mice; SCI:  $n=7$  mice). **(C)** Two-photon fluorescence images of lumbar cord  
500 showing identified astrocytes (tdTomato) and cells loaded with pH-sensitive BCECF.  
501 Scale bar: 50  $\mu m$ ; inset: BCECF-loaded astrocytes (white arrows). Scale bar : 15  $\mu m$ .  
502 **(D)** Pseudocolor images of BCECF signals in tdTomato(+) astrocytes before (left) and

503 after 40s of L-glutamate application (right). ROI#1 shows increased pixel intensity,  
504 while ROIs #2 and #3 show decreased signal intensity in response to L-glutamate. **(E)**  
505 BCECF signal changes in response to L-glutamate in astrocytic ROIs from the same  
506 FOV in (D), showing alkalinization (top) or acidification (bottom). **(F)** Percentage of  
507 alkalinizing and acidifying responses to glutamate in astrocytes from CTL (top, black)  
508 and SCI (bottom, red) mice (CTL: n=263 ROIs, 4 mice; SCI: n=172 ROIs, 4 mice). **(G)**  
509 (Top) Average astrocyte acidic responses during L-glutamate exposure in aCSF  
510 showing enhanced acidosis post-SCI; (Bottom) Reduced response with NBCE1  
511 blocker, S0859 (100  $\mu$ M). **(H)** Violin plots of maximum BCECF acidic changes during  
512 L-glutamate exposure in CTL (left, black) and SCI (right, red) mice without (left) or with  
513 (right) S0859 (CTL: n=158 ROIs, 4 mice; SCI: n=139 ROIs, 4 mice; with S0859, CTL:  
514 n=64 ROIs, 4 mice; SCI: n=32 ROIs, 3 mice). Data: mean  $\pm$  S.E.M, hatched line in  
515 violin plots are median. ns: not significant, \*P < 0.05, \*\*\*P < 0.001. Mann-Whitney test  
516 for B; Kruskal-Wallis one-way ANOVA followed by Dunn's post-hoc test for H; Fisher's  
517 test for F. See also Figure S3.

518

519 **Figure 5. Increased NBCE1-mediated astrocytic acidosis following SCI impairs**  
520 **Kir4.1 function (A)** Confocal images showing endogenous GFP (A1), and  
521 immunofluorescent staining for Kir4.1 (A2), NeuN (A3), and merged image (A4) in the  
522 ventro-lateral lumbar cord of an Aldh1L1-eGFP mouse. Scale bar: 120  $\mu$ m. Higher  
523 magnification (middle) shows astrocytes (A1') expressing Kir4.1 (A2') among neurons  
524 (A3'). Scale bar: 50  $\mu$ m. Insets: close-up of astrocytes (A1'') expressing Kir4.1 (A2'')  
525 enwrapping a large ventro-lateral neuron, likely motoneurons (A3''). Scale bar: 10  $\mu$ m.  
526 GM: gray matter; WM: white matter. **(B)** Superimposed Ba<sup>2+</sup>-sensitive currents (top)  
527 from GFP+ astrocytes clamped at RMP in response to voltage pulses (bottom) in CTL

528 mice with classic (pH=7.4, left) and acidic (pH=6.9, right) intracellular solution. **(C)**  
529 Superimposed Ba<sup>2+</sup>-sensitive currents (top) from a GFP+ astrocyte at RMP with  
530 voltage pulses (bottom) in an SCI mouse without (left) and with (right) NBCe1 blocker,  
531 S0859. **(D)** I/V plots of Ba<sup>2+</sup>-sensitive currents in CTL (black), CTL acidosis (yellow),  
532 SCI (red), and SCI + S0859 (blue). **(E)** Peak amplitude at -140 mV from Ba<sup>2+</sup>-sensitive  
533 currents (CTL: n=23 astrocytes, 5 mice; CTL pH acidosis: n=14 astrocytes, 4 mice;  
534 SCI: n=25 astrocytes, 5 mice; SCI + S0859: n=18 astrocytes, 5 mice). **(F)** Experimental  
535 design diagram for recording K<sup>+</sup>-inward currents in astrocytes. **(G)** Typical K<sup>+</sup>-inward  
536 current traces following a K<sup>+</sup> puff (12mM) in astrocytes from CTL mice without (black)  
537 and with (yellow) acidic solution, and SCI mice without (red) and with (blue) S0859. **(H)**  
538 Peak amplitude of K<sup>+</sup> puff-mediated currents (CTL: n=14 astrocytes, 5 mice; CTL pH  
539 acidosis: n=12 astrocytes, 4 mice; SCI: n=14 astrocytes, 5 mice; SCI + S0859: n=12  
540 astrocytes, 4 mice). Data: mean ± S.E.M. ns: not significant, \*P < 0.05, \*\*P < 0.01, \*\*\*P  
541 < 0.001, Kruskal-Wallis one-way ANOVA followed by Dunn's post-hoc test for E and  
542 H. See also Figure S4.

543

544 **Figure 6. Impaired K<sup>+</sup> homeostasis mediates MN hyperexcitability after SCI (A)**  
545 Schematic of experimental procedure. **(B)** Extracellular potassium concentration ([K<sup>+</sup>]<sub>o</sub>)  
546 increase following glutamate puff in the ventro-lateral horn of the lumbar cord from  
547 control (black) and SCI (red) mice. **(C)** Quantification of peak amplitude (left), area  
548 under the curve (AUC) (middle left), rise slope (middle right), and decay time (right) of  
549 [K<sup>+</sup>]<sub>o</sub> variation mediated by glutamate (CTL: n=16 slices, 7 mice; SCI: n=15 slices, 5  
550 mice). **(D)** High magnification bright field image of an acute lumbar slice from a control  
551 mouse showing a pool of motoneurons (MNs) under IR-DIC. Scale bar : 40µm. **(E)**  
552 Illustration (left) and quantification (right) of reversible MN membrane depolarization

553 mediated by  $[K^+]_o = 6\text{mM}$  (green) (n=12 neurons, 4 mice). **(F)** Quantification of  
554 rheobase before (black) and during (green)  $[K^+]_o = 6\text{mM}$ . **(G)** Voltage traces from MNs  
555 recorded in response to incremented current steps before (black) and during (green)  
556 bath perfusion of  $[K^+]_o = 6\text{mM}$ . **(H)** Quantification of the firing gain before (black) and  
557 during (green)  $[K^+]_o = 6\text{mM}$  (n=11 neurons, 4 mice). **(I)** Superimposed outward  $K^+$   
558 currents in presence of TTX ( $1\mu\text{M}$ ) and TEA ( $10\text{mM}$ ) elicited by a depolarizing step  
559 ( $60\text{mV}$ ) before (black) and during (green)  $[K^+]_o = 6\text{mM}$  (n=8 neurons, 3 mice). Data:  
560 mean  $\pm$  S.E.M. ns: not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , Mann-Whitney  
561 test for C, Wilcoxon test for E, F, H, and I.

562

563 **Figure 7. Kir4.1 gain of function in astrocytes reduces spastic-like symptoms in**  
564 **SCI mice (A)** Experimental design for AAV targeting astrocyte therapy and  
565 *gastrocnemius* EMG recordings. **(B)** Typical *gastrocnemius* spontaneous EMG traces  
566 from SCI mice infected with control-AAV (grey) or Kir4.1-AAV (green). **(C)**  
567 Quantification of spontaneous muscular event frequency (left) and duration (right) at  
568 three different ages in SCI mice injected with control (grey) or Kir4.1-AAV (green). **(**  
569 **(D)** Example of *gastrocnemius* EMGs following tail pinch stimulation from SCI mice  
570 infected with control-AAV (left, grey) or Kir4.1-AAV (right, green). **(E)** Quantification of  
571 tail pinch-evoked muscular event duration at different ages in SCI mice injected with  
572 control-AAV (grey) and Kir4.1-AAV (green). For (C-E), SCI + control-AAV: n=7 mice;  
573 SCI + Kir4.1-AAV: n=7 mice. **(F)** Example of DeepLabCut tracking of joint markers on  
574 hindlimbs. Colored circles show digital markers for analysis. **(G)** Quantification of  
575 spontaneous hindlimb motion event frequency (left) and acceleration (right) at different  
576 ages in SCI mice injected with control-AAV (grey) or Kir4.1-AAV (green). **(H)**  
577 Quantification of ankle angle variation (left) and hindlimb hyperextension duration

578 (right) triggered by tail-pinch stimulation at different ages in SCI mice injected with  
579 control-AAV (grey) or Kir4.1-AAV (green). For (G-H), SCI + control-AAV: n=10 mice;  
580 SCI + Kir4.1-AAV: n=15 mice. Data: mean  $\pm$  S.E.M. ns: not significant, \*P < 0.05, \*\*P  
581 < 0.01, \*\*\*P < 0.001, Two-way ANOVA, Sidak's multiple comparison test. See also  
582 Figures S5 and S6.

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## 610 **MATERIALS AND METHODS**

### 611 Experimental model

#### 612 Mice and ethical standards

613 CD1 mice of either sex (P12-P18 for patch-clamp recording, P13-P20 for two-photon  
614 Ca<sup>2+</sup> imaging, P10, P15 and P20 for EMG recordings and DeepLabCut procedure)  
615 were housed under a 12h light/dark cycle in a temperature-controlled area with *ad*  
616 *libitum* access to water and food. *Aldh1L1*-eGFP mice (CD1 background) expressing  
617 GFP in astrocytes were kindly provided by Nathalie Rouach (Collège de France,  
618 CNRS, INSERM, Labex Memolife, Université PSL, Paris, France). Animals from  
619 different litters were used for each experiment. Sample sizes (number of spinal cords  
620 and/or cells) for each experiment are indicated in figure legends. All animal care and  
621 use were conformed to the French regulations and approved by the local ethics  
622 committee (Comité d’Ethique en experimentation animale CE n°071 (INT-Marseille, Nb  
623 B 13 014 04), APAFIS authorizations: Nb 17485-2018110819197361 and Nb 44572-  
624 2023072115506121).

#### 625 Experimental procedures

##### 626 Spinal cord injury (SCI)

627 The spinal cord transection was performed on P0-P1 neonatal mice, including both  
628 wild-type and *Aldh1L1*-eGFP mice. The animals were anesthetized *via* hypothermia  
629 and received a subcutaneous injection of buprenorphine (0.025 mg/kg; Vetergesic,  
630 CEVA Santé Animale, France). After a midline skin incision, a laminectomy was  
631 performed to expose lower thoracic segments of the spinal cord. The dura was opened  
632 and the spinal cord was completely transected at the T8-T9 segmental level. The lesion  
633 cavity was filled with sterile absorbable collagen contact hemostat (Pangen, Urgo  
634 Medical, France). One drop (ca 20-30 µl) of the antibiotic amoxicillin (Citramox,  
635 Axience, France) was subcutaneously applied at the incision site. Finally, the wound  
636 was closed with 6-0 absorbable suture (Z1032H, Ethicon, NJ) and covered with  
637 adhesive suture (Steri-Strip, R1546, 3M Health Care, MN). Animals were kept in a  
638 warm and wet chamber for 2 hr in cotton-wool swab impregnated with their mother  
639 smell before they returned to the home cage with their mother. Sham animals were  
640 submitted to all procedures except the laminectomy and the spinal cord transection.



641 Adeno-associated virus (AAV) constructs

642 AAV2/5-gfaABC1D-cyto-GCaMP6f (52925-AAV5, titer  $\geq 1 \cdot 10^{13}$  vg/mL), AAV2/9-  
643 Syn.NES-jRGECO1a.WPRE.SV40 (100854-AAV9, titer  $\geq 1 \cdot 10^{13}$  vg/mL), AAV2/5-  
644 gfaABC1D-tdTomato (44332-AAV5, titer  $\geq 7 \cdot 10^{12}$  vg/mL) were sourced from Addgene  
645 (<https://www.addgene.org/>). AAV2/9-GFAP-eGFP-Cre (titer  $\geq 2.5 \cdot 10^{10}$  vg/mL)  
646 AAV2/9-gfaABC1D-eGFP-Kir4.1 (titer  $\geq 2.3 \cdot 10^{11}$  vg/mL) were kindly provided by the  
647 Hu lab<sup>76</sup>.

648

649 Intrathecal AAV delivery

650 A minimally invasive technique was employed to micro-inject AAV vectors through the  
651 intervertebral space. Briefly, in cryoanesthetized pups, a beveled microcapillary (30-  
652 60  $\mu\text{m}$  in diameter) preloaded with the AAV particles was lowered to reaches the  
653 subarachnoidal space at the L3-L4 segments. A total volume of 2  $\mu\text{L}$  per animal was  
654 then progressively injected (1  $\mu\text{L}/5\text{s}$ ).

655

656 Lumbar slice preparation and artificial cerebro-spinal fluid (aCSF) solution

657 Mice were anaesthetized with intraperitoneal injection of a mixture of  
658 ketamine/xylazine (100mg/kg and 10 mg/kg, respectively). They were then  
659 decapitated, eviscerated and the spinal cord removed by laminectomy, and placed in  
660 a Sylgard-lined petri dish with ice-cold (1-2°) aCSF containing (in mM): sucrose (252),  
661 KCl (3),  $\text{NaH}_2\text{PO}_4$  (1.25),  $\text{MgSO}_4$  (4),  $\text{CaCl}_2$  (0.2),  $\text{NaHCO}_3$  (26), D-glucose (25), pH  
662 7.4. The lumbar spinal cord was mounted on an agar block, embedded in a 4% agarose  
663 solution, quickly cooled, and then sliced into 325  $\mu\text{m}$  sections through the L4–L5  
664 lumbar segments using a vibrating microtome (Leica, VT1000S). Slices were  
665 immediately transferred into the holding chamber filled with bubbled (95%  $\text{O}_2$  and 5%  
666  $\text{CO}_2$ ) standard aCSF composed of (in mM): NaCl (120), KCl (3),  $\text{NaH}_2\text{PO}_4$  (1.25),  
667  $\text{MgSO}_4$  (1.3),  $\text{CaCl}_2$  (1.2),  $\text{NaHCO}_3$  (25), D-glucose (20), pH 7.4, 30-32°C. After a 30-  
668 60 min resting period, individual slices were transferred to a recording chamber  
669 continuously perfused with standard aCSF heated to 32-34°C.

670



671 Ex vivo electrophysiological recordings

672 Aldh1L1-eGFP positive astrocytes were visualized in the ventrolateral region of lamina  
673 IX in L4-L5 horizontal slices using a Nikon FN1 microscope with appropriate filters.  
674 Whole-cell patch-clamp recordings of GFP(+) astrocytes and surrounding large  
675 motoneurons (MNs) (soma area  $>800 \mu\text{m}^2$ )<sup>72</sup> were performed using a Multiclamp 700B  
676 amplifier (Molecular Devices) with electrodes pulled from borosilicate glass capillaries  
677 (1.5 mm OD, 1.12 mm ID; World Precision Instruments) on a Sutter P-97 puller (Sutter  
678 Instruments Company). For MN recordings, electrodes (2-4 M $\Omega$ ) were filled with an  
679 intracellular solution containing (in mM): K<sup>+</sup>-gluconate (140), NaCl (5), MgCl<sub>2</sub> (2),  
680 HEPES (10), EGTA (0.5), ATP (2), GTP (0.4), pH 7.3. For GFP+ astrocyte recordings,  
681 electrodes (6-8 M $\Omega$ ) were filled with an intracellular solution containing (in mM): K<sup>+</sup>-  
682 gluconate (105), NaCl (10), KCl (20), MgCl<sub>2</sub> (0.15), HEPES (10), EGTA (0.5), ATP (4),  
683 GTP (0.3), pH 7.3. In some experiments, the intracellular pH was lowered to 6.9 by the  
684 addition of HCl. Patch clamp recordings were made using a Multiclamp 700B amplifier  
685 driven by PClamp 10 software (Molecular Devices). Recordings were digitized on-line  
686 and filtered at 10 kHz (Digidata 1550B, Molecular Devices). Pipette and neuronal  
687 capacitive currents were canceled and, after breakthrough, the access resistance was  
688 compensated. All experiments were designed to gather data within a stable period (i.e.,  
689 at least 1-2 min after establishing whole-cell access).

690

691 Ex vivo two-photon Ca<sup>2+</sup> imaging

692 Two-photon fluorescence measurements were obtained with a dual-scanhead two-  
693 photon microscope (FemtoS-Dual, Femtonics Ltd, Budapest, Hungary) and made  
694 using an Olympus XLUMPlanFLN 20X, 1.00 NA objective (Olympus America, Melville,  
695 NY) or a Nikon LWD 16X/0.80W objective (Nikon Instruments Inc. Melville, NY). For  
696 two-photon Ca<sup>2+</sup> imaging, excitation of GCaMP6f and jRGECO1a was simultaneously  
697 evoked with a femtosecond pulsed laser (Chameleon Ultra II; Coherent, Santa Clara,  
698 CA) tuned to 960 nm. Recordings were performed at ~32–34 °C in standard aCSF  
699 saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (pH 7.4). The microscope system was controlled by  
700 MESc acquisition software (<https://femtonics.eu/femtosmart-software/>, Femtonics Ltd,  
701 Budapest, Hungary). A single acquisition plane was selected and full-frame imaging  
702 was started in a resonant scanning mode at 30.5202 Hz. Scan parameters were

703 [pixels/line × lines/frame (frame rate in Hz)]: [512 × 519 (30.5202)]. Scanning area was  
704 468 μm \* 474 μm. This microscope was equipped with two detection channels for  
705 fluorescence imaging.

706

#### 707 Ex vivo two-photon imaging of intracellular pH (pH<sub>i</sub>) in astrocytes

708 Lumbar slices were incubated in standard aCSF containing BCECF (5 μM) and  
709 Pluronic® F-127 (0.02%) for ≈ 45 min followed by a thorough washing out to allow de-  
710 esterification of the dye. Two-photon fluorescence measurements were obtained with  
711 a dual-scanhead two-photon microscope (FemtoS-Dual, Femtonics Ltd, Budapest,  
712 Hungary) and made using an Olympus XLUMPlanFLN 20X, 1.00 NA objective  
713 (Olympus America, Melville, NY) or a Nikon LWD 16X/0.80W objective (Nikon  
714 Instruments Inc. Melville, NY). For two-photon BCECF signal imaging, excitation was  
715 simultaneously evoked with a femtosecond pulsed laser (Chameleon Ultra II;  
716 Coherent, Santa Clara, CA) tuned to 820 nm. Recordings were performed at  
717 ~32–34 °C in aCSF saturated with 95% O<sub>2</sub>/5% CO<sub>2</sub> (pH 7.4). Astroglial loading of  
718 BCECF was confirmed by analysing the characteristic morphology of the BCECF-  
719 stained cells featuring astrocytic phenotype from mice injected at birth with AAV  
720 AAV2/5-gfaABC1D-tdTomato. Changes in pH<sub>i</sub> are expressed as changes in BCECF  
721 fluorescence at the maximum of the fluorescent signal over the baseline ( $\Delta F/F_0$ ). The  
722 microscope system was controlled by MESc acquisition software  
723 (<https://femtonics.eu/femtosmart-software/>, Femtonics Ltd, Budapest, Hungary). A  
724 single acquisition plane was selected and full-frame imaging was started in a resonant  
725 scanning mode at 30.5202 Hz. Scan parameters were [pixels/line × lines/frame (frame  
726 rate in Hz)]: [512 × 519 (30.5202)]. Scanning area was 468 μm \* 474 μm. This  
727 microscope was equipped with two detection channels for fluorescence imaging.

728

#### 729 Puff-application of extracellular K<sup>+</sup>

730 To measure the astrocytes' potassium (K<sup>+</sup>) uptake capacity, a high extracellular  
731 potassium concentration ([K<sup>+</sup>]<sub>o</sub>, 12mM) was added to standard aCSF and delivered via  
732 a pipette similar to the recording pipette, controlled by a Picospritzer (Picospritzer II,

733 General Valve Corporation) set at 10 psi. The electrode was positioned in the ventro-  
734 lateral part (lamina IX) of a lumbar slice, within ~60  $\mu\text{m}$  of a GFP+ astrocyte soma.

#### 735 Measurement of extracellular $\text{K}^+$ concentration

736  $\text{K}^+$ -sensitive microelectrodes were made from thin-walled borosilicate theta tubes  
737 (GC150T-10, Harvard Apparatus, MA) following the procedure described in<sup>77</sup>. Briefly,  
738 the theta tubes were washed in 70% ethanol for 1 hour, then for 1 hour in ultra-pure  
739 water, and dried in the oven overnight at 60°C. The double-channel pipettes were  
740 pulled to obtain a tip diameter of 3-5  $\mu\text{m}$ . One channel was filled with 150 mM NaCl  
741 and the second one with 100 mM KCl. The inside of the tip of the KCl-containing  
742 channel was silanized using Silanization Solution I (85126, Merck-Sigma Aldrich) and  
743 then filled with Potassium Ionophore I Cocktail A (99311, Merck- Sigma Aldrich). The  
744 electrode tip was immersed in a 100 mM KCl solution for 3 hours or longer for  
745 stabilization. Before each recording session, the electrode was calibrated using ACSF  
746 solutions enriched with gradually decreasing KCl concentrations: 20 mM, 10 mM, 6  
747 mM, 3 mM, 1.25 mM, 0.6 mM, 0.3 mM. The  $\text{K}^+$  sensor was accepted for the experiment  
748 if it generated a stable potential at each  $\text{K}^+$  concentration and displayed a >540 mV  
749 increase at 30 mM  $\text{K}^+$  drop.

750 The extracellular potassium concentration ( $[\text{K}^+]_o$ ) was measured in acute lumbar slices  
751 from control and spinal cord injury (SCI) mice. Experiments were conducted in  
752 standard aCSF, saturated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  (pH 7.4) and heated to 32-34°C. L-  
753 glutamate (1 mM, 5 s, at 10 psi) was puffed using a glass pipette connected to a  
754 picospritzer (Picospritzer II, General Valve Corporation). Both the puff electrode and  
755 the  $\text{K}^+$ -sensitive microelectrode were aligned and positioned in the ventro-lateral part  
756 (lamina IX) of the lumbar slice, within ~50  $\mu\text{m}$  of each other in the z axis. Basal  $[\text{K}^+]_o$   
757 was measured after proper positioning and stabilization of the  $\text{K}^+$ -sensitive  
758 microelectrode in the lumbar slice.

759 Data acquisition was performed in current-clamp mode using a Multiclamp 700B  
760 amplifier (Molecular Devices), sampled at 10 kHz, filtered at 4 kHz, and digitized with  
761 a Digidata 1550B (Molecular Devices). The peak amplitude, area under the curve  
762 (AUC) of  $[\text{K}^+]_o$  transients, the rise slope from glutamate puff onset to peak amplitude,  
763 and decay time from peak amplitude to baseline recovery were measured.

764 Pharmacological compounds and dyes

765 All solutions were oxygenated with 95% O<sub>2</sub>/5% CO<sub>2</sub>. Barium chloride dihydrate (Ba<sup>2+</sup>,  
766 100µM; 217565), Pluronic® F-127 (0.02%; P2443), DMSO (0.1%; D4540), L-glutamic  
767 acid (L-glutamate, 1 mM; G1251) and Sodium Propionate (5mM; P1880) were  
768 obtained from Merck Sigma-Aldrich. Tetrodotoxin (TTX, 0.5-1µM; 1078) from Tocris  
769 Bioscience. S0859 (50-100 µM, M5069) from Abmole Europe Branch (Forlab). 2',7'-  
770 Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetomxymethyl Ester (BCECF-  
771 AM, 5 µM) from Invitrogen. All drugs were dissolved in water and added to the standard  
772 aCSF except BCECF-AM incubated in Pluronic® F-127 (0.02%) and DMSO (0.2%).

773

774 Immunohistochemistry

775 Spinal cords of 14-16 days-old mice were dissected out and fixed for 5-6 h in 4%  
776 paraformaldehyde (PFA), then rinsed in phosphate buffered saline (PBS) and  
777 cryoprotected overnight in 20% sucrose at 4°C. Spinal cords were frozen in OCT  
778 medium (Tissue Tek) and 30 µm cryosections were collected from the L4-L5 segments.  
779 After having been washed in PBS 3x5 min, the slides were incubated for 1 h in a  
780 blocking solution (BSA 1%, Normal Donkey Serum 3% in PBS) with 0.2% triton X-100  
781 and for 24 h at 4 °C in a humidified chamber with the following primary antibodies  
782 diluted in the blocking solution with triton x-100: rabbit anti-Kir4.1 (1/1000; APC-035,  
783 Alomone, Israel), mouse anti-NeuN (1/1000; MAB377, Merck Millipore, MA), rabbit  
784 anti-GFAP (1/1000; Z0334, Dako, Agilent, CA) and/or goat anti-C3d (1/40; AF2655,  
785 R&D Systems, MN). For STAT-3 staining, slices were pretreated with a drop of MetOH  
786 100% (34860-1L-R; Merck Sigma-Aldrich, MA) for 10 minutes at -20°C. After 3 washes  
787 of 5 min in PBS, they were incubated for 1h in a blocking solution (Normal Donkey  
788 Serum 5% in PBS 0.1M) with 0.3% triton X-100. The primary antibody anti-STAT3  
789 (1/300; 8768, Cell Signaling Technology, MA) was incubated with Cell signal blocking  
790 solution (8112, Cell Signaling Technology, MA) for 48 h at 4°C. Slides were then  
791 washed 3x5 min in PBS and incubated for 2 h with the appropriate secondary  
792 antibodies diluted in the blocking solution: Alexa Fluor® Plus 555- donkey anti-rabbit  
793 (1/400; A32794, Invitrogen, MA), Cy5- donkey anti-mouse (1/400; 715-175-151,  
794 Jackson ImmunoResearch, PA) and/or Alexa Fluor® Plus 555- donkey anti-goat  
795 (1/400; A32816, Invitrogen, MA). After 3 washes of 5 min in PBS, slides were incubated

796 with Hoechst 33342 (2  $\mu$ M in PBS; 62249, Thermo Fisher Scientific, MA) for 5 min and  
797 washed again 3x5 min before being mounted with a homemade gelatinous aqueous  
798 medium. Images were acquired using a confocal microscope (LSM700, Zeiss)  
799 equipped with either a 20x air objective, a 40x oil objective or a 63x oil objective and  
800 processed with the Zen software (Zeiss).

801

## 802 Protein analysis by capillary Western Blot

803 The lumbar parts of the spinal cords were dissected in aCSF at 4°C and conserved at  
804 -80°C until protein extraction. Tissues were homogenized in ice-cold lysis buffer (250  
805 mM sucrose, 3.9 mM Tris pH 7.5, 10 mM iodoacetamide) supplemented with protease  
806 inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, 11836170001,  
807 Roche Diagnostics, Germany). Unsolubilized material was pelleted by centrifugation  
808 at 7,000 x g for 5 min at 4°C and discarded. The supernatants were subjected to an  
809 additional centrifugation step at 19,000 x g for 70 min at 4°C. The resulting pellets  
810 corresponding to the membrane-enriched fraction were resuspended in ice-cold lysis  
811 buffer (PBS 1X, IGEPAL® CA-630 1%, SDS 0.1%) supplemented with protease  
812 inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, 11836170001,  
813 Roche Diagnostics, Germany). Protein concentrations were determined using the  
814 Pierce™ BCA Protein Assay Kit (23227, Thermo Fisher Scientific, MA). Kir4.1 and  
815 NBCe1 expressions were then analyzed using the 12-230 kDa and the 66-440 kDa  
816 separation modules respectively (SM-W004 and SM-W008, ProteinSimple, Bio-  
817 Techne, MN) on an automated capillary western blotting system ('Jess', ProteinSimple,  
818 Bio-Techne, MN) according to the manufacturer's protocol with small modifications.  
819 Samples were not implemented with DTT and they were not submitted to heat  
820 denaturation in order to keep proteins in a more 'native' form. Total protein  
821 concentrations of 0.1 mg/mL and 0.5 mg/mL were used for Kir4.1 and NBCe1  
822 respectively. Samples were probed with a rabbit polyclonal Kir4.1 antibody (1:60; APC-  
823 035, Alomone, Israel) or a rabbit polyclonal NBCe1 antibody (1:90; ANT-075, Alomone,  
824 Israel) and revealed with the appropriate detection module (DM-001, ProteinSimple,  
825 Bio-Techne, MN). Loaded samples were normalized to their own total protein content  
826 using the 'total protein detection module' (DM-TP01, ProteinSimple, Bio-Techne, MN).

827

828 Assesment of electromyography (EMG) recordings

829 Mice were tested at 10, 15, and 20 days post-SCI, when signs of spastic motor  
830 behaviors, such as excessive involuntary twitches/movements and exaggerated  
831 reflexes, were evident. For electrophysiological assessment of spasticity, a stainless  
832 steel needle electrode was transcutaneously inserted into the lateral gastrocnemius  
833 muscle (ankle extensor). Two reference electrodes were placed subcutaneously on  
834 the back and in contact with the patellar tendon. Because mice were spinalized, they  
835 did not feel any pain during this procedure. Following a 30-minute acclimation period  
836 to the recording environment, mice were immobilized with tape. Spontaneous motor  
837 responses were recorded for 15 minutes. Motor responses evoked by tail pinch ( $50.5$   
838  $\pm 5.6$  g,  $0.5 \pm 0.2$  s) were recorded until baseline activity returned. Each mouse  
839 experienced five consecutive sensory stimuli, each separated by a resting period of at  
840 least 30 seconds. The proximal ends of the recording wires were connected to a A-M  
841 Systems Amplifier (Model 1700, Everett, WA). EMG signals were amplified (1000x)  
842 and bandpass filtered (100 Hz to 5 kHz), then sampled at 13.5 kHz (Digidata 1440A,  
843 Molecular Devices). The applied pressure (measured in grams) to the distal third of the  
844 tail was recorded by a miniature pressure sensor (#FS1901-000-0500-G, Farnell)  
845 placed between the thumb and the tail, and monitored in real-time.

846

847 Assessment of DeepLabCut video acquisition

848 Animals were tested at P10, P15, and P20, when signs of a spastic motor phenotype,  
849 such as excessive involuntary hindlimb twitches and exaggerated reflexes, were  
850 visible.

851 **Assessment of mice hindlimb spontaneous activity:** Animals were removed from  
852 their home cages, weighted, and individually subjected to a single 20-second video  
853 recording in a standardized homemade setup. Video acquisition was performed at 240  
854 frames per second (FPS) to allow complete visualization of hindlimb spasms from a  
855 lateral view, at a distance of 10 cm from the mouse.

856 **Assessment of mice hindlimb induced activity:** For the behavioral assessment of  
857 induced activity, the same procedure was applied as previously described. Induced  
858 activity was initiated by gently pinching the tail (without causing discomfort or signs of



859 physical pain) with the thumb. Hindlimb motor responses following tail pinching were  
860 recorded by a miniature pressure sensor placed between the thumb and the tail and  
861 monitored online. Each animal underwent three consecutive pinching stimulations,  
862 separated by 10 seconds, through a single video recording

863

## 864 **Experimental design and statistical analysis**

865 No statistical method was used to predetermine sample size. Group measurements  
866 are expressed as means  $\pm$  S.E.M. The Mann-Whitney test was used for comparisons  
867 between two groups, while the Fisher's test was employed to compare percentages.  
868 For comparisons between two conditions, the Wilcoxon matched pairs test was  
869 utilized. One-way or two-way ANOVA test was applied for multiple comparisons.  
870 Normality of the data sets was evaluated for all statistical analyses, and the  
871 significance level was set at  $p < 0.05$ . Statistical analyses were conducted using  
872 GraphPad Prism 9 software. Details of the analyses are provided in each figure legend.

## 873 **Data analysis**

### 874 **Assessment of astrocyte reactivity**

875 (i) ***Morphometric analyses.*** Confocal images at 63X magnification,  
876 immunostained with GFAP antibody in Aldh1L1-eGFP mice, were serially  
877 stacked and maximally projected. The maximal projection image of the  
878 GFAP signal in the ventral horn of the spinal cord was used for analysis. The  
879 maximal projection image of the GFAP signal in the ventral horn of the spinal  
880 cord was used for analysis. The Sholl analysis plugin in ImageJ  
881 automatically drew serial concentric circles at 5  $\mu$ m intervals from the center  
882 of the nucleus (Hoechst signal) to the end of the longest process in each  
883 individual astrocyte. This plugin provided key morphometric parameters  
884 such as the number of intercepts of GFAP processes with each circle, the  
885 ramification index, and the ending radius (longest process). Four different  
886 fields from the left and right parts of the ventral spinal cord were analyzed in  
887 at least five different slices from the same mouse, evaluating all astrocytes  
888 within the field. For illustrative purposes, a few representative astrocytes  
889 were fully reconstructed in 3D using Chimera software.

890 (ii) **Evaluation of C3d positive astrocytes:** Immunofluorescence staining was  
891 analyzed from stacked confocal images (15 steps; Z-step, 1  $\mu\text{m}$ , maximum  
892 intensity stack) acquired with a 40 $\times$  objective on five slices per mouse, with  
893 four different fields on each slice. All Aldh1L1-eGFP positive astrocytes in  
894 the field, including those co-expressing C3d markers (Aldh1L1+ C3d+), were  
895 evaluated.

896 (iii) **Quantification of astrocytic STAT3 nuclear fraction.** To evaluate the  
897 nuclear fraction of the STAT3 signal, we measured the STAT3 signal  
898 intensity within the Hoechst (nuclei) signal. Immunofluorescence staining  
899 was analyzed from stacked confocal images (15 steps; Z-step, 1  $\mu\text{m}$ ,  
900 maximum intensity stack) acquired with a 40 $\times$  objective on five slices per  
901 mouse, with four different fields on each slice. All Aldh1L1-eGFP positive  
902 astrocytes in the field with a clear Hoechst signal were included. Nuclear  
903 masks were manually segmented using the Hoechst signal, applied to the  
904 STAT3 channel, and the mean fluorescence intensity was measured (see  
905 <sup>30</sup>).

#### 906 Analysis of *ex vivo* electrophysiological (Patch-clamp) recordings

907 Electrophysiological data were analyzed offline using Clampfit 10.7 software  
908 (Molecular Devices). To ensure optimum quality of intracellular recordings, several  
909 criteria were established: only cells exhibiting a stable resting membrane potential  
910 (RMP), access resistance with less than 20% variation, and an action potential  
911 amplitude larger than 40 mV under standard aCSF were considered.

912 **Passive Membrane Properties:** Passive membrane properties of astrocytes and  
913 motoneurons (MNs) were measured by determining the largest voltage deflections  
914 induced by small current pulses from the holding potential, avoiding activation of  
915 voltage-sensitive currents. Input resistance was determined by the slope of linear fits  
916 to voltage responses evoked by small positive and negative current injections.

917 **Recording Kir4.1-Currents in GFP (+) Astrocytes.** Voltages were repeatedly  
918 stepped for 2.5s from -140 to 0 mV in 10 mV increments, with astrocytes initially  
919 clamped at their RMP. Approximately 5 minutes after the start of the recording, barium  
920 chloride ( $\text{Ba}^{2+}$ , 100  $\mu\text{M}$ ) was bath-applied to the slice. The current amplitude was



921 measured as the difference between the baseline level before the initial voltage step  
922 and the mean amplitude over a 10 ms window starting 10 ms after the onset of the  
923 hyperpolarizing voltage step. The Ba<sup>2+</sup>-sensitive current was obtained by subtracting  
924 the current after Ba<sup>2+</sup> application from the current before application.

925 **MNs excitability.** For MNs, firing properties were measured from depolarizing current  
926 pulses of varying amplitudes. The rheobase was defined as the minimum step current  
927 intensity required to induce an action potential from the membrane potential held at  
928 RMP. The instantaneous discharge frequency was determined as the inverse of the  
929 interspike interval. The firing gain refers to the ratio between the measured  
930 instantaneous firing frequency (Hz) and the injected current (pA).

### 931 Analysis of two-photon Ca<sup>2+</sup> and BCECF pHi changes imaging

932 The fluorescent time series depicting Ca<sup>2+</sup> and BCECF changes in ex vivo neurons  
933 and/or astrocytes were analyzed using MESc data acquisition software (Femtonics Ltd,  
934 Budapest, Hungary) and the MES curve analyzer tool. Regions of interest (ROIs) for  
935 neurons and astrocytic soma were selected manually, while ROIs for astrocytic  
936 processes and microdomains were selected automatically using a custom script in  
937 ImageJ (Fiji). A signal was declared as a Ca<sup>2+</sup> transient if it exceeded the baseline by  
938 more than twice the baseline noise (standard deviation, SD). We defined active  
939 astrocytes as astrocytes displaying at least one Ca<sup>2+</sup> transient during the recording  
940 session.

941 In Ca<sup>2+</sup> imaging experiments, ROIs included : (i) active soma for neurons (jRGECO1a),  
942 (ii) active soma, processes and microdomains for astrocytes (GCaMP6f). In BCECF  
943 experiments, ROIs corresponded to astrocytic soma double-labeled with Td-tomato  
944 (AAV2/5-gfaABC1D-TdTomato).

945 For the two-photon Ca<sup>2+</sup> imaging analysis, raw fluorescence traces were extracted to  
946 Excel and analyzed using a custom Matlab script. These traces were converted to  
947 show fluorescence changes according to Equation (1):

$$948 \quad \Delta F/F = (F_1 - F_0)/F_0 \quad (1)$$

949 where  $F$  is the fluorescence at any given time, and  $F_0$  is the mean fluorescence value  
950 for the 5 to 10 s range, preceding the bath perfusion of L-Glutamate (1 mM). The signal-  
951 to-noise ratio (SNR) was calculated as:

$$952 \quad \text{SNR} = (\Delta F/F)_{\text{peak}} / \sigma F$$

953 where  $\sigma F$  is the SD of the baseline period. Events were considered as  $\text{Ca}^{2+}$  response  
954 if they exceeded twice the SD of the baseline period. The peak amplitude, duration and  
955 frequency of both spontaneous and glutamate-evoked  $\text{Ca}^{2+}$  transients were analysed  
956 by using a Matlab script.

957 For the two-photon pH<sub>i</sub> imaging analysis, raw fluorescence traces were extracted to  
958 Excel. The proportion of Tdtomato (+) astrocytes showing increased or decreased  
959 fluorescence ( $\Delta F/F$ ) and the peak amplitude of acidic pH<sub>i</sub> responses to L-glutamate  
960 were analyzed from both control and SCI mice.

#### 961 Analysis of *in vivo* electromyography (EMG) recordings

962 Custom-built amplifiers enabled simultaneous online rectification and integration  
963 (100ms time constant) of the raw AC-mode signals. Data analysis was performed  
964 offline with the automatic event detection plugin in Clampfit 11 software. Spontaneous  
965 muscle contractions were identified when the signal envelope exceeded a  
966 predetermined threshold, and the contraction was considered to end when the signal  
967 fell below this threshold. This threshold was adjusted for each signal, typically set at  
968 1.3 times the background noise level. For an event to be classified as a valid muscle  
969 spasm, it needed to remain above the threshold for at least 250 ms. We quantified both  
970 the average and cumulative distribution of the duration and frequency of these muscle  
971 events over a 15 min intervals. For responses triggered by tail stimulation, spasm  
972 duration was measured from the stimulus artefact until the recovery of a stable  
973 baseline. The experimenter was blinded during both the procedure and the data  
974 analysis of the electrophysiological experiments.

975

#### 976 Analysis of mice spastic motor behaviors with DeepLabCut

977 **Analysis of kinematics hindlimbs spontaneous activity.** For hindlimb spasms  
978 analysis, videos captured during experiments were analyzed using the markerless  
979 pose estimation software DeepLabCut 2.2 (DLC)<sup>39</sup> to extract tracks based on digital

980 markers placed on the mice (Figure 7). For tracking DeepLabCut was installed on a  
981 PC equipped with a NVIDIA Ge-Force RTX 3060 12Go graphics card. DeepLabCut  
982 was used to identify four reference points among the mouse body (hip, knee, ankle  
983 and foot) on a side view (Figure 7) for kinematic analysis. The ResNet-50 based neural  
984 network (imgaug augmentation, 500,000 iterations) was used to identify points of  
985 interest on the mice. The training was performed on a set of 800 frames randomly  
986 selected by DeepLabCut from 40 videos representatives of experimentation conditions  
987 (same background, luminosity, distance from the mouse and the camera).

988 Note that two separate training has been performed between the first post-natal and  
989 second post-natal week face to the very fast morpho-physical changes occurring in  
990 mice at this period. Network was trained until it reaches a stable error rate plateau (>  
991 400,000 iterations, 0.0005 error per frame). Next network was evaluated (test error =  
992 2.89 pixels 0,19 mm), and videos analysis was consecutively performed. Raw data  
993 provide by DeepLabCut was extracted and proceeded to collect key parameters such  
994 as the number of events, acceleration of the spasms, maximum angle of the ankle and  
995 the mean ankle angle using custom scripts in Python (see below).

996 (i) *Acceleration*. Sudden increase in the speed of the hindlimb was quantify by  
997 estimating the instantaneous acceleration derived from the instantaneous  
998 speed estimates:

999 
$$at=\Delta vt/\Delta t(\text{cms}^{-2})$$

1000 (ii) *Number of events*. Hindlimbs motion event was considered as ankle angle  
1001 variation of more than 10° degree from the initial position through the entire  
1002 video recording

1003

#### 1004 **Analysis of hindlimb evoked activity.**

1005 (i) *Measure of the ankle angle variation following stimulation*. Angle of the ankle  
1006 was measured using the angle tool of Fiji (ImageJ) before stimulation and  
1007 immediately following the stimulation. Each measure is performed for the 3  
1008 consecutives stimulation for each animal.

1009

1010 (ii) *Measure of spasms duration.* Spasms duration was measured from the end  
1011 of the stimulation until the recovery of a stable hindlimb motion activity (e.g  
1012 no hindlimb twitches) using the free and open source cross-platform  
1013 multimedia player and framework, VLC.

1014

1015 (iii) *Measure of tail pinch force.* Stimulation intensity was recorded by a miniature  
1016 pressure sensor (#FS1901-000-0500-G, Farnell) placed between the thumb  
1017 and the tail, and monitored in real-time. The applied pressure measurements  
1018 were compared between the groups (SCI + control-GFP vs SCI + Kir4.1-  
1019 GFP) and were similar.

1020

1021 **Data Source File:**

1022 [https://github.com/remibos/Data\\_Source\\_File\\_Barbay\\_SCI\\_Kir4.1](https://github.com/remibos/Data_Source_File_Barbay_SCI_Kir4.1)

1023 **Code/software**

1024 MATLAB scripts :

1025 [https://github.com/remibos/Analysis\\_2P.git](https://github.com/remibos/Analysis_2P.git)

1026

1027 Python script :

1028 [https://github.com/remibos/Analysis\\_Spasms\\_DLC](https://github.com/remibos/Analysis_Spasms_DLC)

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## 1314 **Data and code availability**

- 1315 • All data reported in this paper will be shared by the lead contact upon request
- 1316 • All original codes have been deposited at
- 1317 [https://github.com/remibos/Analysis\\_2P.git](https://github.com/remibos/Analysis_2P.git) or
- 1318 [https://github.com/remibos/Analysis\\_Spasms\\_DLC](https://github.com/remibos/Analysis_Spasms_DLC) and are publicly available
- 1319 as of the date of publication.
- 1320 • Any additional information required to reanalyse the data reported in this paper
- 1321 is available from the lead contact upon request

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## 1334 **Author contributions**

1335  
1336 Research design, T.B., E.P. and R.B.; Methodology, T.B., E.P., J.J.R.F., A.I. and R.B.;  
1337 Investigation and data analysis, T.B., E.P., J.J.R.F. and R.B.; Figures design, T.B. and  
1338 R.B.; Consulting/Editing, N.R., F.B.; Writing, T.B. and R.B.; Funding Acquisition, R.B.;  
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1342 **Declaration of interests**

1343  
1344 The authors declare no competing interests.

1346 **Inclusion and diversity**

1347  
1348 We support inclusive, diverse, and equitable conduct of research.

1350 **Supplemental information**

1351 Document S1. Figures S1–S6

1352 **Figure S1:** Increased GFAP staining is associated with increased inflammatory  
1353 markers the lumbar segments after thoracic SCI. Related to Figure 1.

1354 **Figure S2:** Spontaneous and glutamate-evoked  $\text{Ca}^{2+}$  transients in astrocytic soma and  
1355 microdomains after SCI. Related to Figures 2 and 3.

1356 **Figure S3:** Effect of propionic acid on astrocytic intracellular pH monitored with with  
1357 the pH-sensitive probe BCECF. Related to Figure 4.

1358 **Figure S4:** Blockade of Kir4.1 channels affects astrocytic  $\text{K}^+$  uptake. Related to Figure  
1359 5.

1360 **Figure S5:** Efficiency, specificity and diffusion of the viral strategy for boosting  
1361 astrocytic Kir4.1. Related to Figure 7.

1362 Figure S6: Complementary metrics for assessing spasticity using EMG recordings and  
1363 DLC tracking. Related to Figure 7.

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