Two opposite voltage-dependent currents control the unusual early development pattern of embryonic Renshaw cell electrical activity

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Abstract

Renshaw cells (V1^R) are excitable as soon as they reach their final location next to the spinal motoneurons and are functionally heterogeneous. Using multiple experimental approaches, in combination with biophysical modeling and dynamical systems theory, we analyzed, for the first time, the mechanisms underlying the electrophysiological properties of V1R during early embryonic development of the mouse spinal cord locomotor networks (E11.5-E16.5). We found that these interneurons are subdivided into several functional clusters from E11.5 and then display an unexpected transitory involution process during which they lose their ability to sustain tonic firing. We demonstrated that the essential factor controlling the diversity of the discharge pattern of embryonic V1^R is the ratio of a persistent sodium conductance to a delayed rectifier potassium conductance. Taken together, our results reveal how a simple mechanism, based on the synergy of two voltage-dependent conductances that are ubiquitous in neurons, can produce functional diversity in embryonic V1^R and control their early developmental trajectory.

Keywords: development, spinal cord, embryo, Renshaw cell, firing pattern, functional involution, electrophysiology, biophysical modeling.

Introduction

49

50   The development of the central nervous system (CNS) follows complex steps, which

51   depend on genetic and environmental factors and involve interactions between multiple

52   elements of the neural tissue. Remarkably, emergent neurons begin to synchronize soon

53   after the onset of synapse formation, generating long episodes of low frequency (<0.01 Hz)

54   correlated spontaneous network activity (SNA) [1-8]. In the mouse embryonic spinal cord

55   (SC), SNA is driven by an excitatory cholinergic-GABAergic loop between motoneurons

56   (MNs) and interneurons (INs), GABA being depolarizing before embryonic day 16.5 (E16.5)

57   [9]. SNA emerges around E12.5 [4, 6, 10-12], at a time when functional neuromuscular

58   junctions are not yet established [13], and sensory and supraspinal inputs have not yet

59   reached the spinal motor networks [14-17].

60   Several studies pointed out that SNA is an essential component in neuronal networks

61   formation. [18-21]. In the SC, pharmacologically-induced disturbances of SNA between

62   E12.5 and E14.5 induce defects in the formation of motor pools, in motor axon guidance to

63   their target muscles and in the development of motor networks [4, 21-23]. During SNA

64   episodes, long lasting giant depolarization potentials (GDPs) are evoked in the SC, mainly

65   by the massive release of GABA onto MNs [12]. Immature Renshaw cells (V1[R]) are likely the

66   first GABAergic partners of MNs in the mouse embryo [24, 25], and the massive release of

67   GABA during SNA probably requires that many of them display repetitive action potential

68   firing or plateau potential activity [25].

69   However, little is known about the firing pattern of embryonic V1[R] and the maturation

70   of their intrinsic properties. We recently found that V1[R] exhibit heterogeneous excitability

71   properties when SNA emerges in the SC [25] in contrast to adult Renshaw cells that

72   constitute a functionally homogeneous population [26, 27]. Whether this early functional

2

73 diversity really reflects distinct functional classes of V1[R], how this diversity evolves during

74 development, and what are the underlying biophysical mechanisms remain open questions.

75 The present study addresses these issues using multiple approaches, including patch-clamp

76 recordings, cluster analysis, biophysical modeling and dynamical systems theory. The firing

77 patterns of V1[R] and the mechanisms underlying their functional diversity are analyzed

78 during a developmental period covering the initial phase of development of SC activity in

79 the mouse embryo (E11.5-E14.5), when SNA is present, and during the critical period

80 (E14.5-E16.5), when GABAergic neurotransmission gradually shifts from excitation to

81 inhibition [28] and locomotor-like activity emerges [4, 10, 11].

82   We discover that the balance between the slowly inactivating subthreshold persistent

83 sodium inward current (I_{Nap}) [29] and the delayed rectifier potassium outward current

84 (I_{Kdr}), accounts for the heterogeneity of embryonic V1[R] and the changes in firing pattern

85 during development. The heterogeneity of V1[R] at E12.5 arises from the existence of distinct

86 functional groups. Surprisingly, and in opposition to the classically accepted development

87 scheme [30-35], we show that the embryonic V1[R] population loses its ability to support

88 tonic firing from E13.5 to E15.5, exhibiting a transient functional involution during its

89 development. Our experimental and theoretical results provide a global view of the

90 developmental trajectories of embryonic V1[R]. They demonstrate that a simple mechanism,

91 based on the synergy of only two major opposing voltage-dependent currents, accounts for

92 functional diversity in these immature neurons.

93

94 **Results**

95 **The delayed rectifier potassium current I_{Kdr} is a key partner of the persistent sodium**

96 **current I_{Nap} in controlling embryonic V1[R] firing patterns during development**

97   We previously highlighted that V1[R] are spontaneously active at E12.5. Their response to

98   a 2 s suprathreshold depolarizing current steps revealed four main patterns, depending of

99   the recorded interneuron [25]: i) single spiking (SS) V1[R] that fires only 1-3 APs at the onset

100   of the depolarizing pulse, ii) repetitive spiking (RS) V1[R], iii) mixed events (ME) V1[R] that

101   shows an alternation of action potentials (APs) and plateau potentials or, iv) V1[R] that

102   displays a long-lasting sodium-dependent plateau potential (PP) (***Figure 1A1–A4***).

103   We also uncovered a relationship between I_{Nap} and the ability of embryonic V1[R] to

104   sustain repetitive firing [25]. However, the heterogeneous firing patterns of V1[R] observed

105   at E12.5 could not be fully explained by variations in I_{Nap} [25], suggesting the involvement

106   of other voltage-gated channels in the control of the firing pattern of V1[R], in particular

107   potassium channels, known to control firing and AP repolarization. Our voltage clamp

108   protocol, performed in the presence of TTX (1 μM), did not disclose any inward rectifying

109   current (hyperpolarizing voltage steps to -100 mV from V_H = -20 mV, data not shown), but

110   revealed two voltage-dependent outward potassium currents, a delayed rectifier current

111   (I_{Kdr}) and a transient potassium current (I_A) in all embryonic V1[R], whatever the firing

112   pattern (***Figure 1B1–B4***). These currents are known to control AP duration (I_{Kdr}) or firing

113   rate (I_A), respectively [36]. The activation threshold of I_{Kdr} lied between -30 mV and -20

114   mV and the threshold of I_A between -60 mV and -50 mV, (n = 27; N = 27 embryos) (***Figure***

115   ***1C1–C4***). Removing external calcium had no effect on potassium current I/V curves (data

116   not shown), suggesting that calcium-dependent potassium currents are not yet present at

117   E12.5.

118   It was unlikely that the heterogeneity of V1[R] firing patterns resulted from variations in

119   the intensity of I_A. Indeed, its voltage-dependent inactivation (time constant: 23.3 ± 2.6 ms,

120   n = 8; N = 8), which occurs during the depolarizing phase of an AP, makes it ineffective to

4

121   control AP or plateau potential durations. This was confirmed by our theoretical analysis

122   (*see Figure 7—figure supplement 1*). We thus focused our study on I_{Kdr}. At E12.5, PP V1[R]

123   had a significantly lower G_{Kdr} (2.12 ± 0.44 nS, n = 6; N = 6) than SS V1[R] (5.57 ± 0.56 nS, n =

124   9; N = 9) and RS V1[R] (6.39 ± 0.83 nS, n = 7; N = 7) (*Figure 1D*). However, there was no

125   significant difference in G_{Kdr} between SS V1[R] and RS V1[R] at E12.5 (*Figure 1D*), which

126   indicated that variations in G_{Kdr} alone could not explain all the firing patterns observed at

127   E12.5. Similarly, there was no significant difference in G_{Nap} between RS V1[R] (0.91 ± 0.21nS,

128   n = 8; N = 8) and PP V1[R] (1.24 ± 0.19 nS, n = 6; N = 6) at E12.5 (*Figure 1E*), indicating that

129   variations in G_{Nap} alone could not explain all the firing patterns of V1[R] at E12.5 [25]. In

130   contrast G_{Nap} measured in SS V1[R] at E12.5 (0.21 ± 0.20 nS, n = 9; N = 9) were significantly

131   lower compared to G_{Nap} measured in RS V1[R] and in PP V1[R] at E12.5 (*Figure 1E*).

132   Mature neurons often display multiple stable firing patterns [37-39]. This usually

133   depends on the combination of several outward and inward voltage- or calcium-dependent

134   conductances and on their spatial localization [37-39]. In contrast, immature V1[R] have a

135   limited repertoire of voltage-dependent currents (I_{Nat} and I_{Nap}, I_{Kdr} and I_A) at E12.5, and

136   we did not find any evidence of voltage-dependent calcium currents at this age [25].

137   Blocking I_{Nap} prevented plateau potential activity, PP-V1[R] becoming unexcitable, and

138   turned repetitive spiking V1[R] into single spiking V1[R] [25]. Therefore, we hypothesized that

139   the different firing patterns of V1[R] observed at E12.5 were related to the G_{Nap} / G_{Kdr} ratio

140   only, with variations in the intensity of I_A being unlikely to account for the heterogeneity of

141   firing pattern. We found that this ratio was significantly lower for SS V1[R] recorded at E12.5

142   (G_{Nap} / G_{Kdr} = 0.043 ± 0.015, n = 9) compared to RS V1[R] (0.154 ± 0.022, n = 8) and PP V1[R]

143   (0.66 ± 0.132, n = 6) (*Figure 1F*). We also found that the G_{Nap} / G_{Kdr} ratio was significantly

144   lower for RS V1[R] compared to PP V1[R] (*Figure 1F*).

145   Altogether, these results strongly suggest that, although the presence of I_{Nap} is

146   required for embryonic V1^R to fire repetitively or to generate plateau potentials [25], the

147   heterogeneity of the firing pattern observed between E12.5 is not determined by I_{Nap} *per*

148   *se* but likely by the balance between I_{Nap} and I_{Kdr}.

149

150   **Manipulating the balance between G$_{Nap}$ and G$_{Kdr}$ changes embryonic V1^R firing patterns**

151   We previously showed that blocking I_{Nap} with riluzole converted PP V1^R or RS V1^R into

152   SS V1^R [25]. To confirm further that the balance between G_{Nap} and G_{Kdr} was the key factor

153   in the heterogeneity of V1^R firing patterns, we assessed to what extent a given E12.5 SS V1^R

154   cell could change its firing pattern when I_{Kdr} was gradually blocked by 4-aminopiridine (4-

155   AP). We found that I_{Kdr} could be blocked by micromolar concentrations of 4-AP without

156   affecting I_A (***Figure 2—figure supplement 1***). 4-AP, applied at concentrations ranging from

157   0.3 μM to 300 μM, specifically inhibited I_{Kdr} with an IC$_{50}$ of 2.9 μM (***Figure 2—figure***

158   ***supplement 1C1***).

159   We then determined to what extent increasing the concentration of 4-AP modified the

160   firing pattern of V1^R at E12.5. Applying 4-AP at concentrations ranging from 3 μM to 300

161   μM changed the firing pattern of SS V1^R (n = 10; N = 10) in a concentration-dependent

162   manner (***Figure 2A1–A3***). In 50% of the recorded V1^R, increasing 4-AP concentrations

163   successfully transformed SS V1^R into PP V1^R with the following sequence: SS → RS → ME

164   → PP (***Figure 2A1***). In a second group of embryonic V1^R (25%), 4-AP application only

165   evoked mixed activity, with the same sequence as aforementioned (SS → RS → ME) (data

166   not shown). In the remaining SS V1^R (25%), increasing 4-AP concentration only led to

167   sustained AP firing (***Figure 2A2***). Application of 300 μM 4-AP on RS V1^R at E12.5 evoked

168   mixed events or plateau potentials (***Figure 2—figure supplement 2***). Plateau potentials and

6

169   repetitive spiking evoked in the presence of 300 μM 4-AP were fully blocked by 0.5-1 μM

170   TTX, indicating that they were generated by voltage-gated Na$^+$ channels (***Figure 2B,C*** and

171   ***Figure 2—figure supplement 2***). It should be noted that the application of 300 μM of 4-AP

172   induced a significant 30.5 ± 12.4 % increase (P = 0.0137; Wilcoxon test) of the input

173   resistance (1.11 ± 0.08 GΩ versus 1.41 ± 0.12 GΩ; n = 11; N = 11).

174   These results show that, in addition to I_{Nap}, I_{Kdr} is also a major determinant of the

175   firing pattern of embryonic V1^R. The above suggests that the firing patterns depend on a

176   synergy between I_{Nap} and I_{Kdr} and that the different patterns can be ordered along the

177   following sequence SS → RS → ME → PP when the ratio G_{Nap}/G_{Kdr} is increased.

178

179   **The heterogeneity of the** V1^R **firing patterns decreases during embryonic development**

180   It was initially unclear whether these different firing patterns corresponded to well

181   separated classes within the E12.5 V1^R population or not. To address this question, we

182   performed a hierarchical cluster analysis on 163 embryonic V1^R, based on three

183   quantitative parameters describing the firing pattern elicited by the depolarizing pulse: the

184   mean duration of evoked APs or plateau potentials measured at half-amplitude (mean

185   ½Ad), the variability of the event duration during repetitive firing (coefficient of variation of

186   ½Ad: CV ½Ad) and the total duration of all events, expressed in percentage of the pulse

187   duration (depolarizing duration ratio: ddr) (***Figure 3A inserts***). In view of the large

188   dispersion of mean ½Ad and ddr values, cluster analysis was performed using the (decimal)

189   logarithm of these two quantities [40]. The analysis of the distribution of log mean ½Ad, CV

190   ½Ad and log ddr revealed multimodal histograms that could be fitted with several

191   Gaussians (***Figure 3—figure supplement 1A1–C1***). Cluster analysis based on these three

192   parameters showed that the most likely number of clusters was 5 (***Figure 3A,B***), as

193 determined by the silhouette width measurement (**Figure 3B**). Two clearly separated

194 embryonic V1^R groups with CV ½Ad = 0 stood out, as shown in the 3D plot in **Figure 5C**. The

195 cluster with the largest ½Ad (mean ½Ad = 833.5 ± 89.99 ms) and the largest ddr (0.441 ±

196 0.044) contained all PP V1^R (n = 35; N = 29) (**Figure 3C-D** and **Figure 3—figure supplement**

197 **1A2,C2**). Similarly, the cluster with the shortest ½Ad (9.73 ± 0.66 ms) and the lowest ddr

198 (0.0051 ± 0.0004) contained all SS V1^R (n = 46; N = 37) (**Figure 3C-D** and **Figure 3—figure**

199 **supplement 1A2,C2**).

200   The three other clusters corresponded to V1^R with nonzero values of CV ½Ad (**Figure 3C**).

201 A first cluster regrouping all RS V1^R (n = 69; N = 61) was characterized by smaller values of

202 ½Ad (23.91 ± 1.43 ms), CV ½Ad (27.36 ± 1.64%) and ddr (0.11 ± 0.01) (**Figure 3C-D** and

203 **Figure 3—figure supplement 1A2,C2**). The last two clusters corresponded to ME V1^R

204 (**Figure 3C,D**). The smaller cluster, characterized by a larger CV ½Ad (170.9 ± 8.9%; n= 4; N =

205 4), displayed a mix of APs and short plateau potentials, while the second cluster, with

206 smaller CV ½Ad (87.61 ± 7.37%; n = 9; N = 9), displayed a mix of APs and long-lasting

207 plateau potentials (**Figure 3D** and **Figure 3—figure supplement 1B2**). Their ½Ad and ddr

208 values were not significantly different (**Figure 3—figure supplement 1A2,C2**).

209   It must be noted that three embryonic V1^R (1.8%) were apparently misclassified since

210 they were aggregated within the RS cluster although having zero CV ½Ad (**Figure 3C;**

211 **arrows**). Examination of their firing pattern revealed that this was because they generated

212 only two APs, although their ddr (0.16 to 0.2) and ½ Ad values (31.6 to 40.3 ms) were well

213 in the range corresponding of the RS cluster.

214   These different firing patterns of V1^R might reflect different states of neuronal

215 development [31, 41-43]. Single spiking and/or plateau potentials are generally believed to

216 be the most immature forms of firing pattern, repetitive spiking constituting the most

217   mature form [19, 44]. If it were so, the firing patterns of embryonic V1[R] would evolve

218   during embryonic development from single spiking or plateau potential to repetitive

219   spiking, this latter firing pattern becoming the only one in neonates [26] and at early

220   postnatal stages [27]. However, RS neurons already represent 41% of V1[R] at E12.5. We

221   therefore analyzed the development of firing patterns from E11.5, when V1[R] terminate

222   their migration and reach their final position [45], to E16.5. This developmental period

223   covers a first phase of development (E11.5-E14.5), where lumbar spinal networks exhibit

224   SNA, and a second phase (E14.5-E16.5), where locomotor-like activity emerges [4, 11, 46,

225   47]. We first analyzed changes in the intrinsic properties (input capacitance C_{in}, input

226   resistance R_{in} = $1/G_{in}$ and spike voltage threshold) of V1[R]. C_{in} did not change significantly

227   from E11.5 to E13.5 (**Figure 4A1**), remaining of the order of 12 pF, in agreement with our

228   previous work [25]. However, it increased significantly at the transition between the two

229   developmental periods (E13.5-E15.5) to reach about 23.5 pF at E15.5 (**Figure 4A1**). A

230   similar developmental pattern was observed for R_{in}, which remained stable during the

231   first phase from E11.5 to E14.5 ($R_{in} \approx$ 1-1.2 GΩ) but decreased significantly after E14.5 to

232   reach about 0.7 GΩ at E15.5 (**Figure 4A2**). Spike threshold also decreased significantly

233   between the first and the second developmental phases, dropping from about -34 mV at

234   E12.5 to about -41 mV at E16.5 (**Figure 4A3**). Interestingly, this developmental transition

235   around E14.5 correspond to the critical stage at which SNA gives way to a locomotor-like

236   activity [11, 46, 47] and rhythmic activity becomes dominated by glutamate release rather

237   than acetylcholine release [4].

238   This led us to hypothesize that this developmental transition could be also critical for

239   the maturation of V1[R] firing patterns. The distinct firing patterns observed at E12.5 were

240   already present at E11.5 (**Figure 4B1,C**), but the percentage of RS V1[R] strongly increased

241 from E11.5 to E12.5, while the percentage of ME V1[R] decreased significantly (***Figure 4C***).

242 The heterogeneity of V1[R] firing patterns then substantially diminished. Plateau potentials

243 were no longer observed at E13.5 (***Figure 4B2,C***), and ME V1[R] disappeared at E14.5 (***Figure***

244 ***4B3,C***). Interestingly, the proportion of SS V1[R] remained high from E13.5 to E15.5 and even

245 slightly increased (91.23% at E14.5 and 93.33% at E15.5; ***Figure 4C***). This trend was partially

246 reversed at E16.5, as the percentage of RS V1[R] increased at the expense of SS V1[R] (67.86%

247 SS V1[R] and 32.34% RS V1[R]; ***Figure 4B5,C***). This decrease in repetitive firing capability after

248 E13.5 was surprising in view of what is classically admitted on the developmental pattern

249 of neuronal excitability [18, 48]. Therefore, we verified that it did not reflect the death of

250 some V1[R] after E13.5. Our data did not reveal any activated caspase3 (aCaspase3) staining

251 in V1[R] (FoxD3 staining) at E14.5 (n = 10 SCs; N = 10) (***Figure 5***), in agreement with previous

252 reports showing that developmental cell death of V1[R] does not occur before birth [49].

253   To determine whether G_{Nap} and G_{Kdr} also controlled the firing pattern of V1[R] at E14.5

254 (see ***Figure 4B3,C***), we assessed the presence of I_{Nap} and I_{Kdr} in single spiking V1[R] at this

255 embryonic age. Both I_{Nap} and I_{Kdr} were present in V1[R] at E14.5 (***Figure 6—figure***

256 ***supplement 1*** and ***Figure 6—figure supplement 2***) whereas, as in V1[R] at E12.5, no calcium-

257 dependent potassium current was detected at this developmental age (not shown). In SS

258 V1[R], G_{Kdr} was significantly higher at E14.5 (11.11 ± 1.12 nS, n = 10; N = 10) than at E12.5

259 (***Figure 1D***). In contrast, G_{Nap} was similar at E14.5 (0.13 ± 0.14 nS, n = 10; N = 10) and E12.5

260 (***Figure 1E***). We also found that the G_{Nap} / G_{Kdr} ratio was significantly lower for SS V1[R]

261 recorded at E14.5 (0.012 ± 0.004, n = 10) compared to RS V1[R] (0.154 ± 0.022, n = 8) and PP

262 V1[R] (0.66 ± 0.132, n = 6) recorded at E12.5 (***Figure 1F***).

263   We tested the effect of 4-AP in SS V1[R] at E14.5. At this embryonic age, 300 μM 4-AP

264 inhibited only 59.2% of I_{Kdr}. Increasing 4-AP concentration to 600 μM did not inhibit I_{Kdr}

265 significantly more (60.2%) (**Figure 6—figure supplement 2**), indicating that inhibition of

266 I_{Kdr} by 4-AP reached a plateau at around 300 µM. 600 µM 4-AP application had no

267 significant effect on I_A (**Figure 6—figure supplement 2**). The application of the maximal

268 concentration of 4-AP tested (600 µM) converted SS V1^R (n = 13; N = 13) to PP V1^R (23.1%;

269 **Figure 6A1,B**), RS V1^R (38.5%; **Figure 6A2,B**) or ME V1^R (38.4%; **Figure 6B**), as was observed

270 at E12.5, thus indicating that the firing pattern of V1^R depends on the balance between

271 I_{Nap} and I_{Kdr} also at E14.5. Plateau potential and repetitive spiking recorded in the

272 presence of 4-AP at E14.5 were fully blocked by 0.5-1 µM TTX indicating that they were

273 generated by voltage-gated sodium channels (**Figure 6A1,A2**), as observed at E12.5.

274

275 **Theoretical analysis: the basic model**

276 As shown in **Figure 7A** for 26 cells, in which both G_{Nap} and G_{Kdr} were measured, the

277 three largest clusters revealed by the hierarchical clustering analysis (SS, RS and PP, which

278 account together for the discharge of more than 95% of cells, see **Figure 5**) correspond to

279 well defined regions of the G_{Nap}- G_{Kdr} plane. Single spiking is observed only when G_{Nap} is

280 smaller than 0.6 nS. For larger values of G_{Nap}, repetitive spiking occurs when G_{Kdr} is larger

281 than 3.5 nS, and V1^R display plateau potentials when G_{Kdr} is smaller than 3.5 nS. Mixed

282 events (ME, 4.5% of the 163 cells used in the cluster analysis), where plateaus and spiking

283 episodes alternate, are observed at the boundary of RS and PP clusters. This suggested to

284 us that a conductance-based model incorporating only the leak current, I_{Nat}, I_{Nap} and I_{Kdr}

285 (see Materials and Methods) could account for most experimental observations, the

286 observed zonation being explained in terms of bifurcations between the different stable

287 states of the model. Therefore, we first investigated a simplified version of the model

288 without I_A and slow inactivation of I_{Nap}.

289   A one-parameter bifurcation diagram of this "basic" model is shown in ***Figure 7B*** for two

290   values of G_{Kdr} (2.5 nS and 10 nS) and a constant injected current I = 20 pA. In both cases,

291   the steady-state membrane voltage (stable or unstable) and the peak and trough voltages

292   of stable and unstable periodic solutions are shown as the function of the maximal

293   conductance G_{Nap} of the I_{Nap} current, all other parameters being kept constant. For G_{Kdr}

294   = 10 nS, the steady-state membrane voltage progressively increases (in gray) with G_{Nap},

295   but repetitive spiking (in red, see voltage trace for G_{Nap} = 1.5 nS) is not achieved until G_{Nap}

296   reaches point SN_1, where a saddle node (SN) bifurcation of limit cycles occurs. This fits with

297   the experimental data, where a minimal value of G_{Nap} is required for repetitive spiking

298   (see also [25]), and is in agreement with the known role of I_{Nap} in promoting repetitive

299   discharge [50, 51]. Below SN_1, the model responds to the onset of a current pulse by firing

300   only one spike before returning to quiescence (see voltage trace for G_{Nap}= 0.2 nS), or a few

301   spikes when close to SN_1 (not shown) before returning to quiescence. The quiescent state

302   becomes unstable through a subcritical Hopf bifurcation (HB) at point HB_1, with bistability

303   between quiescence and spiking occurring between SN_1 and HB_1 points. Repetitive firing

304   persists when G_{Nap} is increased further and eventually disappears at point SN_2. The firing

305   rate does not increase much throughout the RS range (***Figure 7—figure supplement 1C***),

306   remaining between 20.1 Hz (at SN_1) and 28.7 Hz (at SN_2). A stable plateau appears at point

307   HB_2 through a subcritical HB. The model is bistable between HB_2 and SN_2, with plateau and

308   large amplitude APs coexisting in this range.

309   The model behaves very differently when G_{Kdr} is reduced to 2.5 nS (gray-blue curve in

310   ***Figure 7B***). It exhibits a unique stable fixed point whatever the value of G_{Nap} is, and the

311   transition from quiescence to plateau is gradual as G_{Nap} is increased. No repetitive spiking

312   is ever observed. This indicates that the activity pattern is controlled not only by G_{Nap} but

313   also by G_{Kdr}. This is demonstrated further in **Figure 7C**, where G_{Nap} was fixed at 1.2 nS

314   while G_{Kdr} was increased from 0 to 25 nS. The model exhibits a plateau potential until

315   G_{Kdr} is increased past point the subcritical HB point HB_2, repetitive spiking sets in before at

316   point SN_2 *via* a SN of limit cycles bifurcation. When G_{Kdr} is further increased, repetitive

317   firing eventually disappears through a SN bifurcation of limit cycles at point SN_1, the

318   quiescent state becomes stable through a subcritical HB at point HB_1, and bistability occurs

319   between these two points. This behavior is in agreement with **Figure 7A**.

320   Since both conductances G_{Nap} and G_{Kdr} control the firing pattern of embryonic V1[R] cells,

321   we computed a two-parameters bifurcation diagram (**Figure 7D**), where the stability

322   regions of the different possible activity states and the transition lines between them are

323   plotted in the G_{Nap} - G_{Kdr} plane. The black curves correspond to the bifurcations HB_1 and

324   HB_2 and delimit a region where only repetitive firing occurs. The red curves correspond to

325   the SN bifurcations of periodic orbits associated with the transition from quiescence to

326   firing (SN_1) and the transition from plateau to firing (SN_2). They encompass a region

327   (shaded area) where repetitive firing can be achieved but may coexist with quiescence

328   (between the HB_1 and SN_1 lines) or plateau potential (in the narrow region between the

329   HB_2 and SN_2 lines).

330   Some important features of the diagram must be emphasized: 1) minimal values of both

331   G_{Nap} (to ensure sufficient excitability) and G_{Kdr} (to ensure proper spike repolarization) are

332   required for repetitive spiking, 2) quiescence and plateau can be clearly distinguished only

333   when they are separated by a region of repetitive spiking (see also **Figure 7B** for G_{Kdr} = 10

334   nS), otherwise the transition is gradual (**Figure 7B** for G_{Kdr}= 2.5 nS), 3) only oblique lines

335   with an intermediate slope cross the bifurcation curve and enter the RS region (see, for

336   example, the red line in **Figure 7D**). This means that repetitive spiking requires an

337   appropriate balance between I_{Nap} and I_{Kdr}. If the ratio G_{Nap}/G_{Kdr} is too large (blue line)

338   or too small (gray line), only plateau potentials or quiescence will be observed at steady

339   state. This is exactly what is observed in experiments, as shown by the cumulative

340   distribution function of the ratio G_{Nap}/G_{Kdr} for the different clusters of embryonic V1[R] in

341   **Figure 7E** (same cells as in **Figure 7A**). The ratio increases according to the sequence SS →

342   RS → ME → PP, with an overlap of the distributions for SS V1[R] and RS V1[R]. Note also that

343   the ratio for ME cells (around 0.25) corresponds to the transition between repetitive

344   spiking and plateau potentials (more on this below).

345   Embryonic V1[R] cells display voltage fluctuations that may exceed 5 mV and are

346   presumably due to channel noise. The relatively low number of sodium and potassium

347   channels (of the order of a few thousands) led to voltage fluctuations in the stochastic

348   version of our model comparable to those seen experimentally when the cell was

349   quiescent (top voltage trace in **Figure 7D**) or when a voltage plateau occurred (bottom

350   trace). Channel noise caused some jitter during repetitive spiking (middle trace), and

351   induced clearly visible variations in the amplitude of APs. However, repetitive firing proved

352   to be very robust and was not disrupted by voltage fluctuations. Altogether, channel noise

353   little alters the dynamics (compare the deterministic voltage traces in **Figure 7B** and the

354   noisy traces in **Figure 7D**). This is likely because channel noise has a broad power spectrum

355   and displays no resonance with the deterministic solutions of the model.

356   The one-parameter bifurcation diagram of the model was not substantially modified

357   when we took I_A into account, as shown in **Figure 6—figure supplement 1**. It just elicited a

358   slight membrane hyperpolarization, an increase in the minimal value of G_{Nap} required for

359   firing, and a decrease of the firing frequency. The transition from repetitive firing to

360   plateau was not affected because I_A is then inactivated by depolarization.

361   The bifurcation diagram of **Figure 7D** accounts *qualitatively* for the physiological data on

362   V1^R at E12.5 presented in **Figure 7A**, as shown in **Figure 7F** where the conductance data of

363   **Figure 7A** were superimposed on it. However, one must beware of making a more

364   *quantitative* comparison because the theoretical bifurcation diagram was established for a

365   constant injected current of 20 pA, whereas the current injected in experiments data

366   varied from neuron to neuron and ranged from 10 to 30 pA in the sample shown in **Figure**

367   **7A**. The position of bifurcation lines in the G_{Nap} - G_{Kdr} plane depends not only on the value

368   of the injected current, but on the values chosen for the other parameters, which also vary

369   from cell to cell but were kept at fixed values in the model [52]. For instance, the diagrams

370   were computed in **Figure 7D,F** for G_{in}= 1 nS and C_{in}= 13 pF, the median values of the input

371   conductance and capacitance at E12.5, taking no account of the cell-to-cell variations of

372   these quantities. Between E12.5 and E14.5, C_{in} which provides an estimate of the cell size,

373   increases by 38% in average, whereas G_{in} is not significantly modified (see **Figure 4**). As

374   illustrated in **Figure 7G** the two-parameters bifurcation diagram is then shifted upward and

375   rightward compared to **Figure 7F**, because larger conductances are required to obtain the

376   same firing pattern. The observed regression of excitability from E12.5 to E14.5-E15.5 (see

377   **Figure 4C**) thus comes from a decrease in G_{Nap} density (see presumable developmental

378   trajectories indicated by arrows in **Figure 7F**) together with a shift of the RS region as cell

379   size increases. As a result, all 10 cells shown in **Figure 7G** are deeply inside the SS region at

380   E14.5.

381   It is less straightforward to explain on the basis of our model the experiments where 4-

382   AP changed the firing pattern of SS V1^R (**Figure 2**). Indeed, the decrease of G_{Kdr} (**Figure 6—**

383   **figure supplement 2**), although it may exceed 70% at the higher concentrations of 4-AP we

384   used, is not sufficient by itself to account for the change in the firing pattern of V1^R (**Figure**

385 ***6—figure supplement 2***) because data points in the SS cluster will not cross the bifurcation

386 lines between SS and RS (SN1) and between RS and PP (SN2) when displaced downward in

387 the G_{Nap}- G_{Kdr} plane. However, 4-AP at a 300 μM concentration also decreases G_{in} (by 23%

388 in average and up to 50% in some neurons), the rheobase current with it, and the current

389 that was injected in cells during experiments was reduced accordingly. hen we take into

390 account this reduction of both G_{in} and I the two parameters bifurcation diagram pf the

391 model remains qualitatively the same, but it is shifted leftward and downward in the G_{Nap}-

392 G_{Kdr} plane (***Figure 6—figure supplement 2***). As a consequence, the bifurcation lines

393 between SS and RS and between RS and PP (SN2) are then successively crossed when

394 G_{Kdr} is reduced, in accordance with experimental results.

395

396 **Theoretical analysis: slow inactivation of I_{Nap} and bursting**

397   Our basic model accounts for the firing pattern of 73% of the 163 cells used in the

398 cluster analysis. However, bursting, under the form of recurring plateaus separated by brief

399 repolarization episodes (see a typical trace in ***Figure 8A left***), was experimentally observed

400 in half of PP V1[R] (24 out of 46), and plateaus intertwined with spiking episodes were

401 recorded in the 13 cells of the ME cluster (8% of the total sample, see ***Figure 8A right*** for a

402 typical example). Recurrent plateaus indicate membrane bistability and require that the

403 $I - V$ curve be S-shaped. This occurs when G_{Nap} is large and G_{Kdr} small. (***Figure 8B1,B2***).

404 However, our basic model lacks a mechanism for switching between quiescent state and

405 plateau, even in this case. Channel noise might induce such transitions, but our numerical

406 simulations showed that this is too infrequent to account for bursting (see voltage trace in

407 ***Figure 8B1*** where the plateau state is maintained despite channel noise).

408   To explain recurrent plateaus during a constant current pulse, we have to incorporate in

409   our model an additional slow dynamical process. Therefore, we took into account the slow

410   inactivation of I_{Nap} that is observed in experiments. I_{Kdr} also inactivates slowly but over

411   times that are much longer than the timescale of bursting, which is why we did not take its

412   slow inactivation into account. The one-parameter bifurcation diagram of the basic model

413   without slow inactivation of I_{Nap} is shown in **Figure 8C** for G_{Kdr} = 5 nS and an injected

414   current reduced to 10 pA (as compared to 20 pA in the previous section), so as to allow for

415   bistability (see **Figure 8B2**). The $G_{Nap} - V$ curve is then S-shaped, as shown in **Figure 8B1**,

416   with a bistability region for G_{Nap} between 1.36 and 1.85 nS. This is in contrast with **Figure**

417   **7B** where the $G_{Nap} - V$ curve was monotonic. Adding the slow (de)inactivation of

418   I_{Nap} then causes periodic transitions between up (plateau) and down (quiescent) states, as

419   illustrated by the top voltage trace on the right of **Figure 8C**, and the model displayed a

420   stable limit cycle (shown in black in the bifurcation diagram on the left of **Figure 8C**). This

421   mechanism is known as pseudo-plateau or plateau-like bursting (a.k.a. fold-subcritical HB

422   bursting) [53]. In contrast with square wave bursting [54-57], where the up-state is a stable

423   limit cycle arising from a supercritical Hopf bifurcation [58-60], the up-state here is a stable

424   fixed point (which coexists with an unstable limit cycle). This is why one does not observe

425   bursts of APs separated by quiescent periods as, for instance, observed in postnatal CA1

426   Pyramidal cells [61] and in neurons of neonatal pre-Bötzinger Complex [62, 63], but

427   recurrent plateaus. The duration of the plateaus and repolarization episodes depends on

428   the values of G_{Nap} and G_{Kdr}. A voltage-independent time constant $\tau_s = 2\ ms$ leads to up

429   and down states of comparable durations (see top left voltage trace in **Figure 8C**). In

430   agreement with the bifurcation diagram of **Figure 8C**, the persistent sodium current

431   inactivates during plateaus (phase 1, see bottom right trace in in **Figure 8C**) and de-

432   inactivates during quiescent episodes (phase 3, see bottom right trace). Transitions from

433   the down-state to the up-state occurs when inactivation has reached its maximal value

434   (phase 2) and transition from the up-state to the down state when it has reached its

435   maximum (phase 4). Adding channel noise preserves bursting but introduces substantial

436   randomness in the duration of plateaus and repolarization episodes (bottom left voltage

437   trace in **Figure 8C**). Moreover, it substantially decreases the duration of both plateaus and

438   quiescent episodes by making transition between the two states easier (compare the top

439   and bottom voltage traces on the left, both computed for $\tau_s = 2\ ms$).

440   Increasing G_{Nap} (or decreasing G_{Kdr}) makes plateaus much longer than quiescent

441   episodes (see bottom right voltage trace in **Figure 8C**). This again points out to the fact that

442   the ratio is an important control parameter. We also noted that adding the I_A current

443   lengthened the quiescence episodes (**Figure 6–figure supplement 1**).

444   Slow inactivation of I_{Nap} also provides an explanation for mixed patterns, where

445   plateaus alternate with spiking episodes (**Figure 8A, right**). They take place in our model

446   near the transition between repetitive spiking and plateau, as in experiments (see **Figure

447   8A**). Slow inactivation can lead to elliptic bursting, notably when the bifurcation HB$_2$ is

448   subcritical [64, 65], which is the case here (**Figure 8D**). The model then displays a stable

449   limit cycle with alternating plateaus and spiking episodes, arising from crossing the

450   bifurcation points HB$_2$ and SN$_2$ back and forth (see bifurcation diagram in **Figure 8D** and

451   top voltage trace). We note that sufficient de-inactivation of I_{Nap} for triggering a new

452   plateau (phase 3 in the bottom trace of **Figure 8D**) may be difficult to achieve during

453   spiking episodes, because voltage oscillates over a large range, which tends to average out

454   the variations of the inactivation level. If de-inactivation is not sufficient, the model keeps

455   on spiking repetitively without returning to the plateau state. This is what occurs for cells

456 well within the RS region, far away from the RS-PP transition. It also probably explains why

457 it was difficult in many recorded cells to elicit plateaus by increasing the injected current,

458 inactivation balancing then the increase of I_{Nap} induced by the larger current.

459   Altogether, our study shows that a model incorporating the slow inactivation of

460 I_{Nap} accounts for all the firing patterns displayed by cells from the PP and ME clusters.

461

Discussion

463   V1[R] constitute a homogeneous population when referring to their transcription factor

464 program during development [24, 66], their physiological function [67] and their firing

465 pattern at postnatal stages [27]. Surprisingly, our electrophysiological recordings and our

466 cluster analysis clearly indicate that distinct functional classes of V1[R] are transiently

467 present during development at the onset of the SNA (E11.5-E12.5). Five different groups of

468 embryonic V1[R] were defined using cluster analysis, according to their firing properties.

469

Development of the firing pattern of embryonic V1[R] during SNA

471   It is generally assumed that, during early development, newborn neurons cannot sustain

472 repetitive firing [35, 48]. Later on, neurons progressively acquire the ability to fire

473 repetitively, APs become sharper, and neurons eventually reach their mature firing pattern,

474 due to the progressive appearance of a panoply of voltage-gated channels with different

475 kinetics [18, 35, 48]. Our results challenge the general view that single spiking is a more

476 primitive form of excitability [35]. Indeed, we show that repetitive firing and plateau

477 potentials dominated at early stages (E11.5-E12.5), while single spiking was prevailing only

478 later (E13.5- E16.5).

19

479　　　The different V1^R firing patterns observed at E11.5-E12.5 might reflect variability in the

480　　maturation level between V1^R at a given developmental stage, as suggested for developing

481　　MNs [68, 69]. However, this is unlikely since V1^R transiently lose their ability to sustain

482　　tonic firing or plateau potential after E13.5. The heterogeneous discharge patterns of V1^R

483　　observed before E13.5 contrasts with the unique firing pattern of V1^R at postnatal age [27].

484　　Accordingly, the transient functional heterogeneity of V1^R rather reflects an early initial

485　　developmental stage (E11.5-E13.5) of intrinsic excitability.

486　　　The physiological meaning of the transient functional involution of V1^R that follows,

487　　after E12.5, is puzzling. To our knowledge, such a phenomenon was never described in

488　　vertebrates during CNS development. So far, a functional involution was described only for

489　　inner hair cells between E16 and P12 [70, 71] and cultured oligodendrocytes [72], and it

490　　was irreversible. Because most V1^R cannot sustain tonic firing after E12.5, it is likely that

491　　their participation to SNA is limited to the developmental period before other GABAergic

492　　interneuron subtypes mature and start to produce GABA and glycine [73]. Interestingly,

493　　embryonic V1^R begin to recover their capability to sustain tonic firing when locomotor-like

494　　activity emerges [4, 11], a few days before they form their recurrent synaptic loop with

495　　MNs (around E18.5 in the mouse embryos, [74]). One possible function of the transient

496　　involution between E12.5 and E15.5 could be to regulate the growth of V1^R axons toward

497　　their targets. It is indeed known that low calcium fluctuations within growth cones are

498　　required for axon growth while high calcium fluctuations stop axon growth and promote

499　　growth cone differentiation [75].

500

501　　**Ion channels mechanisms underlying the functional heterogeneity of embryonic V1^R**

502   Blockade of I_{Nap} leads to single spiking [25], which emphasizes the importance of this

503   current for the occurrence of repetitive firing and plateau potentials in V1[R] at early

504   developmental stages. But these neurons can also switch from one firing pattern to

505   another, when G_{Kdr} is decreased by 4-AP, which emphasizes the importance of I_{Kdr}. We

506   found that the main determinant of embryonic V1[R] firing pattern is the balance between

507   G_{Nap} and G_{Kdr}.

508   A Hodgkin-Huxley-type model incorporating a persistent sodium current I_{Nap} provided a

509   parsimonious explanation of all five firing patterns recorded in the V1[R] population at E12.5.

510   It provided a mathematical interpretation for the clustering of embryonic V1[R] shown by the

511   hierarchical analysis and accounted for the effect of 4-AP and riluzole [25] on the discharge.

512   Remarkably, it highlighted how a simple mechanism involving only the two opposing

513   currents I_{Nap} and I_{Kdr}, but not I_A, could produce functional diversity in a population of

514   developing neurons. The model explained why minimal G_{Nap} and G_{Kdr} are required for

515   firing, and how a synergy between G_{Nap} and G_{Kdr} controls the firing pattern and accounts

516   for the zonation of the $G_{Nap} - G_{Kdr}$ plane that is observed experimentally.

517   Taking into account the slow inactivation of I_{Nap} to the model allowed us to explain the

518   bursting patterns displayed by cells of the PP and ME clusters. We showed, in particular,

519   that mixed events arose from elliptic bursting at the repetitive spiking-plateau transition

520   and that smooth repetitive plateaus could be explained by a pseudo-plateau bursting

521   mechanism [53, 59]. Such bursting scenario has been previously studied in models of

522   endocrine cells [58, 76, 77] and adult neurons [78], but rarely observed in experiments [79].

523   It contrasts with the more common square wave bursting at firing onset, i.e. alternating

524   bursts of APs and quiescent episodes, on which most studies of bursting focused [61-63].

525   Our model can also display such square wave bursting, but this occurs for physiologically

526   realistic parameter values, and did not dwell on that bursting mode that we never

527   observed in embryonic V1^R. The model also provides a mathematical explanation for mixed

528   events, where bursts of APs alternate with plateau episodes. It is due to an elliptic bursting

529   scenario at the RS-PP transition, a firing range that the afore-mentioned studies did not

530   examine. This further emphasizes the capacity of our simple model to account for a wide

531   diversity of firing patterns.

532   Pseudo-plateau bursting has also been observed in the embryonic pre-Bötzinger

533   network [79]. However, it is produced there by the calcium-activated nonselective cationic

534   current I_{CAN}, while I_{Nap} leads to square wave bursting. Pseudo-plateau bursting, displayed

535   by half of the cells at E16.5 largely disappears at E18.5 because of the change in the

536   balance between I_{CAN} and I_{Nap} during embryonic maturation [79]. Such a scenario cannot

537   account for the variety of discharge patterns observed in embryonic V1^R at the E11.5-12.5

538   stage of development. Our theoretical analysis and our experimental data clearly indicate

539   that the interplay between two opposing currents is necessary to explain all the firing

540   patterns of V1^R. Our model is of course not restricted to embryonic V1^R, but may also apply

541   to any electrically compact cell, the firing activity of which is dominated by I_{Nap} and

542   delayed rectifier potassium currents. This is the case of many classes of embryonic cells in

543   mammals at an early stage of their development. It can also apply to the axon initial

544   segment, where G_{Nap} and G_{Kdr} are known to play the major role in the occurrence of

545   repetitive firing [80].

546   Altogether our experimental and theoretical results provide a global view of the

547   developmental trajectories of embryonic V1^R (see **Figure 7F, G**). At E12.5, conductances of

548   embryonic V1^R are widely spread in the $G_{Nap} - G_{Kdr}$ plane, which explains the

549   heterogeneity of their firing patterns. This likely results from the random and uncorrelated

550 expression of sodium and potassium channels from cell to cell at this early stage. Between

551 E12.5 and E14.5-15.5 cell size increases, and G_{Kdr} with it, while the density of sodium

552 channels decreases (see **Figures 1 and 4**). The functional involution displayed by V1[R]

553 between E12.5 and E15.5 thus mainly results from a decrease of G_{Nap} coordinated with an

554 increase of G_{Kdr}. How these synergistic processes are controlled during this developmental

555 period remains an open issue.

556   It is important to note that the presence of I_{Nap} is required for the functional diversity

557 of V1[R]. Indeed, in the absence of I_{Nap}, V1[R] lose their ability to generate plateau potentials

558 or to fire repetitively. More generally, when the diversity of voltage-gated channels is

559 limited, as observed in embryonic neurons [18], changes in the balance between I_{Kdr} and

560 non (or poorly) inactivating inward current can modify the firing pattern. This can be

561 achieved not only by I_{Nap}, but also by other slowly or non-inactivating inward

562 conductances, such as I_{CAN} [79]. Our work also clearly indicates that a change in the firing

563 pattern can only occur if a change in inward conductances cannot be counterbalanced by a

564 corresponding change in outward conductances. This implies that there is no homeostatic

565 regulation of channel density to ensure the robustness of V1[R] excitability during its early

566 development, contrarily to the mature CNS [37]. In addition, the poor repertoire of

567 voltage-gated channels at this developmental stage precludes channel degeneracy, which

568 is also known to ensure the robustness of excitability in mature neurons [37].

569   In conclusion, our study shows that there is no universal pattern of development in

570 embryonic neurons, and it demonstrates that a simple general mechanism involving only

571 two slowly inactivating voltage-gated channels with opposite effects is sufficient to

572 produce

573   a wide variety of firing patterns in immature neurons having a limited repertoire of

574   voltage-gated channels.

575

Materials and Methods

Key Resources Table				
Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifiers**	**Additional information**
genetic reagent (M. musculus Swiss) male and female	GAD1^{GFP}	PMID: 14574680		a cDNA encoding enhanced GFP (eGFP) was targeted to the locus encoding the gene Gad1.
Antibody	Anti-FoxD3 (Guinea pig polyclonal)	PMID:19088088		IF(1:5000)
Antibody	Anti-cleaved Caspase-3 (Asp175) (Rabbit polyclonal)	Cell Signaling Technology	Cat# 9661, RRID: AB_2341188	IF(1:1000)
chemical compound, drug	Tetrodotoxin	Alomone labs	Cat# T550, CAS No.: 18660-81-6	1 µM
chemical compound, drug	4-aminopyridine	Sigma-Aldrich	Cat# A78403, CAS No.: 504-24-5	0.3 - 600 µM
software, algorithm	pCLAMP 10.5	Molecular devices	RRID:SCR_014284	
software, algorithm	Axograph 1.7.2	AxoGraph	RRID:SCR_014284	
software, algorithm	PRISM 7.0e	GraphPad Software	RRID:SCR_002798)	
software, algorithm	ImageJ 1.5	N.I.H. (USA)	RRID:SCR_003070	

software, algorithm	Adobe Photoshop CS6	Adobe, USA	RRID:SCR_014199	
software, algorithm	R software 3.3.2	Cran project (https://cran.r-project.org/)	RRID:SCR_001905	
software, algorithm	XPP-Aut 8.0	University of Pittsburgh; Pennsylvania; USA	RRID:SCR_001996	

578

Isolated spinal cord preparation

Experiments were performed in accordance with European Community guiding principles on the care and use of animals (86/609/CEE, CE Off J no. L358, 18 December 1986), French decree no. 97/748 of October 19, 1987 (J Off République Française, 20 October 1987, pp. 12245-12248). All procedures were carried out in accordance with the local ethics committee of local Universities and recommendations from the CNRS. We used Gad1^{GFP} knock-in mice to visualize putative GABAergic INs [81], as in our previous study [25]. To obtain E12.5-E16.5 Gad1^{GFP} embryos, 8 to 12 weeks old wild-type Swiss female mice were crossed with Gad1^{GFP} Swiss male mice.

Isolated mouse SCs from 420 embryos were used in this work and obtained as previously described [28, 82]. Briefly, pregnant mice were anesthetized by intramuscular injection of a mix of ketamine and xylazine and sacrificed using a lethal dose of CO_2 after embryos of either sex were removed. Whole SCs were isolated from eGFP-positive embryos and maintained in an artificial cerebrospinal fluid (ACSF) containing 135 mM NaCl, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, 3 mM KCl, 11 mM glucose, 2 mM $CaCl_2$, and 1 mM $MgCl_2$ (307 mOsm/kg H_2O), continuously bubbled with a 95% O_2-5% CO_2 gas mixture.

595 In the lumbar SC of Gad1^{GFP} mouse embryos, eGFP neurons were detected using 488 nm

596 UV light. They were localized in the ventro-lateral marginal zone between the motor

597 columns and the ventral funiculi [66]. Embryonic V1^R identity was confirmed by the

598 expression of the forkhead transcription factor Foxd3 [25].

599

600 **Whole-cell recordings and analysis**

601 The isolated SC was placed in a recording chamber and was continuously perfused (2

602 ml/min) at room temperature (22-26°C) with oxygenated ACSF. Whole-cell patch-clamp

603 recordings of lumbar spinal embryonic V1^R were carried out under direct visualization using

604 an infrared-sensitive CCD video camera. Whole-cell patch-clamp electrodes with a

605 resistance of 4-7 MΩ were pulled from thick-wall borosilicate glass using a P-97 horizontal

606 puller (Sutter Instrument Co., USA). They were filled with a solution containing (in mM):

607 96.4 K methanesulfonate, 33.6 KCl, 4 MgCl$_2$, 4 Na$_2$ATP, 0.3 Na$_3$GTP, 10 EGTA, and 10 HEPES

608 (pH 7.2; 290 mOsm/kg-H$_2$O). This intracellular solution led to an equilibrium potential of

609 chloride ions, E_{Cl}, of about -30 mV, close to the physiological values measured at E12.5 in

610 spinal MNs [28]. The junction potential (6.6 mV) was systematically corrected offline.

611 Signals were recorded using Multiclamp 700B amplifiers (Molecular Devices, USA). Data

612 were low-pass filtered (2 kHz), digitized (20 kHz) online using Digidata 1440A or 1550B

613 interfaces and acquired using pCLAMP 10.5 software (Molecular Devices, USA). Analyses

614 were performed off-line using pCLAMP 10.5 software packages (Molecular devices;

615 RRID:SCR_014284) and Axograph 1.7.2 (AxoGraph; RRID:SCR_002798).

616 In voltage-clamp mode, voltage-dependent K$^+$ currents (I_{Kv}) were elicited in the presence

617 of 1 μM tetrodotoxin (TTX, Alomone lab, Cat# T550, CAS No.: 18660-81-6) by 500 ms

618 depolarizing voltage steps (10 mV increments, 10 s interval) after a prepulse of 300 ms at

619   V_H = -100 mV. To isolate I_{Kdr}, voltage steps were applied after a 300 ms prepulse at V_H = -

620   30 mV that inactivated the low threshold transient potassium current I_A. I_A was then

621   obtained by subtracting offline I_{Kdr} from the total potassium current I_{Kv}. Capacitance and

622   leak current were subtracted using on-line P/4 protocol provided by pCLAMP 10.5.

623   In current-clamp mode, V1^R discharge was elicited using 2 s depolarizing current steps

624   (from 0 to ≈ 50 pA in 5-10 pA increments, depending on the input resistance of the cell)

625   with an 8 s interval to ensure that the membrane potential returned to V_H. When a cell

626   generated a sustained discharge, the intensity of the depolarizing pulse was reduced to the

627   minimal value compatible with repetitive firing.

628   I_{Nap} was measured in voltage-clamp mode using a 70 mV/s depolarizing voltage ramp [83].

629   This speed was slow enough to preclude substantial contamination by the inactivating

630   transient current and fast enough to avoid substantial inactivation of I_{Nap}. Subtraction of

631   the current evoked by the voltage ramp in the presence of 1 μM TTX from the control

632   voltage ramp-evoked current revealed I_{Nap}.

633

634   **Pharmacological reagents**

635   During patch-clamp recordings, bath application of TTX (1 μM, Alomone lab, Cat# T550,

636   CAS No.: 18660-81-6) or 4-aminopyridine (4-AP, Sigma Aldrich Cat# T550, CAS No.: 18660-

637   81-6) was done using 0.5 mm diameter quartz tubing positioned, under direct visual

638   control, 50 μm away from the recording area. The quartz tubing was connected to 6

639   solenoid valves linked with 6 reservoirs *via* a manifold. Solutions were gravity-fed into the

640   quartz tubing. Their application was controlled using a VC-8 valve controller (Warner

641   Instruments, USA).

642   4-AP was used to block I_{Kdr}. To determine the concentration–response curve, $I - V$ curves

643   of I_{Kdr} for different concentrations of 4-AP (0.3 to 300 μM) were compared to the control

644   curve obtained in the absence of 4-AP. The percentage of inhibition for a given

645   concentration was calculated by dividing the peak intensity of I_{Kdr} by the peak value

646   obtained in control condition. The obtained normalized concentration–response curves

647   were fitted using the Hill equation:

648    $\frac{100 - I_{min}}{1 + ([4-AP]/IC_{50})^{n_H}} + I_{min}$,

649   where [4-AP] is the 4-AP concentration, I_{min} is the residual current (in percentage of the

650   peak I_{Kdr}), $100 - I_{min}$ is the maximal inhibition achieved for saturating concentration of

651   4-AP, IC_{50} is the 4-AP concentration producing half of the maximal inhibition, and n_H is

652   the Hill coefficient. Curve fitting was performed using KaleidaGraph 4.5 (Synergy Software,

653   USA).

654

655   **Immunohistochemistry and confocal microscopy**

656   E14.5 embryos were collected from pregnant females. Once dissected out of their yolk sac,

657   SCs were dissected and immediately immersion-fixed in phosphate buffer (PB 0.1 M)

658   containing 4% paraformaldehyde (PFA; freshly prepared in PB, pH 7.4) for 1 h at 4°C. Whole

659   SCs were then rinsed out in 0.12 M PB at 4°C, thawed at room temperature, washed in PBS,

660   incubated in NH_4Cl (50 mM), diluted in PBS for 20 min and then permeabilized for 30 min in

661   a blocking solution (10% goat serum in PBS) with 0.2% Triton X-100. They were incubated

662   for 48 h at 4°C in the presence of the following primary antibodies: guinea pig anti-FoxD3

663   (1:5000, gift from Carmen Birchmeier and Thomas Müller of the Max Delbrück Center for

664   Molecular Medicine in Berlin) and rabbit anti-cleaved Caspase-3 (1:1000, Cell Signaling

665   Technology Cat# 9661, RRID:AB_2341188). SCs were then washed in PBS and incubated for

666 2 h at RT with secondary fluorescent antibodies (goat anti-rabbit-conjugated 649; donkey

667 anti-guinea pig-conjugated Alexa Fluor 405 [1:1000, ThermoFisher]) diluted in 0.2% Triton

668 X-100 blocking solution. After washing in PBS, SCs were dried and mounted in Mowiol

669 medium (Millipore, Molsheim, France). Preparations were then imaged using a Leica SP5

670 confocal microscope. Immunostaining was observed using a 40X oil-immersion objective

671 with a numerical aperture of 1.25, as well as with a 63X oil-immersion objective with a

672 numerical aperture of 1.32. Serial optical sections were obtained with a Z-step of 1 μm

673 (40X) and 0.2-0.3 μm (63X). Images (1024x1024; 12-bit color scale) were stored using Leica

674 software LAS-AF and analyzed using ImageJ 1.5 (N.I.H., USA, RRID:SCR_003070) and Adobe

675 Photoshop CS6 (Adobe, USA, RRID:SCR_014199) software.

676

677 **Cluster analysis**

678 To classify the firing patterns of embryonic $V1^R$, we performed a hierarchical cluster

679 analysis on a population of 163 cells. Each cell was characterized by three quantitative

680 measures of its firing pattern (see legend of Figure 5). After normalizing these quantities to

681 zero mean and unit variance, we performed a hierarchical cluster analysis using the hclust

682 function in R 3.3.2 software (Cran project ; https://cran.r-project.org/; RRID:SCR_001905)

683 that implements the complete linkage method. The intercluster distance was defined as

684 the maximum Euclidean distance between the points of two clusters, and, at each step of

685 the process, the two closest clusters were merged into a single one, thus constructing

686 progressively a dendrogram. Clusters were then displayed in data space using the

687 dendromat function in the R package 'squash' dedicated to color-based visualization of

688 multivariate data. The best clustering was determined using the silhouette measure of

689 clustering consistency [84]. The silhouette of a data point, based on the comparison of its

690   distance to other points in the same cluster and to points in the closest cluster, ranges

691   from -1 to 1. A value near 1 indicates that the point is well assigned to its cluster, a value

692   near 0 indicates that it is close to the decision boundary between two neighboring clusters,

693   and negative values may indicate incorrect assignment to the cluster. This allowed us to

694   identify an optimal number k of clusters by maximizing the overall average silhouette over

695   a range of possible values for k [84], using the silhouette function in the R package 'cluster'.

696

697   **Biophysical modeling**

698   To understand the relationship between the voltage-dependent membrane conductances

699   and the firing patterns of embryonic V1^R, we relied on a single compartment conductance-

700   based model that included the leak current, the transient and persistent components of

701   the sodium current, I_{Nat} and I_{Nap}, a delayed rectifier potassium current I_{Kdr} and the

702   inactivating potassium current I_A revealed by experiments. Voltage evolution then

703   followed the equation

704   $C_{in}\frac{dV}{dt} = G_{in}(V_r - V) + G_{Nat}m^3h(E_{Na} - V) + G_{Nap}m_p^3s(V_{Na} - V) + G_{Kdr}n^3(E_K - V) +$

705   $G_A m_A h_A(E_K - V) + I$ (1),

706   ,

707   where C_{in} was the input capacitance; G_{in} the input conductance; G_{Nat}, G_{Nap}, G_{Kdr} and

708   G_A the maximal conductances of the aforementioned currents; m, m_p, n and m_A their

709   activation variables; h the inactivation variable of I_{Nat}, s the slow inactivation variable of

710   I_{Nap}, and m_A the inactivation variable of I_A. V_r is the baseline potential imposed by *ad hoc*

711   current injection in current-clamp experiments; E_{Na} and E_K are the Nernst potentials of

712   sodium and potassium ions, and I the injected current. All gating variables satisfied

713   equations of the form:

714 $\quad \tau_x \frac{dx}{dt} = x_\infty(V) - x,$

715 where the (in)activation curves were modeled by a sigmoid function of the form:

716 $\quad x_\infty = \frac{1}{1+\exp(-(V-V_x)/k_x)}$

717 with k_x being positive for activation and negative for inactivation. The time constant τ_x

718 was voltage-independent except for the inactivation variables h and s. The activation

719 variable m_A of I_A was assumed to follow instantaneously voltage changes.

720 The effect of channel noise was investigated with a stochastic realization of the model,

721 where channels kinetics were described by Markov-type models, assuming a unitary

722 channel conductance of 10 pS for all channels.

723

724 **Choice of model parameters**

725 Most model parameters were chosen on the basis of experimental measurements

726 performed in the present study or already reported [25]. Parameters that could not be

727 constrained from our experimental data were chosen from an experimentally realistic

728 range of values. V_r was set at -60 mV as in experiments (see **table 1**). C_{in} (average 13.15

729 pF, 50% between 11.9 and 15.1 pF, only 18 cells out of 246 in the first quartile below 7.2 pF

730 or in the fourth quartile above 19 pF) and G_{in} (50% of cells between 0.71 and 1.18 nS, only

731 7 out of 242 with input conductance above 2 nS) were not spread much in the cells

732 recorded at E12.5, which showed that most embryonic V1^R were of comparable size.

733 Interestingly, C_{in} and G_{in} were not correlated, which indicated that the input conductance

734 was determined by the density of leak channels rather than by the sheer size of the cell.

735 Moreover, no correlation was observed between the passive properties and the firing

736 pattern [25]. Therefore, we always set G_{in} and C_{in} to 1 nS and 13 pF in the model (except

737　in **Figure 6—figure supplement 2**), close to the experimental medians (0.96 nS and 13.15

738　pF, respectively). The membrane time constant C_{in}/G_{in} was then equal to 13 ms, which

739　was also close to the experimental median (13.9 ms, N=241).

740　E_{Na} was set to 60 mV (see [25]). The activation curve of I_{Nap} was obtained by fitting

741　experimental data, leading to an average mid-activation of -36 mV and an average

742　steepness of 9.5 mV. The experimentally measured values of G_{Nap} were in the range 0-2.2

743　nS. We assumed that the activation curve of I_{Nat} was shifted rightward by 10 mV in

744　comparison to I_{Nap}. No experimental data was available for the inactivation of I_{Nat}. We

745　chose a mid-inactivation voltage V_h= -45 mV and a steepness k_h=-5 mV. We also assumed

746　that the activation time constant of both I_{Nat} and I_{Nap} was 1.5 ms, and that the

747　inactivation　　　time　　　constant　　　was　　　voltage-dependent:

748　$\tau_h(V) = 16.5 - 13.5\tanh((V+20)/15)$, decreasing by one order of magnitude (from 30

749　ms down to 3 ms) with the voltage increase. This enabled us to account for the shape of

750　the action potentials recorded in experiments, showing a slow rise time and rather long

751　duration. The conductance G_{Nat} was not measured experimentally. When choosing a

752　reasonable value of 20 nS for G_{Nat}, the model behaved very much as recorded embryonic

753　V1[R]: with similar current threshold (typically 10-20 pA) and stable plateau potential

754　obtained for the largest values of G_{Nap}.

755　When taking into account slow inactivation of I_{Nap} (see **Figure 8**), we chose V_s = -30 mV

756　for the mid-inactivation voltage and set the steepness k_s at -5 mV (as for the inactivation

757　of I_{Nat}). For simplicity, we assumed that the inactivation time constant was voltage-

758　independent and set it at a value of 2 s.

759　E_K was set to the experimentally measured value of -96 mV [25]. The activation

760　parameters of I_{Kdr} were obtained by fitting the experimental data: V_n = -20 mV, k_n = 15

761 mV, τ_n = 10 ms and an activation exponent of 3. The activation and inactivation properties

762 of I_A were also chosen based on experimental measurements. Accordingly, V_{m_A} = -30 mV,

763 k_{m_A} = -12 mV, V_{h_A} = -70 mV, k_{h_A} = -7 mV, and τ_{h_A} = 23 ms. When I_A was taken into

764 account, we assumed that $G_A = G_{Kdr}$, consistently with experimental data (see **_Figure 6—_**

765 **_figure supplement 1_**).

766

767 **Numerical simulations and dynamical systems analysis**

768 We integrated numerically the deterministic model using the freeware XPP-Aut 8.0

769 (University of Pittsburgh; Pennsylvania; USA; RRID:SCR_001996) [85] and a standard

770 fourth-order Runge-Kutta algorithm. XPP-Aut was also used to compute one-parameter

771 and two-parameters bifurcation diagrams. The stochastic version of the model was also

772 implemented in XPP-Aut and computed with a Gillespie's algorithm [86].

773 To investigate the dynamics of the model with slow inactivation of I_{Nap}, we relied on

774 numerical simulations together with fast/slow dynamics analysis [87]. In this approach, one

775 distinguishes slow dynamical variables (here only s) and fast dynamical variables. Slow

776 variables vary little at the time scale of fast variables and may therefore be considered as

777 constant parameters of the fast dynamics in first approximation. In contrast, slow variables

778 are essentially sensitive to the time average of the fast variables, much more than to their

779 instantaneous values. This separation of time scales allows one to conduct a phase plane

780 analysis of the full dynamics.

781

782 **Statistics**

783 Samples sizes (n) were determined based on previous experience. The number of embryos

784 (N) is indicated in the main text and figure captions. No power analysis was employed, but

785 sample sizes are comparable to those typically used in the field. All values were expressed

786 as mean with standard error of mean (SEM). Statistical significance was assessed by non-

787 parametric Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons, Mann-

788 Whitney test for unpaired data or Wilcoxon matched pairs test for paired data using

789 GraphPad Prism 7.0e Software (USA). Significant changes in the proportions of firing

790 patterns with age were assessed by chi-square test for large sample and by Fisher's exact

791 test for small sample using GraphPad Prism 7.0e Software (GraphPad Software, USA;

792 RRID:SCR_002798). Significance was determined as $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$

793 (***). The exact p value was mentioned in the result section or in the figure captions.

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Additional information

Competing interests

The authors declare no competing interests

806

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Figure captions

Figure 1. G_{Kdr} and G_{Nap} in embryonic V1^R at E12.5 and E14.5

(A) Representative traces of voltage responses showing single-spiking activity in E12.5 SS

V1^R (A1), repetitive action potential firing in RS V1^R (A2), Mixed of plateau potential activity

and repetitive action potential firing in ME V1^R (A3) and plateau potential activity in PP V1^R

(A4). (B) Representative examples of the total outward K$^+$ currents (IKv total) obtained

from V_H = -100 mV (left traces), of I_{Kdr} (V_H = -30 mV, middle traces) and of isolated I_A (left

traces) recorded at E12.5 in SS V1^R (B1), RS V1^R (B2), ME V1^R (B3) and PP V1^R (B4). Voltage-

dependent potassium currents were evoked in response to 10 mV membrane potential

steps (200 ms) from -100 or from -30 mV to +40 mV (10 s interval between pulses). V1^R

were voltage clamped at V_H = -60 mV. A prepulse of -40 mV (300 ms) was applied to

activate both I_A and I_{Kdr}. I_{Kdr} was isolated by applying a prepulse of 30 mV (300 ms) to

inactivate I_A (B1 insert). I_A was isolated by subtracting step-by-step the currents obtained

using a pre-pulse of 30 mV (V_H = - 30 mV) from the currents obtained using a pre-pulse of -

40 mV (V_H = -100 mV). (C) Current-voltage relationship ($I - V$ curves) of I_{Kdr} (filled circles)

and of I_A (open circles) recorded in SS V1^R (C1), RS V1^R (C2), ME V1^R (C3) and PP V1^R (C4).

$I - V$ curves were obtained from currents shown in B1, B2, B3 and B4. Note that $I -$

V curves are similar between SS V1^R, RS V1^R, ME V1^R and PP V1^R. (D) Bar graph showing

maximal G_{Kdr} value (Max G_{Kdr}) in SS V1^R at E12.5 (n = 9; N = 9; gray bar) and at E14.5 (n =

10; N = 10 gray bar), and in RS V1^R (n = 7; N = 7; red bar), ME V1^R (n = 3; N = 3 purple bar)

and PP V1^R at E12.5 (n = 7; N = 7 blue bar). G_{Kdr} was calculated from I_{Kdr} at V_H = + 20 mV,

assuming a K$^+$ equilibrium potential of -96 mV. There is no significant difference

in G_{Kdr} between SS V1^R and RS V1^R, while G_{Kdr} is significantly smaller in PP V1^R as

compared to SS V1^R and RS V1^R. G_{Kdr} was significantly higher in SS V1^R at E14.5 than in SS

1143   V1^R, RS V1^R and PP V1^R at E12.5. (Kruskall-Wallis test $P < 0.0001$; SS V1^R versus RS V1^R at

1144   E12.5, $P = 0.5864$; SS V1^R versus PP V1^R at E12.5, $P = 0.0243$; RS V1^R versus PP V1^R at E12.5,

1145 $P = 0.0086$; E14.5 SS V1^R versus E12.5 SS V1^R, $P = 0.0048$; E14.5 SS V1^R versus E12.5 RS V1^R,

1146 $P = 0.0384$, E14.5 SS V1^R versus E12.5 PP V1^R, $P < 0.0001$). The increase in G_{Kdr} between

1147   E12.5 and E14.5 is likely to be due to the increase in neuronal size (input capacitance;

1148   Figure 2A). Indeed, there was no significant difference (Mann Whitney test, $P = 0.133$)

1149   in G_{Kdr} density between SS V1^R at E12.5 (n = 9; N = 9 gray bar) and at E14.5 (n = 10; N = 10

1150   gray bar). (E) Bar graph showing the maximal G_{Nap} value (Max G_{Nap}) in SS V1^R at E12.5 (n =

1151   9; N = 9 gray bar) and E14.5 (n = 10; N = 10 gray bar), and in RS V1^R (n = 8; N = 8 red bar),

1152   ME V1^R (n = 3; N = 3 purple bar) and PP V1^R (n = 6; N = 6 blue bar) at E12.5. Max G_{Nap} was

1153   calculated from maximal I_{Nap} value measured on current evoked by assuming a Na$^+$

1154   equilibrium potential of +60 mV. There was no difference in G_{Nap} between RS V1^R and PP

1155   V1^R. On the contrary, G_{Nap} measured in SS V1^R at E12.5 or at E14.5 was significantly

1156   smaller as compared to G_{Nap} measured at E12.5 in RS V1^R or in PP V1^R. G_{Nap} measured at

1157   E12.5 and E14.5 in SS V1^R were not significantly different (Kruskall-Wallis test $P < 0.0001$;

1158   E12.5 SS V1^R versus E12.5 RS V1^R, $P = 0.0034$; E12.5 SS V1^R versus E12.5 PP V1^R, $P = 0.0006$;

1159   E12.5 RS V1^R versus E12.5 PP V1^R, $P = 0.5494$; E14.5 SS V1^R versus E12.5 SS V1^R, $P = 0.5896$;

1160   E14.5 SS V1^R versus E12.5 RS V1^R, $P = 0.0005$; E14.5 SS V1^R versus E12.5 PP V1^R, $P < 0.0001$).

1161   (F) Histograms showing the G_{Nap} / G_{Kdr} ratio in SS V1^R at E12.5 (n = 9; gray bar) and E14.5

1162   (n = 10; green bar) and in RS V1^R (n = 8; red bar), ME V1^R (n = 3; purple bar) and PP V1^R (n =

1163   6; blue bar) at E12.5. Note that the G_{Nap} / G_{Kdr} ratio differs significantly between SS V1^R,

1164   RS V1^R and PP V1^R at E12.5, while it is not different between SS V1^R recorded at E12.5 and

1165   at E14.5 (Kruskall-Wallis test $P < 0.0001$; SS V1^R versus RS V1^R at E12.5, $P = 0.0367$; SS V1^R

1166   versus PP V1^R at E12.5, $P < 0.0001$; RS V1^R versus PP V1^R at E12.5, $P = 0.0159$; E14.5 SS V1^R

1167   versus E12.5 SS V1^R, P = 0.2319; E14.5 SS V1^R versus E12.5 RS V1^R, P = 0.0017; E14.5 SS V1^R

1168   versus E12.5 PP V1^R P < 0.0001). Data shown in A and B were used to

1169   calculate G_{Nap} / G_{Kdr} ratio shown in C. (*P < 0.05, ** P < 0.01, *** P < 0.001).

1170

1171   **Figure 2. Increasing 4-AP concentration changed the firing pattern of single spiking**

1172   **embryonic V1^R recorded at E12.5**

1173   The firing pattern of embryonic V1^R was evoked by 2 s suprathreshold depolarizing current

1174   steps. (A) Representative traces showing examples of the effect of increasing concentration

1175   of 4-AP (from 3 to 300 μM) on the firing pattern of a SS V1^R recorded at E12.5. Note that in

1176   A1, increasing 4-AP concentration converted single spiking (gray trace) to repetitive spiking

1177   (red trace), repetitive spiking to a mixed event pattern (purple trace) and mixed events to

1178   plateau potential (blue trace). (A2) Example of SS V1^R in which increasing 4-AP

1179   concentration converted single spiking to repetitive spiking only. (A3) Bar plots showing the

1180   change in the firing pattern of SS V1^R according to 4-AP concentrations (control n = 10; N =

1181   10, 3 μM 4-AP n = 8; N = 8, 10 μM 4-AP n = 10; N = 10, 30 μM 4-AP n = 10; N = 10, 100 μM

1182   4-AP n = 10; N = 10, 300 μM 4-AP n = 8; N = 8). (B) Representative traces showing the effect

1183   of 0.5 μM TTX on a plateau potential evoked in a SS V1^R in the presence of 300 μM 4-AP. (C)

1184   Representative traces showing the effect of 0.5 μM TTX on repetitive AP firing evoked in a

1185   SS V1^R in the presence of 300 μM 4-AP. In both cases, the application of TTX fully blocked

1186   the responses evoked in the presence of 4-AP, indicating that they were underlain by the

1187   activation of voltage-gated Na$^+$ channels.

1188

1189   **Figure 3. Cluster analysis of V1^R firing pattern at E12.5**

49

1190   (A, inserts) Cluster analysis of embryonic V1^R firing pattern was performed using three

1191   parameters that describe the firing pattern during a 2 s suprathreshold depolarizing pulses:

1192   the mean of the half-amplitude event duration (mean ½Ad), the coefficient of variation of

1193   ½ Ad (CV ½Ad) allowing to quantify the AP variation within a train (CV was set to 0 when

1194   the number of spikes evoked by a depolarizing pulse was ≤ 3) and the duration ratio ddr =

1195   Σ½ Ad/Pw, obtained by dividing the sum of ½ Ad by the pulse duration Pw, that indicates

1196   the total time spent in the depolarized state. For example, ddr = 1 when a plateau potential

1197   lasts as long as the depolarizing pulse. Conversely, its value is low when the depolarizing

1198   pulse evokes a single AP only. (A) Dendrogram for complete linkage hierarchical clustering

1199   of 164 embryonic V1^R (N = 140) according to the values of log mean ½Ad, of CV ½Ad and of

1200   log ddr. The colored matrix below the dendrogram shows the variations of these three

1201   parameters for all the cells in the clusters (colored trees) extracted from the dendrogram.

1202   (B) The number of clusters was determined by analyzing the distribution of silhouette

1203   width values (see Material and Methods). The boxplots show the distribution of silhouette

1204   width values when the number of clusters k varies from 2 to 12. The mean silhouette width

1205   values (red diamond shaped points) attained their maximum when the estimated cluster

1206   number was 5. (C) 3D plot showing cluster distribution of embryonic V1^R according to log

1207   mean ½Ad, CV ½Ad and log ddr. Each cluster corresponds to a particular firing pattern as

1208   illustrated in D. V1^R that cannot sustain repetitive firing of APs (1 to 3 AP/pulse only, gray,

1209   Single spiking, SS), V1^R that can fire tonically (red, Repetitive spiking, RS), V1^R with a firing

1210   pattern characterized by a mix of APs and relatively short plateau potentials (dark purple,

1211   Mixed event short PP, ME short PP), V1^R with a firing pattern characterized by a mix of APs

1212   and relatively long plateau potentials (light purple, Mixed event long PP, ME long PP) and

1213   V1^R with evoked plateau potentials only (blue, Plateau potential, PP). The arrow in C

1214   indicates 3 misclassified V1^R that could not sustain repetitive firing although they were

1215   assigned to the cluster of repetitively firing V1^R (see text).

1216

1217   **Figure 4. Developmental changes of embryonic V1^R firing patterns from E11.5 to E16.5**

1218   (A1) Graph showing how the input capacitance C_{in} of V1^R changes with embryonic age. C_{in}

1219   significantly increased between E12.5 or E13.5 and E14.5 (Kruskall-Wallis test $P < 0.0001$;

1220   E12.5 versus E11.5 $P = 0.258$, E12.5 versus E13.5 $P = 0.904$, E12.5 versus E14.5 $P < 0.0001$,

1221   E12.5 versus E15.5 $P < 0.0001$, E12.5 versus E16.5 $P < 0.0001$, E13.5 versus E14.5 $P < 0.0001$,

1222   E13.5 versus E15.5 $P < 0.0001$, E13.5 versus E16.5 $P < 0.0001$; E11.5 n = 31; N = 27, E12.5 n

1223   = 267; N = 152, E13.5 n = 43; N = 40, E14.5 n = 61; N = 49, E15.5 n = 16; N = 4, E16.5 n = 30;

1224   N = 9). (A2) Graph showing how the input resistance R_{in} of V1^R changes with embryonic

1225   age. R_{in} significantly decreased between E12.5 or E14.5 and E15.5 (Kruskall-Wallis test $P <$

1226 0.0001; E12.5 versus E11.5 $P > 0.999$, E12.5 versus E13.5 $P = 0.724$, E12.5 versus E14.5 $P >$

1227 0.999, E12.5 versus E15.5 $P = 0.0004$, E12.5 versus E16.5 $P = 0.0005$, E14.5 versus E15.5 $P =$

1228 0.0019, E14.5 versus E16.5 $P < 0.0058$; E11.5 n = 31, E12.5 n = 261; N = 146, E13.5 n = 43; N

1229   = 40, E14.5 n = 60; N = 48, E15.5 n = 16; N = 4, E16.5 n = 30; N = 9). (A3) Graph showing

1230   how the threshold of regenerative events (APs and plateau potentials) of V1^R changes with

1231   embryonic age. The average threshold became significantly more hyperpolarized after

1232   E12.5 (Kruskall-Wallis test $P < 0.0001$; E12.5 versus E11.5 $P = 0.676$, E12.5 versus E13.5 $P =$

1233 0.0039, E12.5 versus E14.5 $P < 0.0001$, E12.5 versus E15.5 $P < 0.0001$, E12.5 versus E16.5 P

1234 < 0.0001, E13.5 versus E14.5 $P > 0.999$, E13.5 versus E15.5 $P = 0.1398$, E13.5 versus E16.5 P

1235 $= 0.0013$; E14.5 versus E15.5 $P > 0.999$, E14.5 versus E16.5 $P = 0.0634$, E15.5 versus E16.5 $P >$

1236 0.999; E11.5 n = 20; N = 16, E12.5 n = 162; N = 139, E13.5 n = 31; N = 28, E14.5 n = 30; N =

1237   26, E15.5 n = 16; N = 4, E16.5 n = 30; N = 9). Yellow and purple bars below the graphs

1238 indicate the two important phases of the functional development of spinal cord networks.

1239 The first one is characterized by synchronized neuronal activity (SNA) and the second one is

1240 characterized by the emergence of a locomotor-like activity (see text). Note that changes in

1241 C_{in} and R_{in} occurred at the end of the first developmental phase. (*$P < 0.05$, ** $P < 0.01$,

1242 *** $P < 0.001$; control, E12.5). The intrinsic activation properties were analyzed using 2 s

1243 suprathreshold depolarizing current steps. (B) Representative traces of voltage responses

1244 showing Single Spiking (SS) V1[R] (gray), Repetitive Spiking (RS) V1[R] (red), ME V1[R] (purple)

1245 and Plateau Potential (PP) V1[R] (blue) at E11.5 (B1), E13.5 (B2), E14.5 (B3) E15.5 (B4) and

1246 E16.5 (B5). (C) Bar graph showing how the proportions of the different firing patterns

1247 change from E11.5 to E16.5 (E11.5 n = 22; N = 18, E12.5 n = 163; N = 140, E13.5 n = 32; N =

1248 29, E14.5 n = 57; N = 45, E15.5 n = 15; N = 4, E16.5 n = 28; N = 9). Yellow and purple bars

1249 below the graphs indicate the first and the second phase of functional embryonic spinal

1250 cord networks. The proportions of the different firing patterns significantly changed

1251 between E11.5 to E12.5 (Fisher's exact test, $P = 0.0052$) with a significant increase in the

1252 proportion of RS V1[R] (Fisher's exact test, $P = 0.0336$) and a significant decrease in the

1253 proportion of ME V1[R] (Fisher's exact test, $P = 0.01071$) at E12.5. Only two firing patterns (SS

1254 and RS) were observed after E13.5 and most embryonic V1[R] lost their ability to sustain

1255 tonic firing after E13.5. However, at E16.5 the proportion of RS V1[R] significantly increased

1256 at the expense of SS V1[R] when compared to E14.5 (Fisher's exact test, $P = 0.0112$),

1257 indicating that embryonic V1[R] began to recover the ability to sustain tonic firing after E15.5.

1258

1259 **Figure 5**. **Activated caspase-3 is not observed in embryonic V1[R] at E14.5**

1260 Representative confocal image of the ventral part of an isolated lumbar spinal cord of

1261 E14.5 GAD67-eGFP mouse embryo showing immunostainings using antibodies against

1262   eGFP (A), FoxD3 (B) and activated Caspase 3 (aCaspace 3) (C). (D) Superimposition of the

1263   three stainings shows that embryonic V1^R (eGFP+ and FoxD3+) were not aCaspase 3

1264   immunoreactive. (A1, B1, C1 and D1). Enlarged images from A, B and C showing that

1265   aCaspase 3 staining is localized in areas where eGFP and Foxd3 staining were absent. (A2,

1266   B2, C2 and D2) Enlarged images from A, B and C showing that aCaspase 3 staining is absent

1267   in the area where V1^R (eGFP+ and FoxD3+) are located. aCaspase 3 staining that did not co-

1268   localize with GAD67eGFP likely indicates MN developmental cell death.

1269

1270   **Figure 6. 600 μM 4-AP changed the firing pattern of single spiking embryonic V1^R**

1271   **recorded at E14.5**

1272   The firing pattern of embryonic V1^R was evoked by 2 s suprathreshold depolarizing current

1273   steps. (A) Representative traces showing the effect of 4-AP application (600 μM) on the

1274   firing pattern of single spiking (SS) V1^R recorded at E14.5. Note that the applications of 600

1275   μM 4-AP evoked either a plateau potential (A1) or repetitive spiking (A2), both fully

1276   blocked by TTX. (B) Bar plots showing the proportions of the different firing patterns

1277   observed in the presence of 600 μM 4-AP versus control recorded in SS V1^R at E14.5 (n = 14;

1278   N = 14). Single Spiking (SS) V1^R (gray), Repetitive Spiking (RS) V1^R (red), Mixed Events (ME)

1279   V1^R (purple), Plateau Potential (PP) V1^R (blue).

1280

1281   **Figure 7. Embryonic V1^R firing patterns predicted by computational modeling**

1282   (A) Firing patterns of 27 recorded cells, in which both G_{Nap} and G_{Kdr} were measured. Gray:

1283   SS, red: RS, blue: PP. The three purple points located at the boundary between the RS and

1284   PP regions correspond to mixed events (ME) where plateau potentials alternate with

1285   spiking episodes. Note that no cell exhibited low values of both G_{Nap} and G_{Kdr} (lower left),

1286   or large values of both conductances (upper right). (B) Bifurcation diagram of the

1287   deterministic model when G_{Kdr} is kept fixed to 2.5 nS or 10 nS while G_{Nap} is varied

1288   between 0 and 2.5 nS. G_{in} = 1 nS and I = 20 pA. For G_{Kdr} = 10 nS (i.e., in the top

1289   experimental range), the red curves indicate the maximal and minimal voltages achieved

1290   on the stable limit cycle associated with repetitive firing (solid lines) and on the unstable

1291   limit cycle (dashed lines). The fixed point of the model is indicated by a gray solid line when

1292   it corresponds to the stable quiescent state, a gray dashed line when it is unstable and a

1293   solid blue line when it corresponds to a stable plateau potential. The two HB corresponding

1294   to the change of stability of the quiescence state (HB$_1$, G_{Nap}= 0.81 nS) and of the voltage

1295   plateau (HB$_2$, G_{Nap}= 2.13 nS) are indicated, as well as the two SN bifurcations of limit cycles

1296   associated with the onset (SN$_1$, $G_{Nap} = 0.65$ nS) and offset (SN$_2$, G_{Nap}= 2.42 nS) of

1297   repetitive spiking as G_{Nap} is increased. For G_{Kdr} = 2.5 nS, the model does not display

1298   repetitive firing; it possesses a unique fixed point, which is always stable (blue-gray curve).

1299   The transition from quiescence to plateau is gradual with no intervening bifurcation.

1300   Representative voltage traces of the three different activity patterns are shown: single

1301   spiking in response to a 2 s current pulse (gray, G_{Nap} = 0.2 nS, G_{Kdr} = 10 nS), repetitive

1302   spiking (red, G_{Nap} = 1.2 nS, G_{Kdr} = 10 nS) and plateau potential (blue, G_{Nap} = 1.2 nS, G_{Kdr} =

1303   2.5 nS). Note that the plateau never outlasts the current pulse. (C) Bifurcation diagram

1304   when G_{Nap} is kept fixed at 1.2 nS and G_{Kdr} is varied between 0 and 25 nS (I = 20 pA). Same

1305   conventions as in B. Plateau potential is stable until the subcritical Hopf bifurcation HB$_2$

1306   (G_{Kdr} = 6.34 nS) is reached, repetitive firing can be observed between SN$_2$ (G_{Kdr} = 5.93 nS)

1307   and SN$_1$ (G_{Kdr} = 22.65 nS). The quiescent state is stable from point HB$_1$ ($G_{Kdr} = 17.59$ nS)

1308   onward. (D) Two-parameters bifurcation diagram of the model in the G_{Nap} - G_{Kdr} plane (I

1309   = 20 pA). The black merged curves indicate the bifurcations HB$_1$ and HB$_2$. The red curves

1310   indicate the SN bifurcations of limit cycles SN_1 and SN_2. The shaded area indicates the

1311   region where repetitive firing can occur. The oblique lines through the points labeled 1, 2

1312   and 3, the same as in B, correspond to three different values of the ratio G_{Nap}/G_{Kdr} : 0.02

1313   (gray), 0.12 (red) and 0.48 (blue). Voltage traces on the right display the response to a 2 s

1314   current pulse when channel noise is taken into account for the three regimes: quiescence

1315   (top, gray trace and dot in the diagram), repetitive firing (middle, red) and plateau

1316   potential (bottom, blue). They correspond to the three deterministic voltage traces shown

1317   in B. Note that the one-parameter bifurcation diagrams shown in B correspond to

1318   horizontal lines through points 1 and 2 (G_{Kdr} = 10 nS) and through point 3 (G_{Kdr} = 2.5 nS),

1319   respectively. The bifurcation diagram in C corresponds to a vertical line through point 2 and

1320   3 (G_{Nap} = 1.2 nS). (E) Cumulative distribution function of the ratio G_{Nap}/G_{Kdr} for the four

1321   clusters in A, showing the sequencing SS (gray) → RS (red) → ME (purple, 3 cells only) →

1322   PP (blue) predicted by the two-parameters bifurcation diagram in D. The wide PP range, as

1323   compared to SS and RS, merely comes from the fact that G_{Kdr} is small for cells in this

1324   cluster. The three colored points indicate the slopes of the oblique lines displayed in D

1325   (0.02, 0.12 and 0.48, respectively). (F) The data points in A are superimposed on the two-

1326   parameters bifurcation diagram shown in D, demonstrating a good agreement between

1327   our basic model and experimental data (the same color code as in A for the different

1328   clusters). The bifurcation diagram is simplified compared to A, only the region where

1329   repetitive spiking is possible (i.e. between the lines SN_1 and SN_2 in A) being displayed

1330   (shaded area). Notice that 3 ME cells (purple dots) are located close to the transition

1331   between the RS and PP regions. The four arrows indicate the presumable evolution of G_{Nap}

1332   and G_{Kdr} for SS, RS, ME and PP cells between E12.5 and E14.5-15.5. G_{Nap} eventually

1333   decreases while G_{Kdr} keeps on increasing. (G) Distribution of a sample of cells in the G_{Kdr} -

1334 G_{Kdr} plane at E14.5. All the cells are located well within the SS region far from bifurcation

1335 lines because of the decreased G_{Nap} compared to E12.5, the increased G_{Kdr}, and the shift

1336 of the RS region (shaded) due to capacitance increase (18 versus 13 pF).

1337

1338 **Figure 8. Effects of the slow inactivation of I_{Nap} on firing patterns predicted by**

1339 **computational modeling**

1340 (A) Examples of repetitive plateaus (left) and mixed events (right) recorded in V1[R] at E12.5

1341 during a 2 s current pulse. (B1) Current-voltage curve of the basic model (without slow

1342 inactivation of I_{Nap}, and without I_A or channel noise) for G_{Kdr} = 5 nS and for G_{Nap} = 1.65

1343 nS (lower curve) and 2 nS (upper curve). Solid lines denote stable fixed points and dashed

1344 lines unstable ones. For G_{Nap} =1.65 nS, bistability between quiescence and plateau occurs

1345 between 1.39 and 10.48 pA. When G_{Nap} is increased to 2 nS, the bistability region ranges

1346 from -10.84 to 9.70 pA, thus extending into the negative current range. This implies that

1347 once a plateau has been elicited, the model will stay in that stable state and not return to

1348 the resting state, even though current injection is switched off (see insert). B1 Insert.

1349 Voltage response to a 2 s current pulse of 15 pA for G_{Nap} = 2 nS. The resting state (gray dot

1350 on the lower curve in B1 is destabilized at pulse onset and a plateau is elicited (blue dot on

1351 the upper curve in B1). At pulse offset, the plateau is maintained, even though the injected

1352 current is brought back to zero, and channel noise is not sufficient to go back to the resting

1353 state. (B2) Domain of bistability between quiescence and plateau (shaded) in the I-

1354 G_{Nap} plane for G_{Kdr}= 5 nS. It is delimited by the line SN$_2$ where a SN bifurcation of fixed

1355 points occurs and by the subcritical Hopf bifurcation line HB where the plateau becomes

1356 unstable. Bistability requires that G_{Nap} exceeds 1.35 nS, and the domain of bistability

1357 enlarges as G_{Nap} is increased further. The two horizontal lines correspond to the two cases

1358   shown in B1: G_{Nap} =1.65 nS and 2 nS. (C) Behavior of the model when slow inactivation is

1359   incorporated. The bifurcation diagram of the basic model (without slow inactivation) for I =

1360   10 pA and G_{Kdr} = 5 nS (same conventions as in Fig 7B) and the stable limit cycle (black solid

1361   curve) obtained when slow inactivation is added are superimposed. The limit cycle is

1362   comprised of four successive phases (see labels): 1) long plateau during which I_{Nap} slowly

1363   inactivates, 2) fast transition to the quiescent state, 3) repolarization episode during which

1364 I_{Nap} slowly deinactivates, 4) fast transition back to the plateau. Each plateau starts with a

1365   full-blown action potential followed by rapidly decaying spikelets. Note that the bifurcation

1366   HB is subcritical here (unstable limit cycle shown by dashed red curve), at variance with

1367   square wave bursting (supercritical bifurcation and stable limit cycle); this is a characteristic

1368   feature of pseudo-plateau bursting. Note also that the plateau extends beyond the

1369   bifurcation HB because it is only weakly unstable then. Responses to a 15 s current pulse

1370   are shown on the right side. Top left: voltage response (G_{Nap}= 2.5 nS), Top right: behavior

1371   of the "effective" conductance of I_{Nap} channel, i.e., the maximal conductance G_{Nap}

1372   multiplied by the slow inactivation variable s. Bottom left: voltage trace when channel

1373   noise is added to fast and slow gating variables, Bottom right: Voltage trace when G_{Nap} is

1374   increased by 50% to 3.75 nS. (D) Mixed events. The bifurcation diagram of the basic model

1375   for G_{Kdr} = 5 nS and I = 12 pA and the stable limit cycle obtained in the presence of slow

1376   inactivation (G_{Nap} = 2.5 nS) are superimposed. Here again, the limit cycle is comprised of

1377   four successive phases (see labels): 1) slow inactivation of I_{Nap} that leads to the crossing of

1378   the bifurcation point HB_2 and then to the destabilization of the plateau potential, 2) fast

1379   transition to the spiking regime, 3) repetitive spiking during which I_{Nap} slowly de-

1380   inactivates, which leads to the crossing of the bifurcation point SN_2 and terminates the

1381   spiking episode, 4) fast transition back to the stable plateau potential. Response to a 15 s

1382   current pulse of 12 pA is shown on the right in the absence of any channel noise. Top:

1383   Voltage trace (same labels as in the bifurcation diagram on the left), Bottom: Variations of

1384   the "effective" conductance $G_{Nap}s$ (same labels as in the voltage trace). Note that de-

1385   inactivation sufficient to trigger a new plateau occurs over a series of successive spikes,

1386   hence the small oscillations are visible on the trace. Note also that in C and D the first

1387   plateau lasts longer than the following ones, as in electrophysiological recordings of

1388   embryonic V1[R] cells displaying repetitive plateaus. This form of adaptation is caused by the

1389   slow inactivation of the persistent sodium current.

1390

1391

Supplementary legends

Figure 2—figure supplement 1. Effect of 4-AP on I_{Kdr} and I_A in embryonic V1^R

(A1) Example of voltage-dependent potassium currents evoked in response to 10 mV

membrane potential steps (200 ms) from -100 mV or from -30 mV to +40 mV (10 s interval

between pulses). V1^R were voltage clamped at V_H = -60 mV. A prepulse of -40 mV (300 ms)

was applied to activate both I_A and I_{Kdr}. I_{Kdr} was evoked in response to 10 mV membrane

potential steps (200 ms) from -100 mV to +40 mV. V1^R were voltage clamped at V_H = -60

mV. A prepulse of 30 mV (V_H = - 30 mV) was applied to isolate I_{Kdr}. (A1) Representative

example of the effect of 300 μM 4-AP application on I_{Kdr} recorded from embryonic V1^R at

E12.5. (B1) Curves showing current-voltage relationships of I_{Kdr} in control and in the

presence of 300 μM 4-AP. Measurements were performed on traces shown in A1. (C1)

Dose-response relationship of 4-AP-evoked I_{Kdr} inhibition (mean + SE). Data were

normalized to I_{Kdr} amplitude measured in the absence of 4-AP (V_H = 40mV) and fitted as

explained in Materials and Methods. Note that 4-AP IC$_{50}$ is in μM range (2.9 μM). 0.3 μM 4-

AP n = 3; N = 3, 1 μM 4-AP n = 3; N = 3, 3 μM 4-AP n = 9; N = 9, 10 μM 4-AP n = 13; N = 13,

30 μM 4-AP n = 7; N = 7, 100 μM 4-AP n = 7; N = 7, 300 μM 4-AP n = 7; N = 7. (A2) I_A was

obtained as the difference between currents evoked from V_H = -100 mV and currents

evoked from V_H = -30 mV (10 mV voltage step). (A2) Representative example of the effect

of 300 μM 4-AP on I_A in V1^R recorded at E12.5. (B2) I_A Current-voltage ($I - V$) relationship

in control conditions and in the presence of 300 μM 4-AP. The $I - V$ curves were obtained

from the traces shown in A1. (C2) Bar graph showing the percentage of I_A block elicited by

4-AP. Note that 4-AP did not significantly block I_A (Wilcoxon test P = 0.065, n = 10).

1415 **Figure 2—figure supplement 2. Relates to Fig 2. Effect of 4-AP application in repetitively**

1416 **spiking V1^R at E12.5**

1417 (A) Representative traces showing the effect of 4-AP application (300 µM) on Repetitive

1418 Spiking (RS) V1^R at E12.5. Note that plateau potential activity evoked in the presence of 4-

1419 AP (middle trace) was blocked by 0.5 µM TTX (right trace). (B) Bar plots showing the

1420 changes in the firing pattern of RS V1^R evoked by 300 µM 4-AP application (n = 14). 4-AP

1421 application evoked a plateau potential in 71.4 % of the recorded neurons (10/14) and

1422 mixed events in 14.3% of the recorded neurons (2/14). The excitability pattern was not

1423 modified in 2 neurons. Repetitive Spiking (RS) V1^R (red), Mixed events (ME) V1^R (purple),

1424 Plateau Potential (PP) V1^R (blue).

1425

1426 **Figure 3—figure supplement 1. Distributions of log ½Ad, CV ½Ad and log ddr values**

1427 **related to the cluster analysis of embryonic V1^R firing patterns**

1428 (A1) Histogram of log mean ½Ad (mean half amplitude event duration) for the whole V1^R

1429 population at E12.5 (n= 164; bin width 0.1). The histogram was well fitted by the sum of

1430 three Gaussian curves with means and SDs of 1.135, 2.046 & 2.84, and 0.316, 0.181 & 0.21,

1431 respectively. (A2) Histogram of the values of log mean ½Ad sorted after cluster analysis

1432 showing single spiking (SS) V1^R (gray), repetitive spiking (RS) V1^R (red), mixed events (ME)

1433 V1^R with short plateau potentials (ME short PP V1^R, light purple), ME V1^R with long plateau

1434 potentials (ME long PP V1^R, dark purple) and plateau potential (PP) V1^R (blue). log mean

1435 ½Ad was significantly different between SS V1^R, PP V1^R, the whole ME V1^R population (ME$_s$

1436 and ME$_l$ V1^R) and PP V1^R (Kruskall-Wallis test $P < 0.0001$; SS V1^R versus RS V1^R, $P < 0.0001$;

1437 SS V1^R versus ME V1^R, $P < 0.0001$; SS V1^R versus PP V1^R, $P < 0.0001$; RS V1^R versus ME V1^R, P

1438 $= 0.0004$; RS V1^R versus PP V1^R, $P < 0.0001$; ME V1^R versus PP V1^R, $P = 0.018$; SS V1^R n = 46,

60

1439 RS V1^R n = 69, ME$_s$ V1^R n = 9, ME$_l$ V1^R n = 4, PP V1^R n = 35). (B1) Histogram of CV ½Ad for

1440 the whole V1^R population at E12.5 (n= 164; bin width 5%). Note that a large population of

1441 V1^R had zero CV ½Ad (n = 83). The histogram for CV ½Ad ≠ 0 was fitted by the sum of three

1442 Gaussian curves with means and SDs of 23.4, 68.4 & 117 (%) and 8.9, 6.8 & 4.1, respectively.

1443 (B2) Histograms of the values of CV ½Ad sorted after cluster analysis showing SS V1^R (black),

1444 RS V1^R (red), ME$_s$ V1^R (light purple), ME$_l$ V1^R (dark purple) and PP V1^R. CV ½Ad was not

1445 significantly different between SS V1^R and PP V1^R (CV ½Ad of SS V1^R and PP V1^R = 0.682 %

1446 and 0% respectively: only one of the 46 SS V1^R displayed 3 PA and had a CV ½Ad of 31.37).

1447 CV ½Ad was significantly different between RS V1^R and the whole ME V1^R population and

1448 also between SS V1^R or PP V1^R and RS V1^R or ME V1^R (Kruskall-Wallis test $P < 0.0001$; SS V1^R

1449 versus RS V1^R $P < 0.0001$, SS V1^R versus ME V1^R $P < 0.0001$, SS V1^R versus PP V1^R $P = 0.846$,

1450 RS V1^R versus ME V1^R $P = 0.0003$, RS V1^R versus PP V1^R $P < 0.0001$, ME V1^R versus PP V1^R P

1451 < 0.0001). (C1) Histogram of log ddr (sum of ½Ad divided by pulse duration) for the whole

1452 V1^R population at E12.5 (n= 164; bin width 0.2). The histogram was fitted by the sum of

1453 two Gaussian curves with means and SDs of -2.51 & -0.851, and 0.2 & 0.46, respectively.

1454 (C2) Histograms of the values of log ddr sorted after cluster analysis showing SS V1^R (black),

1455 RS V1^R (red), ME$_s$ V1^R (light purple), ME$_l$ V1^R (dark purple) and PP V1^R. log (ddr) was not

1456 significantly different between ME V1^R and PP V1^R, while it was significantly different

1457 between SS V1^R and RS V1^R, SS V1^R and the whole ME V1^R population, SS V1^R and PP V1^R,

1458 RS V1^R and the whole ME V1^R population, RS V1^R and PP V1^R (Kruskall-Wallis test $P < 0.0001$;

1459 SS V1^R versus RS V1^R, $P < 0.0001$; SS V1^R versus ME V1^R, $P < 0.0001$; SS V1^R versus PP V1^R, P

1460 < 0.0001; RS V1^R versus ME V1^R, $P < 0.0001$; RS V1^R versus PP V1^R, $P < 0.0001$; ME V1^R

1461 versus PP V1^R, $P = 0.977$). ME$_s$ V1^R and ME$_l$ V1^R differed only by their CV ½Ad (Mann-

1462 Whitney test, log mean ½Ad for ME$_s$ V1^R versus log mean ½Ad for ME$_l$ V1^R, $P = 0.26$; CV

1463   ½Ad for ME$_s$ V1^R versus CV ½Ad ME$_l$ V1^R, P = 0.0028 and log ddr for ME$_s$ V1^R versus log ddr

1464   for ME$_l$ V1^R, P = 0.1483). It is noteworthy that the distribution of the values of each metric

1465   was multimodal thus indicating that each of them could partially discriminate different

1466   groups of embryonic V1^R according to their firing pattern.

1467

1468   **Figure 6—figure supplement 1. I_{Nap} is present in embryonic V1^R recorded at E14.5**

1469   (A) Representative trace of I_{Nap} evoked by a slow depolarizing voltage ramp (70 mV/s,

1470   upper insert) in SS embryonic V1^R (lower insert). I_{Nap} was isolated by subtracting currents

1471   evoked by depolarizing ramps in the presence of 1 μM TTX to the control current evoked in

1472   the absence of TTX (upper insert). (B) Voltage dependence of G$_{Nap}$ conductance calculated

1473   from the trace shown in A. The activation curve was obtained by transforming the current

1474   evoked by a depolarizing voltage ramp from -100 mV to 20 mV (70 mV/s) using the

1475   following equation: G$_{NaP}$ = -I$_{Nap}$/((-Vh)+E$_{Na}$) where Vh is the holding potential at time t

1476   during a depolarizing voltage ramp and E$_{Na}$ is the equilibrium potential for sodium (E$_{Na}$ = 60

1477   mV). The G$_{NaP}$/Vh curve was fitted with the following Boltzmann function: G =

1478   G$_{MAX}$/(1+exp(-(V-V$_{HALF}$)/k))) (Boeri et al. 2018), where V$_{half}$ is the Vh value for G$_{Nap}$ half

1479   activation, k the slope factor of the curve and G$_{max}$ the maximum conductance. We found

1480   no significant difference between the values of V$_{half}$ (Mann-Whitney test: P = 0.8518) and

1481   of k (Mann-Whitney test: P = 0.7546) obtained at E12.5 (Boeri et al. 2018) and those

1482   obtained at E14.5. At E14.5 V$_{half}$ = -27 ± 5.1 mV and k = 7.73 ± 0.78 (n = 6).

1483

1484   **Figure 6—figure supplement 2. I_{Kdr} was inhibited by 4-AP in V1^R recorded at E14.5**

1485   (A1) Representative examples of the total outward K$^+$ currents obtained from V_H = -100 mV

1486   (left traces), of I_{Kdr} (V_H = -30 mV, middle traces) and of isolated I_A (left traces) recorded in

1487 single spiking (SS) V1^R at E14.5. (A2) Current-voltage relationship of I_{Kdr} (filled circle) and

1488 of I_A (open circle) in SS V1^R at E14.5. $I-V$ curves were obtained from currents shown in

1489 A1. (B1) Representative example of the effect of 4-AP at 600 µM in V1^R at E14.5. (B2)

1490 Current-voltage curves in control condition and in the presence of 600 µM 4-AP. (B3) Bar

1491 plots showing the percentage of I_{Kdr} inhibition evoked by 300 µM 4-AP application (n = 8)

1492 and by 600 µM 4-AP application (n = 7). The percentages of I_{Kdr} inhibition evoked by 300

1493 µM 4-AP and by 600 µM 4-AP applications were not significantly different (P = 0.574). (C1)

1494 Representative example of the effect of 600 µM 4-AP on I_A in V1^R recorded at E14.5. (C2)

1495 $I-V$ curves in control conditions and in the presence of 600 µM 4-AP. These curves were

1496 obtained from the traces shown in B1. (C3) Bar graph showing the percentage of I_A block

1497 elicited by 4-AP. 4-AP did not significantly block I_A (Wilcoxon test P = 0.11, n = 6).

1498

1499 **Figure 7—figure supplement 1. Effects of I_A on embryonic V1^R firing patterns predicted**

1500 **by computational modeling**

1501 (A) The maximal conductances of I_{Kdr} and I_A at E12.5 are linearly correlated. Best fit: G_A =

1502 1.09 G_{Kdr} (R^2 = 0.81, N=44). (B) Effect of I_A on the dynamics of the basic model. The one-

1503 parameter bifurcation diagrams in control condition (black, I = 20 pA, G_{Kdr} = 10 nS, no

1504 I_A, same as in Fig 7B) and with I_A added (orange, G_A = 10 nS) are superimposed. The

1505 I_A current shifts the firing threshold SN$_1$ to the right by 0.18 nS (see also C) as indicated by

1506 the orange arrow, with little effect on the amplitude of action potentials (see also insert in

1507 C). In contrast, I_A shifts SN$_2$ by only 0.03 nS because it is inactivated by depolarization. (C)

1508 I_A also slows down the discharge frequency, as shown by comparing the $G_{Nap}-V$ curves

1509 without I_A (black) and with I_A (orange). For G_{Nap} = 1 nS, for instance, the firing frequency is

1510 reduced by 31%, from 15 to 10.4 Hz. Here again, the effect of I_A progressively decreases as

1511 G_{Nap} increases because of the membrane depolarization elicited by I_{Nap}. For G_{Nap} = 2.4 nS,

1512   for instance, the firing frequency is reduced by 11% only, from 19.1 to 17 Hz. This

1513   frequency reduction elicited by I_A does not merely result from the increased firing

1514   threshold. Note also that the latency of the first spike is increased (see voltage trace in

1515   insert), which is a classical effect of I_A. (D) I_A reduces the frequency of pseudo-plateau

1516   bursting by lengthening quiescent episodes (doubling their duration in the example shown)

1517   without affecting the duration of plateaus much (here a mere 5% increase), as shown by

1518   the comparison of the voltage traces obtained without I_A (control, $G_{Kdr} = 2.5$ nS, black)

1519   and with I_A ($G_{Kdr} = G_A = 2.5$ nS, orange). This is because I_A is activated near rest but

1520   inactivated during voltage plateaus. Note that increasing G_{Kdr}, in the absence of I_A has not

1521   the same effect; it shortens both plateaus and quiescent episodes (see Fig 8C, where

1522 $G_{Kdr} = 5$ nS). Again, this is because I_{Kdr} does not inactivate (or does it only very slowly), in

1523   contrast to I_A.

1524

1525   **Figure 7—figure supplement 2. Explaining the effect of 4-AP on the firing pattern**

1526   The RS region of the basic model, where repetitive firing may occur, is displayed in the

1527 $G_{Nap} - G_{Kdr}$ plane in control condition for E12.5 V1[R] (C_{in} = 13 pF, G_{in} = 1 nS, I = 20 pA,

1528   shaded area), and when G_{in} and I were both reduced by 25% (middle curve) or by 50% (left

1529   curve). The reduced I accounts for the decrease in rheobase, and thus in the current

1530   injected in the experiments, following the decrease in G_{in}. If 4-AP reduced only G_{Kdr} (as

1531   indicated by the downward arrow) the firing pattern of SS V1[R] would not change, the RS

1532   region being too far to the right to be visited. In contrast, when the effects of 4-AP on the

1533   input conductance and rheobase are taken into account, the bifurcation diagram moves

1534   leftwards and downwards, as indicated by the oblique black arrow, and the RS and PP

1535   regions are then successively entered as G_{Kdr} is reduced. The same explanation holds at

1536   E14.5.

1537

1538   **Figure 3-souce data-1**

1539   Numerical data used to perform cluster analysis shown in Figure 3

1540

TABLE 1 Model parameters

Parameter	Basic model	Model with slow inactivation of I_{Nap}
Passive parameters		
Input conductance G_{in}	1 nS	same
Input capacitance C_{in}	13 pF (E12.5, Figs. 7B, C, D and F and 8B to D) or 18 pF (E14.5, Fig. 7G)	13 pF
Resting potential V_r	-60 mV	same
Injected current I	20 pA (Fig. 7B to G)	10 pA (Fig. 8C) or 12 pA (Fig. 8D) variable in Fig. 8B
Transient sodium current I_{nat}		
Maximal conductance G_{Nat}	20 nS	same
Reversal potential E_{Na}	60 mV	
Activation exponent	3	
Mid-activation V_m	-26 mV	
Steepness of activation k_m	9.5 mV	
Activation time constant	1.5 ms	
Mid-inactivation V_h	-45 mV	
Steepness of inactivation K_h	-5 mV	
Inactivation time constant τ_m	Voltage-dependent (see Material and Methods)	
Persistent sodium current I_{Nap}		
Maximal conductance	variable (see text and figure captions)	same
Mid-activation voltage	-36 mV	same
Mid-inactivation V_s		-30 mV
Steepness of inactivation k_s		-5 mV
Inactivation time constant	Slow inactivation not included	2 s
Delayed rectifier potassium current I_{Kdr}		
Maximal conductance G_{Kdr}	variable (see text and figure captions)	same
Reversal potential E_K	-96 mV	
Activation exponent	3	
Mid-activation V_n	-20 mV	
Steepness of activation k_n	15 mV	
Activation time constant τ_m	10 ms	
Potassium A current I_A (when included in the basic model)		
Maximal conductance G_A	Equal to G_{Kdr}	never included
Mid-activation V_{mA}	-30 mV	
Steepness of activation k_{mA}	12 mV	
Activation time constant	Instantaneous activation	
Mid-inactivation V_{hA}	-70 mV	

Steepness of inactivation k_{hA}	-7 mV	
Inactivation time constant τ_{hA}	23 ms	

1542
1543

A1 Single-spiking (SS)

0 mV

40 mV

30 pA

1 s

A2 Repetitive-spiking (RS)

0 mV

40 mV

30 pA

1 s

A3 Mixed events (ME)

0 mV

40 mV

30 pA

1 s

A4 Plateau potential (PP)

0 mV

40 mV

30 pA

1 s

B1

+40

-60 mV

-100

+40

-60 mV

- 30

IKv total   IKdr   IA = IKv-IKdr

400 pA

50 ms

B2

400 pA

50 ms

B3

400 pA

50 ms

B4

200 pA

50 ms

C1

IA
IKdr

1.5 nA

mV

-60 -40 -20   0   20   40

C2

IA
IKdr

1.5 nA

1

0.5

mV

-60 -40 -20   0   20   40

C3

IA
IKdr

1.5 nA

1

0.5

mV

-60 -40 -20   0   20   40

C4

IA
IKdr

250 pA

150

50

mV

-60 -40 -20   0   20   40

D

G_{Kdr} (nS)

20

15

10

5

*
**
*
**
***

E12.5   E14.5
Embryonic age (days)

E

G_{Nap} (nS)

2.5

1.5

0.5

**
***
**
***

E12.5   E14.5
Embryonic age (days)

F

G_{Nap}/G_{Kdr}

1.5

1.0

0.5

*
***
*
***
***

E12.5   E14.5
Embryonic age (days)

■ SS   ■ RS   ■ ME   ■ PP

Fig 1

Fig 2

A

1/2 amplitude duration (1/2Ad)

$\Sigma\ 1/2Ad = 1/2Ad + 1/2Ad + 1/2Ad$

Pulse width (Pw)

$$\text{mean } 1/2Ad = \frac{\Sigma\ 1/2Ad}{n\ 1/2Ad}$$

$$CV\ 1/2Ad = \frac{SD\ 1/2Ad}{\text{mean } 1/2Ad}$$

$$ddr = \frac{\Sigma\ 1/2Ad}{Pw}$$

CV 1/2Ad (%)
log mean 1/2Ad
log ddr

B

Silhouette width vs Number of clusters

C

- PP
- RS
- ME short PP
- ME long PP
- SS 1 - 3 AP(s)

CV 1/2 Ad (%)

log ddr

log mean 1/2Ad

D

SS   RS   ME short PP   ME long PP   PP

0 mV

40 mV

30 pA

1 s

Fig 3

Fig 4

Fig 5

Fig 6

Fig 7

A

Plateau potential (PP)

Mixed events (ME)

B1

B2

C

D

Fig 8

A1 E12.5

I_{Kdr} control

I_{Kdr} 300 µM 4-AP

400 pA
50 ms

B1

I_{Kdr} (pA)

- control
- 4-AP

C1

% control

IC$_{50}$ = 2.9 ± 1.23 µM
Hill = 0.63 ± 0.14
Max inhibition = 73.2 ± 6.4%

4-AP concentration (µM)

A2 E12.5

I_A control

I_A 300 µM 4-AP

400 pA
50 ms

B2

I_A (pA)

- control
- 4-AP

C2

% control

300 µM 4-AP

Fig 2 figure supplement 1

A E12.5

Control   300 µM 4-AP   300 µM 4-AP
                                      + TTX

0 mV —

20 mV

20 pA

1 s

B

%

100
80
60
40
20
0

Control   300 µM
                 4-AP

■ RS
■ ME
■ PP

Fig 2 figure supplement 2

Fig 3 figure supplement 1

A

+20 mV

-100 mV   70 mV/s

nA

1 μM TTX

Control

mV   -60   -40

-20

0 mV

20 mV

50 pA

1 s

20 pA

-30

B

G_{max} = 0.27 pS

V_{half} = -33.1 mV

k = 8.2

Conductance (nS)

Vh (mV)
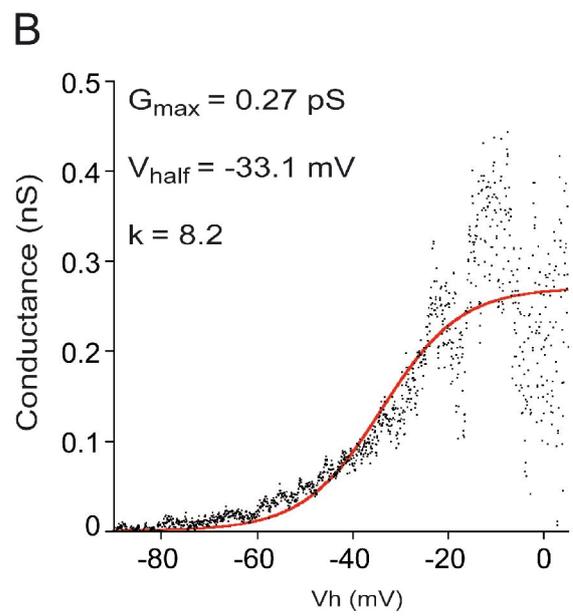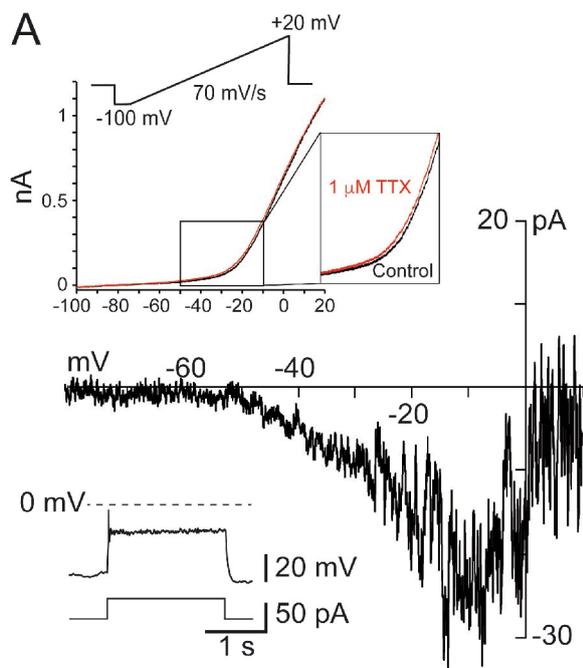
Fig 6 figure supplement 1
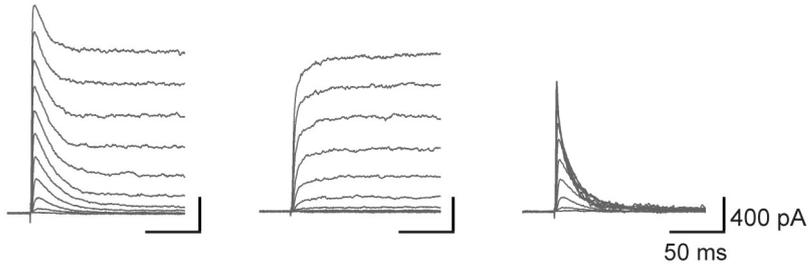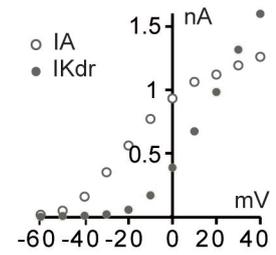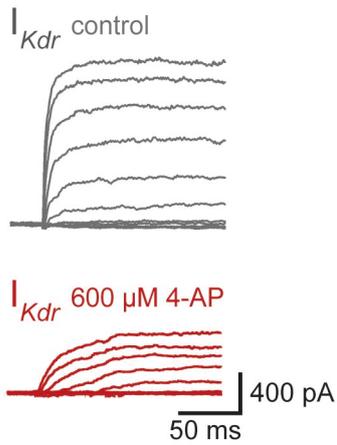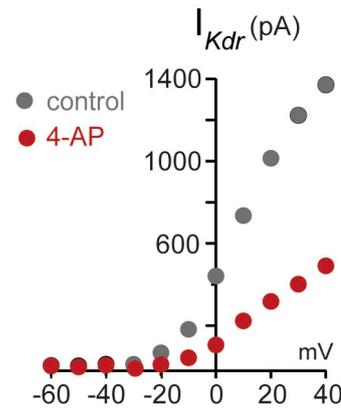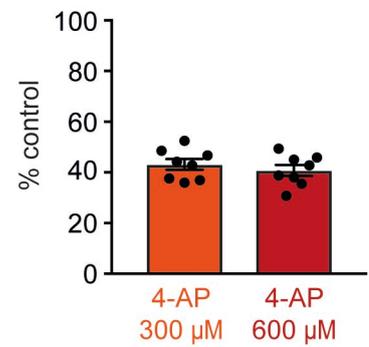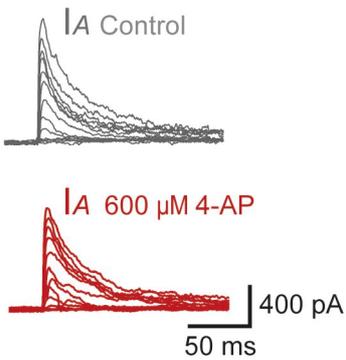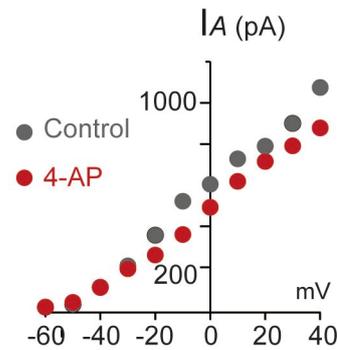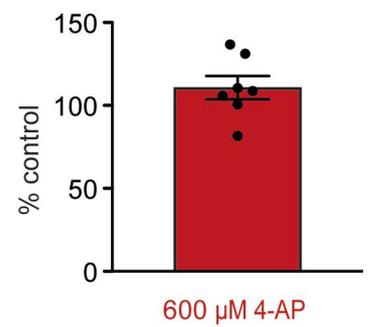
Fig 6 figure supplement 2
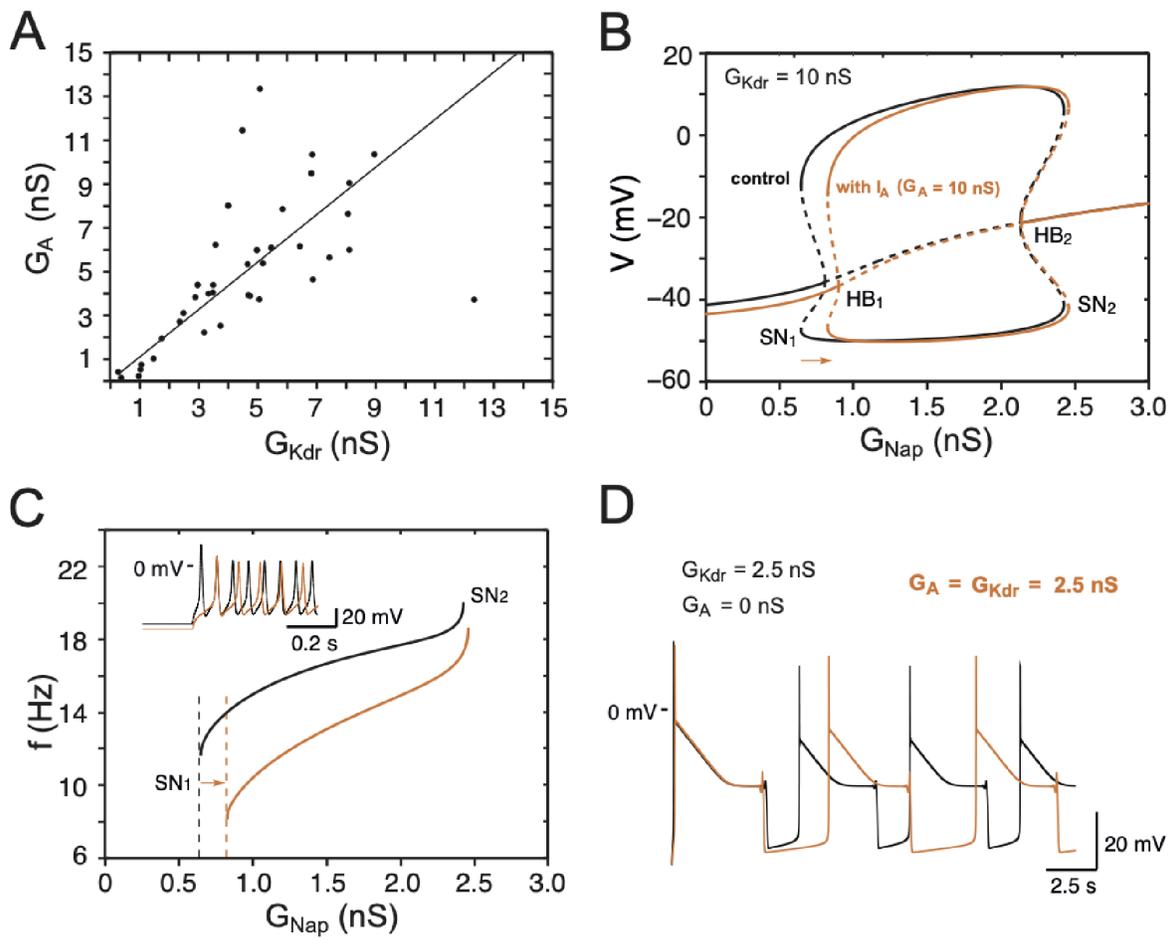
Fig 7 figure supplement 1

Fig 7 figure supplement 2