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

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Abstract

Renshaw cells (V1R) are excitable as soon as they reach their final location next to the spinal motoneurons and display heterogeneous activity patterns at the onset of the synaptogenesis (12 days post-fertilization; E12.5). 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 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. V1R begin to recover this capability when a locomotor-like activity emerges (after E15.5). Our computational analysis, closely based on experimental data, demonstrated that the essential factor controlling the diversity of the discharge pattern of embryonic V1R is the ratio of a persistent sodium conductance to a delayed rectifier potassium conductance. We also found that V1R involution is due to a decrease of the persistent sodium conductance as V1R develop and their size increases. 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 V1R and control their early developmental trajectory. Our findings also indicate that homeostatic regulation of channel density or channel degeneracy known to maintain homogeneous firing pattern within specific populations of mature neurons, are unlikely to occur at these early developmental stages.

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

Introduction

The development of the central nervous system (CNS) follows complex steps, which depend on genetic and environmental factors and involve interactions between multiple elements of the neural tissue. Remarkably, emergent neurons begin to synchronize soon after the onset of synapse formation, generating long episodes of low frequency (<0.01 Hz) correlated spontaneous network activity (SNA) [1-8]. In the mouse embryonic spinal cord (SC), SNA is driven by an excitatory cholinergic-GABAergic loop between motoneurons (MNs) and interneurons (INs), GABA being depolarizing before embryonic day 16.5 (E16.5) [9]. SNA emerges around E12.5 [4, 6, 10-12], at a time when functional neuromuscular junctions are not yet established [13], and sensory and supraspinal inputs have not yet reached the spinal motor networks [14-17].

Several studies pointed out that SNA is an essential component in neuronal networks formation. [18-21]. In the SC, pharmacologically-induced disturbances of SNA between E12.5 and E14.5 induce defects in the formation of motor pools, in motor axon guidance to their target muscles and in the development of motor networks [4, 21-23]. During SNA episodes, long lasting giant depolarization potentials (GDPs) are evoked in the SC, mainly by the massive release of GABA onto MNs [12]. Immature Renshaw cells ($V1^R$) are likely the first GABAergic partners of MNs in the mouse embryo [24, 25], and the massive release of GABA during SNA probably requires that many of them display repetitive action potential firing or plateau potential activity [25].

However, little is known about the firing pattern of embryonic $V1^R$ and the maturation of their intrinsic properties. We recently found that $V1^R$ exhibit heterogeneous excitability properties when SNA emerges in the SC [25] in contrast to adult Renshaw cells that constitute a functionally homogeneous population [26, 27]. Whether this early functional diversity really reflects distinct functional classes of $V1^R$, how this diversity evolves during development, and

what are the underlying biophysical mechanisms remain open questions. The present study addresses these issues using multiple approaches, including patch-clamp recordings, cluster analysis, biophysical modeling and dynamical systems theory. The firing patterns of $V1^R$ and the mechanisms underlying their functional diversity are analyzed during a developmental period covering the initial phase of development of SC activity in the mouse embryo (E11.5-E14.5), when SNA is present, and during the critical period (E14.5-E16.5), when GABAergic neurotransmission gradually shifts from excitation to inhibition [28] and locomotor-like activity emerges [4, 10, 11].

We discover that the balance between the slowly inactivating subthreshold persistent sodium inward current (I_{Nap}) [29] and the delayed rectifier potassium outward current (I_{Kdr}), accounts for the heterogeneity of embryonic $V1^R$ and the changes in firing pattern during development. The heterogeneity of $V1^R$ at E12.5 arises from the existence of distinct functional groups. Surprisingly, and in opposition to the classically accepted development scheme [30-35], we show that the embryonic $V1^R$ population loses its ability to support tonic firing from E13.5 to E15.5, exhibiting a transient functional involution during its development. Our experimental and theoretical results provide a global view of the developmental trajectories of embryonic $V1^R$. They demonstrate that a simple mechanism, based on the synergy of only two major opposing voltage-dependent currents, accounts for functional diversity in these immature neurons.

Results

The delayed rectifier potassium current I_{Kdr} is a key partner of the persistent sodium current I_{Nap} in controlling embryonic V1^R firing patterns during development

We previously highlighted that V1^R are spontaneously active at E12.5. Their response to a 2 s suprathreshold depolarizing current steps revealed four main patterns, depending of the recorded interneuron [25]: i) single spiking (SS) V1^R that fires only 1-3 APs at the onset of the depolarizing pulse, ii) repetitive spiking (RS) V1^R, iii) mixed events (ME) V1^R that shows an alternation of action potentials (APs) and plateau potentials or, iv) V1^R that displays a long-lasting sodium-dependent plateau potential (PP) (**Fig 1A1–A4**).

We also uncovered a relationship between I_{Nap} and the ability of embryonic V1^R to sustain repetitive firing [25]. However, the heterogeneous firing patterns of V1^R observed at E12.5 could not be fully explained by variations in I_{Nap} [25], suggesting the involvement of other voltage-gated channels in the control of the firing pattern of V1^R, in particular potassium channels, known to control firing and AP repolarization. Our voltage clamp protocol, performed in the presence of TTX (1 μM), did not disclose any inward rectifying current (hyperpolarizing voltage steps to -100 mV from V_H = -20 mV, data not shown), but revealed two voltage-dependent outward potassium currents, a delayed rectifier current (I_{Kdr}) and a transient potassium current (I_A) in all embryonic V1^R, whatever the firing pattern (**Fig 1B1–B4**). These currents are known to control AP duration (I_{Kdr}) or firing rate (I_A), respectively [36]. The activation threshold of I_{Kdr} lied between -30 mV and -20 mV and the threshold of I_A between -60 mV and -50 mV, (n = 27; N = 27 embryos) (**Fig 1C1–C4**). Removing external calcium had no effect on potassium current I/V curves (data not shown), suggesting that calcium-dependent potassium currents are not yet present at E12.5.

It was unlikely that the heterogeneity of V1^R firing patterns resulted from variations in the intensity of I_A. Indeed, its voltage-dependent inactivation (time constant: 23.3 ± 2.6 ms, n = 8;

N = 8), which occurs during the depolarizing phase of an AP, makes it ineffective to control AP or plateau potential durations. This was confirmed by our theoretical analysis (**see S6 Fig**). We thus focused our study on I_{Kdr}. At E12.5, PP V1^R 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 = 9; N = 9) and RS V1^R (6.39 ± 0.83 nS, n = 7; N = 7) (**Fig 1D**). However, there was no significant difference in G_{Kdr} between SS V1^R and RS V1^R at E12.5 (**Fig 1D**), which indicated that variations in G_{Kdr} alone could not explain all the firing patterns observed at E12.5. Similarly, there was no significant difference in G_{Nap} between RS V1^R (0.91 ± 0.21nS, n = 8; N = 8) and PP V1^R (1.24 ± 0.19 nS, n = 6; N = 6) at E12.5 (**Fig 1E**), indicating that variations in G_{Nap} alone could not explain all the firing patterns of V1^R at E12.5 [25]. In contrast G_{Nap} measured in SS V1^R at E12.5 (0.21 ± 0.20 nS, n = 9; N = 9) were significantly lower compared to G_{Nap} measured in RS V1^R and in PP V1^R at E12.5 (**Fig 1E**).

Mature neurons often display multiple stable firing patterns [37-39]. This usually depends on the combination of several outward and inward voltage- or calcium-dependent conductances and on their spatial localization [37-39]. In contrast, immature V1^R have a limited repertoire of voltage-dependent currents (I_{Nat} and I_{Nap}, I_{Kdr} and I_A) at E12.5, and we did not find any evidence of voltage-dependent calcium currents at this age [25]. Blocking I_{Nap} prevented plateau potential activity, PP-V1^R becoming unexcitable, and turned repetitive spiking V1^R into single spiking V1^R [25]. Therefore, we hypothesized that the different firing patterns of V1^R observed at E12.5 were related to the G_{Nap} / G_{Kdr} ratio only, with variations in the intensity of I_A being unlikely to account for the heterogeneity of firing pattern. We found that this ratio was significantly lower for SS V1^R recorded at E12.5 (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 (0.66 ± 0.132, n = 6) (**Fig 1F**). We also found that the G_{Nap} / G_{Kdr} ratio was significantly lower for RS V1^R compared to PP V1^R (**Fig 1F**).

Altogether, these results strongly suggest that, although the presence of I_{Nap} is required for embryonic V1^R to fire repetitively or to generate plateau potentials [25], the heterogeneity of the firing pattern observed between E12.5 is not determined by I_{Nap} *per se* but likely by the balance between I_{Nap} and I_{Kdr}.

Manipulating the balance between G_{Nap} and G_{Kdr} changes embryonic V1^R firing patterns

We previously showed that blocking I_{Nap} with riluzole converted PP V1^R or RS V1^R into SS V1^R [25]. To confirm further that the balance between G_{Nap} and G_{Kdr} was the key factor in the heterogeneity of V1^R firing patterns, we assessed to what extent a given E12.5 SS V1^R cell could change its firing pattern when I_{Kdr} was gradually blocked by 4-aminopiridine (4-AP). We found that I_{Kdr} could be blocked by micromolar concentrations of 4-AP without affecting I_A (**S1 Fig**). 4-AP, applied at concentrations ranging from 0.3 μM to 300 μM, specifically inhibited I_{Kdr} with an IC_{50} of 2.9 μM (**S1C1 Fig**).

We then determined to what extent increasing the concentration of 4-AP modified the firing pattern of V1^R at E12.5. Applying 4-AP at concentrations ranging from 3 μM to 300 μM changed the firing pattern of SS V1^R (n = 10; N = 10) in a concentration-dependent manner (**Fig 2A1–A3**). In 50% of the recorded V1^R, increasing 4-AP concentrations successfully transformed SS V1^R into PP V1^R with the following sequence: SS → RS → ME → PP (**Fig 2A1**). In a second group of embryonic V1^R (25%), 4-AP application only evoked mixed activity, with the same sequence as aforementioned (SS → RS → ME) (data not shown). In the remaining SS V1^R (25%), increasing 4-AP concentration only led to sustained AP firing (**Fig 2A2**). Application of 300 μM 4-AP on RS V1^R at E12.5 evoked mixed events or plateau potentials (**S2 Fig**). Plateau potentials and repetitive spiking evoked in the presence of 300 μM 4-AP were fully blocked by 0.5-1 μM TTX, indicating that they were generated by voltage-gated Na^+ channels (**Fig 2B and 2C**; **S2 Fig**). It should be noted that the application

of 300 μM of 4-AP induced a significant 30.5 ± 12.4 % increase (P = 0.0137; Wilcoxon test) of the input resistance (1.11 ± 0.08 GΩ versus 1.41 ± 0.12 GΩ; n = 11; N = 11).

These results show that, in addition to I_{Nap}, I_{Kdr} is also a major determinant of the firing pattern of embryonic $V1^R$. The above suggests that the firing patterns depend on a synergy between I_{Nap} and I_{Kdr} and that the different patterns can be ordered along the following sequence SS → RS → ME → PP when the ratio $G_{Nap}/\ G_{Kdr}$ is increased.

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

It was initially unclear whether these different firing patterns corresponded to well separated classes within the E12.5 $V1^R$ population or not. To address this question, we performed a hierarchical cluster analysis on 163 embryonic $V1^R$, based on three quantitative parameters describing the firing pattern elicited by the depolarizing pulse: the mean duration of evoked APs or plateau potentials measured at half-amplitude (mean ½Ad), the variability of the event duration during repetitive firing (coefficient of variation of ½Ad: CV ½Ad) and the total duration of all events, expressed in percentage of the pulse duration (depolarizing duration ratio: ddr) (**Fig 3A inserts**). In view of the large dispersion of mean ½Ad and ddr values, cluster analysis was performed using the (decimal) logarithm of these two quantities [40]. The analysis of the distribution of log mean ½Ad, CV ½Ad and log ddr revealed multimodal histograms that could be fitted with several Gaussians (**S3A1-C1 Fig)**. Cluster analysis based on these three parameters showed that the most likely number of clusters was 5 (**Fig 3A and 3B**), as determined by the silhouette width measurement (**Fig 3B**). Two clearly separated embryonic $V1^R$ groups with CV ½Ad = 0 stood out, as shown in the 3D plot in **Fig 5C**. The cluster with the largest ½Ad (mean ½Ad = 833.5 ± 89.99 ms) and the largest ddr (0.441 ± 0.044) contained all PP $V1^R$ (n = 35; N = 29) (**Fig 3C and 3D; S3A2 and S3C2 Fig**).

Similarly, the cluster with the shortest ½Ad (9.73 ± 0.66 ms) and the lowest ddr (0.0051 ± 0.0004) contained all SS $V1^R$ (n = 46; N = 37) (**Fig 3C and 3D; S3A2 and S3C2 Fig**).

The three other clusters corresponded to $V1^R$ with nonzero values of CV ½Ad (**Fig 3 C**). A first cluster regrouping all RS $V1^R$ (n = 69; N = 61) was characterized by smaller values of ½Ad (23.91 ± 1.43 ms), CV ½Ad (27.36 ± 1.64%) and ddr (0.11 ± 0.01) (**Fig 3C and 3D; S3 A2 and S3C2 Fig**). The last two clusters corresponded to ME $V1^R$ (**Fig 3C and 3D**). The smaller cluster, characterized by a larger CV ½Ad (170.9 ± 8.9%; n= 4; N = 4), displayed a mix of APs and short plateau potentials, while the second cluster, with smaller CV ½Ad (87.61 ± 7.37%; n = 9; N = 9), displayed a mix of APs and long-lasting plateau potentials (**Fig 3D; S3B2 Fig**). Their ½Ad and ddr values were not significantly different (**S3A2 and S3C2 Fig**).

It must be noted that three embryonic $V1^R$ (1.8%) were apparently misclassified since they were aggregated within the RS cluster although having zero CV ½Ad (**Fig 3C; arrows**). Examination of their firing pattern revealed that this was because they generated only two APs, although their ddr (0.16 to 0.2) and ½ Ad values (31.6 to 40.3 ms) were well in the range corresponding of the RS cluster.

These different firing patterns of $V1^R$ might reflect different states of neuronal development [31, 41-43]. Single spiking and/or plateau potentials are generally believed to be the most immature forms of firing pattern, repetitive spiking constituting the most mature form [19, 44]. If it were so, the firing patterns of embryonic $V1^R$ would evolve during embryonic development from single spiking or plateau potential to repetitive spiking, this latter firing pattern becoming the only one in neonates [26] and at early postnatal stages [27]. However, RS neurons already represent 41% of $V1^R$ at E12.5. We therefore analyzed the development of firing patterns from E11.5, when $V1^R$ terminate their migration and reach their final position [45], to E16.5. This developmental period covers a first phase of

development (E11.5-E14.5), where lumbar spinal networks exhibit SNA, and a second phase (E14.5-E16.5), where locomotor-like activity emerges [4, 11, 46, 47]. We first analyzed changes in the intrinsic properties (input capacitance C_{in} , input resistance $R_{in} = 1/G_{in}$ and spike voltage threshold) of $V1^R$. C_{in} did not change significantly from E11.5 to E13.5 (**Fig 4A1**), remaining of the order of 12 pF, in agreement with our previous work [25]. However, it increased significantly at the transition between the two developmental periods (E13.5-E15.5) to reach about 23.5 pF at E15.5 (**Fig 4A1**). A similar developmental pattern was observed for R_{in} , which remained stable during the first phase from E11.5 to E14.5 ($R_{in} \approx$ 1-1.2 GΩ) but decreased significantly after E14.5 to reach about 0.7 GΩ at E15.5 (**Fig 4A2**). Spike threshold also decreased significantly between the first and the second developmental phases, dropping from about -34 mV at E12.5 to about -41 mV at E16.5 (Figure 4 A3). Interestingly, this developmental transition around E14.5 correspond to the critical stage at which SNA gives way to a locomotor-like activity [11, 46, 47] and rhythmic activity becomes dominated by glutamate release rather than acetylcholine release [4].

This led us to hypothesize that this developmental transition could be also critical for the maturation of $V1^R$ firing patterns. The distinct firing patterns observed at E12.5 were already present at E11.5 (**Fig 4B1 and 4C**), but the percentage of RS $V1^R$ strongly increased from E11.5 to E12.5, while the percentage of ME $V1^R$ decreased significantly (**Fig 4C**). The heterogeneity of $V1^R$ firing patterns then substantially diminished. Plateau potentials were no longer observed at E13.5 (**Fig 4B2 and 4C**), and ME $V1^R$ disappeared at E14.5 (**Fig 4B3 and 4C**). Interestingly, the proportion of SS $V1^R$ remained high from E13.5 to E15.5 and even slightly increased (91.23% at E14.5 and 93.33% at E15.5; **Fig 4C**). This trend was partially reversed at E16.5, as the percentage of RS $V1^R$ increased at the expense of SS $V1^R$ (67.86% SS $V1^R$ and 32.34% RS $V1^R$; **Fig 4B5 and 4C**). This decrease in repetitive firing capability after E13.5 was surprising in view of what is classically admitted on the developmental

pattern of neuronal excitability [18, 48]. Therefore, we verified that it did not reflect the death of some $V1^R$ after E13.5. Our data did not reveal any activated caspase3 (aCaspase3) staining in $V1^R$ (FoxD3 staining) at E14.5 (n = 10 SCs; N = 10) (**Fig 5**), in agreement with previous reports showing that developmental cell death of $V1^R$ does not occur before birth [49].

To determine whether G_{Nap} and G_{Kdr} also controlled the firing pattern of $V1^R$ at E14.5 (**see Fig 4B3 and 4C**), we assessed the presence of I_{Nap} and I_{Kdr} in single spiking $V1^R$ at this embryonic age. Both I_{Nap} and I_{Kdr} were present in $V1^R$ at E14.5 (**S4 and S5 Fig)** whereas, as in $V1^R$ at E12.5, no calcium-dependent potassium current was detected at this developmental age (not shown). In SS $V1^R$, G_{Kdr} was significantly higher at E14.5 (11.11 ± 1.12 nS, n = 10; N = 10) than at E12.5 (**Fig 1D**). In contrast, G_{Nap} was similar at E14.5 (0.13 ± 0.14 nS, n = 10; N = 10) and E12.5 **(Fig 1E**). We also found that the G_{Nap} / G_{Kdr} ratio was significantly lower for SS $V1^R$ recorded at E14.5 (0.012 ± 0.004, n = 10) compared to RS $V1^R$ (0.154 ± 0.022, n = 8) and PP $V1^R$ (0.66 ± 0.132, n = 6) recorded at E12.5 (**Fig 1F**).

We tested the effect of 4-AP in SS $V1^R$ at E14.5. At this embryonic age, 300 µM 4-AP inhibited only 59.2% of I_{Kdr}. Increasing 4-AP concentration to 600 µM did not inhibit I_{Kdr} significantly more (60.2%) (**S5 Fig**), indicating that inhibition of I_{Kdr} by 4-AP reached a plateau at around 300 µM. 600 µM 4-AP application had no significant effect on I_A (**S5 Fig**). The application of the maximal concentration of 4-AP tested (600 µM) converted SS $V1^R$ (n = 13; N = 13) to PP $V1^R$ (23.1%; **Fig 6A1 and 6B**), RS $V1^R$ (38.5%; **Fig 6A2 and 6B**) or ME $V1^R$ (38.4%; **Fig 6B**), as was observed at E12.5, thus indicating that the firing pattern of $V1^R$ depends on the balance between I_{Nap} and I_{Kdr} also at E14.5. Plateau potential and repetitive spiking recorded in the presence of 4-AP at E14.5 were fully blocked by 0.5-1 µM TTX indicating that they were generated by voltage-gated sodium channels (**Fig 6A1and 6A2**), as observed at E12.5.

Theoretical analysis: the basic model

As shown in **Fig 7A** for 26 cells, in which both G_{Nap} and G_{Kdr} were measured, the three largest clusters revealed by the hierarchical clustering analysis (SS, RS and PP, which account together for the discharge of more than 95% of cells, see **Fig 5**) correspond to well defined regions of the G_{Nap}- G_{Kdr} plane. Single spiking is observed only when G_{Nap} is smaller than 0.6 nS. For larger values of G_{Nap}, repetitive spiking occurs when G_{Kdr} is larger than 3.5 nS, and $V1^R$ display plateau potentials when G_{Kdr} is smaller than 3.5 nS. Mixed events (ME, 4.5% of the 163 cells used in the cluster analysis), where plateaus and spiking episodes alternate, are observed at the boundary of RS and PP clusters. This suggested to us that a conductance-based model incorporating only the leak current, I_{Nat} , I_{Nap} and I_{Kdr} (see Materials and Methods) could account for most experimental observations, the observed zonation being explained in terms of bifurcations between the different stable states of the model. Therefore, we first investigated a simplified version of the model without I_A and slow inactivation of I_{Nap}.

A one-parameter bifurcation diagram of this "basic" model is shown in **Fig 7B** for two values of G_{Kdr} (2.5 nS and 10 nS) and a constant injected current I = 20 pA. In both cases, the steady-state membrane voltage (stable or unstable) and the peak and trough voltages of stable and unstable periodic solutions are shown as the function of the maximal conductance G_{Nap} of the I_{Nap} current, all other parameters being kept constant. For G_{Kdr} = 10 nS, the steady-state membrane voltage progressively increases (in gray) with G_{Nap}, but repetitive spiking (in red, see voltage trace for G_{Nap} = 1.5 nS) is not achieved until G_{Nap} reaches point SN_1, where a saddle node (SN) bifurcation of limit cycles occurs. This fits with the experimental data, where a minimal value of G_{Nap} is required for repetitive spiking (see also [25], and is in agreement with the known role of I_{Nap} in promoting repetitive discharge [50,

51]. Below SN_1, the model responds to the onset of a current pulse by firing only one spike before returning to quiescence (see voltage trace for G_{Nap}= 0.2 nS), or a few spikes when close to SN_1 (not shown) before returning to quiescence. The quiescent state becomes unstable through a subcritical Hopf bifurcation (HB) at point HB_1, with bistability between quiescence and spiking occurring between SN_1 and HB_1 points. Repetitive firing persists when G_{Nap} is increased further and eventually disappears at point SN_2. The firing rate does not increase much throughout the RS range (**S6C Fig**), remaining between 20.1 Hz (at SN_1) and 28.7 Hz (at SN_2). A stable plateau appears at point HB_2 through a subcritical HB. The model is bistable between HB_2 and SN_2, with plateau and large amplitude APs coexisting in this range.

The model behaves very differently when G_{Kdr} is reduced to 2.5 nS (gray-blue curve in **Fig 7B**). It exhibits a unique stable fixed point whatever the value of G_{Nap} is, and the transition from quiescence to plateau is gradual as G_{Nap} is increased. No repetitive spiking is ever observed. This indicates that the activity pattern is controlled not only by G_{Nap} but also by G_{Kdr}. This is demonstrated further in **Fig 7C**, where G_{Nap} was fixed at 1.2 nS while G_{Kdr} was increased from 0 to 25 nS. The model exhibits a plateau potential until G_{Kdr} is increased past point the subcritical HB point HB_2, repetitive spiking sets in before at point SN_2 *via* a SN of limit cycles bifurcation. When G_{Kdr} is further increased, repetitive firing eventually disappears through a SN bifurcation of limit cycles at point SN_1, the quiescent state becomes stable through a subcritical HB at point HB_1, and bistability occurs between these two points. This behavior is in agreement with **Fig 7A**.

Since both conductances G_{Nap} and G_{Kdr} control the firing pattern of embryonic $V1^R$ cells, we computed a two-parameters bifurcation diagram (**Fig 7D**), where the different possible activity states and the transitions between them are plotted in the G_{Nap} - G_{Kdr} plane. The black curves correspond to the bifurcations HB_1 and HB_2 and delimit a region where only

repetitive firing occurs. The red curves correspond to the SN bifurcations of periodic orbits associated with the transition from quiescence to firing (SN_1) and the transition from plateau to firing (SN_2). They encompass a region (shaded area) where repetitive firing can be achieved but may coexist with quiescence (between the HB_1 and SN_1 lines) or plateau potential (in the narrow region between the HB_2 and SN_2 lines).

Some important features of the diagram must be emphasized: 1) minimal values of both G_{Nap} (to ensure sufficient excitability) and G_{Kdr} (to ensure proper spike repolarization) are required for repetitive spiking, 2) quiescence and plateau can be clearly distinguished only when they are separated by a region of repetitive spiking (see also **Fig 7B** for G_{Kdr} = 10 nS), otherwise the transition is gradual (**Fig 7B** for G_{Kdr}= 2.5 nS), 3) only oblique lines with an intermediate slope cross the bifurcation curve and enter the RS region (see, for example, the red line in **Fig 7D**). This means that repetitive spiking requires an appropriate balance between I_{Nap} and I_{Kdr}. If the ratio G_{Nap}/G_{Kdr} is too large (blue line) or too small (gray line), only plateau potentials or quiescence will be observed at steady state. This is exactly what is observed in experiments, as shown by the cumulative distribution function of the ratio G_{Nap}/G_{Kdr} for the different clusters of embryonic $V1^R$ in **Fig 7E** (same cells as in **Fig 7A**). The ratio increases according to the sequence SS → RS → ME → PP, with an overlap of the distributions for SS $V1^R$ and RS $V1^R$. Note also that the ratio for ME cells (around 0.25) corresponds to the transition between repetitive spiking and plateau potentials (more on this below).

Embryonic $V1^R$ cells display voltage fluctuations that may exceed 5 mV and are presumably due to channel noise. The relatively low number of sodium and potassium channels (of the order of a few thousands) led to voltage fluctuations in the stochastic version of our model comparable to those seen experimentally when the cell was quiescent (top voltage trace in **Fig 7D**) or when a voltage plateau occurred (bottom trace). Channel noise

caused some jitter during repetitive spiking (middle trace), and induced clearly visible variations in the amplitude of APs. However, repetitive firing proved to be very robust and was not disrupted by voltage fluctuations. Altogether, channel noise little alters the dynamics (compare the deterministic voltage traces in **Fig 7B** and the noisy traces in **Fig 7D**). This is likely because channel noise has a broad power spectrum and displays no resonance with the deterministic solutions of the model.

The one-parameter bifurcation diagram of the model was not substantially modified when we took I_A into account, as shown in **S6 Fig**. It just elicited a slight membrane hyperpolarization, an increase in the minimal value of G_{Nap} required for firing, and a decrease of the firing frequency. The transition from repetitive firing to plateau was not affected because I_A is then inactivated by depolarization.

The bifurcation diagram of **Fig 7D** accounts *qualitatively* for the physiological data on $V1^R$ at E12.5 presented in **Fig 7A**, as shown in **Fig 7F** where the conductance data of **Fig 7A** were superimposed on it. However, one must beware of making a more *quantitative* comparison because the theoretical bifurcation diagram was established for a constant injected current of 20 pA, whereas the current injected in experiments data varied from neuron to neuron and ranged from 10 to 30 pA in the sample shown in **Fig 7A**. The position of bifurcation lines in the G_{Nap} - G_{Kdr} plane also depends on the values chosen for the other parameters, which vary from cell to cell but were kept at fixed values in the model (Ori et al. 2018). For instance, the diagrams were computed in **Fig 7D** and **F** for G_{in} = 1 nS and C_{in} = 13 pF, the median values of the input conductance and capacitance at E12.5, taking no account of the cell-to-cell variations of these quantities. Between E12.5 and E14.5, C_{in} which provides an estimate of the cell size, increases by 38% in average, whereas G_{in} is not significantly modified (see **Fig 4**). As illustrated in **Fig 7G** the two-parameters bifurcation diagram is then shifted upward and rightward compared to **Fig 7F**, because larger conductances are required to obtain the

same firing pattern. The observed regression of excitability from E12.5 to E14.5-E15.5 (see **Fig 4C**) thus comes from a decrease in G_{Nap} density (see presumable developmental trajectories indicated by arrows in **Fig 7F**) together with a shift of the RS region as cell size increases. As a result, all 10 cells shown in **Fig 7G** are deeply inside the SS region at E14.5.

It is less straightforward to explain the experiments where 4-AP changed the firing pattern of SS $V1^R$ (**Fig 2**). The decrease of G_{Kdr} (**S7 Fig**), although it may exceed 70% at the higher concentrations of 4-AP we used, is not sufficient by itself to account for the change in the firing pattern of $V1^R$ (**S7 Fig**). However, 4-AP at a 300 μM concentration also decreases G_{in} (by 23% in average and up to 50% in some neurons) and therefore the rheobase current. The bifurcation diagram remains qualitatively the same, but it is then shifted in the G_{Nap}- G_{Kdr} plane, and the bifurcation lines between SS and RS (SN1) and between RS and PP (SN2) are successively crossed when G_{Kdr} is reduced, in accordance with experimental results.

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

Our basic model accounts for the firing pattern of 73% of the 163 cells used in the cluster analysis. However, bursting, under the form of recurring plateaus separated by brief repolarization episodes (see a typical trace in **Fig 8A left**), was experimentally observed in half of PP $V1^R$ (24 out of 46), and plateaus intertwined with spiking episodes were recorded in the 13 cells of the ME cluster (8% of the total sample, see **Fig 8A right** for a typical example). Recurrent plateaus indicate membrane bistability and require that the $I - V$ curve be S-shaped. This occurs when G_{Nap} is large and G_{Kdr} small. (**Fig 8B1 and 8B2**). However, our basic model lacks a mechanism for switching between quiescent state and plateau, even in this case. Channel noise might induce such transitions, but our numerical simulations showed that this is too infrequent to account for bursting (see voltage trace in **Fig 8B1** where the plateau state is maintained despite channel noise).

To explain recurrent plateaus, we took into account slow inactivation of I_{Nap}, in our model. The one-parameter bifurcation diagram of the basic model without slow inactivation is shown in **Fig 8C** for G_{Kdr} = 5 nS and an injected current reduced to 10 pA (as compared to 20 pA in the previous section), so as to allow for bistability (see **Fig 8B2**). The $G_{Nap} - V$ curve is then S-shaped, as shown in **Fig 8B1**, with a bistability region for G_{Nap} between 1.36 and 1.85 nS. This is in contrast with **Fig 7B** where the $G_{Nap} - V$ curve was monotonic. Adding the slow (de)inactivation of I_{Nap} then caused periodic transitions between up (plateau) and down (quiescent) states, and the model displayed a stable limit cycle (shown in black in **Fig 8C**), as illustrated by the top voltage trace on the right. This mechanism is known as pseudo-plateau or plateau-like bursting (a.k.a. fold-subcritical HB bursting) [52]. It is akin to square wave bursting [53-56], but the up-state is a stable fixed point rather than a limit cycle [57-59], which is why recurrent plateaus are obtained rather than bursts of APs. The duration of the plateaus and repolarization episodes depends on the values of G_{Nap} and G_{Kdr}. A voltage-independent time constant $\tau_s = 2\,ms$ leads to up and down states of comparable durations (see top left voltage trace in **Fig 8C**). In agreement with the bifurcation diagram of **Fig 8C**, the persistent sodium current inactivates during plateaus (phase 1, see bottom right trace in in **Fig 8C**) and de-inactivates during quiescent episodes (phase 3, see bottom right trace). Transitions from the down-state to the up-state occurs when inactivation has reached its maximal value (phase 2) and transition from the up-state to the down state when it has reached its maximum (phase 4).

Increasing G_{Nap} by 50% (or decreasing G_{Kdr}) makes plateaus much longer than quiescent episodes (see top right voltage trace in **Fig 8C**). This again points out to the fact that the ratio is an important control parameter. Adding channel noise preserves bursting but introduces substantial randomness in the duration of plateaus and repolarization episodes (bottom right

voltage trace in **Fig 8C**). Moreover, it substantially decreases the duration of both plateaus and quiescent episodes by making transition between the two states easier (compare the two top voltage traces in **Fig 8C**, both computed for $\tau_s = 2\,ms$). We also noted that adding the I_A current lengthened the quiescence episodes (**S6 Fig**).

Slow inactivation of I_{Nap} also provides an explanation for mixed patterns, where plateaus alternate with spiking episodes (**Fig 8A, right**). They take place in our model near the transition between repetitive spiking and plateau, as in experiments (see **Fig 8A**). Slow inactivation can lead to elliptic bursting, notably when the bifurcation HB_2 is subcritical [60, 61], which is the case here (**Fig 8D**). The model then displays a stable limit cycle with alternating plateaus and spiking episodes, arising from crossing the bifurcation points HB_2 and SN_2 back and forth (see bifurcation diagram in **Fig 8D** and top voltage trace). We note that sufficient de-inactivation of I_{Nap} for triggering a new plateau (phase 3 in the bottom trace of **Fig 8D**) may be difficult to achieve during spiking episodes, because voltage oscillates over a large range, which tends to average out the variations of the inactivation level. If de-inactivation is not sufficient, the model keeps on spiking repetitively without returning to the plateau state. This is probably why it was difficult in many recorded cells to elicit plateaus by increasing the injected current, an experimental observation that could not be explained by our basic model.

Altogether, our study shows that a model incorporating the slow inactivation of I_{Nap} accounts for all the firing patterns displayed by cells from the PP and ME clusters.

Discussion

$V1^R$ constitute a homogeneous population when referring to their transcription factor program during development [24, 62], their physiological function [63] and their firing pattern at postnatal stages [27]. Surprisingly, our electrophysiological recordings and our cluster analysis clearly indicate that distinct functional classes of $V1^R$ are transiently present during development at the onset of the SNA (E11.5-E12.5). Five different groups of embryonic $V1^R$ were defined using cluster analysis, according to their firing properties.

Development of the firing pattern of embryonic $V1^R$ during SNA

It is generally assumed that, during early development, newborn neurons cannot sustain repetitive firing [35, 48]. Later on, neurons progressively acquire the ability to fire repetitively, APs become sharper, and neurons eventually reach their mature firing pattern, due to the progressive appearance of a panoply of voltage-gated channels with different kinetics [18, 35, 48]. Our results challenge the general view that single spiking is a more primitive form of excitability [35]. Indeed, we show that repetitive firing and plateau potentials dominated at early stages (E11.5-E12.5), while single spiking was prevailing only later (E13.5- E16.5).

The different $V1^R$ firing patterns observed at E11.5-E12.5 might reflect variability in the maturation level between $V1^R$ at a given developmental stage, as suggested for developing MNs [64, 65]. However, this is unlikely since $V1^R$ transiently lose their ability to sustain tonic firing or plateau potential after E13.5. The heterogeneous discharge patterns of $V1^R$ observed before E13.5 contrasts with the unique firing pattern of $V1^R$ at postnatal age [27]. Accordingly, the transient functional heterogeneity of $V1^R$ rather reflects an early initial developmental stage (E11.5-E13.5) of intrinsic excitability.

The physiological meaning of the transient functional involution of $V1^R$ that follows, after E12.5, is puzzling. To our knowledge, such a phenomenon was never described in vertebrates

during CNS development. So far, a functional involution was described only for inner hair cells between E16 and P12 [66, 67] and cultured oligodendrocytes [68], and it was irreversible. Because most $V1^R$ cannot sustain tonic firing after E12.5, it is likely that their participation to SNA is limited to the developmental period before other GABAergic interneuron subtypes mature and start to produce GABA and glycine [69]. Interestingly, embryonic $V1^R$ begin to recover their capability to sustain tonic firing when locomotor-like activity emerges [4, 11], a few days before they form their recurrent synaptic loop with MNs (around E18.5 in the mouse embryos, [70]). One possible function of the transient involution between E12.5 and E15.5 could be to regulate the growth of $V1^R$ axons toward their targets. It is indeed known that low calcium fluctuations within growth cones are required for axon growth while high calcium fluctuations stop axon growth and promote growth cone differentiation [71].

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

Blockade of I_{Nap} leads to single spiking [25], which emphasizes the importance of this current for the occurrence of repetitive firing and plateau potentials in $V1^R$ at early developmental stages. But these neurons can also switch from one firing pattern to another, when G_{Kdr} is decreased by 4-AP, which emphasizes the importance of I_{Kdr}. We found that the main determinant of embryonic $V1^R$ firing pattern is the balance between G_{Nap} and G_{Kdr}.

A Hodgkin-Huxley-type model incorporating a persistent sodium current I_{Nap} provided a parsimonious explanation of all the firing patterns recorded in the $V1^R$ population at E12.5. It provided a mathematical interpretation for the clustering of embryonic $V1^R$ shown by the hierarchical analysis and accounted for the effect of 4-AP and riluzole [25] on the discharge. Remarkably, it highlighted how a simple mechanism involving only the two opposing currents I_{Nap} and I_{Kdr} , but not I_A, could produce functional diversity in a population of

developing neurons. The model explained why minimal G_{Nap} and G_{Kdr} are required for firing, and how a synergy between G_{Nap} and G_{Kdr} controls the firing pattern and accounts for the zonation of the $G_{Nap} - G_{Kdr}$ plane that is observed experimentally.

Taking into account the slow inactivation of I_{Nap} to the model allowed us to explain the bursting patterns displayed by cells of the PP and ME clusters. We showed, in particular, that mixed events arose from elliptic bursting at the repetitive spiking-plateau transition and that smooth repetitive plateaus could be explained by a pseudo-plateau bursting mechanism [52, 58]. Such bursting scenario has been previously studied in models of endocrine cells [57, 72, 73] and adult neurons [74], but rarely observed in experiments.

Heterogeneity of the discharge pattern of pacemaker neurons has also been observed in the embryonic pre-Bötzinger network [75]. However, it was related there to the gradual change of balance between two inward currents, I_{Nap} and the calcium-activated nonselective cationic current I_{CAN} during neuronal maturation, which led to the progressive replacement of pseudo-plateau bursting by elliptic bursting. Such a scenario cannot account for the variety of discharge patterns observed in embryonic $V1^R$ at the E11.5-12.5 stage of development [25]. Our theoretical analysis and our experimental data clearly indicate that the interplay between two opposing currents is necessary to explain all the firing patterns of $V1^R$. Our model is of course not restricted to embryonic $V1^R$, but may also apply to any electrically compact cell, the firing activity of which is dominated by sodium and delayed rectifier potassium currents. This is the case of many classes of embryonic cells in mammals at an early stage of their development. It can also apply to the axon initial segment, where G_{Nap} and G_{Kdr} are known to play the major role in the occurrence of repetitive firing [76].

Altogether our experimental and theoretical results provide a global view of the developmental trajectories of embryonic $V1^R$ (see **Fig 7F and 7G**). At E12.5, conductances of embryonic $V1^R$ are widely spread in the $G_{Nap} - G_{Kdr}$ plane, which explains the heterogeneity

of their firing patterns. This likely results from the random and uncorrelated expression of sodium and potassium channels from cell to cell at this early stage. Between E12.5 and E14.5-15.5 cell size increases, and G_{Kdr} with it, while the density of sodium channels decreases (see **Fig 1 and 4**). The functional involution displayed by $V1^R$ between E12.5 and E15.5 thus mainly results from a decrease of G_{Nap} coordinated with an increase of G_{Kdr}. How these synergistic processes are controlled during this developmental period remains an open issue.

It is important to note that the presence of I_{Nap} is required for the functional diversity of $V1^R$. Indeed, in the absence of I_{Nap}, $V1^R$ lose their ability to generate plateau potentials or to fire repetitively. More generally, when the diversity of voltage-gated channels is limited, as observed in embryonic neurons [18], changes in the balance between I_{Kdr} and non (or poorly) inactivating inward current can modify the firing pattern. This can be achieved not only by I_{Nap}, but also by other slowly or non-inactivating inward conductances, such as I_{CAN} [75]. Our work also clearly indicates that a change in the firing pattern can only occur if a change in inward conductances cannot be counterbalanced by a corresponding change in outward conductances. This implies that there is no homeostatic regulation of channel density to ensure the robustness of $V1^R$ excitability during its early development, contrarily to the mature CNS [37]. In addition, the poor repertoire of voltage-gated channels at this developmental stage precludes channel degeneracy, which is also known to ensure the robustness of excitability in mature neurons [37].

In conclusion, our results demonstrate that a simple mechanism, involving only two classes of voltage-gated channels with opposite effects is sufficient to produce functional diversity in immature neurons.

Materials and Methods

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 GAD67eGFP knock-in mice to visualize putative GABAergic INs [77], as in our previous study [25]. To obtain E12.5-E16.5 transgenic GAD67-eGFP embryos, 8 to 12 weeks old wild-type Swiss female mice were crossed with GAD67-eGFP Swiss male mice.

Isolated mouse SCs from 420 embryos were used in this work and obtained as previously described [28, 78]. 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.

In the lumbar SC of GAD67eGFP mouse embryos, eGFP neurons were detected using 488 nm UV light. They were localized in the ventro-lateral marginal zone between the motor columns and the ventral funiculi [62]. Embryonic $V1^R$ identity was confirmed by the expression of the forkhead transcription factor Foxd3 [25].

Whole-cell recordings and analysis

The isolated SC was placed in a recording chamber and was continuously perfused (2 ml/min) at room temperature (22-26°C) with oxygenated ACSF. Whole-cell patch-clamp recordings of lumbar spinal embryonic $V1^R$ were carried out under direct visualization using

an infrared-sensitive CCD video camera. Whole-cell patch-clamp electrodes with a resistance of 4-7 MΩ were pulled from thick-wall borosilicate glass using a P-97 horizontal puller (Sutter Instrument Co., USA). They were filled with a solution containing (in mM): 96.4 K methanesulfonate, 33.6 KCl, 4 $MgCl_2$, 4 Na_2ATP, 0.3 Na_3GTP, 10 EGTA, and 10 HEPES (pH 7.2; 290 mOsm/kg-H_2O). This intracellular solution led to an equilibrium potential of chloride ions, E_{Cl}, of about -30 mV, close to the physiological values measured at E12.5 in spinal MNs [28]. The junction potential (6.6 mV) was systematically corrected offline.

Signals were recorded using Multiclamp 700B amplifiers (Molecular Devices, USA). Data were low-pass filtered (2 kHz), digitized (20 kHz) online using Digidata 1440A or 1550B interfaces and acquired using pCLAMP 10.5 software (Molecular Devices, USA). Analyses were performed off-line using pCLAMP 10.5 and Axograph 1.7.2 (Molecular devices; RRID:SCR_014284) software packages.

In voltage-clamp mode, voltage-dependent K^+ currents (I_{Kv}) were elicited in the presence of 1 μM TTX by 500 ms depolarizing voltage steps (10 mV increments, 10 s interval) after a prepulse of 300 ms at V_H = -100 mV. To isolate I_{Kdr}, voltage steps were applied after a 300 ms prepulse at V_H = -30 mV that inactivated the low threshold transient potassium current I_A. I_A was then obtained by subtracting offline I_{Kdr} from the total potassium current I_{Kv}. Capacitance and leak current were subtracted using on-line P/4 protocol provided by pCLAMP 10.5.

In current-clamp mode, $V1^R$ discharge was elicited using 2 s depolarizing current steps (from 0 to ≈ 50 pA in 5-10 pA increments, depending on the input resistance of the cell) with an 8 s interval to ensure that the membrane potential returned to V_H. When a cell generated a sustained discharge, the intensity of the depolarizing pulse was reduced to the minimal value compatible with repetitive firing.

I_{Nap} was measured in voltage-clamp mode using a 70 mV/s depolarizing voltage ramp [79]. This speed was slow enough to preclude substantial contamination by the inactivating transient current and fast enough to avoid substantial inactivation of I_{Nap}. Subtraction of the current evoked by the voltage ramp in the presence of 1 µM tetrodotoxin (TTX) from the control voltage ramp-evoked current revealed I_{Nap}.

Pharmacological reagents

During patch-clamp recordings, bath application of TTX (1 µM, Alomone, Israel) or 4-AP (0.3 - 600 µM, Sigma) was done using 0.5 mm diameter quartz tubing positioned, under direct visual control, 50 µm away from the recording area. The quartz tubing was connected to 6 solenoid valves linked with 6 reservoirs *via* a manifold. Solutions were gravity-fed into the quartz tubing. Their application was controlled using a VC-8 valve controller (Warner Instruments, USA).

4-aminopyridine (4-AP; Sigma Aldrich, USA) was used to block I_{Kdr}. To determine the concentration–response curve, $I-V$ curves of I_{Kdr} for different concentrations of 4-AP (0.3 to 300 µM) were compared to the control curve obtained in the absence of 4-AP. The percentage of inhibition for a given concentration was calculated by dividing the peak intensity of I_{Kdr} by the peak value obtained in control condition. The obtained normalized concentration–response curves were fitted using the Hill equation:

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

where [4-AP] is the 4-AP concentration, I_{min} is the residual current (in percentage of the peak I_{Kdr}), $100-I_{min}$ is the maximal inhibition achieved for saturating concentration of 4-AP, IC_{50} is the 4-AP concentration producing half of the maximal inhibition, and n_H is the Hill coefficient. Curve fitting was performed using KaleidaGraph 4.5 (Synergy Software, USA).

Immunohistochemistry and confocal microscopy

E14.5 embryos were collected from pregnant females. Once dissected out of their yolk sac, SCs were dissected and immediately immersion-fixed in phosphate buffer (PB 0.1 M) containing 4% paraformaldehyde (PFA; freshly prepared in PB, pH 7.4) for 1 h at 4°C. Whole SCs were then rinsed out in 0.12 M PB at 4°C, thawed at room temperature, washed in PBS, incubated in NH_4Cl (50 mM), diluted in PBS for 20 min and then permeabilized for 30 min in a blocking solution (10% goat serum in PBS) with 0.2% Triton X-100. They were incubated for 48 h at 4°C in the presence of the following primary antibodies: guinea pig anti-FoxD3 (1:5000, gift from Carmen Birchmeier and Thomas Müller of the Max Delbrück Center for Molecular Medicine in Berlin) and rabbit anti-cleaved Caspase-3 (1:1000, Cell Signaling Technology Cat# 9661, RRID:AB_2341188). SCs were then washed in PBS and incubated for 2 h at RT with secondary fluorescent antibodies (goat anti-rabbit-conjugated 649; donkey anti-guinea pig-conjugated Alexa Fluor 405 [1:1000, ThermoFisher]) diluted in 0.2% Triton X-100 blocking solution. After washing in PBS, SCs were dried and mounted in Mowiol medium (Millipore, Molsheim, France). Preparations were then imaged using a Leica SP5 confocal microscope. Immunostaining was observed using a 40X oil-immersion objective with a numerical aperture of 1.25, as well as with a 63X oil-immersion objective with a numerical aperture of 1.32. Serial optical sections were obtained with a Z-step of 1 µm (40X) and 0.2-0.3 µm (63X). Images (1024x1024; 12-bit color scale) were stored using Leica software LAS-AF and analyzed using ImageJ 1.5 (N.I.H., USA; http//rsb.info.nih.gov/ij/) and Adobe Photoshop CS6 (Adobe, USA) software.

Cluster analysis

To classify the firing patterns of embryonic $V1^R$, we performed a hierarchical cluster analysis on a population of 163 cells. Each cell was characterized by three quantitative

measures of its firing pattern (see legend of Figure 5). After normalizing these quantities to zero mean and unit variance, we performed a hierarchical cluster analysis using the hclust function in R software (version 3.3.2, https://cran.r-project.org/) that implements the complete linkage method. The intercluster distance was defined as the maximum Euclidean distance between the points of two clusters, and, at each step of the process, the two closest clusters were merged into a single one, thus constructing progressively a dendrogram. Clusters were then displayed in data space using the dendromat function in the R package 'squash' dedicated to color-based visualization of multivariate data. The best clustering was determined using the silhouette measure of clustering consistency [80]. The silhouette of a data point, based on the comparison of its distance to other points in the same cluster and to points in the closest cluster, ranges from -1 to 1. A value near 1 indicates that the point is well assigned to its cluster, a value near 0 indicates that it is close to the decision boundary between two neighboring clusters, and negative values may indicate incorrect assignment to the cluster. This allowed us to identify an optimal number k of clusters by maximizing the overall average silhouette over a range of possible values for k [80], using the silhouette function in the R package 'cluster'.

Biophysical modeling

To understand the relationship between the voltage-dependent membrane conductances and the firing patterns of embryonic V1^{R}, we relied on a single compartment conductance-based model that included the leak current, the transient and persistent components of the sodium current, I_{Nat} and I_{Nap}, a delayed rectifier potassium current I_{Kdr} and the inactivating potassium current I_A revealed by experiments. Voltage evolution then followed the equation

$$C_{in}\frac{dV}{dt} = G_{in}(V_r - V) + G_{Nat}m^3h(E_{Na} - V) + G_{Nap}m_p^3s(V_{Na} - V) + G_{Kdr}n^3(E_K - V) + G_A m_A h_A(E_K - V) + I \quad (1),$$

,

where C_{in} was the input capacitance; G_{in} the input conductance; G_{Nat}, G_{Nap}, G_{Kdr} and G_A the maximal conductances of the aforementioned currents; m, m_p, n and m_A their activation variables; h the inactivation variable of I_{Nat}, s the slow inactivation variable of I_{Nap}, and m_A the inactivation variable of I_A. V_r is the baseline potential imposed by *ad hoc* current injection in current-clamp experiments; E_{Na} and E_K are the Nernst potentials of sodium and potassium ions, and I the injected current. All gating variables satisfied equations of the form:

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

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

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

with k_x being positive for activation and negative for inactivation. The time constant τ_x was voltage-independent except for the inactivation variables h and s. The activation variable m_A of I_A was assumed to follow instantaneously voltage changes.

The effect of channel noise was investigated with a stochastic realization of the model, where channels kinetics were described by Markov-type models, assuming a unitary channel conductance of 10 pS for all channels.

Choice of model parameters

Most model parameters were chosen on the basis of experimental measurements. Parameters that could not be constrained from our experimental data were chosen from an experimentally realistic range of values. V_r was set at -60 mV as in experiments (see table 1). C_{in} (average 13.15 pF, 50% between 11.9 and 15.1 pF, only 18 cells out of 246 in the first quartile below 7.2 pF or in the fourth quartile above 19 pF) and G_{in} (50% of cells between 0.71 and 1.18 nS, only 7 out of 242 with input conductance above 2 nS) were not spread

much in the cells recorded at E12.5, showing that most embryonic V1^R were of comparable size. Interestingly, C_{in} and G_{in} were not correlated, which indicated that the input conductance was determined by the density of leak channels rather than by the sheer size of the cell. Moreover, no correlation was observed between the passive properties and the firing pattern [25]. Therefore, we always set G_{in} and C_{in} to 1 nS and 13 pF in the model (except in S7 **Fig**), close to the experimental medians (0.96 nS and 13.15 pF, respectively). The membrane time constant C_{in}/G_{in} was then equal to 13 ms, which was also close to the experimental median (13.9 ms, N=241).

E_{Na} was set to 60 mV, the experimentally measured value. The activation curve of I_{Nap} was obtained by fitting experimental data, leading to an average mid-activation of -36 mV and an average steepness of 9.5 mV. The experimentally measured values of G_{Nap} were in the range 0-2.2 nS. We assumed that the activation curve of I_{Nat} was shifted rightward by 10 mV in comparison to I_{Nap}. No experimental data was available for the inactivation of I_{Nat}. We chose a mid-inactivation voltage V_h= -45 mV and a steepness k_h=-5 mV. We also assumed that the activation time constant of both I_{Nat} and I_{Nap} was 1.5 ms, and that the inactivation time constant was voltage-dependent: $\tau_h(V) = 16.5 - 13.5\tanh((V+20)/15)$, decreasing by one order of magnitude (from 30 ms down to 3 ms) with the voltage increase. This enabled us to account for the shape of the action potentials recorded in experiments, showing a slow rise time and rather long duration. The conductance G_{Nat} was not measured experimentally. When choosing a reasonable value of 20 nS for G_{Nat}, the model behaved very much as recorded embryonic V1^R: with similar current threshold (typically 10-20 pA) and stable plateau potential obtained for the largest values of G_{Nap}.

When taking into account slow inactivation of I_{Nap} (see **Fig 8**), we chose V_s = -30 mV for the mid-inactivation voltage and set the steepness k_s at -5 mV (as for the inactivation of I_{Nat}).

For simplicity, we assumed that the inactivation time constant was voltage-independent and set it at a value of 2 s.

E_K was set to the experimentally measured value of -96 mV. The activation parameters of I_{Kdr} were obtained by fitting the experimental data: V_n = -20 mV, k_n = 15 mV, τ_n = 10 ms and an activation exponent of 3. The activation and inactivation properties of I_A were also chosen based on experimental measurements. Accordingly, V_{m_A} = -30 mV, 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 account, we assumed that $G_A = G_{Kdr}$, consistently with experimental data (see **S6 Fig**).

Numerical simulations and dynamical systems analysis

We integrated numerically the deterministic model using the freeware XPPAUT [81] and a standard fourth-order Runge-Kutta algorithm. XPPAUT was also used to compute one-parameter and two-parameters bifurcation diagrams. The stochastic version of the model was also implemented in XPPAUT and computed with a Gillespie's algorithm [82].

To investigate the dynamics of the model with slow inactivation of I_{Nap}, we relied on numerical simulations together with fast/slow dynamics analysis [83]. In this approach, one distinguishes slow dynamical variables (here only s) and fast dynamical variables. Slow variables vary little at the time scale of fast variables and may therefore be considered as constant parameters of the fast dynamics in first approximation. In contrast, slow variables are essentially sensitive to the time average of the fast variables, much more than to their instantaneous values. This separation of time scales allows one to conduct a phase plane analysis of the full dynamics.

Statistics

Samples sizes (n) were determined based on previous experience. The number of embryos (N) is indicated in the main text and figure captions. No power analysis was employed, but

sample sizes are comparable to those typically used in the field. All values were expressed as mean with standard error of mean (SEM). Statistical significance was assessed by non-parametric Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons, Mann-Whitney test for unpaired data or Wilcoxon matched pairs test for paired data using GraphPad Prism 7.0e Software (USA). Significant changes in the proportions of firing patterns with age were assessed by chi-square test for large sample and by Fisher's exact test for small sample using GraphPad Prism 7.0e Software. Significance was determined as $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***). The exact p value was mentioned in the result section or in the figure captions.

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Declaration of Interests

The authors declare no competing interests

References

Figure captions

Fig 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 $V1^R$, RS $V1^R$ and PP $V1^R$ at E12.5. (Kruskall-Wallis test $P < 0.0001$; SS $V1^R$ versus RS $V1^R$ at 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, $P = 0.0086$; E14.5 SS $V1^R$ versus E12.5 SS $V1^R$, $P = 0.0048$; E14.5 SS $V1^R$ versus E12.5 RS $V1^R$, $P = 0.0384$, E14.5 SS $V1^R$ versus E12.5 PP $V1^R$, $P < 0.0001$). The increase in G_{Kdr} between E12.5 and E14.5 is likely to be due to the increase in neuronal size (input capacitance; Figure 2A). Indeed, there was no significant difference (Mann Whitney test, $P = 0.133$) 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 gray bar). (E) Bar graph showing the maximal G_{Nap} value (Max G_{Nap}) in SS $V1^R$ at E12.5 (n = 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), 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 calculated from maximal I_{Nap} value measured on current evoked by assuming a Na^+ equilibrium potential of +60 mV. There was no difference in G_{Nap} between RS $V1^R$ and PP $V1^R$. On the contrary, G_{Nap} measured in SS $V1^R$ at E12.5 or at E14.5 was significantly smaller as compared to G_{Nap} measured at E12.5 in RS $V1^R$ or in PP $V1^R$. G_{Nap} measured at E12.5 and E14.5 in SS $V1^R$ were not significantly different (Kruskall-Wallis test $P < 0.0001$; E12.5 SS $V1^R$ versus E12.5 RS $V1^R$, $P = 0.0034$; E12.5 SS $V1^R$ versus E12.5 PP $V1^R$, $P = 0.0006$; E12.5 RS $V1^R$ versus E12.5 PP $V1^R$, $P = 0.5494$; E14.5 SS $V1^R$ versus E12.5 SS $V1^R$, $P = 0.5896$; E14.5 SS $V1^R$ versus E12.5 RS $V1^R$, $P = 0.0005$; E14.5 SS $V1^R$ versus E12.5 PP $V1^R$, $P < 0.0001$). (F) Histograms showing the G_{Nap} / G_{Kdr} ratio in SS $V1^R$ at E12.5 (n = 9; gray bar) and E14.5 (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 = 6; blue bar) at E12.5. Note that the G_{Nap} / G_{Kdr} ratio differs significantly between SS $V1^R$, RS $V1^R$ and PP $V1^R$ at E12.5, while it is not different between SS $V1^R$ recorded at E12.5 and at E14.5 (Kruskall-Wallis test $P < 0.0001$; SS $V1^R$ versus RS $V1^R$ at E12.5, $P = 0.0367$; SS $V1^R$ 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 $V1^R$ 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 $V1^R$ versus E12.5 PP $V1^R$ $P < 0.0001$). Data shown in A and B were used to calculate G_{Nap} / G_{Kdr} ratio shown in C. (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Fig 2. Increasing 4-AP concentration changed the firing pattern of single spiking embryonic $V1^R$ recorded at E12.5

The firing pattern of embryonic $V1^R$ was evoked by 2 s suprathreshold depolarizing current steps. (A) Representative traces showing examples of the effect of increasing concentration of 4-AP (from 3 to 300 μM) on the firing pattern of a SS $V1^R$ recorded at E12.5. Note that in A1, increasing 4-AP concentration converted single spiking (gray trace) to repetitive spiking (red trace), repetitive spiking to a mixed event pattern (purple trace) and mixed events to plateau potential (blue trace). (A2) Example of SS $V1^R$ in which increasing 4-AP concentration converted single spiking to repetitive spiking only. (A3) Bar plots showing the change in the firing pattern of SS $V1^R$ according to 4-AP concentrations (control n = 10; N = 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 4-AP n = 10; N = 10, 300 μM 4-AP n = 8; N = 8). (B) Representative traces showing the effect of 0.5 μM TTX on a plateau potential evoked in a SS $V1^R$ in the presence of 300 μM 4-AP. (C) Representative traces showing the effect of 0.5 μM TTX on repetitive AP firing evoked in a SS $V1^R$ in the presence of 300 μM 4-AP. In both cases, the application of TTX fully blocked the responses evoked in the presence of 4-AP, indicating that they were underlain by the activation of voltage-gated Na^+ channels.

Fig 3. Cluster analysis of $V1^R$ firing pattern at E12.5

(A, inserts) Cluster analysis of embryonic $V1^R$ firing pattern was performed using three parameters that describe the firing pattern during a 2 s suprathreshold depolarizing pulses: the

mean of the half-amplitude event duration (mean ½Ad), the coefficient of variation of ½ Ad (CV ½Ad) allowing to quantify the AP variation within a train (CV was set to 0 when the number of spikes evoked by a depolarizing pulse was ≤ 3) and the duration ratio ddr = Σ½ Ad/Pw, obtained by dividing the sum of ½ Ad by the pulse duration Pw, that indicates the total time spent in the depolarized state. For example, ddr = 1 when a plateau potential lasts as long as the depolarizing pulse. Conversely, its value is low when the depolarizing pulse evokes a single AP only. (A) Dendrogram for complete linkage hierarchical clustering of 164 embryonic $V1^R$ (N = 140) according to the values of log mean ½Ad, of CV ½Ad and of log ddr. The colored matrix below the dendrogram shows the variations of these three parameters for all the cells in the clusters (colored trees) extracted from the dendrogram. (B) The number of clusters was determined by analyzing the distribution of silhouette width values (see Material and Methods). The boxplots show the distribution of silhouette width values when the number of clusters k varies from 2 to 12. The mean silhouette width values (red diamond shaped points) attained their maximum when the estimated cluster number was 5. (C) 3D plot showing cluster distribution of embryonic $V1^R$ according to log mean ½Ad, CV ½Ad and log ddr. Each cluster corresponds to a particular firing pattern as illustrated in D. $V1^R$ that cannot sustain repetitive firing of APs (1 to 3 AP/pulse only, gray, Single spiking, SS), $V1^R$ that can fire tonically (red, Repetitive spiking, RS), $V1^R$ with a firing pattern characterized by a mix of APs and relatively short plateau potentials (dark purple, Mixed event short PP, ME short PP), $V1^R$ with a firing pattern characterized by a mix of APs and relatively long plateau potentials (light purple, Mixed event long PP, ME long PP) and $V1^R$ with evoked plateau potentials only (blue, Plateau potential, PP). The arrow in C indicates 3 misclassified $V1^R$ that could not sustain repetitive firing although they were assigned to the cluster of repetitively firing $V1^R$ (see text).

Fig 4. Developmental changes of embryonic $V1^R$ firing patterns from E11.5 to E16.5

(A1) Graph showing how the input capacitance C_{in} of $V1^R$ changes with embryonic age. C_{in} significantly increased between E12.5 or E13.5 and E14.5 (Kruskall-Wallis test $P < 0.0001$; E12.5 versus E11.5 $P = 0.258$, E12.5 versus E13.5 $P = 0.904$, E12.5 versus E14.5 $P < 0.0001$, E12.5 versus E15.5 $P < 0.0001$, E12.5 versus E16.5 $P < 0.0001$, E13.5 versus E14.5 $P < 0.0001$, 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 = 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; N = 9). (A2) Graph showing how the input resistance R_{in} of $V1^R$ changes with embryonic age. R_{in} significantly decreased between E12.5 or E14.5 and E15.5 (Kruskall-Wallis test $P < 0.0001$; E12.5 versus E11.5 $P > 0.999$, E12.5 versus E13.5 $P = 0.724$, E12.5 versus E14.5 $P > 0.999$, E12.5 versus E15.5 $P = 0.0004$, E12.5 versus E16.5 $P = 0.0005$, E14.5 versus E15.5 $P = 0.0019$, E14.5 versus E16.5 $P < 0.0058$; E11.5 n = 31, E12.5 n = 261; N = 146, E13.5 n = 43; N = 40, E14.5 n = 60; N = 48, E15.5 n = 16; N = 4, E16.5 n = 30; N = 9). (A3) Graph showing how the threshold of regenerative events (APs and plateau potentials) of $V1^R$ changes with embryonic age. The average threshold became significantly more hyperpolarized after E12.5 (Kruskall-Wallis test $P < 0.0001$; E12.5 versus E11.5 $P = 0.676$, E12.5 versus E13.5 $P = 0.0039$, E12.5 versus E14.5 $P < 0.0001$, E12.5 versus E15.5 $P < 0.0001$, E12.5 versus E16.5 $P < 0.0001$, E13.5 versus E14.5 $P > 0.999$, E13.5 versus E15.5 $P = 0.1398$, E13.5 versus E16.5 $P = 0.0013$; E14.5 versus E15.5 $P > 0.999$, E14.5 versus E16.5 $P = 0.0634$, E15.5 versus E16.5 $P > 0.999$; E11.5 n = 20; N = 16, E12.5 n = 162; N = 139, E13.5 n = 31; N = 28, E14.5 n = 30; N = 26, E15.5 n = 16; N = 4, E16.5 n = 30; N = 9). Yellow and purple bars below the graphs indicate the two important phases of the functional development of spinal cord networks. The first one is characterized by synchronized neuronal activity (SNA) and the second one is characterized by the emergence of a locomotor-like activity (see text). Note that changes in C_{in} and R_{in} occurred at the end of the first

developmental phase. (*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; control, E12.5). The intrinsic activation properties were analyzed using 2 s suprathreshold depolarizing current steps. (B) Representative traces of voltage responses showing Single Spiking (SS) $V1^R$ (gray), Repetitive Spiking (RS) $V1^R$ (red), ME $V1^R$ (purple) and Plateau Potential (PP) $V1^R$ (blue) at E11.5 (B1), E13.5 (B2), E14.5 (B3) E15.5 (B4) and E16.5 (B5). (C) Bar graph showing how the proportions of the different firing patterns change from E11.5 to E16.5 (E11.5 n = 22; N = 18, E12.5 n = 163; N = 140, E13.5 n = 32; N = 29, E14.5 n = 57; N = 45, E15.5 n = 15; N = 4, E16.5 n = 28; N = 9). Yellow and purple bars below the graphs indicate the first and the second phase of functional embryonic spinal cord networks. The proportions of the different firing patterns significantly changed between E11.5 to E12.5 (Fisher's exact test, $P = 0.0052$) with a significant increase in the proportion of RS $V1^R$ (Fisher's exact test, $P = 0.0336$) and a significant decrease in the proportion of ME $V1^R$ (Fisher's exact test, $P = 0.01071$) at E12.5. Only two firing patterns (SS and RS) were observed after E13.5 and most embryonic $V1^R$ lost their ability to sustain tonic firing after E13.5. However, at E16.5 the proportion of RS $V1^R$ significantly increased at the expense of SS $V1^R$ when compared to E14.5 (Fisher's exact test, $P = 0.0112$), indicating that embryonic $V1^R$ began to recover the ability to sustain tonic firing after E15.5.

Fig 5. **Activated caspase-3 is not observed in embryonic $V1^R$ at E14.5**

Representative confocal image of the ventral part of an isolated lumbar spinal cord of E14.5 GAD67-eGFP mouse embryo showing immunostainings using antibodies against eGFP (A), FoxD3 (B) and activated Caspase 3 (aCaspace 3) (C). (D) Superimposition of the three stainings shows that embryonic $V1^R$ (eGFP+ and FoxD3+) were not aCaspase 3 immunoreactive. (A1, B1, C1 and D1). Enlarged images from A, B and C showing that aCaspase 3 staining is localized in areas where eGFP and Foxd3 staining were absent. (A2, B2,

C2 and D2) Enlarged images from A, B and C showing that aCaspase 3 staining is absent in the area where $V1^R$ (eGFP+ and FoxD3+) are located. aCaspase 3 staining that did not co-localize with GAD67eGFP likely indicates MN developmental cell death.

Fig 6. 600 μM 4-AP changed the firing pattern of single spiking embryonic $V1^R$ recorded at E14.5

The firing pattern of embryonic $V1^R$ was evoked by 2 s suprathreshold depolarizing current steps. (A) Representative traces showing the effect of 4-AP application (600 μM) on the firing pattern of single spiking (SS) $V1^R$ recorded at E14.5. Note that the applications of 600 μM 4-AP evoked either a plateau potential (A1) or repetitive spiking (A2), both fully blocked by TTX. (B) Bar plots showing the proportions of the different firing patterns observed in the presence of 600 μM 4-AP versus control recorded in SS $V1^R$ at E14.5 (n = 14; N = 14). Single Spiking (SS) $V1^R$ (gray), Repetitive Spiking (RS) $V1^R$ (red), Mixed Events (ME) $V1^R$ (purple), Plateau Potential (PP) $V1^R$ (blue).

Fig 7. Embryonic $V1^R$ firing patterns predicted by computational modeling

(A) Firing patterns of 27 recorded cells, in which both G_{Nap} and G_{Kdr} were measured. Gray: SS, red: RS, blue: PP. The three purple points located at the boundary between the RS and PP regions correspond to mixed events (ME) where plateau potentials alternate with spiking episodes. Note that no cell exhibited low values of both G_{Nap} and G_{Kdr} (lower left), or large values of both conductances (upper right). (B) Bifurcation diagram of the deterministic model when G_{Kdr} is kept fixed to 2.5 nS or 10 nS while G_{Nap} is varied between 0 and 2.5 nS. G_{in} = 1 nS and I = 20 pA. For G_{Kdr} = 10 nS (i.e., in the top experimental range), the red curves indicate the maximal and minimal voltages achieved on the stable limit cycle associated with repetitive firing (solid lines) and on the unstable limit cycle (dashed lines). The fixed point of

the model is indicated by a gray solid line when it corresponds to the stable quiescent state, a gray dashed line when it is unstable and a solid blue line when it corresponds to a stable plateau potential. The two HB corresponding to the change of stability of the quiescence state (HB_1, G_{Nap}= 0.81 nS) and of the voltage plateau (HB_2, G_{Nap}= 2.13 nS) are indicated, as well as the two SN bifurcations of limit cycles associated with the onset (SN_1, G_{Nap} = 0.65 nS) and offset (SN_2, G_{Nap}= 2.42 nS) of repetitive spiking as G_{Nap} is increased. For G_{Kdr} = 2.5 nS, the model does not display repetitive firing; it possesses a unique fixed point, which is always stable (blue-gray curve). The transition from quiescence to plateau is gradual with no intervening bifurcation. Representative voltage traces of the three different activity patterns are shown: single spiking in response to a 2 s current pulse (gray, G_{Nap} = 0.2 nS, G_{Kdr} = 10 nS), repetitive spiking (red, G_{Nap} = 1.2 nS, G_{Kdr} = 10 nS) and plateau potential (blue, G_{Nap} = 1.2 nS, G_{Kdr} = 2.5 nS). Note that the plateau never outlasts the current pulse. (C) Bifurcation diagram when G_{Nap} is kept fixed at 1.2 nS and G_{Kdr} is varied between 0 and 25 nS (I = 20 pA). Same conventions as in B. Plateau potential is stable until the subcritical Hopf bifurcation HB_2 (G_{Kdr} = 6.34 nS) is reached, repetitive firing can be observed between SN_2 (G_{Kdr} = 5.93 nS) and SN_1 (G_{Kdr} = 22.65 nS). The quiescent state is stable from point HB_1 (G_{Kdr} = 17.59 nS) onward. (D) Two-parameters bifurcation diagram of the model in the G_{Nap} - G_{Kdr} plane (I = 20 pA). The black merged curves indicate the bifurcations HB_1 and HB_2. The red curves indicate the SN bifurcations of limit cycles SN_1 and SN_2. The shaded area indicates the region where repetitive firing can occur. The oblique lines through the points labeled 1, 2 and 3, the same as in B, correspond to three different values of the ratio G_{Nap}/G_{Kdr} : 0.02 (gray), 0.12 (red) and 0.48 (blue). Voltage traces on the right display the response to a 2 s current pulse when channel noise is taken into account for the three regimes: quiescence (top, gray trace and dot in the diagram), repetitive firing (middle, red) and plateau potential (bottom, blue). They correspond to the three deterministic voltage traces

shown in B. Note that the one-parameter bifurcation diagrams shown in B correspond to horizontal lines through points 1 and 2 (G_{Kdr} = 10 nS) and through point 3 (G_{Kdr} = 2.5 nS), respectively. The bifurcation diagram in C corresponds to a vertical line through point 2 and 3 (G_{Nap} = 1.2 nS). (E) Cumulative distribution function of the ratio G_{Nap}/G_{Kdr} for the four clusters in A, showing the sequencing SS (gray) → RS (red) → ME (purple, 3 cells only) → PP (blue) predicted by the two-parameters bifurcation diagram in D. The wide PP range, as compared to SS and RS, merely comes from the fact that G_{Kdr} is small for cells in this cluster. The three colored points indicate the slopes of the oblique lines displayed in D (0.02, 0.12 and 0.48, respectively). (F) The data points in A are superimposed on the two-parameters bifurcation diagram shown in D, demonstrating a good agreement between our basic model and experimental data (the same color code as in A for the different clusters). The bifurcation diagram is simplified compared to A, only the region where repetitive spiking is possible (i.e. between the lines SN_1 and SN_2 in A) being displayed (shaded area). Notice that 3 ME cells (purple dots) are located close to the transition between the RS and PP regions. The four arrows indicate the presumable evolution of G_{Nap} and G_{Kdr} for SS, RS, ME and PP cells between E12.5 and E14.5-15.5. G_{Nap} eventually decreases while G_{Kdr} keeps on increasing. (G) Distribution of a sample of cells in the G_{Kdr} - G_{Kdr} plane at E14.5. All the cells are located well within the SS region far from bifurcation lines because of the decreased G_{Nap} compared to E12.5, the increased G_{Kdr}, and the shift of the RS region (shaded) due to capacitance increase (18 versus 13 pF).

Fig 8. Effects of the slow inactivation of I_{Nap} on firing patterns predicted by computational modeling

(A) Examples of repetitive plateaus (left) and mixed events (right) recorded in $V1^R$ at E12.5 during a 2 s current pulse. (B1) Current-voltage curve of the basic model (without slow

inactivation of I_{Nap}, and without I_A or channel noise) for G_{Kdr} = 5 nS and for G_{Nap} = 1.65 nS (lower curve) and 2 nS (upper curve). Solid lines denote stable fixed points and dashed lines unstable ones. For G_{Nap} =1.65 nS, bistability between quiescence and plateau occurs between 1.39 and 10.48 pA. When G_{Nap} is increased to 2 nS, the bistability region ranges from -10.84 to 9.70 pA, thus extending into the negative current range. This implies that once a plateau has been elicited, the model will stay in that stable state and not return to the resting state, even though current injection is switched off (see insert). B1 Insert. Voltage response to a 2 s current pulse of 15 pA for G_{Nap} = 2 nS. The resting state (gray dot on the lower curve in B1 is destabilized at pulse onset and a plateau is elicited (blue dot on the upper curve in B1). At pulse offset, the plateau is maintained, even though the injected current is brought back to zero, and channel noise is not sufficient to go back to the resting state. (B2) Domain of bistability between quiescence and plateau (shaded) in the I- G_{Nap} plane for G_{Kdr}= 5 nS. It is delimited by the line SN_2 where a SN bifurcation of fixed points occurs and by the subcritical Hopf bifurcation line HB where the plateau becomes unstable. Bistability requires that G_{Nap} exceeds 1.35 nS, and the domain of bistability enlarges as G_{Nap} is increased further. The two horizontal lines correspond to the two cases shown in B1: G_{Nap} =1.65 nS and 2 nS. (C) Behavior of the model when slow inactivation is incorporated. The bifurcation diagram of the basic model (without slow inactivation) for I = 10 pA and G_{Kdr} = 5 nS (same conventions as in Fig 7B) and the stable limit cycle (black solid curve) obtained when slow inactivation is added are superimposed. The limit cycle is comprised of four successive phases (see labels): 1) long plateau during which I_{Nap} slowly inactivates, 2) fast transition to the quiescent state, 3) repolarization episode during which I_{Nap} slowly deinactivates, 4) fast transition back to the plateau. Each plateau starts with a full-blown action potential followed by rapidly decaying spikelets. Note that the bifurcation HB is subcritical here (unstable limit cycle shown by dashed red curve), at variance with square wave bursting (supercritical bifurcation and stable

limit cycle); this is a characteristic feature of pseudo-plateau bursting. Note also that the plateau extends beyond the bifurcation HB because it is only weakly unstable then. Responses to a 15 s current pulse are shown on the right side. Top left: voltage response, Top right: behavior of the "effective" conductance of I_{Nap} channel, i.e., the maximal conductance G_{Nap} multiplied by the slow inactivation variable s. Bottom left: voltage trace when channel noise is added to fast and slow gating variables, Bottom right: Voltage trace when G_{Nap} is increased by 50%. (D) Mixed events. The bifurcation diagram of the basic model for G_{Kdr} = 5 nS and I = 12 pA and the stable limit cycle obtained in the presence of slow inactivation (G_{Nap} = 2.5 nS) are superimposed. Here again, the limit cycle is comprised of four successive phases (see labels): 1) slow inactivation of I_{Nap} that leads to the crossing of the bifurcation point HB_2 and then to the destabilization of the plateau potential, 2) fast transition to the spiking regime, 3) repetitive spiking during which I_{Nap} slowly de-inactivates, which leads to the crossing of the bifurcation point SN_2 and terminates the spiking episode, 4) fast transition back to the stable plateau potential. Response to a 15 s current pulse of 12 pA is shown on the right in the absence of any channel noise. Top: Voltage trace (same labels as in the bifurcation diagram on the left), Bottom: Variations of the "effective" conductance $G_{Nap}s$ (same labels as in the voltage trace). Note that de-inactivation sufficient to trigger a new plateau occurs over a series of successive spikes, hence the small oscillations are visible on the trace. Note also that in C and D the first plateau lasts longer than the following ones, as in electrophysiological recordings of embryonic $V1^R$ cells displaying repetitive plateaus. This form of adaptation is caused by the slow inactivation of the persistent sodium current.

Figure supplements

S1 Fig. Relates to Fig 2. 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).

S2 Fig. Relates to Fig 2. Effect of 4-AP application in repetitively spiking $V1^R$ at E12.5

(A) Representative traces showing the effect of 4-AP application (300 μM) on Repetitive Spiking (RS) $V1^R$ at E12.5. Note that plateau potential activity evoked in the presence of 4-AP (middle trace) was blocked by 0.5 μM TTX (right trace). (B) Bar plots showing the changes in the firing pattern of RS $V1^R$ evoked by 300 μM 4-AP application (n = 14). 4-AP application evoked a plateau potential in 71.4 % of the recorded neurons (10/14) and mixed events in 14.3% of the recorded neurons (2/14). The excitability pattern was not modified in 2 neurons. Repetitive Spiking (RS) $V1^R$ (red), Mixed events (ME) $V1^R$ (purple), Plateau Potential (PP) $V1^R$ (blue).

S3 Fig. Relates to Fig 3. Distributions of log ½Ad, CV ½Ad and log ddr values related to the cluster analysis of embryonic $V1^R$ firing patterns

(A1) Histogram of log mean ½Ad (mean half amplitude event duration) for the whole $V1^R$ population at E12.5 (n= 164; bin width 0.1). The histogram was well fitted by the sum of three Gaussian curves with means and SDs of 1.135, 2.046 & 2.84, and 0.316, 0.181 & 0.21, respectively. (A2) Histogram of the values of log mean ½Ad sorted after cluster analysis showing single spiking (SS) $V1^R$ (gray), repetitive spiking (RS) $V1^R$ (red), mixed events (ME) $V1^R$ with short plateau potentials (ME short PP $V1^R$, light purple), ME $V1^R$ with long plateau potentials (ME long PP $V1^R$, dark purple) and plateau potential (PP) $V1^R$ (blue). log mean ½Ad was significantly different between SS $V1^R$, PP $V1^R$, the whole ME $V1^R$ population (ME_s and ME_l $V1^R$) and PP $V1^R$ (Kruskall-Wallis test $P < 0.0001$; SS $V1^R$ versus RS $V1^R$, $P < 0.0001$; 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 $V1^R$, $P = 0.0004$; 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, 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 the whole $V1^R$ population at E12.5 (n= 164; bin width 5%). Note that a large population of $V1^R$ had zero CV ½Ad (n = 83). The histogram for CV ½Ad ≠ 0 was fitted by the sum of three Gaussian curves with means and SDs of 23.4, 68.4 & 117 (%) and 8.9, 6.8 & 4.1, respectively. (B2) Histograms of the values of CV ½Ad sorted after cluster analysis showing SS $V1^R$ (black), RS $V1^R$ (red), ME_s $V1^R$ (light purple), ME_l $V1^R$ (dark purple) and PP $V1^R$. CV ½Ad was not significantly different between SS $V1^R$ and PP $V1^R$ (CV ½Ad of SS $V1^R$ and PP $V1^R$ = 0.682 % and 0% respectively: only one of the 46 SS $V1^R$ displayed 3 PA and had a CV ½Ad of 31.37). CV ½Ad was significantly different between RS $V1^R$ and the whole ME $V1^R$ population and also between SS $V1^R$ or PP $V1^R$ and RS $V1^R$ or ME $V1^R$ (Kruskall-Wallis test $P < 0.0001$; SS $V1^R$ versus RS $V1^R$ $P < 0.0001$, SS $V1^R$ versus ME $V1^R$ $P < 0.0001$, SS $V1^R$ versus PP $V1^R$ $P = 0.846$, 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 $V1^R$ $P < 0.0001$). (C1) Histogram of log ddr (sum of ½Ad divided by pulse duration) for the whole $V1^R$ population at E12.5 (n= 164; bin width 0.2). The histogram was fitted by the sum of two Gaussian curves with means and SDs of -2.51 & -0.851, and 0.2 & 0.46, respectively. (C2) Histograms of the values of log ddr sorted after cluster analysis showing SS $V1^R$ (black), RS $V1^R$ (red), ME_s $V1^R$ (light purple), ME_l $V1^R$ (dark purple) and PP $V1^R$. log (ddr) was not significantly different between ME $V1^R$ and PP $V1^R$, while it was significantly different between SS $V1^R$ and RS $V1^R$, SS $V1^R$ and the whole ME $V1^R$ population, SS $V1^R$ and PP $V1^R$, RS $V1^R$ and the whole ME $V1^R$ population, RS $V1^R$ and PP $V1^R$ (Kruskall-Wallis test $P < 0.0001$; 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 $V1^R$, $P < 0.0001$; RS $V1^R$ versus ME $V1^R$, $P < 0.0001$; RS $V1^R$ versus PP $V1^R$, $P < 0.0001$; ME $V1^R$ versus PP $V1^R$, $P = 0.977$). ME_s $V1^R$ and ME_l $V1^R$ differed only by their CV ½Ad (Mann-Whitney test, log mean ½Ad for ME_s $V1^R$ versus log mean ½Ad for ME_l $V1^R$, $P = 0.26$; CV 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 for ME_l $V1^R$, P = 0.1483). It is noteworthy that the distribution of the values of each metric was multimodal thus indicating that each of them could partially discriminate different groups of embryonic $V1^R$ according to their firing pattern.

S4 Fig. Relates to Fig 6. I_{Nap} is present in embryonic $V1^R$ recorded at E14.5

(A) Representative trace of I_{Nap} evoked by a slow depolarizing voltage ramp (70 mV/s, upper insert) in SS embryonic $V1^R$ (lower insert). I_{Nap} was isolated by subtracting currents evoked by depolarizing ramps in the presence of 1 μM TTX to the control current evoked in the absence of TTX (upper insert). (B) Voltage dependence of G_{Nap} conductance calculated from the trace shown in A. The activation curve was obtained by transforming the current evoked by a depolarizing voltage ramp from -100 mV to 20 mV (70 mV/s) using the following equation: $G_{NaP} = -I_{Nap}/((-Vh)+E_{Na})$ where Vh is the holding potential at time t during a depolarizing voltage ramp and E_{Na} is the equilibrium potential for sodium (E_{Na} = 60 mV). The G_{NaP}/Vh curve was fitted with the following Boltzmann function: $G = G_{MAX}/(1+\exp(-(V-V_{HALF})/k)))$ (Boeri et al. 2018), where V_{half} is the Vh value for G_{Nap} half activation, k the slope factor of the curve and G_{max} the maximum conductance. We found no significant difference between the values of V_{half} (Mann-Whitney test: P = 0.8518) and of k (Mann-Whitney test: P = 0.7546) obtained at E12.5 (Boeri et al. 2018) and those obtained at E14.5. At E14.5 V_{half} = -27 ± 5.1 mV and k = 7.73 ± 0.78 (n = 6).

S5 Fig. Relates to Fig 6. I_{Kdr} was inhibited by 4-AP in $V1^R$ recorded at E14.5

(A1) Representative examples of the total outward K^+ currents 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 in single spiking (SS) $V1^R$ at E14.5. (A2) Current-voltage relationship of I_{Kdr} (filled circle) and of I_A (open circle) in SS $V1^R$ at E14.5. $I - V$ curves were obtained from currents shown in A1.

(B1) Representative example of the effect of 4-AP at 600 μM in $V1^R$ at E14.5. (B2) Current-voltage curves in control condition and in the presence of 600 μM 4-AP. (B3) Bar plots showing the percentage of I_{Kdr} inhibition evoked by 300 μM 4-AP application (n = 8) and by 600 μM 4-AP application (n = 7). The percentages of I_{Kdr} inhibition evoked by 300 μM 4-AP and by 600 μM 4-AP applications were not significantly different (P = 0.574). (C1) Representative example of the effect of 600 μM 4-AP on I_A in $V1^R$ recorded at E14.5. (C2) $I - V$ curves in control conditions and in the presence of 600 μM 4-AP. These curves were obtained from the traces shown in B1. (C3) Bar graph showing the percentage of I_A block elicited by 4-AP. 4-AP did not significantly block I_A (Wilcoxon test P = 0.11, n = 6).

S6 Fig. Relates to Fig7. Effects of I_A on embryonic $V1^R$ firing patterns predicted by computational modeling

(A) The maximal conductances of I_{Kdr} and I_A at E12.5 are linearly correlated. Best fit: G_A = 1.09 G_{Kdr} (R^2 = 0.81, N=44). (B) Effect of I_A on the dynamics of the basic model. The one-parameter bifurcation diagrams in control condition (black, I = 20 pA, G_{Kdr} = 10 nS, no I_A, same as in Fig 7B) and with I_A added (orange, G_A = 10 nS) are superimposed. The I_A current shifts the firing threshold SN_1 to the right by 0.18 nS (see also C) as indicated by the orange arrow, with little effect on the amplitude of action potentials (see also insert in C). In contrast, I_A shifts SN_2 by only 0.03 nS because it is inactivated by depolarization. (C) I_A also slows down the discharge frequency, as shown by comparing the $G_{Nap} - V$ curves without I_A (black) and with I_A (orange). For G_{Nap} = 1 nS, for instance, the firing frequency is reduced by 31%, from 15 to 10.4 Hz. Here again, the effect of I_A progressively decreases as G_{Nap} increases because of the membrane depolarization elicited by I_{Nap}. For G_{Nap} = 2.4 nS, for instance, the firing frequency is reduced by 11% only, from 19.1 to 17 Hz. This frequency reduction elicited by I_A does not merely result from the increased firing threshold. Note also

that the latency of the first spike is increased (see voltage trace in insert), which is a classical effect of I_A. (D) I_A reduces the frequency of pseudo-plateau bursting by lengthening quiescent episodes (doubling their duration in the example shown) without affecting the duration of plateaus much (here a mere 5% increase), as shown by the comparison of the voltage traces obtained without I_A (control, $G_{Kdr} = 2.5$ nS, black) and with I_A ($G_{Kdr} = G_A = 2.5$ nS, orange). This is because I_A is activated near rest but inactivated during voltage plateaus. Note that increasing G_{Kdr}, in the absence of I_A has not the same effect; it shortens both plateaus and quiescent episodes (see Fig 8C, where $G_{Kdr} = 5$ nS). Again, this is because I_{Kdr} does not inactivate (or does it only very slowly), in contrast to I_A.

S7 Fig. Relates to Fig7. Explaining the effect of 4-AP on the firing pattern

The RS region of the basic model, where repetitive firing may occur, is displayed in the G_{Nap} – G_{Kdr} plane in control condition for E12.5 V1^R (C_{in} = 13 pF, G_{in} = 1 nS, I = 20 pA, shaded area), and when G_{in} and I were both reduced by 25% (middle curve) or by 50% (left curve). The reduced I accounts for the decrease in rheobase, and thus in the current injected in the experiments, following the decrease in G_{in}. If 4-AP reduced only G_{Kdr} (as indicated by the downward arrow) the firing pattern of SS V1^R would not change, the RS region being too far to the right to be visited. In contrast, when the effects of 4-AP on the input conductance and rheobase are taken into account, the bifurcation diagram moves leftwards and downwards, as indicated by the oblique black arrow, and the RS and PP regions are then successively entered as G_{Kdr} is reduced. The same explanation holds at E14.5.

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Table 1

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	

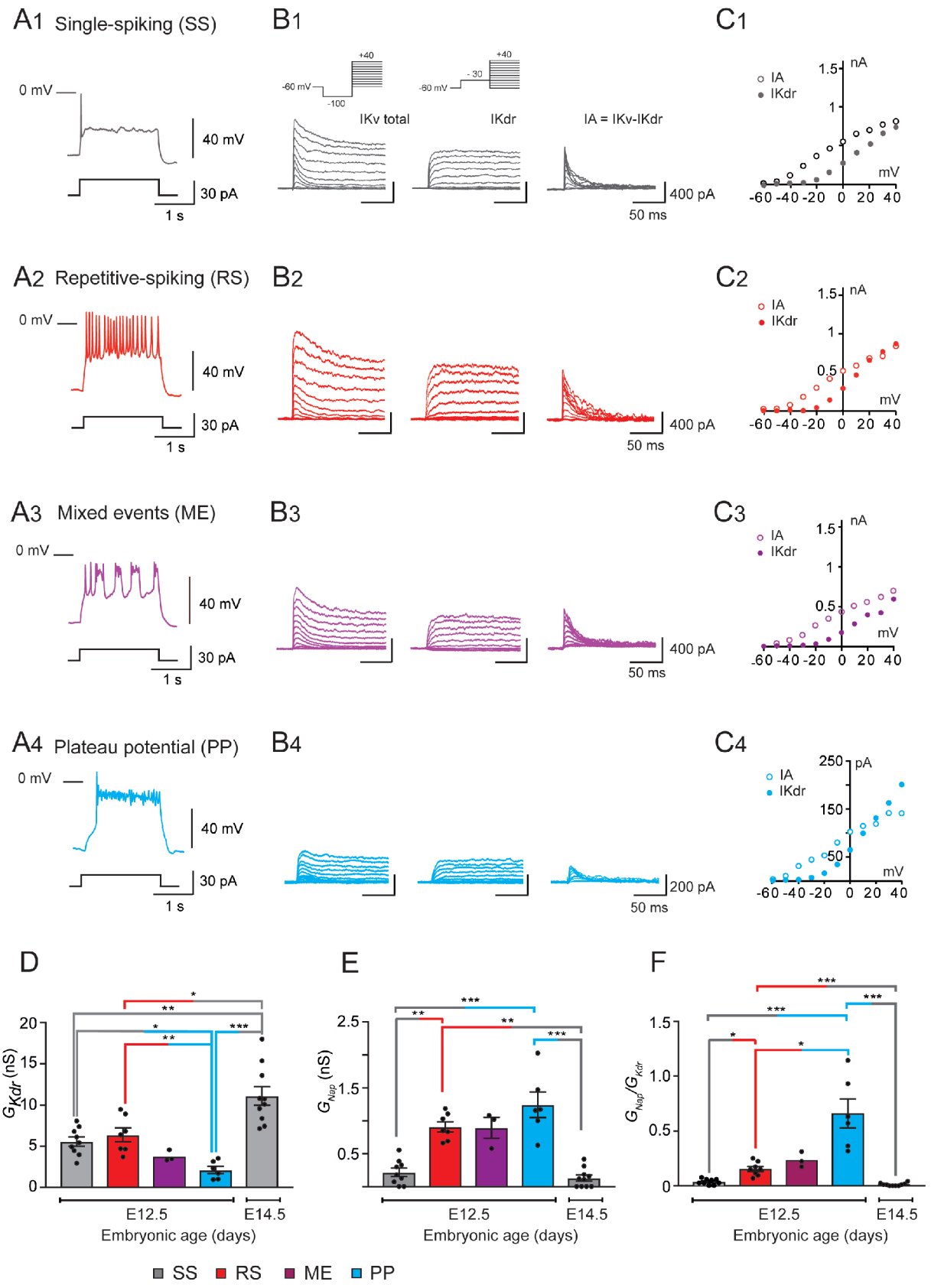

A1 Single-spiking (SS)
B1
C1
0 mV
40 mV
30 pA
1 s
+40
-60 mV
-100
-80 mV
- 30
IKv total
IKdr
IA = IKv-IKdr
400 pA
50 ms
IA
IKdr
nA
mV
A2 Repetitive-spiking (RS)
B2
C2
A3 Mixed events (ME)
B3
C3
A4 Plateau potential (PP)
B4
C4
200 pA
pA
D
E
F
G_{Kdr} (nS)
G_{Nap} (nS)
G_{Nap}/G_{Kdr}
E12.5
E14.5
Embryonic age (days)
SS
RS
ME
PP

Fig 1

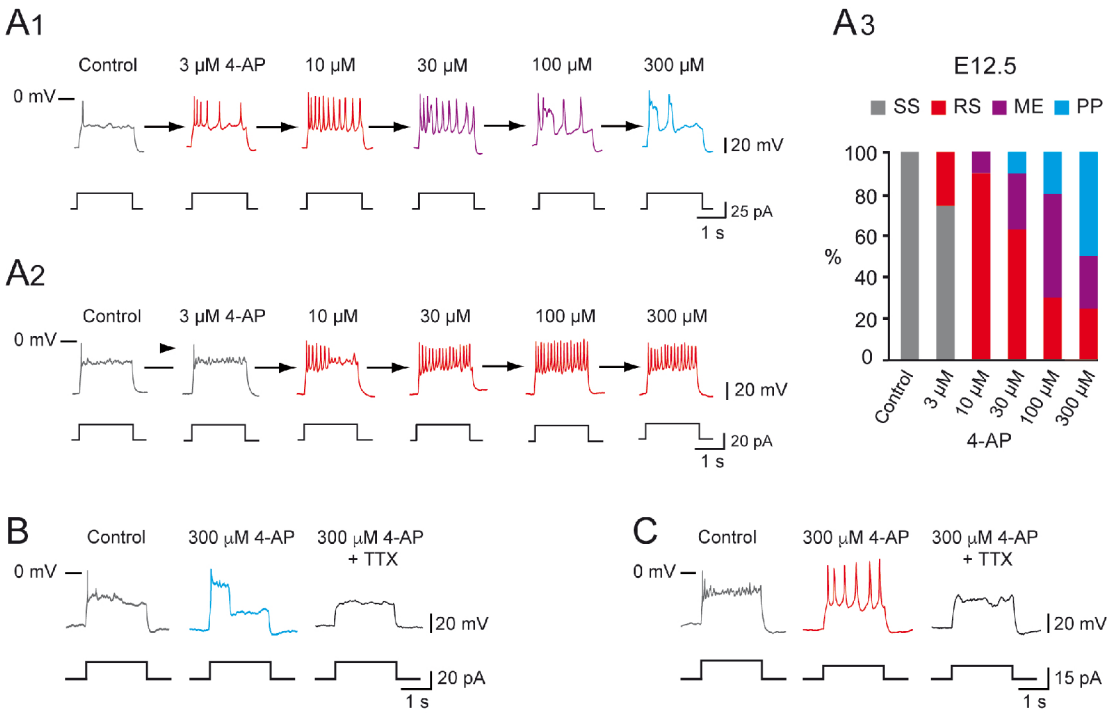

A1
Control
3 μM 4-AP
10 μM
30 μM
100 μM
300 μM
0 mV
20 mV
25 pA
1 s
A2
Control
3 μM 4-AP
10 μM
30 μM
100 μM
300 μM
0 mV
20 mV
20 pA
1 s
A3
E12.5
SS
RS
ME
PP
100
80
60
40
20
0
%
Control
3 μM
10 μM
30 μM
100 μM
300 μM
4-AP
B
Control
300 μM 4-AP
300 μM 4-AP
+ TTX
0 mV
20 mV
20 pA
1 s
C
Control
300 μM 4-AP
300 μM 4-AP
+ TTX
0 mV
20 mV
15 pA
1 s

Fig 2

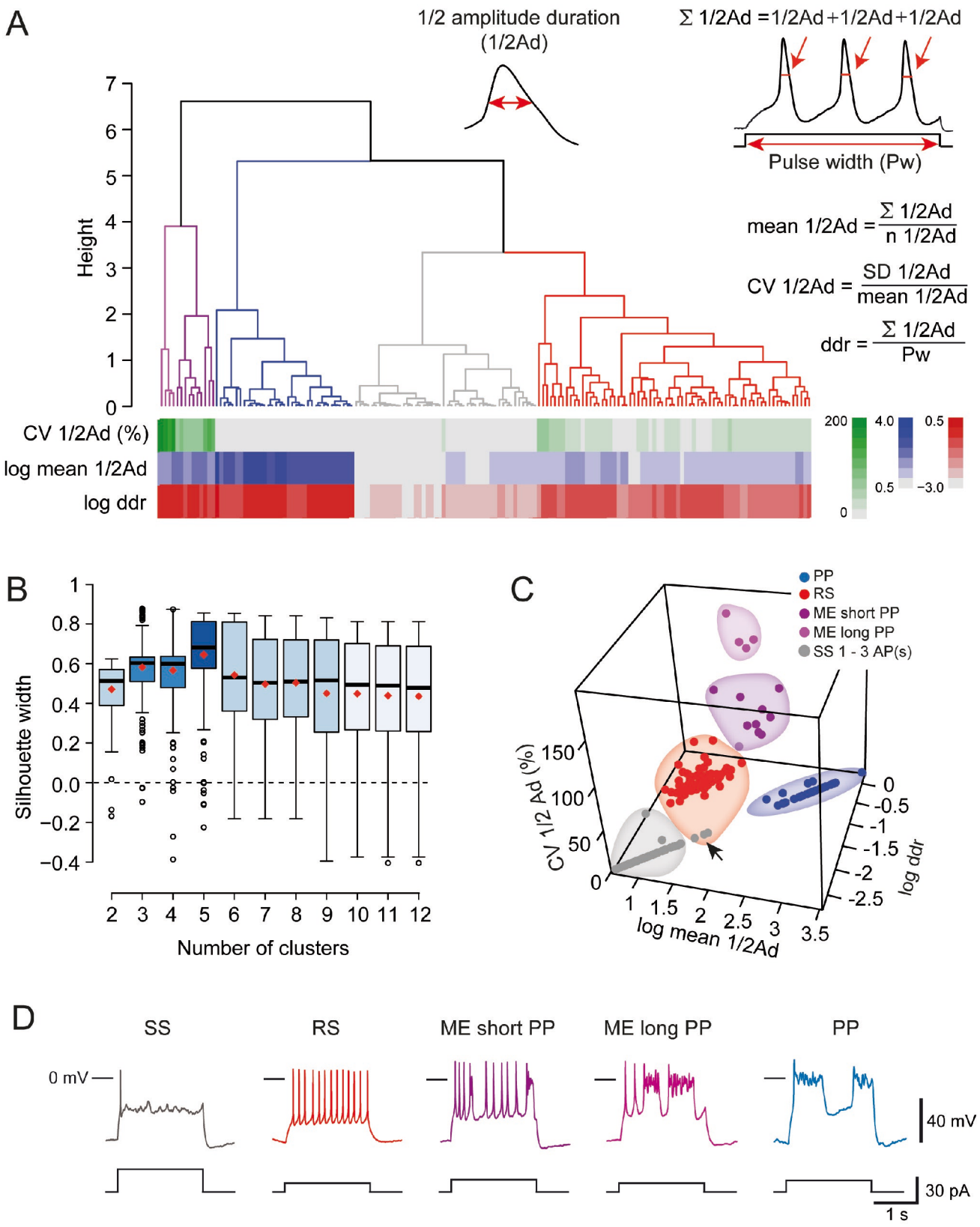

A
1/2 amplitude duration
(1/2Ad)
Σ 1/2Ad =1/2Ad+1/2Ad+1/2Ad
Pulse width (Pw)
mean 1/2Ad = Σ 1/2Ad / n 1/2Ad
CV 1/2Ad = SD 1/2Ad / mean 1/2Ad
ddr = Σ 1/2Ad / Pw
Height
CV 1/2Ad (%)
log mean 1/2Ad
log ddr
B
Silhouette width
Number of clusters
C
PP
RS
ME short PP
ME long PP
SS 1 - 3 AP(s)
CV 1/2 Ad (%)
log mean 1/2Ad
log ddr
D
SS
RS
ME short PP
ME long PP
PP
0 mV
40 mV
30 pA
1 s

Fig 3

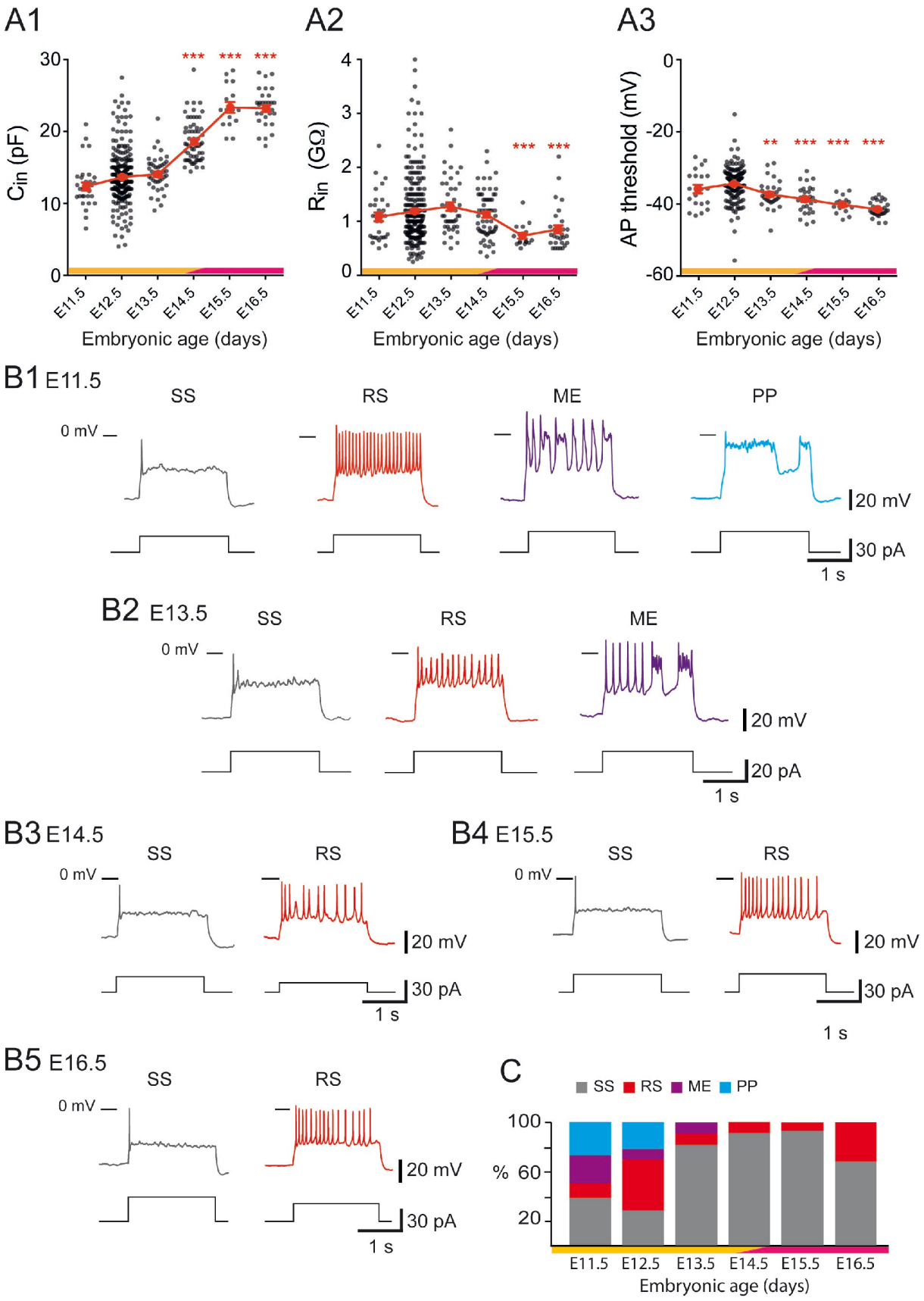

A1
A2
A3
Cin (pF)
Rin (GΩ)
AP threshold (mV)
Embryonic age (days)
E11.5
E12.5
E13.5
E14.5
E15.5
E16.5
B1 E11.5
B2 E13.5
B3 E14.5
B4 E15.5
B5 E16.5
SS
RS
ME
PP
0 mV
20 mV
30 pA
20 pA
1 s
C
%

Fig 4

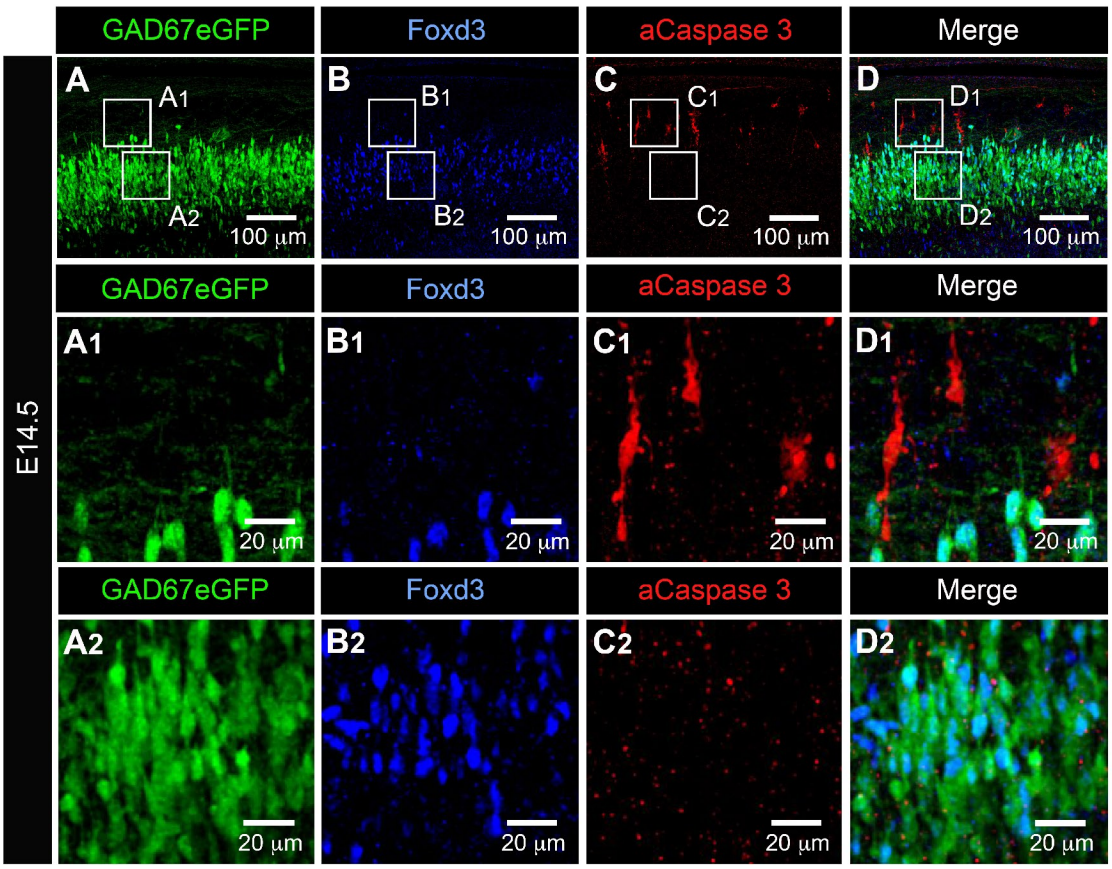

GAD67eGFP
Foxd3
aCaspase 3
Merge
E14.5
A
A1
A2
100 μm
B
B1
B2
100 μm
C
C1
C2
100 μm
D
D1
D2
100 μm
A1
B1
C1
D1
20 μm
A2
B2
C2
D2
20 μm

Fig 5

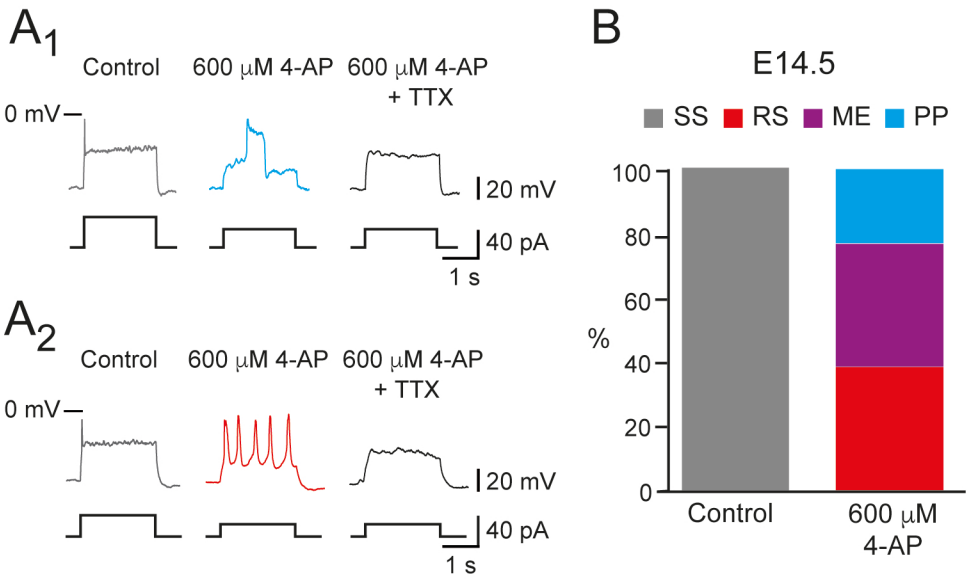

A1
Control
600 μM 4-AP
600 μM 4-AP
+ TTX
0 mV
20 mV
40 pA
1 s
A2
Control
600 μM 4-AP
600 μM 4-AP
+ TTX
0 mV
20 mV
40 pA
1 s
B
E14.5
SS
RS
ME
PP
100
80
60
40
20
0
%
Control
600 μM
4-AP

Fig 6

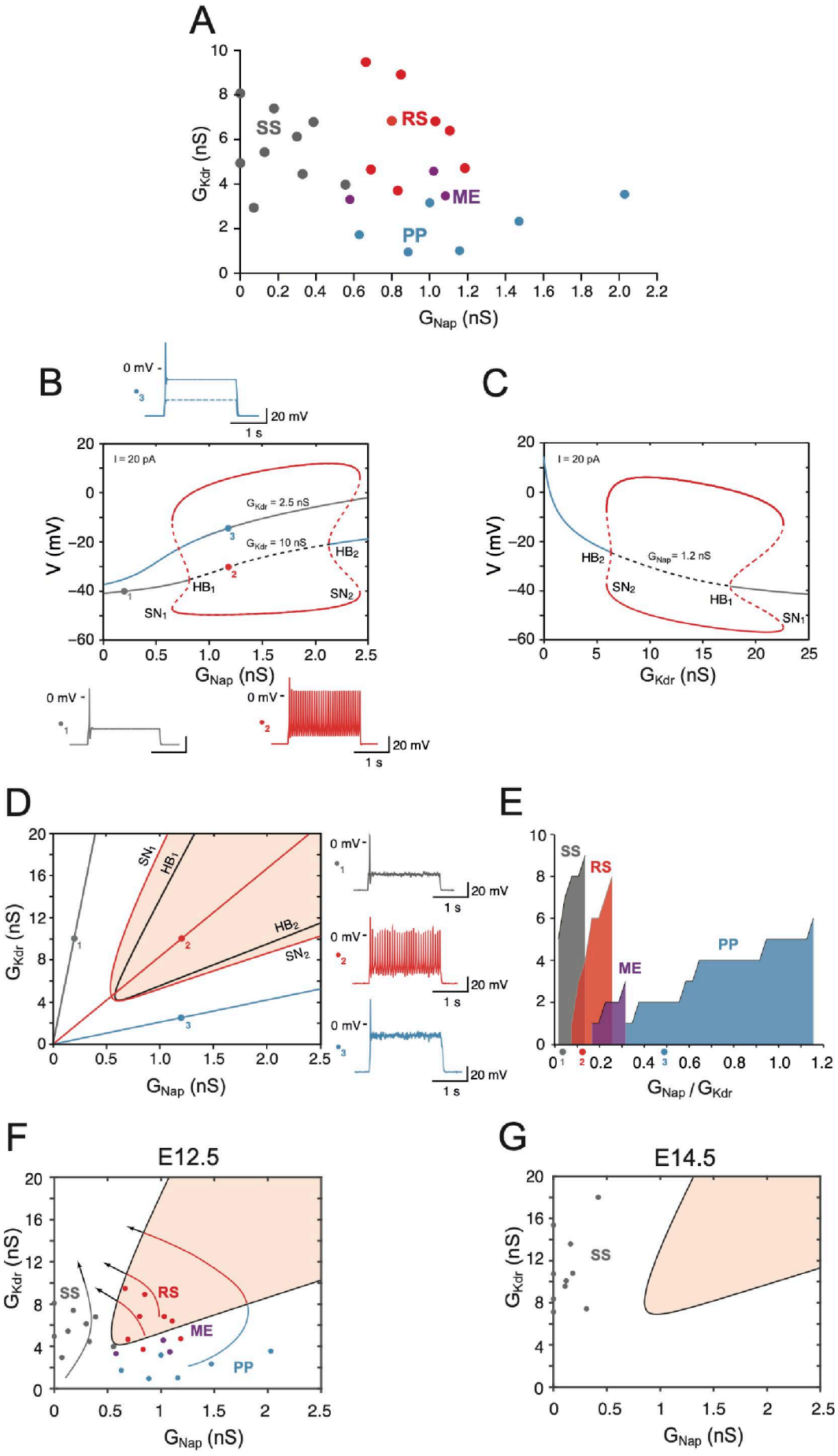

A
SS
RS
ME
PP
G_{Kdr} (nS)
G_{Nap} (nS)
B
0 mV
20 mV
1 s
I = 20 pA
G_{Kdr} = 2.5 nS
G_{Kdr} = 10 nS
HB1
HB2
SN1
SN2
V (mV)
G_{Nap} (nS)
C
I = 20 pA
G_{Nap} = 1.2 nS
HB2
SN2
HB1
SN1
V (mV)
G_{Kdr} (nS)
D
SN1
HB1
HB2
SN2
G_{Kdr} (nS)
G_{Nap} (nS)
E
SS
RS
ME
PP
G_{Nap} / G_{Kdr}
F
E12.5
SS
RS
ME
PP
G_{Kdr} (nS)
G_{Nap} (nS)
G
E14.5
SS
G_{Kdr} (nS)
G_{Nap} (nS)

Fig 7

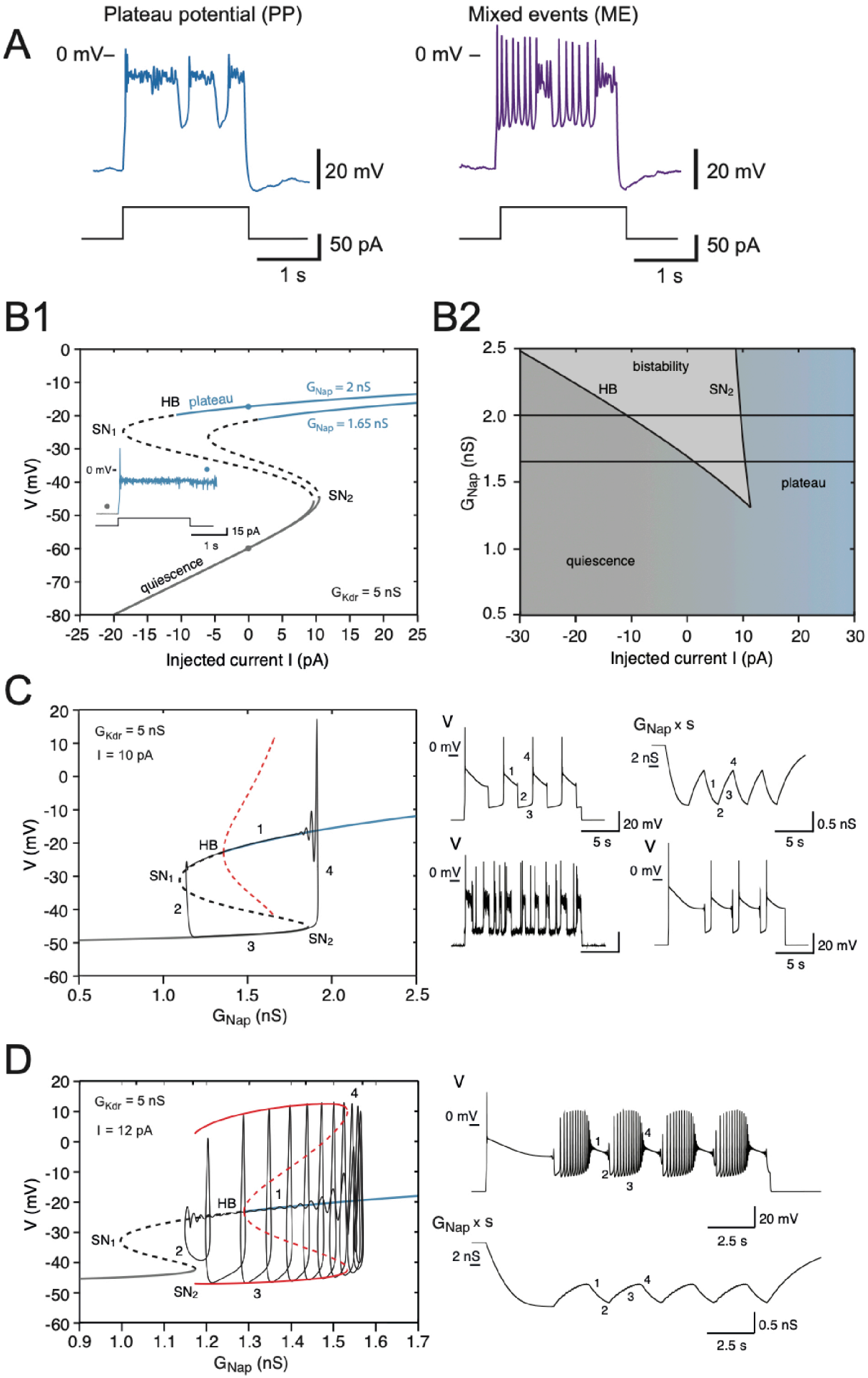
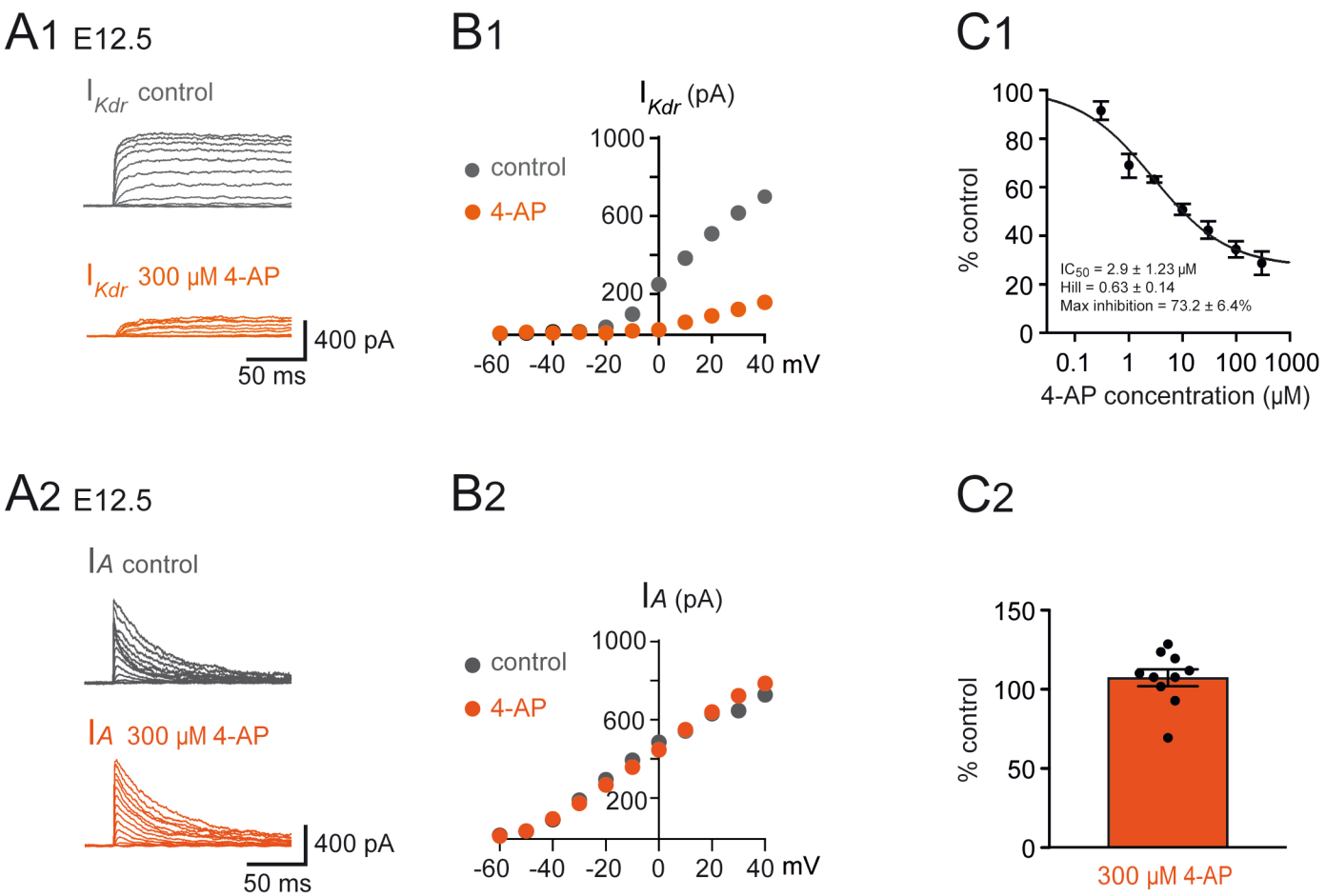
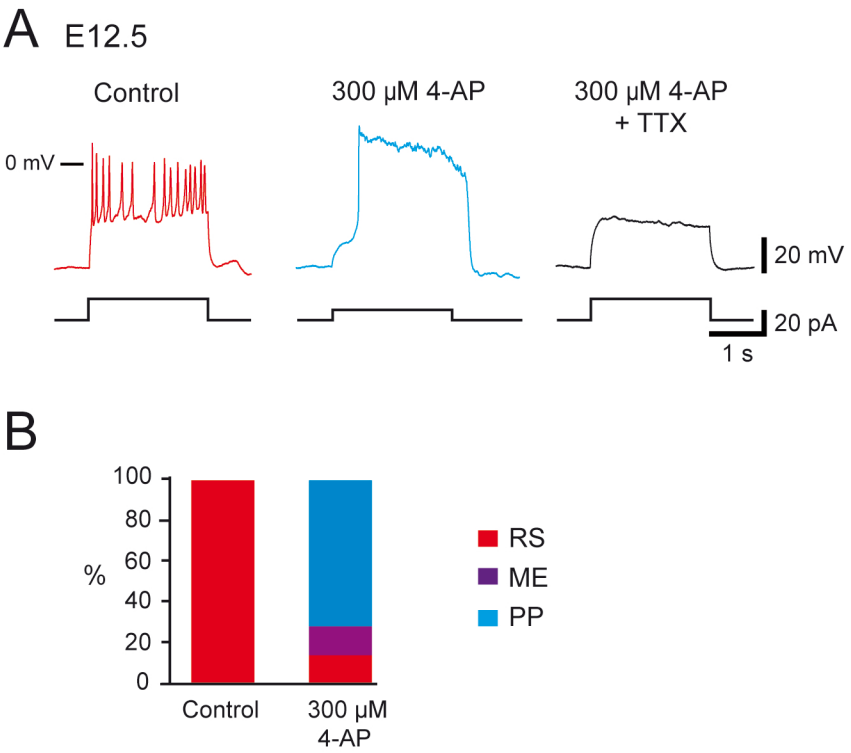

A
Plateau potential (PP)
Mixed events (ME)
0 mV
20 mV
50 pA
1 s
B1
B2
HB
plateau
SN1
SN2
quiescence
GNap = 2 nS
GNap = 1.65 nS
GKdr = 5 nS
15 pA
V (mV)
Injected current I (pA)
bistability
GNap (nS)
C
GKdr = 5 nS
I = 10 pA
GNap x s
2 nS
0.5 nS
5 s
20 mV
D
I = 12 pA
2.5 s

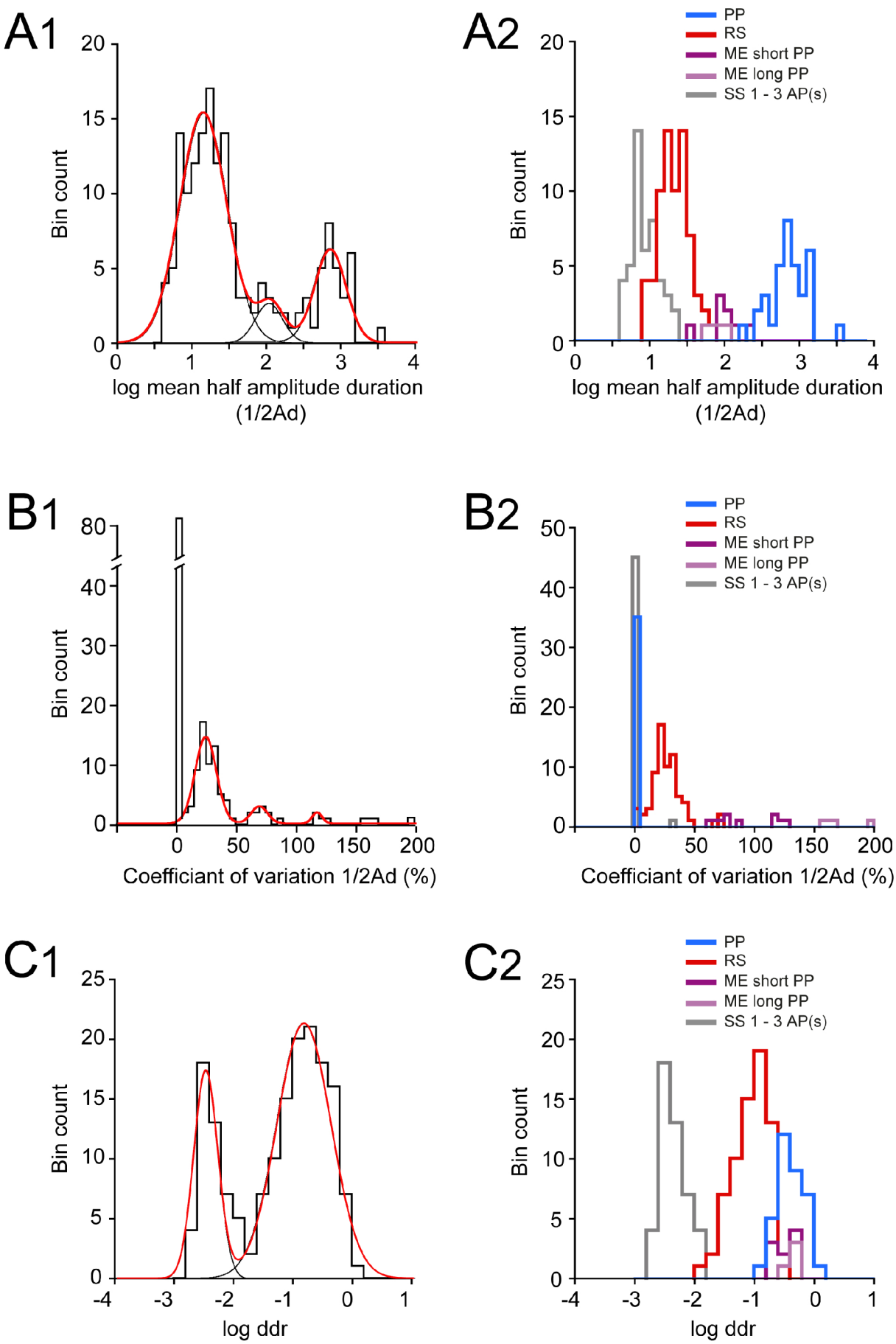
Fig 8


A1 E12.5
I Kdr control
I Kdr 300 μM 4-AP
400 pA
50 ms
B1
I Kdr (pA)
control
4-AP
1000
600
200
-60 -40 -20 0 20 40 mV
C1
% control
100
80
60
40
20
0
IC50 = 2.9 ± 1.23 μM
Hill = 0.63 ± 0.14
Max inhibition = 73.2 ± 6.4%
0.1 1 10 100 1000
4-AP concentration (μM)
A2 E12.5
IA control
IA 300 μM 4-AP
400 pA
50 ms
B2
IA (pA)
control
4-AP
1000
600
200
-60 -40 -20 0 20 40 mV
C2
% control
150
100
50
0
300 μM 4-AP

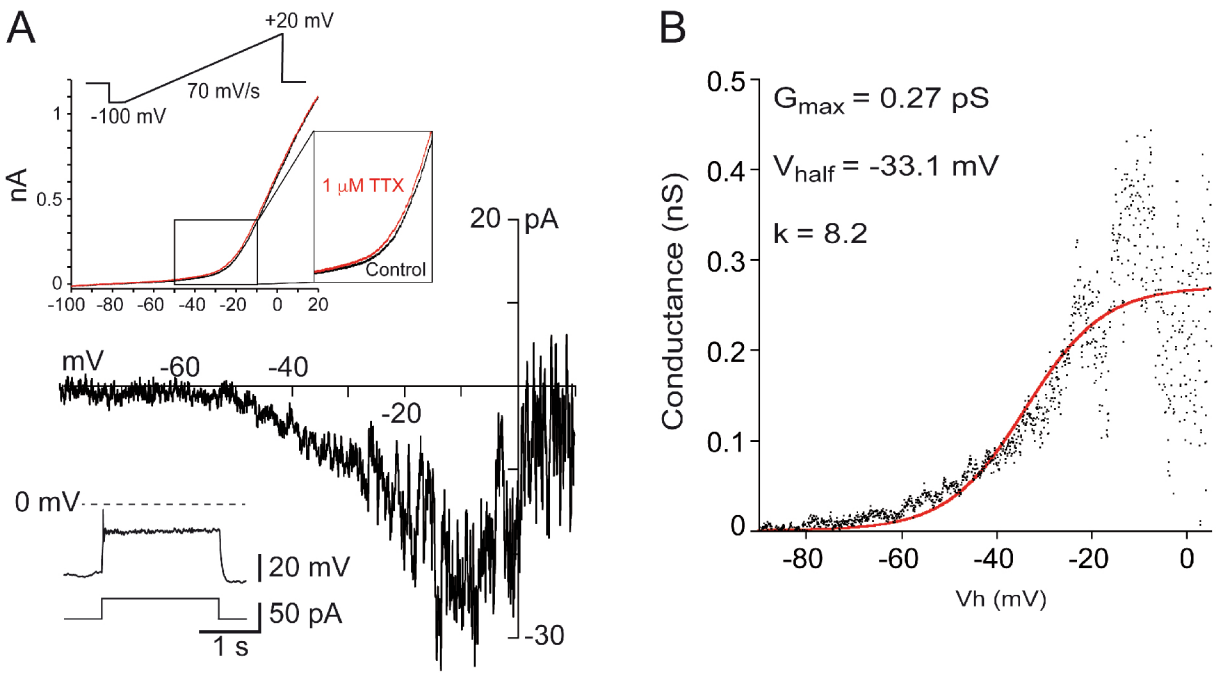
S1 Fig


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

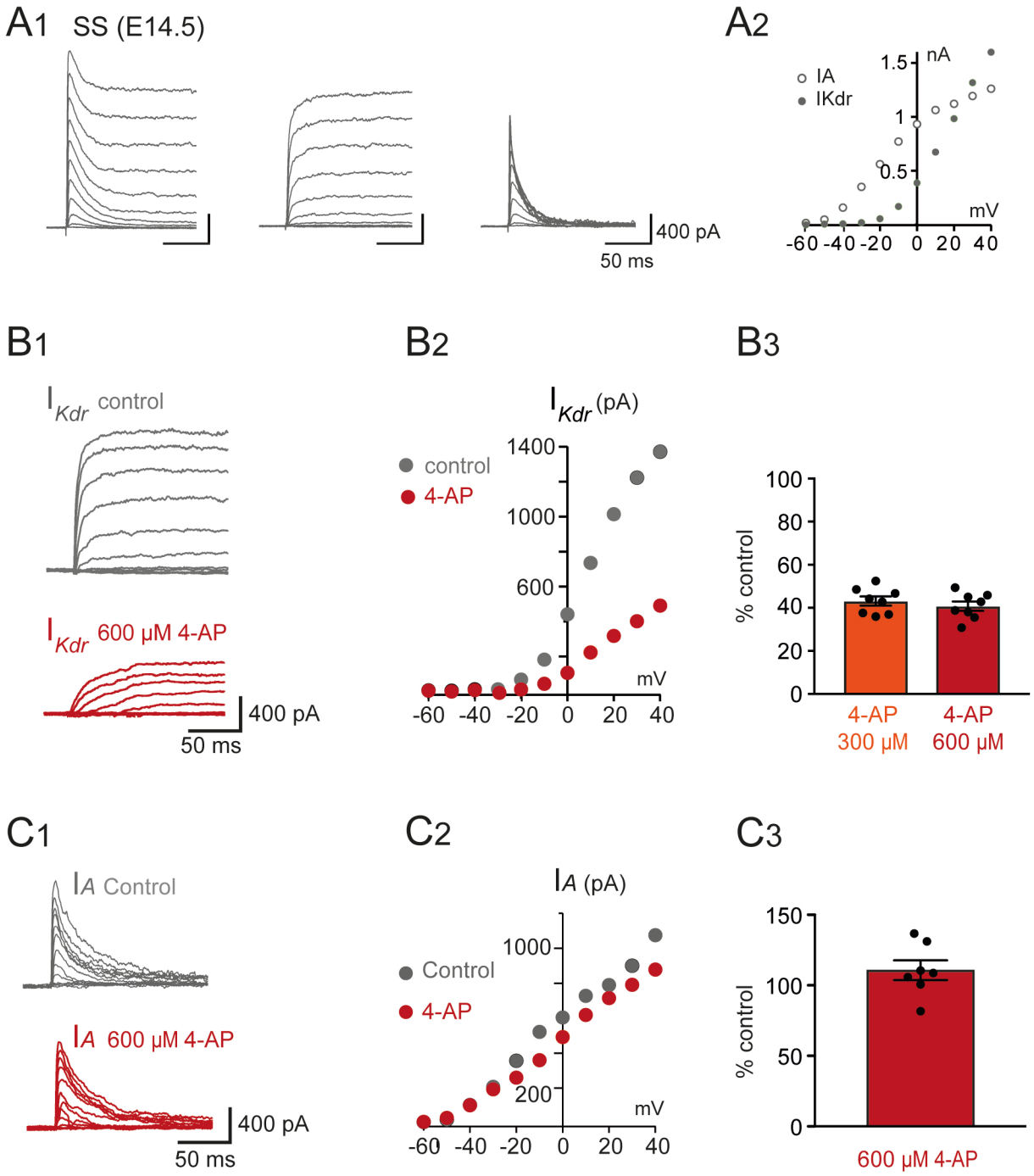
S2 Fig


A1
Bin count
log mean half amplitude duration
(1/2Ad)
A2
PP
RS
ME short PP
ME long PP
SS 1 - 3 AP(s)
Bin count
log mean half amplitude duration
(1/2Ad)
B1
Bin count
Coefficiant of variation 1/2Ad (%)
B2
PP
RS
ME short PP
ME long PP
SS 1 - 3 AP(s)
Bin count
Coefficiant of variation 1/2Ad (%)
C1
Bin count
log ddr
C2
PP
RS
ME short PP
ME long PP
SS 1 - 3 AP(s)
Bin count
log ddr

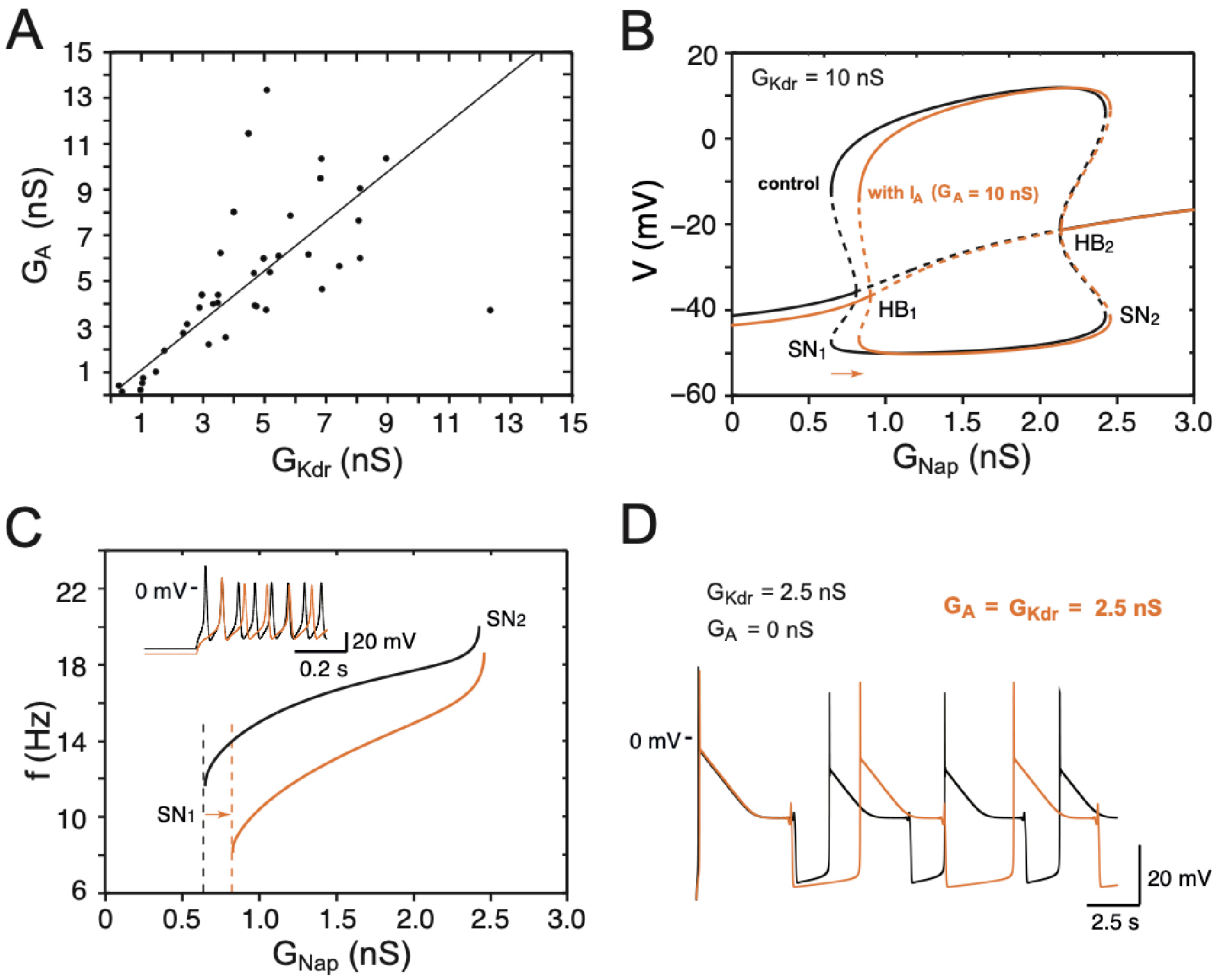
S3 Fig


A
+20 mV
70 mV/s
-100 mV
nA
1
0.5
0
-100 -80 -60 -40 -20 0 20
1 μM TTX
Control
20 pA
mV
-60
-40
-20
0 mV
20 mV
50 pA
1 s
-30
B
G_{max} = 0.27 pS
V_{half} = -33.1 mV
k = 8.2
Conductance (nS)
0.5
0.4
0.3
0.2
0.1
0
-80 -60 -40 -20 0
Vh (mV)

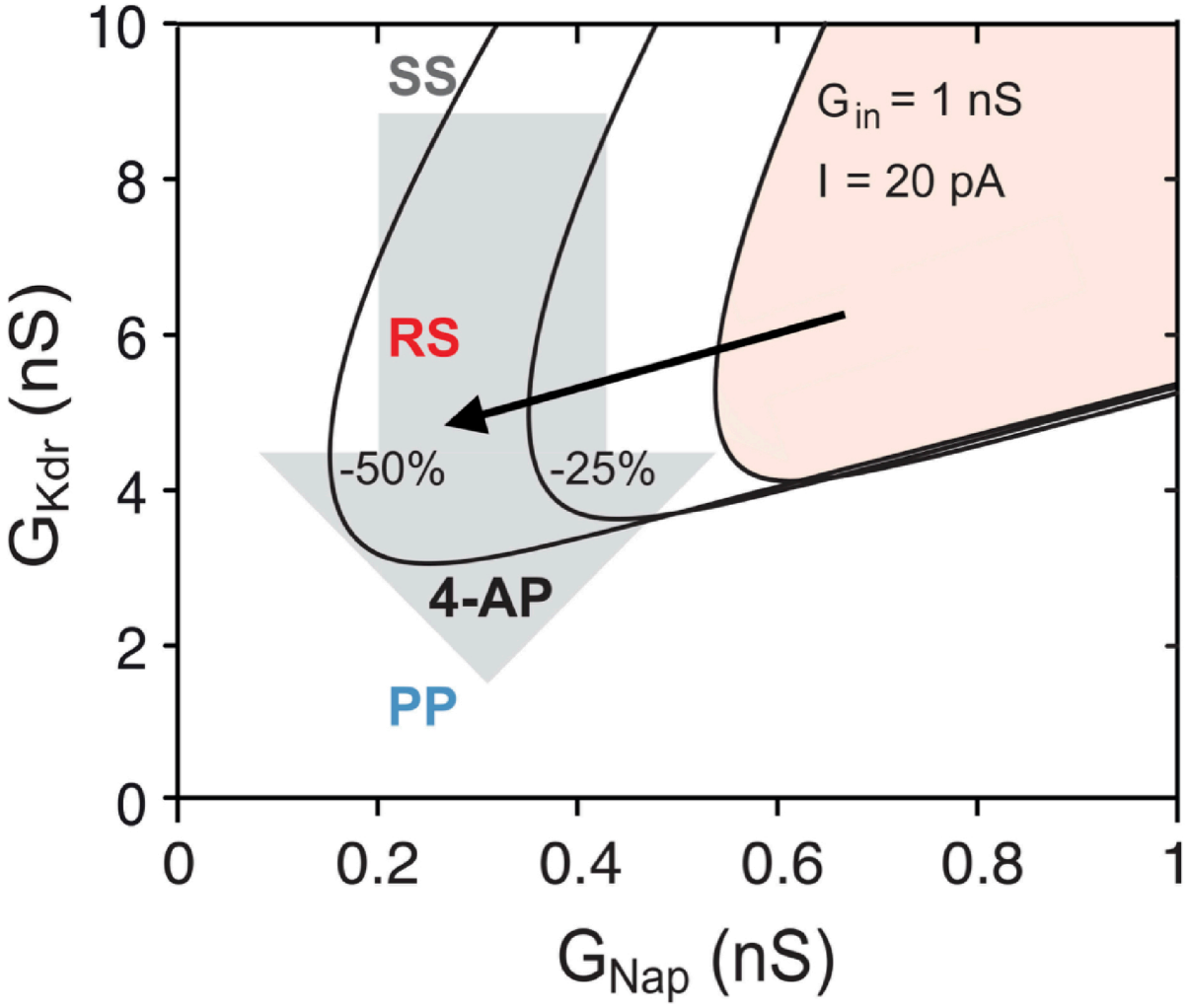
S4 Fig


A1
SS (E14.5)
400 pA
50 ms
A2
IA
IKdr
1.5
nA
1
0.5
mV
-60 -40 -20 0 20 40
B1
IKdr control
IKdr 600 μM 4-AP
400 pA
50 ms
B2
IKdr (pA)
control
4-AP
1400
1000
600
mV
-60 -40 -20 0 20 40
B3
% control
100
80
60
40
20
0
4-AP 300 μM
4-AP 600 μM
C1
IA Control
IA 600 μM 4-AP
400 pA
50 ms
C2
IA (pA)
Control
4-AP
1000
200
mV
-60 -40 -20 0 20 40
C3
% control
150
100
50
0
600 μM 4-AP

S5 Fig


A
B
C
D
G_A (nS)
G_{Kdr} (nS)
G_{Kdr} = 10 nS
control
with I_A (G_A = 10 nS)
HB1
HB2
SN1
SN2
V (mV)
G_{Nap} (nS)
0 mV
20 mV
0.2 s
f (Hz)
G_{Kdr} = 2.5 nS
G_A = 0 nS
G_A = G_{Kdr} = 2.5 nS
2.5 s

S6 Fig


SS
RS
PP
4-AP
-50%
-25%
G in = 1 nS
I = 20 pA
G Kdr (nS)
G Nap (nS)
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8
1

S7 Fig