Persistent Sodium Current Drives Excitability of Immature Renshaw Cells in Early Embryonic Spinal Networks

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Persistent sodium current drives excitability of immature Renshaw cells in early embryonic spinal networks

Abbreviated title: I_{Nap} drives the excitability of newborn $V1^R$

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ABSTRACT

Spontaneous network activity (SNA) emerges in the spinal cord (SC) before the formation of peripheral sensory inputs and central descending inputs. SNA is characterized by recurrent giant depolarizing potentials (GDPs). Because GDPs in motoneurons (MNs) are mainly evoked by prolonged release of GABA, they likely necessitate sustained firing of interneurons. To address this issue we analyzed, as a model, embryonic Renshaw cell ($V1^R$) activity at the onset of SNA (E12.5) in the embryonic mouse SC (both sexes). $V1^R$ are one of the interneurons known to contact MNs, which are generated early in the embryonic SC. Here, we show that $V1^R$ already produce GABA in E12.5 embryo, and that $V1^R$ make synaptic-like contacts with MNs and have putative extrasynaptic release sites, while paracrine release of GABA occurs at this developmental stage. In addition, we discovered that $V1^R$ are spontaneously active during SNA and can already generate several intrinsic activity patterns including repetitive-spiking and sodium-dependent plateau potential that rely on the presence of persistent sodium currents (I_{Nap}). This is the first demonstration that I_{Nap} is present in the embryonic SC and that this current can control intrinsic activation properties of newborn interneurons in the SC of mammalian embryos. Finally, we found that 5 μM riluzole, which is known to block I_{NaP}, altered SNA by reducing episode duration and increasing inter-episode interval. Because SNA is essential for neuronal maturation, axon pathfinding and synaptogenesis, the presence of I_{NaP} in embryonic SC neurons may play a role in the early development of mammalian locomotor networks.

64 **SIGNIFICANCE STATEMENT**

65 The developing spinal cord (SC) exhibits spontaneous network activity (SNA) involved in the
66 building of nascent locomotor circuits in the embryo. Many studies suggest that SNA depends
67 on the rhythmic release of GABA, yet intracellular recordings of GABAergic neurons have
68 never been performed at the onset of SNA in the SC. We first discovered that embryonic
69 Renshaw cells ($V1^R$) are GABAergic at E12.5 and spontaneously active during SNA. We
70 uncover a new role for persistent sodium currents (I_{NaP}) in driving plateau potential in $V1^R$
71 and in SNA patterning in the embryonic SC. Our study thus sheds light on a role for I_{NaP} in
72 the excitability of $V1^R$ and the developing SC.
73

INTRODUCTION

A remarkable feature of the developing central nervous system (CNS) is its capacity to generate spontaneous network activity (SNA) at the end of neuronal migration. SNA occurs in the absence of any external inputs and is not experience-driven or use-dependent (Moody, 1998; Feller, 1999). SNA has been described as playing an essential role in several areas of the developing CNS, including the neocortex, the thalamus, the hippocampus, the locus coeruleus, the retina and the spinal cord (Landmesser and O'Donovan, 1984; Ben-Ari et al., 1989; Garaschuk et al., 1998; Feller, 1999; Garaschuk et al., 2000; Gust et al., 2003; Corlew et al., 2004; Marder and Rehm, 2005; Myers et al., 2005; Gonzalez-Islas and Wenner, 2006; Hanson et al., 2008; Rockhill et al., 2009; Watt et al., 2009). SNA regulates the development of neural circuits by influencing synaptogenesis, neuronal maturation, axonal guidance and axonal pathfinding (Zhang and Poo, 2001; Hanson et al., 2008; Kirkby et al., 2013).

In the embryonic spinal cord (SC), SNA is characterized by long-lasting bursts of action potentials (APs) occurring every 2-4 min that can propagate along the cord (Hanson and Landmesser, 2003). SNA occurs at the onset of synaptogenesis at E12.5, before the emergence of functional neuromuscular junction and the formation of sensory and supra-spinal inputs (Hanson and Landmesser, 2003). Bursts of APs recorded on ventral roots (Hanson and Landmesser, 2003) or in the whole SC (Yvert et al., 2004) are long-lasting episodes characterizing SNA in the embryonic SC. We previously showed that most individual motoneurons (MNs) only generate a single AP during each episode of SNA (Czarnecki et al., 2014) and we demonstrated that acetylcholine (ACh) release could not directly synchronize MN firing during SNA as MNs do not express functional acetylcholine receptors (Czarnecki et al., 2014). Therefore, MNs must rely on other mechanisms to synchronize their firing during SNA. We have recently shown that MN activity occurring during SNA at the onset of synaptogenesis (E12.5) is generated by giant depolarizing potentials (GDPs) involving a massive and long-lasting release of GABA, as well as a

moderate release of glutamate and glycine (Czarnecki et al., 2014). It is therefore likely that newborn GABAergic interneurons (INs) play an essential role in the generation of the sustained episodes of depolarization necessary to synchronize MN firing during SNA.

To gain insight into the excitability pattern of spinal GABAergic INs involved in the release of GABA during SNA, we used multiple approaches to examine the intrinsic activation properties of immature Renshaw cells ($V1^R$) in the lumbar SC. Renshaw cells are known to regulate MN activity in the adult through recurrent synaptic inhibition (Eccles et al., 1956) and play an important role in the regulation of SC activity at late developmental stages in the chicken embryo (Wenner and O'Donovan, 2001). $V1^R$ are the first V1 INs to be generated during neurogenesis in the mouse embryo (Benito-Gonzalez and Alvarez, 2012). From E9.5 to E12.5, $V1^R$ migrate toward their final location between MN columns and the ventrolateral funiculus (Benito-Gonzalez and Alvarez, 2012; Alvarez et al., 2013). Because glycine, unlike GABA, is nearly absent in SC INs of E12.5 mouse embryo (Allain et al., 2004, 2006; Scain et al., 2010), $V1^R$ are likely to be GABAergic neurons at the early developmental stage.

Here, we show that, like MNs, $V1^R$ display GDPs during SNA at E12.5. However, we discovered that, unlike MNs, most $V1^R$ are able to produce repetitive spiking or sodium-dependent plateau potentials in response to GDPs (Czarnecki et al, 2014). Remarkably, these sustained discharges depend on the presence of a persistent sodium current (I_{Nap}). In addition, we also demonstrate that I_{Nap} already has important functions at the onset of SNA. Inhibition of I_{Nap} alters SNA episode duration and inter-episode interval duration, which reveals that the ability of embryonic SC neurons to generate sustained discharge is required for a correct SNA pattern (Hanson and Landmesser, 2006).

MATERIALS AND METHODS

Isolated spinal cord preparation

These 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) and recommendations from the CNRS. We used GAD67eGFP knock-in mice to visualize putative GABAergic INs (Tamamaki et al., 2003). Briefly, a cDNA encoding enhanced GFP (eGFP) was targeted to the locus encoding the gene Gad1. GAD67 is a rate-limiting enzyme of GABA biosynthesis and is known to be a marker for GABAergic neurons (Le Corronc et al. 2011). To obtain E12.5 GAD67-eGFP embryos, 8- to 12-week-old wild-type Swiss female mice were crossed with heterozygous GAD67-eGFP Swiss male mice. HB9-eGFP mouse embryos were used to visualize MNs (Wichterle et al., 2002). To obtain E12.5 transgenic HB9-eGFP embryos, 8- to 12-week-old wild-type Swiss female mice were crossed with heterozygous HB9-eGFP C57BL/6Jrj male mice.

269 embryos obtained from 152 pregnant mice were used. Isolated embryonic mouse SCs were obtained as previously described (Delpy et al., 2008; Scain et al., 2010). Briefly, pregnant mice were anesthetized by intramuscular injection of a mix of ketamine and xylazine and sacrificed using a lethal dose of CO_2. Embryos of either sex were removed and the SC was isolated from eGFP-positive embryos. Whole SCs were 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 mosmol/kg H_2O), continuously bubbled with a 95% O_2-5% CO_2 gas mixture.

Whole-cell recordings and analysis

The isolated SC was placed in a recording chamber and continuously perfused (2 mL/min) at room temperature (20-24°C) with the oxygenated ACSF described above. Whole-

cell patch-clamp recordings of lumbar spinal $V1^R$ and MNs were carried out under direct visualization using an infrared-sensitive CCD video camera.

In the SC of GAD67eGFP mouse embryos, eGFP neurons were detected using UV light. MNs were identified by their size, their location in the ventral area of the SC parenchyma (Czarnecki et al., 2014) and by the lack of eGFP expression. These neurons localized in this SC area express the MN transcription factors Islet1/2, as shown in a previous study (Scain et al, 2010). Recorded eGFP interneurons were localized in the ventrolateral area of the SC at the marginal zone between motor columns and the ventral funiculus, which is the known location of developing $V1^R$ (Stam et al., 2012). To confirm $V1^R$ identity, recorded cells were filled with neurobiotin (0.5-1 mg/mL) and stained with an antibody directed against Foxd3. Foxd3 is a specific transcription factor of V1 INs (Dottori et al., 2001; Stam et al., 2012) and the hallmark of $V1^R$ localized in the ventrolateral area of the embryonic SC (Carr et al., 1998; Benito-Gonzalez and Alvarez, 2012).

Whole-cell patch-clamp electrodes were pulled from thick-wall borosilicate glass using a Brown-Flaming puller (Sutter Instrument Co., USA). The tip of the electrode was fire-polished using a microforge (Narishige, Japan). Patch-clamp electrodes had resistances of 4-7 MΩ. The electrode was 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 mosmol/kg-H_2O). Using these potassium methanesulfonate solutions, the equilibrium potential for chloride ions (ECl) ≈ -30 mV was close to the physiological values measured at E12.5 on spinal MNs (Delpy et al., 2008). The junction potential (6.6 mV) was systematically corrected offline. In some voltage-clamp experiments, the electrode contained: (in mM) 130 CsCl, 4 $MgCl_2$, 4 Na_2ATP, 10 EGTA and 10 HEPES (pH 7.2; 290 mosmol/kg-H_2O).

Signals were recorded using Axopatch 200B or Multiclamp 700B amplifiers (Molecular Devices, USA). Data were low-pass filtered (2 kHz), digitized (20 kHz) online using a Digidata 1440A interface and acquired using PClamp 10.5 software (pClamp,

RRID:SCR_011323). Analyses were performed off-line using PClamp 10.5 software and Axograph X.1.6.4 (Axograph , RRID:SCR_014284).

In voltage-clamp or current-clamp experiments, neurons were recorded at a holding potential (Vh) of -60 mV. Series resistance (10-20 MΩ) was monitored throughout the experiments and was 50-80% compensated. Data were discarded if series resistance varied by more than ≈ 30% from the initial value. In current-clamp mode, $V1^R$ intrinsic discharge patterns were elicited using depolarizing current steps (from 0 to ≈ 50 pA, 2-10 pA increments depending on the input resistance of the cell, 2 seconds) or depolarizing current triangular-ramp (from 0 to ≈ 50 pA, 5 pA increments, 20 seconds) with an 8-second interval to ensure that the membrane potential returned to baseline Vh. In voltage-clamp mode, whole-cell currents were elicited by a depolarizing voltage ramp. The 70 mV/s speed was chosen to elicit and measure persistent inward currents (I_{Nap}) (Huang and Trussell, 2008). Subtraction of the current evoked by the voltage ramp in the presence of 1 µM TTX to control voltage ramp-evoked current revealed I_{Nap}.

Action potentials or plateau potentials were analyzed based on the following parameters during a 2-second current step: threshold potential, peak amplitude, half amplitude duration, rate of rise of events (by dividing the amplitude of the event by the duration from its onset to the peak; 2 seconds step width).

Boltzmann functions were used to describe I_{Nap} activation on current evoked by voltage ramp. For the fit current (20 kHz sampling 2 KHz filter):

$$G = G_{MAX}/(1+\exp(-(V-V_{HALF})/k))$$

where G is conductance in nS, G_{Max} is the maximal conductance, V is the potential in mV, V_{HALF} is the voltage for half-maximal activation in mV, and k is the slope factor in mV. Threshold for activation I_{Nap} was determined by eye from Boltzmann curves.

Extracellular recordings

In the same experimental conditions as for whole-cell recordings, spontaneous activity was recorded extracellularly from E12.5 SCs using glass electrodes. Extracellular electrodes were placed under visual control on the superficial part of the ventral horn both at the cervical and the lumbar levels. Targeted networks likely include MNs as well as surrounding INs (Czarnecki et al., 2014). Electrodes were connected to a high-gain a.c. amplifier (ISO-DAM8A-4 Bio-amplifier System, World Precision Instruments Ltd, Stevenage, UK). Filtered (Filter cutoff frequency: 0.3-3 kHz) raw signals were integrated off-line and analyzed using Spike2 software (Spike2 Software, RRID:SCR_000903). Changes in burst duration and maximum instantaneous frequency were calculated from cervical and lumbar recordings.

Pharmacological agents

During patch-clamp recordings, drugs were applied using 0.5 mm diameter quartz tubing positioned 50 µm away from the recording area under direct visual control. The quartz tubing was connected using a manifold to 6 solenoid valves linked with 6 reservoirs. Solutions were gravity-fed into the quartz tubing. Drug application was controlled using a VC-8 valve controller (Warner Instruments, USA). The following pharmacological agents were used: tetrodotoxin (TTX) (1 µM, Alomone, Israel) and riluzole (5-10 µM Tocris Bioscience, UK). Both were dissolved in the bath solution.

Immunohistochemistry

All primary antibodies used and their respective dilutions are listed in Table 1. E12.5 embryos were collected from pregnant females. Once dissected out of their yolk sac, these embryos were immediately immersion-fixed in phosphate buffer (PB 0.12 M) with 4% paraformaldehyde (PFA; freshly prepared in PB, pH 7.4) for 1 h at 4°C. Embryos were then

rinsed with PB and cryoprotected in PB-15% sucrose at 4°C for 24 h and then in PB-30% sucrose at 4°C for 24 h. Embryos were embedded in OCT medium (VWR, Fontenay-sous-Bois, France) and quickly frozen. Serial sections 20 µm thick were collected onto slides using a cryostat. Immunostaining was processed on SC transverse sections and on whole SC post-recording to confirm IN identity. To reveal neurobiotin-labeled cells, SCs were fixed for 1 h in 4% PFA and were then incubated in 0.12 M PB at 4°C until immunohistochemical studies. Tissues (embryo slices and whole SC) were 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 and then for 48 h at 4°C with the primary antibodies, which were diluted in the 0.2% Triton X-100 blocking solution. Slices or whole SCs were then washed in PBS and incubated for 2 h at RT in the secondary antibodies (diluted at 1/1000 in the 0.2% Triton X-100 blocking solution): Alexa Fluor 405 (Thermo Fisher Scientific Cat# A-31556, RRID:AB_221605) or 647 goat anti-rabbit (Thermo Fisher Scientific Cat# A-21244, RRID:AB_2535812); Alexa Fluor 594 goat anti-guinea pig (Thermo Fisher Scientific Cat# A-11076, RRID:AB_2534120); Alexa Fluor 649 donkey anti-guinea pig (Jackson ImmunoResearch Labs Cat# 706-605-148, RRID:AB_2340476); Alexa Fluor 594 goat anti-mouse (Thermo Fisher Scientific Cat# A-11005, RRID:AB_2534073); Alexa Fluor 488 goat anti-chicken (Thermo Fisher Scientific Cat# A-11039, RRID:AB_2534096) and streptavidin-conjugated Alexa Fluor 405 (1:1000, Thermo Fisher Scientific Cat# S32351). After washing in PBS, slides or whole SCs were dried and mounted in Mowiol medium (Millipore, Molsheim, France).

Confocal microscopy and image analysis

Preparations were analyzed 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 and a 4X digital

zoom magnification. 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 softwares (Wright Cell Imaging Facility, RRID:SCR_008488). Colocalization of synaptophysin staining with calbindin staining, eGFP staining (MNs; HB9eGFP mouse embryos) or neurobiotin staining was assessed in 3 axes using a single confocal slice and X and Y orthogonal views of the stack (ImageJ 1.5).

Sniffer recordings.

To make a "sniffer" electrode (Young and Poo, 1983) for GABA, an outside-out patch was pulled from a transfected HEK293 cell line expressing the $GABA_AR$ subunits $\alpha3\beta2\gamma2$ (kindly provided by Michel Partiseti, Sanofi Advantis R&D LGCR/LIT, France). This $GABA_AR$ is highly specific for GABA and is characterized by slow desensitization, which makes it a good sensor for GABA paracrine release (Barberis et al., 2007). As a control, the electrode was first positioned outside the SC to verify any GABA contamination in the recording medium. The electrode was then pushed inside the SC and positioned within the motoneuronal area and the ventral funiculi. Outside-out patch-clamp electrodes (5-10 MΩ) were pulled from thick-wall borosilicate glass, fire-polished and filled with (in mM): CsCl 130, $MgCl_2$ 4, Na_2ATP 4, EGTA 10, HEPES 10 (pH 7.2, osmolarity 290 mosmol/kg-H_2O). Single channel currents were recorded using an Axopatch 200B amplifier (Molecular Devices, USA). Recordings were filtered at 10 kHz, sampled at 50 kHz and stored on a PC computer. Membrane potential was held at -50 mV throughout the experiment. Analysis of the sniffer currents was performed using Axograph X.1.6.4 software.

Human embryonic kidney 293 cells (HEK293) were maintained in a 95% air - 5% CO_2 humidified incubator, at 35°C, in Dulbecco's modified Eagle's medium supplemented with 0.11 g/L sodium pyruvate, 6 g/L D-glucose, 10% (v/v) heat-inactivated fetal bovine serum (all from Gibco BRL). Cells were passaged every 5-6 days (up to maximum 20 times). For

electrophysiological recordings, cells were seeded onto glass coverslips coated with poly-L-ornithine (0.1 mg/mL).

Statistics

All values were expressed as mean ± standard deviation. Statistical significance was assessed by the non-parametric Kruskal-Wallis test with Dunn's post tests, Mann-Whitney test and Wilcoxon matched pairs test (GraphPad Prism 5.0 Software, USA). Significance was determined as $P < 0.05$ (*), $P < 0.01$ (**) or $P < 0.001$ (***).

RESULTS

$V1^R$ are identified with calbindin, Foxd3 and MafB at the onset of SNA.

In the present study, we used GAD67-enhanced green fluorescent protein (eGFP) knock-in mice (Tamamaki et al., 2003) to visualize putative GABAergic INs (Figures 1A1, 1B1 and 1C1). $V1^R$ can be unambiguously identified by their characteristic position in the ventral horn both in the adult (Geiman et al., 2000) and in the embryo (Sapir et al., 2004; Stam et al., 2012). Most $V1^R$ were localized between the ventral border of MN columns and the ventral funiculi. To identify $V1^R$ in the lumbar SC of E12.5 GAD67eGFP embryos, we first performed immunostaining using anti-calbindin, anti-Foxd3 and anti-MafB antibodies (Figure 1). Calbindin is a calcium-binding protein exclusively expressed by $V1^R$ when looking in the ventrolateral area of the SC between the MN columns and the funiculus (Stam et al., 2012). The forkhead transcription factor Foxd3 controls the early phase of $V1^R$ differentiation, whereas MafB is required to maintain $V1^R$ at later developmental stages, and both are therefore expressed at E12.5 in $V1^R$ (Stam et al., 2012). At E12.5 INs located in the ventrolateral marginal zone between the motor columns and the ventral funiculi express Foxd3 (Figures 1A2, 1B2 and 1C2) and calbindin (Figures 1A3 and 1B3), as previously described (Stam et al., 2012; Benito-Gonzalez et al, 2012). $Foxd3^+$ INs were also observed in neurons positioned dorsally to the motor columns (Figures 1A2 and 1B2). However, these INs were not stained by calbindin antibody, hence this population of INs was not included in our study. In the $V1^R$ area, double immunostaining (Figures 1A4 and 1B4) indicated that 81.7 ± 15.0% of $Foxd3^+$ INs localized in the ventral marginal zone were also immunoreactive to calbindin at E12.5 (n=4 embryos, 2 sections/embryo). To ensure that all $Foxd3^+$ INs localized in the area are $V1^R$, we performed double immunostaining using anti-Foxd3 and anti-MafB antibodies (Figures 1C). We found that 100% of $Foxd3^+$ $eGFP^+$ INs localized in the ventral marginal zone were also immunoreactive to anti-MafB antibody (n = 4 embryos, 2 sections/embryo), thus confirming their $V1^R$ fate (Figure 1C4). It should also be noted that at

this developmental stage we detected MafB^{+} eGFP^{-} neurons localized within the motor columns (Figures 1C1 and 1C3) that are likely MNs (Stam et al., 2012**)**. Therefore, we used Foxd3 immunostaining to confirm that the recorded IN belonged to the V1^{R} population (Figures 1D1, 1D2, 1D3 and 1D4). Whereas Calbindin is a good marker for V1^{R}, it was not used to identify recorded V1^{R} because of cytoplasm-calbindin dilution complications during whole-cell recording (Muller et al., 2005), caused by the high nucleus/cytoplasm volume ratio in embryonic neurons.

V1^{R} already produce GABA at E12.5.

As calbindin is localized within the cytoplasm of V1^{R}, we used calbindin immunostaining to visualize V1^{R} morphology (Stam et al., 2012). 96.6 ± 7.2% of calbindin^{+} neurons in the ventrolateral marginal zone were also eGFP^{+} in E12.5 GAD67eGFP knock-in mouse embryos (n = 4 embryos, 2 sections/embryo), which could suggest that most V1^{R} are likely to produce GABA at this developmental stage. To determine to what extent V1^{R} already produce GABA at E12.5, we performed double immunostaining using anti-calbindin and anti-GABA antibodies (Figures 2A and 2B). We found that 90.1 ± 10.5% (n = 8 embryos, 2 sections/embryo) of calbindin^{+} INs localized in the ventrolateral marginal zone (Figures 2A2 and 2B2) were also immunoreactive to GABA antibody (Figures 2A3, 2A4, 2B3 and 2B4), suggesting that V1^{R} already produced enough GABA to be detected by immunohistochemistry at E12.5.

It is important to note that many eGFP^{+} GABA^{-} INs were detected within the ventral parenchyma at E12.5, as previously observed (Allain et al., 2004). This could result from the production of the GAD67 splice variant GAD25. At early developmental stages, GAD1 first encodes the truncated 25-kDa leader (GAD25) without GAD enzymatic activity and then the enzymatically active protein GAD44 (see for review (Le-Corronc et al., 2011). These two GAD isoforms are down-regulated during neuronal differentiation concomitantly with up-

regulation of GAD67 expression.

Synaptic-like contacts are detected between $V1^R$ and MNs at the onset of SNA

At E12.5, $V1^R$ axons are localized in ventral funiculi and extend for a few segments without forming collateral branches entering the motor columns (Alvarez et al., 2013). The first evidence for collateral $V1^R$ axons innervating the soma of lumbar MNs was found at E15.5 only while synaptic-like connections from MNs onto the $V1^R$ cell body were already observed at E12.5 (Alvarez et al., 2013). Accordingly, it was proposed that MNs can control $V1^R$ activity at E12.5 while $V1^R$ are unable to control MN activity at this time point (Alvarez et al., 2013). However, it should be noted that synaptophysin staining was found mainly in the ventral funiculus at E12.5 (Figure 3A1) (Alvarez et al., 2013; Czarnecki et al., 2014), where IN axonal projections and MN dendrite-like processes were observed (Czarnecki et al., 2014). Accordingly, we hypothesized that $V1^R$ already make some synaptic-like contacts with MN projections within the ventral funiculus.

To address this issue we first performed multiple immunostaining using anti-synaptophysin antibody (putative release site), anti-calbindin antibody ($V1^R$) and anti-eGFP antibody on coronal sections of the SC HB9eGFP mouse embryos to visualize MNs (Figures 3A2, 3A3, 3A4, 3B1, 3B2 and 3B3). As shown in Figure 3B3, many calbindin$^+$ fibers were apposed to MN neurites (eGFP staining) within the ventral funiculus. We found 21.7 ± 8.4 synaptophysin$^+$ punctates within calbindin$^+$ fibers apposed to MN neurites per hemi section in coronal slices (n = 9; 5 embryos) (Figures 3B1, 3B2 and 3B3; inserts, arrow). Conversely, synaptophysin punctates were also found in MN fibers (eGFP$^+$) apposed to calbindin$^+$ fibers (Figures 3B1, 3B2 and 3B3; insert, arrow head), which indicate the presence of MN putative release sites apposed to $V1^R$ within the ventral funiculus. We also found synaptophysin punctates within calbindin$^+$ fibers that were not apposed to MN neurites (Figures 3B1, 3B2 and 3B3; barred arrow). This may indicate the presence of non-synaptic release sites and/or

that $V1^R$ can make contact with fibers of neurons other than MNs. To further confirm the presence of $V1^R$ synaptic-like contacts on MNs, we performed multiple immunostaining using anti-synaptophysin antibody (putative release site) and anti-eGFP antibody on isolated SC after patch-clamp injection of neurobiotin in $V1^R$ (Figures 3C). Recorded INs (eGFP negative) in eGFP HB9 embryos were identified as $V1^R$ using Foxd3 immunostaining (Figure 3C1; insert). We found 3 to 5 putative release sites per $V1^R$ (n = 4 cells) that were apposed to MN fibers within the ventral funiculus (Figures 3C1, 3C2, 3C3 and 3C4). We did not find any release site within the $V1^R$ axonal growth cone (data not shown).

Paracrine release of GABA occurred in the SC of E12.5 mouse embryos.

Paracrine release of GABA may occur from non-synaptic release sites and/or may reflect neurotransmitter spillover of GABA from immature synapses (Safiulina and Cherubini, 2009). Therefore, we hypothesized that GABA release can occur at this developmental stage. To detect the presence of GABA paracrine release, we used the sniffer technique (Scain et al., 2010). Outside-out patches from HEK cells expressing the $GABA_AR$ subunits $\alpha3\beta2\gamma2$ were used as a "sniffer" to detect the presence of GABA in the extracellular space (Figure 4 A1). When inserting the sniffer electrode in the vicinity of MNs, we did not detect any $GABA_AR$, thus indicating that basal GABA concentration is below the detection threshold of the sniffer patch (data not shown). The possibility of evoking GABA release, and hence $GABA_AR$ activation, by inducing a global cell membrane depolarization was tested using bath application of 30 mM KCl (Scain et al., 2010; Czarnecki et al., 2014). Increasing $[K^+]_o$ evoked a significant $GABA_AR$ activation in the sniffer patch (n = 9), indicating that GABA can be released in the extracellular space in response to cell membrane depolarization (Figure 4 A1). We estimated the concentration of released GABA around MNs by comparing the peak amplitude of the sniffer response evoked by KCl application to sniffer responses evoked by exogenous application of 3, 10 and 30 μM GABA onto the same outside-out patch

(n = 9). We found that the GABA concentration accounting for $GABA_AR$ activity evoked by KCl application was close to 3 μM (Figure 4 A2). Although the estimated concentration of non-synaptic release of GABA was relatively low, the exogenous application of 3 μM GABA in the presence of 1 μM TTX was sufficient to evoke a 20.6 ± 6.1 mV MN depolarization (Vh = -60 mV; ECl = -30 mV; n = 7) (Figures 4B2).

$V1^R$ exhibit heterogeneous excitability patterns at the onset of SNA.

Spontaneous or evoked RC activity was assessed using whole-cell current-clamp recordings and the open-book SC preparation (Scain et al., 2010). To determine the identity of recorded INs, GAD67eGFP neurons were filled with neurobiotin (≈ 1 mg/mL) in combination with post-hoc Foxd3 staining (Figures 1D). At E12.5, $V1^R$ had a whole-cell capacitance of 13.7 ± 3.8 pF (n = 192) and an input resistance of 1242 ± 620 MΩ (n = 192). Spontaneous activity recorded in 37 $V1^R$ was characterized by recurrent GDPs (Figure 5), occurring every 3.7 ± 2.4 min (n = 30), similar to what has been observed in MNs (~3 min) (Czarnecki et al., 2014). Interestingly, in 21.6% (n = 8/37) of the recorded $V1^R$, GDPs evoked long-lasting depolarizing plateau potentials (Figure 5A), while 37.8% (n = 14/37) of the recorded $V1^R$ had the ability to spike repetitively during GDPs (Figure 5B). Plateau potentials had an absolute amplitude of 53.4 ± 11.5 mV (n = 8) and a half amplitude duration ranging from 0.12 to 0.86 s (0.44 ± 0.22 s; n = 8). In 16.2% (n = 6/37) of the recorded $V1^R$, GDPs evoked a single spike or a doublet (figure 5C), while the remaining 24.3% (n = 9/37) of the recorded $V1^R$ did not spike during GDPs (data not shown). These data show that $V1^R$ can generate various spontaneous activity patterns and raise the question of their intrinsic activation properties.

To analyze the intrinsic activation properties of embryonic $V1^R$, we depolarized the cells from a holding potential of -60 mV using 2 s step current pulses or 20 s depolarizing current ramps. At E12.5, all $V1^R$ were excitable (n = 164), but depolarizing current injections triggered various and complex excitability patterns (Figure 6). 28.7% (n = 47/164) of the

417 analyzed $V1^R$ were able to generate 1 to 3 action potentials (APs) in response to
418 suprathreshold depolarizing steps (Figure 6A). When a depolarizing ramp was applied, only
419 fast membrane potential oscillations of small amplitude were observed. We called these $V1^R$,
420 SS-$V1^R$, ("single" spiking $V1^R$,). In contrast, 21.2% (n = 36/164) of the recorded $V1^R$ were
421 able to generate long-lasting events defined as plateau potentials in response to depolarizing
422 current pulses and in response to depolarizing current ramps. These plateau potentials were
423 all-or-none events (Figure 6B). They can be evoked by long depolarizing pulses (2s) (Figure
424 6B) and short depolarizing current pulses (Figure 6F2). They had a half amplitude duration of
425 ≈ 700 ms and an absolute peak amplitude of ≈ 3 mV (Table 2). Remarkably, these plateau
426 potentials were sodium-dependent events as they were fully blocked by the application of 1
427 μM TTX (Figure 6F1). Unlike SS-$V1^R$, these $V1^R$ can generate repetitive plateau potentials
428 when depolarized by a suprathreshold depolarizing current ramp (Figure 6B). We called these
429 $V1^R$, PP-$V1^R$ (plateau potential $V1^R$). A third group of $V1^R$ (41.5%; n = 68/164) was
430 identified based on their ability to generate repetitive AP firing in response to suprathreshold
431 depolarizing current steps or to depolarizing current ramps. In these cells, APs had a half
432 amplitude duration ≈ 13 ms (Table 2). We called these $V1^R$, RS-$V1^R$ (repetitive spiking $V1^R$)
433 (Figure 6C). Finally, a fourth group of cells (8.5%; n = 14/164) was determined according to
434 their ability to generate both APs and plateau potential-like events (Figure 6D). We called
435 these $V1^R$, ME-$V1^R$ (mixed event $V1^R$). SS-$V1^R$ are unlikely to be dying neurons.
436 Developmental cell death (DCD) of V1 INs occurs after E14.5 in mouse embryos (Prasad et
437 al., 2008) and SS-$V1^R$ passive electrical properties did not differ from those of more active
438 $V1^R$ (Table 2).

439 Considering that the different types of intrinsic activation pattern may reflect a
440 continuum between a single spike and a plateau potential, we chose to focus our study on the
441 three clear patterns of intrinsic activation, SS-$V1^R$, RS-$V1^R$ and PP-$V1^R$, and to not study the
442 mixed regime in depth. Table 2 summarizes passive and active intrinsic electrical properties

of recorded SS-V1^R, RS-V1^R and PP-V1^R. Note that the difference in intrinsic excitability in these different classes of V1^R is not due to difference in their membrane resistance ($P > 0.1$).

These results reveal that V1^R can have different regimes of activity in the embryonic SC at the onset of synaptogenesis and SNA (E12.5) (Scain et al., 2010; Czarnecki et al., 2014).

Sustained discharge in embryonic V1^R depends on persistent sodium current.

A key element in neurons, which exhibit sustained discharges, is the expression of subthreshold slow-inactivating inward currents. Among these inward currents, the TTX-sensitive persistent inward current (I_{Nap}) plays a fundamental role in controlling sustained discharge in INs and MNs in the SC at postnatal developmental stages (Lee and Heckman, 1998; Kuo et al., 2006; Theiss et al., 2007; Ziskind-Conhaim et al., 2008).

To determine whether I_{Nap} is already expressed by V1^R in E12.5 mouse embryo, we made whole-cell voltage-clamp recordings. In this set of experiments, the electrodes were filled with a Cs-based intracellular solution to minimize contamination of the recorded current by potassium currents. A test pulse from -100 to -20 mV evoked TTX-sensitive sodium currents: a large amplitude transient current (I_{NaT}) followed by a smaller amplitude current persisting over 0.5 s was also fully blocked by TTX (Figure 7A1, inset), which indicates the presence of I_{Nap}. This also indicates that sodium-independent voltage-gated inward currents were not detected at this developmental stage. To characterize I_{Nap} better, we used a slow voltage ramp (70 mV/s) from -100 to +20 mV designed to inactivate transient currents (Huang and Trussell, 2008). Subtraction of the trace after TTX application revealed the I_{Nap} on the I-V curve (Figure 7A1). I_{Nap} was observed in all recorded RCs. We further investigated the activation properties of the I_{Nap}. The Boltzmann fit of the voltage dependence of the I_{Nap} generated by voltage ramps revealed a V_{half} of -22.9 ± 5.7 mV and a slope factor (k) of 7.0 ± 1.8 mV in RCs (n = 8) (Figure 7A2). In RCs, the I_{Nap} activated at Vh = -67.4 ± 6.2 mV (n = 8)

and the mean persistent sodium conductance density (g_{NaP}/pF) was 41.8 ± 22.7 pS/pF (n = 12) (Figure 7B).

To further confirm that this persistent inward current truly reflects the presence of I_{Nap}, we tested the effect of a low concentration of riluzole (5 μM), which is known to block I_{Nap} (Urbani and Belluzzi, 2000). Indeed, 5 μM riluzole suppressed 81.5 ± 14.6% (n = 15; P = 0.0005) of the I_{Nap} in $V1^R$ (Figure 7C), whereas at this low concentration riluzole only slightly reduced the peak amplitude of I_{NaT} (see red arrows in Figure 7A1, inset). We confirmed on MNs that this low concentration had a minimal effect on the sodium-dependent AP, which was not the case when riluzole concentration was increased to 10 μM (Figures 8A and 8B1-B3). AP amplitude was significantly reduced to 83.2 ± 12.6% of control in the presence of 5 μM riluzole (n = 7; P = 0.008), but was reduced to 67.5 ± 17.0% of control when riluzole concentration was increased to 10 μM (n = 7, P = 0.0078). We then reasoned that the lack of repetitive spiking or plateau potential activity observed in some $V1^R$ might depend on a reduced level of I_{Nap}. To test this hypothesis, we compared the presence of I_{Nap} in $V1^R$ that cannot generate repetitive spiking (SS-$V1^R$) with $V1^R$ that can generate repetitive spiking (RS-$V1^R$) and with $V1^R$ generating plateau potentials (PP-$V1^R$). We developed a voltage- and current-clamp recording protocol in a given neuron allowing us to associate the amplitude of the I_{Nap} with the excitability pattern in each type of spinal neuron (K-methanesulfonate intracellular solution) (Figure 9 A1-A4). The same voltage ramp (70 mV/s) was used as described above. The I_{Nap} current was revealed by subtracting ramp-evoked current in the presence of 1 μM TTX from controls. In these conditions, SS-$V1^R$ had a significantly lower g_{NaP} density (22.1 ± 12.6 pS/pF, n = 13) when compared to RS-$V1^R$ (57.8 ± 17.4 pS/pF, n = 7) and to PP-$V1^R$ (70.6 ± 34.0 pS/pF, n = 11; P < 0.0001) (Figure 9A4). These results highlight a direct relationship between the strength of I_{Nap} and the ability of $V1^R$ to sustain repetitive spiking and plateau potential activity.

To determine whether I_{Nap} is required for repetitive spiking in embryonic $V1^R$, we assessed the effect of riluzole on $V1^R$ that can sustain AP firing or plateau potential activity. The application of 5 µM riluzole turned all RS-$V1^R$ into SS-$V1^R$ (n = 10) (Figure 9B). In the presence of 5 µM riluzole, the depolarizing current step failed to trigger more than a doublet in these cells. 5 µM riluzole did not significantly change the AP threshold (-37.2 ± 3.2 mV vs -36.8 ± 6.0 mV with 5 µM riluzole, n = 10, $P = 0.70$). In all PP-$V1^R$, riluzole prevented the plateau potential activity (n = 7) (Figure 9C). Increasing the amplitude of the injected current failed to evoke any AP, indicating that PP-RCs were turned into non-excitable neurons when I_{Nap} was blocked.

Altogether, these results indicate that I_{Nap} already plays an important role in $V1^R$ intrinsic activation properties at this early embryonic developmental stage of SC neuronal networks.

Blocking persistent sodium current alters SNA pattern and SNA propagation along the cord.

Synchronous activation of MNs and INs was proposed to drive episodes of activity during SNA (Hanson and Landmesser, 2003; Czarnecki et al., 2014). If intrinsic activation properties of the few INs that produce GABA including $V1^R$, rely on the I_{NaP},, we expected that blocking I_{NaP} would alter episodes of activity during SNA in a manner similar to that observed in the presence of $GABA_AR$ antagonists (Hanson and Landmesser, 2003; Czarnecki et al., 2014). Blocking GABA neurotransmission during SNA evoked an increase in inter-episode interval, but had little effect on the duration of episode of activity and on their propagation along the cord (Hanson and Landmesser, 2003). In addition, we previously showed that blocking GABA neurotransmission decreased the amplitude of spontaneous giant inward currents (sGICs) recorded in MNs during SNA (Czarnecki et al., 2014).

To determine the involvement of I_{Nap} in SNA, we first tested the effect of 5 µM riluzole

519 on the spontaneous activity of MNs using whole-cell voltage-clamp recordings. In control
520 conditions, $_S$GICs occurred every 4 minutes (4.0 ± 0.8 min, n = 9), had mean amplitude of
521 291 ± 200 pA (n= 9) and a half-width of 0.6 ± 0.3 s (Vh = -60 mV; ECl = 3 mV) (Figure
522 10A1-A2). The time course of the effect of riluzole on sGIC occurrence was variable among
523 MNs. In one out of nine recorded MNs, riluzole abolished GIC activity in less than 10 min. In
524 other MNs, 1 to 2 GICs could still be observed during the 20- to 30-min application of
525 riluzole. (Figure 10A2). During this transitory period, riluzole significantly reduced the
526 amplitude of GICs (30.8 ± 32.7% reduction; n= 8; P = 0.039) (Figure 10B1) and the half-
527 duration of GICs (44.0 ± 27.2% reduction; n= 8; P = 0.0078) (Figure 10B2). In order to assess
528 the effect of riluzole on SNA, we made long-lasting extracellular recordings of SC activity
529 using two electrodes positioned on the cervical and lumbar superficial part of the ventral horn
530 of the SC at E12.5 (Czarnecki et al., 2014) (Figure 10C1).

531 These recordings allowed us to monitor SC neuron activity related to GIC activity
532 recorded in MNs (Czarnecki et al., 2014). We found that a 30- to 40-min application of 5 µM
533 riluzole significantly increased the inter-episode interval from 4.18 ± 1.93 min in control
534 conditions to 12.34 ± 9.08 min in the presence of riluzole (P < 0.001, n = 12 paired SCs)
535 (196.3 ± 203% increase) (Figure 10C2-C3), revealing a strong involvement of the I_{Nap} current
536 in the rhythmicity of early SC network activity. We therefore analyzed individual bursts of
537 AP exhibited by the cervical (C) and lumbar (L) SC networks in the presence of riluzole. We
538 found that the duration of bursts (both C and L), compared before (2.31 ± 0.89 s) and after the
539 riluzole application (1.44 ± 0.7 s), was significantly reduced (36.5 ± 18.3% reduction; n = 22;
540 P < 0.001) (Figure 10C4). In addition, the instantaneous AP frequency within a burst at both
541 the cervical level (C level) and the lumbar level (L level) was significantly reduced by 25.3 ±
542 11.9% (P < 0.001) in the presence of riluzole (112.4 ± 39.6 Hz in control conditions and 83.3
543 ± 34.4 Hz with riluzole, n = 22) (Figure 10C5). We also observed a reduction in the speed of
544 propagation of SNA episodes between the C level and L level of the SC in the presence of

545 riluzole. Propagation of SNA along the cord was investigated by analysis of the time needed
546 for a burst of AP recorded at the C level to reach L level (distance between the two recording
547 levels ≈ 4mm) (Yvert et al., 2004). We found that the C-L time was significantly increased (P
548 < 0.001) from 1.56 ± 0.42 s in control conditions to 2.42 ± 0.77 s in the presence of riluzole
549 (56.3 ± 36.1% increase; n = 10) (Figure 10C6).

550 Riluzole had a stronger effect on SNA than previously observed in the presence of
551 $GABA_AR$ antagonists (Hanson and Landmesser, 2003; Czarnecki et al., 2014). Targeted
552 networks during extracellular recordings likely include MNs as well as surrounding INs
553 (Hanson and Landmesser, 2003; Czarnecki et al., 2014) including $V1^R$ GABAergic INs.
554 Because most GABAergic $V1^R$ are able to generate repetitive firing in response to GDP,
555 which is not the case for MNs (Czarnecki et al., 2014), this apparent discrepancy could be
556 explained if blocking GABA neurotransmission does not strongly alter GABAergic INs
557 firing, just as riluzole does. We cannot, however, exclude that I_{Nap} is also present in neurons
558 other than $V1^R$ in the SC at E12.5. Anyhow, these results indicate that I_{Nap} plays an important
559 role in SNA at the onset of synaptogenesis (E12.5) by regulating neuron excitability in the
560 mouse embryonic SC.

DISCUSSION

How excitability of neurons evolves during SC development was extensively studied at developmental stages at which central pattern generators are already functional. At these developmental stages (P0 to P5, postnatal day), I_{Nap} is present in MNs and in SC INs (Tazerart et al., 2007; Zhong et al., 2007) and is required to generate fictive locomotion (Zhong et al., 2007). Here, we show that $V1^R$ already express I_{Nap} at the onset of synaptogenesis in the embryonic (E12.5) SC (Scain et al., 2010). Our results reveal that I_{Nap} already closely controls the $V1^R$ excitability pattern. In addition, we found that low concentrations of riluzole, an I_{Nap} blocker, dramatically altered the SC SNA pattern, suggesting that I_{Nap} is already expressed by neurons in the mammalian embryonic SC well before the control of muscle contraction by MNs (Sanes and Lichtman, 1999).

$V1^R$ display different excitability patterns at the onset of SNA.

Repetitive firing of presynaptic neurons is required for long-lasting neurotransmitter-dependent episodes, as GDPs observed in MNs at E12.5. Previous studies have examined the development of passive and active membrane properties of MNs and INs in late embryonic and newborn rats or mice (Ziskind-Conhaim, 1988; Gao and Ziskind-Conhaim, 1998; Vinay et al., 2000; Theiss et al., 2007; Perry et al., 2015; Bikoff et al., 2016), but there is no information about the excitability of INs at early mammalian embryonic developmental stages when SNA first arises. We found that a majority of $V1^R$ (≈ 60%) can already generate repetitive AP firing or long-lasting sodium-dependent plateau potentials at the onset of SNA, indicating that these cells are already active at this early embryonic developmental stage. Our analysis revealed a strong heterogeneity in $V1^R$ excitability pattern, which can be separated into different independent classes, but such functional diversity is unlikely to persist in the adult. In the adult, $V1^R$ were found to generate two different excitability patterns only, a prominent low-threshold depolarization and burst firing followed by continuous firing

dependent on Vh (Perry et al., 2015; Bikoff et al., 2016). It is therefore likely that the functional heterogeneity we observed at E12.5 reflects an immature form of $V1^R$ excitability (Perry et al., 2015; Bikoff et al., 2016). Sodium-dependent plateau potentials were also observed in ipsilateral caudal SC INs of zebrafish embryos at the appearance of functional neuromuscular junctions and then disappeared at more mature stages (Tong and McDearmid, 2012).

I_{Nap} already regulate excitability patterns of $V1^R$ at early developmental stages.

In this study, we reveal the presence of I_{Nap} in neurons of mammalian embryos at the onset of SNA. Persistent inward currents (PICs) are present in many types of neurons. PICs can be calcium- and/or sodium-dependent depending on the neuron subtypes. We did not find any evidence for a significant calcium component in recorded PICs in $V1^R$ at E12.5. Indeed, the PIC was fully blocked by TTX in $V1^R$, which contrasts with what is known about more mature spinal neurons like MNs (Hounsgaard and Kiehn, 1989; Bui et al., 2006; Carlin et al., 2009). Accordingly, we concluded that immature $V1^R$ at the onset of SNA generate a pure sodium-dependent PIC. I_{Nap} recorded in $V1^R$ of the mouse embryo has an activation onset (≈ -65 mV) similar to that of I_{Nap} at postnatal developmental stages in the rat and mouse SC (Kuo et al., 2006; Tazerart et al., 2007; Theiss et al., 2007; Tazerart et al., 2008; Dai and Jordan, 2010). The Boltzmann constant of activation calculated for I_{Nap} in $V1^R$ (≈ 7 mV) is in the range of the values known for I_{Nap} in INs and MNs (≈ 6 mV) of neonatal rats (Bouhadfane et al., 2013) or in the calyx of Held (≈ 8 mV) (Huang and Trussell, 2008). Small differences in the voltage dependency of I_{Nap} activation may reflect differences in sodium channel α-subunit and/or β-subunit combination expression (Isom et al., 1994; Qu et al., 2001).

I_{Nap} likely has diverse functions and plays an important function in locomotor pattern generation in neonatal rats (Tazerart, S.*, et al.* 2007). In neonate SC, I_{Nap} is present in both INs and MNs (Kuo et al., 2006; Tazerart et al., 2007) and is known to generate pacemaker

activities in central pattern generator INs (Tazerart et al., 2008). Remarkably, I_{Nap} also contributes to lumbar MN activity related to plateau potential in rat neonates (Bouhadfane et al., 2013). However, while in neonate MNs, I_{Nap} is essential for self-sustained firing only during calcium-dependent plateau potential depolarization (Carlin et al., 2009; Bouhadfane et al., 2013), plateau potential activity in $V1^R$ at E12.5.is fully I_{Nap}-dependent

Does I_{Nap} already participate in SC activity at the onset of neuronal network formation?

I_{Nap} plays a crucial role in the regulation of locomotor pattern generation in rat neonates (Tazerart et al., 2007; Zhong et al., 2007; Bouhadfane et al., 2013) and in the SC of the zebrafish embryo during the cooling stage (Tong and McDearmid, 2012). Here, we demonstrated that a low concentration of riluzole dramatically reduced the amplitude and the duration of GICs recorded on MNs and strongly altered SC activity (extracellular recordings) at the onset of SNA.

In our experiments, we used a concentration of riluzole that has a minimal effect on the sodium AP waveform (Figure 6). However, beside its effect on $I_{Nap,}$ riluzole is known to inhibit the release of glutamate (Cheramy et al., 1992), to inhibit AMPA receptor activation (Albo et al., 2004), $GABA_A$ and glycine receptor activation (Mohammadi et al., 2001) and to block several voltage-gated channels including calcium channels (Huang et al., 1997; Ahn et al., 2006). With the exception of AMPA receptor inhibition (Albo et al., 2004), these side effects of riluzole occur at concentrations ≥ 10 µM (Cheramy et al., 1992; Huang et al., 1997; Mohammadi et al., 2001; Ahn et al., 2006). Because the inhibition of glutamate neurotransmission did not alter SNA in the mouse embryonic SC at E12.5 (Czarnecki et al., 2014), it is unlikely that the alteration of SNA we observed in the presence of 5 µM riluzole is caused by the inhibition of glutamatergic synaptic activity (Albo et al., 2004). Accordingly, we propose that I_{Nap} already regulates the SNA pattern at the onset of synaptogenesis in mammalian embryos before the formation of the locomotor SC network and of functional

neuromuscular junctions. However, we cannot exclude that spinal neurons other than $V1^R$ exhibit I_{Nap}-dependent sustain discharge at this developmental stage.

Because $V1^R$ already produce GABA at E12.5 and make synaptic-like contacts with MNs while MNs make synaptic-like contacts with $V1^R$ (see also (Alvarez et al., 2013), it is likely that $V1^R$ participate in early SC SNA. Although correlative, our data reinforce the hypothesis that a primitive $V1^R$-MN recurrent-like circuit may exist at the onset of synaptogenesis in the mouse embryo. This primitive $V1^R$-MN recurrent circuit differs in several ways from the adult RC-MN recurrent circuits. Evoked MN spiking did not trigger a recurrent synaptic response at E12.5 (Le Bras et al., 2014), which suggests that although $V1^R$ projects on MNs and vice versa, it is unlikely that $V1^R$ projecting on MNs receive inputs from these MNs and vice versa. It is also unlikely that MN release sites apposed on $V1^R$ are mixed glutamatergic and cholinergic inputs as observed at postnatal stages (Nishimaru et al., 2005; Lamotte d'Incamps et al., 2017). Glutamatergic vesicular transporters were not observed within MNs at E12.5 (Czarnecki et al., 2014). But we cannot completely exclude that some immature motor axon synapses on $V1^R$ already release aspartate, as suggested in the adult (Richards et al., 2014). However, contrary to what is observed for GABAergic and cholinergic networks, the spontaneous activation of glutamate receptors had a minor role only in the generation of SNA episodes at E12.5 (Czarnecki et al., 2014).

We clearly show that paracrine release of GABA can occur in the embryonic SC, which may explain the smooth shape of GABAergic-dependent GDPs observed in MNs during SNA (Czarnecki et al., 2014). Since $V1^R$ are GABAergic at this embryonic age and possess putative release sites, $V1^R$ may be one of the sources for the paracrine release of GABA we observed. Accordingly, we propose that $V1^R$-MN interactions occur through synaptic and paracrine release and participate in the synchronization of neuronal assembly required for the generation of the propagating waves of activity characterizing SC SNA at E12.5 (Momose-Sato and Sato, 2013).

665

666 ***Conclusions***

667 Taken together, our findings demonstrate that I_{Nap} is already present in developing SC
668 neurons at an early developmental stage and governs $V1^R$ excitability. I_{Nap} plays an important
669 role in the regulation of locomotor pattern generation at postnatal developmental stages in
670 rodents (Tazerart et al., 2008). Because the application of a low concentration of riluzole
671 altered SNA, we propose that I_{Nap} contributes to the patterning of embryonic SC activity at the
672 onset of synaptogenesis. Accordingly, the capacity of SC neurons to generate sustained firing
673 must be crucial for correct embryonic SC patterned activity at the onset of synaptogenesis,
674 which is required for the correct development of MN projections toward their peripheral
675 targets (Hanson and Landmesser, 2006).

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880
881 **LEGENDS**

882 ***Figure 1: $V1^R$ identification in the lumbar spinal cord of E12.5 embryos***

883 A) Coronal slice of the lumbar spinal cord of E12.5 GA67-eGFP mouse embryo showing the
884 distribution of eGFP neurons (A1), FoxD3 (A2) and calbindin (A3) immunoreactive neurons.
885 A4) Superimposed images showing the colocalization of eGFP, FoxD3 immunostaining and
886 calbindin immunostaining. B) Enlarged images from A showing that eGFP neurons localized
887 in the ventrolateral area of the spinal cord (B1) are Foxd3 immunoreactive (B2), most of them
888 being calbindin immunoreactive (B3-B4). C) Coronal slice of the ventrolateral part of the
889 lumbar spinal cord of E12.5 GA67-eGFP mouse embryo showing the distribution of eGFP
890 neurons (C1), FoxD3 (C2) and MafB (C3) immunoreactive neurons. C4) Superimposed
891 images showing the colocalization of eGFP FoxD3 immunostaining and MafB
892 immunostaining. Note that all FoxD3 immunoreactive neurons localized in the marginal zone
893 of the ventrolateral area are also MafB-positive, indicating that they are $V1^R$ neurons. D1)
894 Example of a neuron filled with neurobiotin during the recording at the lumbar level of an
895 embryonic spinal cord open book preparation of GAD67-GFP mice at E12.5. This eGFP
896 neuron (D2) was immunoreactive to Foxd3 antibody (D3), as shown in the merged images
897 (D4). Each image corresponds to a single confocal section.

898

899 ***Figure 2: $V1^R$ already produce GABA in the lumbar spinal cord of E12.5 embryos***

900 A) Single confocal sections of coronal slice of the lumbar spinal cord of E12.5 GA67-eGFP
901 mouse embryo showing the distribution of eGFP neurons (A1), calbindin (A2) and GABA
902 (A3) immunoreactive neurons. A4) Superimposed images showing the colocalization of
903 eGFP, calbindin immunostaining and GABA immunostaining. B1-B3) Enlarged images from
904 A1, A2 and A3 showing eGFP neurons (B1), calbindin immunoreactive neurons (B2), and
905 GABA immunoreactive neurons (B3) in the ventrolateral area of the spinal cord. B4)
906 Superimposed images showing the colocalization of calbindin immunostaining and GABA

immunostaining with z projections within a stack. Note the colocalization of calbindin immunostaining and GABA immunostaining in the three axes, indicating that $V1^R$ already produce GABA at E12.5.

Figure 3: $V1^R$ make synaptic-like contacts with motoneurons (HB9-eGFP) at E12.5.

A) Coronal slice of the lumbar spinal cord of E12.5 HB9-eGFP mouse embryo with cell nucleus staining (Hoesch) and synaptophysin immunostaining (A1). A1) Note that synaptophysin immunostaining is mainly restricted in the ventral funiculus (VF) A2) Calbindin staining showing the distribution of $V1^R$ neurite extensions and synaptophysin immunostaining. A3) eGFP immunostaining showing the distribution of MN neurite extensions and synaptophysin immunostaining. Antibody against GFP was used to visualize MN morphology better (Czarnecki et al., 2014). A4) Superimposition of eGFP fluorescence, calbindin immunostaining and synaptophysin immunostaining (A1, A2, A3, and A4 are confocal stacks). B) Single confocal sections with z projections of enlarged images from A2, A3 and A4 showing the colocalization of synaptophysin punctates with calbindin immunostaining apposed to eGFP immunostaining (B1, B2 and B3; enlarged images in boxes). B2) Note that a synaptophysin punctate colocalized with calbindin immunostaining (B1, arrow) did not colocalize with eGFP immunostaining (B2, arrow). Note that synaptophysin punctate colocalized with eGFP immunostaining (B1, arrow heads) did not colocalize with calbindin immunostaining (B2, arrow head). Barred arrow (B1) shows a colocalization of calbindin and synaptophysin immunostaining not apposed to eGFP immunostaining. B4) superimposed images (B1, B2 and B3) with z projections showing calbindin immunostaining and eGFP appositions. C1) Confocal stacks showing neurobiotin injected Foxd3 immunoreactive $V1^R$, HB9-eGFP immunostaining and synaptophysin immunostaining in an SC open book preparation. C2-C4). Single confocal sections with z projections of enlarged images from C1 (white box) showing the colocalization of

933 synaptophysin punctates with neurobiotin staining (C2), the apposition of the same
934 synaptophysin punctates to eGFP (C3) and the apposition of neurobiotin staining containing
935 synaptophysin punctates to eGFP (C4) (enlarged image in boxes in C2, C3 and C4),
936 indicating the presence of a $V1^R$ synaptic-like contact on an MN neurite.
937
938 ***Figure 4: Paracrine release of GABA detected by a sniffer outside-out patch.***
939 A1) Upper drawing showing the location of the outside-out sniffer to detect paracrine release
940 of GABA (left) and to obtain outside-out currents in response to GABA application (left).
941 Lower traces (purple): example of outside-out sniffer current evoked by the application of 30
942 mM KCl when the sniffer electrode was positioned in the dorsal area of the SC close to motor
943 columns. Enlarged trace shows single channel currents at the onset of the sniffer current.
944 Right traces (green) show outside-out current evoked by the application of 3 μM and 10 μM
945 GABA to a sniffer patch positioned outside the spinal cord. Purple and green traces are from
946 the same outside-out patch. A2) Box plots of normalized maximum outside-out current
947 evoked by KCl application (purple left) and by the application of 3 μM or 10 μM GABA
948 (green right) on the same outside-out sniffer patch (n = 9). Amplitudes of the outside-out
949 currents evoked by the application of 30 mM KCl, 3 μM or 10 μM GABA, were normalized
950 to the amplitude of the outside-out currents evoked by the application of 30 μM GABA (not
951 shown). Note that the normalized amplitude of the outside-out current evoked by 30 mM KCl
952 application (0.118 ± 0.097) was not significantly different ($P > 0.9$) from the normalized
953 amplitude of the outside-out current evoked by the application of 3 μM GABA (0.152 ±
954 0.062). Normalized amplitudes of the outside-out currents evoked by the application of 30
955 mM KCl or of 3 μM GABA were significantly different (KCl: $P = 0.0029$; 3 μM GABA: $P =$
956 0.00665) from the normalized amplitude of the outside-out currents evoked by the application
957 of 10 μM GABA (0.697 ± 0.073). $V1^R$: immature Renshaw cell; MN: motoneuron; ** $P <$
958 0.01. B1) Example of motoneuron membrane potential depolarization evoked by the

application of 3 μM GABA in the presence of 1 μM TTX (current clamp recording: Vh = -60 mV; ECl = - 30 mV). The application of 3 μM GABA evoked a depolarizing response of 20.6 ± 6.1 mV (n = 7).

Figure 5: $V1^R$ display plateau potential, repetitive firing or generate a single action potential during episodes of SNA in E12.5 spinal cord.

Examples of spontaneous activities recorded in $V1^R$ being characterized by GDPs displaying plateau potential (A), repetitive firing (B) or a single action potential (C) activity (V holding = -60 mV). Recordings shown in A, B and C are from different cells.

Figure 6: $V1^R$ display different excitability patterns in E12.5 embryonic spinal cords.

Excitability patterns were analyzed using depolarizing current step (2 sec) and depolarizing current ramp (20 sec). A-D) Representative traces of voltage responses showing single-spiking activity (A), plateau potential activity (B) repetitive AP firing (C) and mixed repetitive-spiking/plateau potential activity (D). E) Proportions of $V1^R$ subtypes according to the observed discharge patterns. 28.6% of $V1^R$ could not sustain repetitive spiking, 41.7% were repetitive-spiking $V1^R$, 8.5% were mixed $V1^R$, 21.2% were plateau potential $V1^R$ (n = 164). F) Plateau potentials are blocked by TTX (1 μM) application (n = 5/5) (F1, F2). F2) Plateau potentials are evoked by short (100 ms) pulses of depolarizing current.

Figure 7: Persistent sodium current (I_{Nap}) is already expressed in $V1^R$

A1) Representative trace of I_{Nap} evoked by a slow depolarizing voltage ramp in a $V1^R$ (CsCl intracellular solution). I_{Nap} (black trace) was isolated by subtracting the current elicited by a voltage ramp (70 mV/s) in the presence of TTX (insert green trace) from the control current (insert black trace). TTX-sensitive current was blocked by 5 μM riluzole (red trace). Left insert shows the protocol to generate voltage-dependent slow inward currents in control

conditions (black), after 5 µM riluzole application (red) or 1 µM TTX application (green). Right insert shows the current evoked by a depolarizing voltage step from -100 mV to 20 mV in the absence and in the presence of 5 µM riluzole. A2) Voltage dependence of I_{Nap} conductance calculated from the trace shown in A1. 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 a Boltzmann function (see methods), where V_{half} is the Vh value for I_{Nap} half activation, k the slope factor of the curve and G_{max} the maximum conductance. B) Box plot showing G_{max} density in RCs (n=12). C) Box plots showing the variation of the % I_{Nap} block by 5 µM riluzole in RC.

Figure 8: Effect of 5 and 10 µM riluzole on action potentials.

A) Effect of 5 µM (red trace) and 10 µM (blue trace) riluzole on the action potential (AP) evoked by a depolarizing current step in an MN. B1) Box plot showing the % changes in AP amplitude (% of control) in the presence of 5 µM riluzole and 10 µM riluzole. B2) Box plot showing the % changes in AP threshold (% of control) in the presence of 5 µM and 10 µM riluzole. B3) Box plot showing the % changes in AP half-width (% of control) in the presence of 5 µM and 10 µM riluzole.

Figure 9: Sustained discharge in embryonic $V1^R$ depends on persistent sodium current (I_{Nap}).

A) Representative traces of I_{Nap} recorded in $V1^R$ that cannot sustain repetitive spiking (SS-$V1^R$) (A1), in a repetitive-spiking $V1^R$ (RS-$V1^R$) (A2) and in a plateau potential $V1^R$ (PP-$V1^R$) (A3). I_{Nap} was isolated by subtracting the current elicited by a slow voltage ramp (-100 to + 20 mV; 70 mV/s) in the presence of 1 µM TTX from the current evoked in the absence of

TTX. A4) Box plots showing Gmax density in SS-$V1^R$ (n=13), RS-RCs (n=8) and PP-$V1^R$ (n = 11). Note that Gmax density is significantly lower in SS-$V1^R$ ($p < 0.01$). B) Representative traces showing the effect of riluzole application (5 µM) on the intrinsic activity pattern evoked by suprathreshold current steps (left traces) or a suprathreshold current ramp (right traces) in an RS-$V1^R$. Note that riluzole blocks repetitive spiking (n= 10/10). C) Representative traces showing the effect of riluzole application on plateau potential evoked by suprathreshold current steps (left traces) or by a suprathreshold current ramp (right traces) in a $V1^R$. Note that riluzole blocks plateau potential activity (n=7/7).

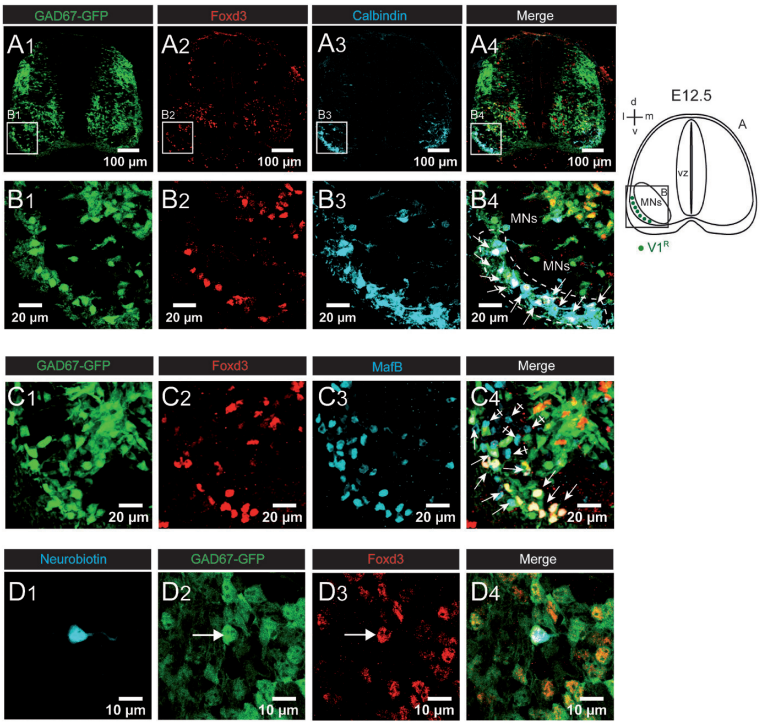
Figure 10: Riluzole dramatically decreases the frequency and duration of episodes of SNA in E12.5 embryonic spinal cord.

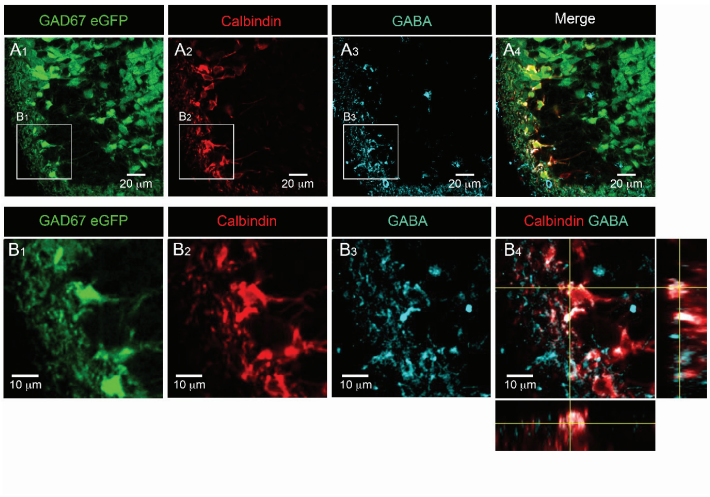
A1) Application of 5 µM riluzole inhibits spontaneous giant inward current (GIC) activity in MNs (voltage clamp recordings; Vh = -60 mV; ECl = -30 mV). A2) Enlarged trace from (A1) showing GIC before (1, black) and at the onset of riluzole application (2, red). Note that the amplitude and the duration of GICs were decreased on riluzole application. B1: Box plots showing the amplitude of the GIC in a control and on riluzole application (n=8). B2: Box plots showing half amplitude durations of the GIC in control and on riluzole application (n=8). Note that the amplitude ($P < 0.05$) and the duration ($P < 0.01$) of GICs were significantly reduced in the presence of riluzole. C) Spontaneous network activity (SNA) recorded at the cervical (C) and lumbar (L) levels (see schematic drawing on the left) in extracellular configuration before (C1) and after 5 µM riluzole (C2). Note that one episode still occurred 35 minutes after riluzole application. C3-C6) Box plots illustrating inter-burst interval, burst duration, intra-burst spike frequency and cervical-lumbar delay of episode propagation.

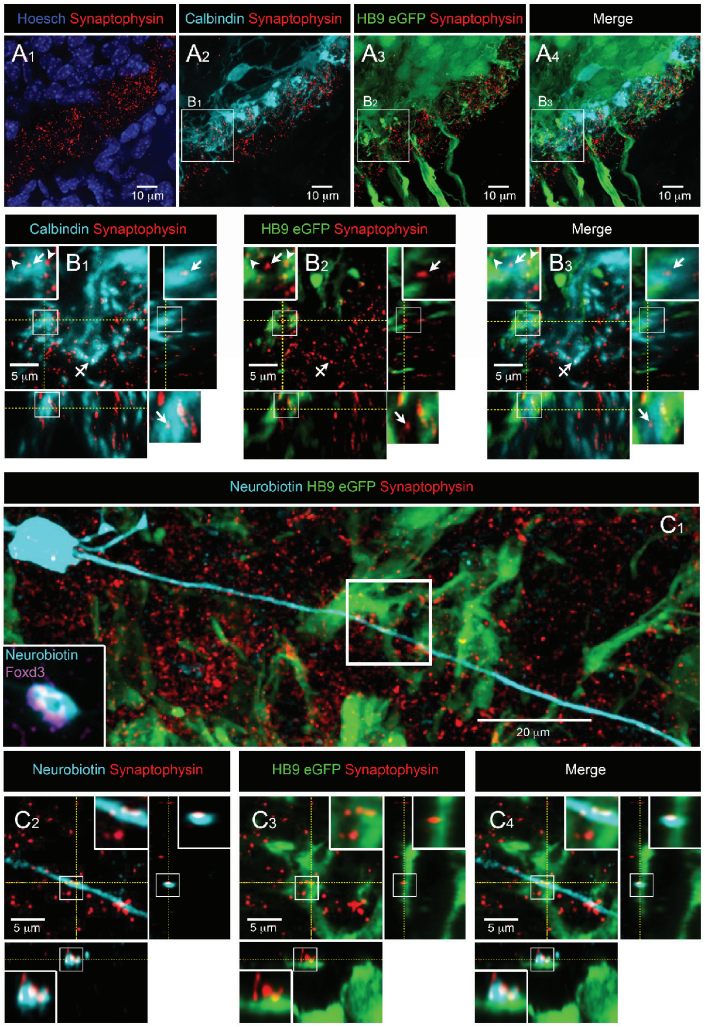
Table 1 Primary antibodies.

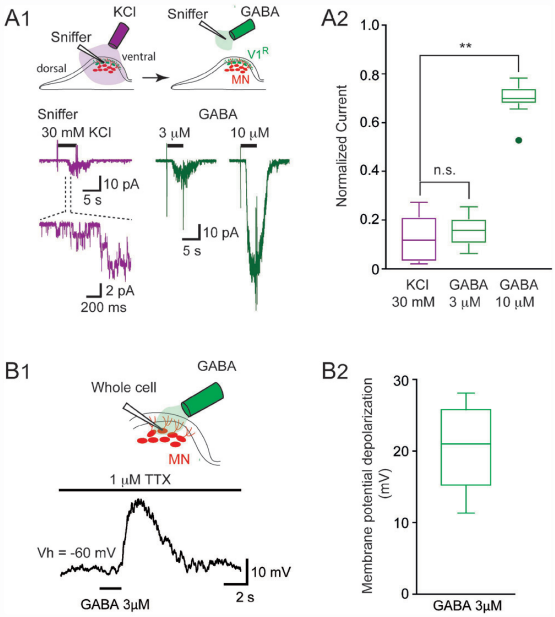
Table 2: Intrinsic membrane properties of Renshaw cells.

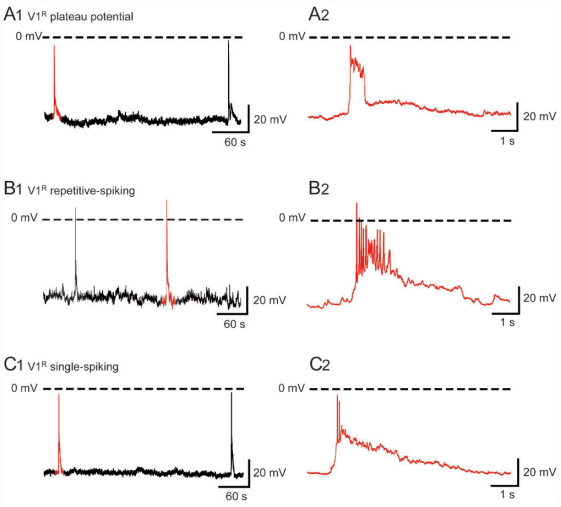
SS-$V1^R$: single-spiking Renshaw cells, RS-$V1^R$: repetitive-spiking Renshaw cells, PP-$V1^R$: plateau potential Renshaw cells. AP: action potential. Threshold values represent the averaged action potential threshold for single spiking $V1^R$ and repetitive spiking $V1^R$ and the averaged plateau potential threshold for plateau potential $V1^R$. Peak amplitude values represent the averaged peak amplitude of the action potential for single spiking $V1^R$, the averaged peak amplitude of the first action potential in a train for repetitive spiking $V1^R$ and the averaged peak amplitude of plateau potentials for plateau potential $V1^R$. Half width values represent the averaged half width of the action potential for single spiking $V1^R$, the averaged half width of the first action potential in a train for repetitive spiking $V1^R$ and the averaged half width of plateau potentials for plateau potential $V1^R$. Rate of rise values represent the averaged rate of rise of the action potential for single spiking $V1^R$, the averaged rate of rise of the first action potential in a train for repetitive spiking $V1^R$ and the averaged rate of rise of plateau potentials for plateau potential $V1^R$. Values are expressed as mean ± SD. Input resistance of SS-$V1^R$, input resistance of RS-$V1^R$ and input resistance of PP-$V1^R$ were not significantly different ($P = 0.17$).

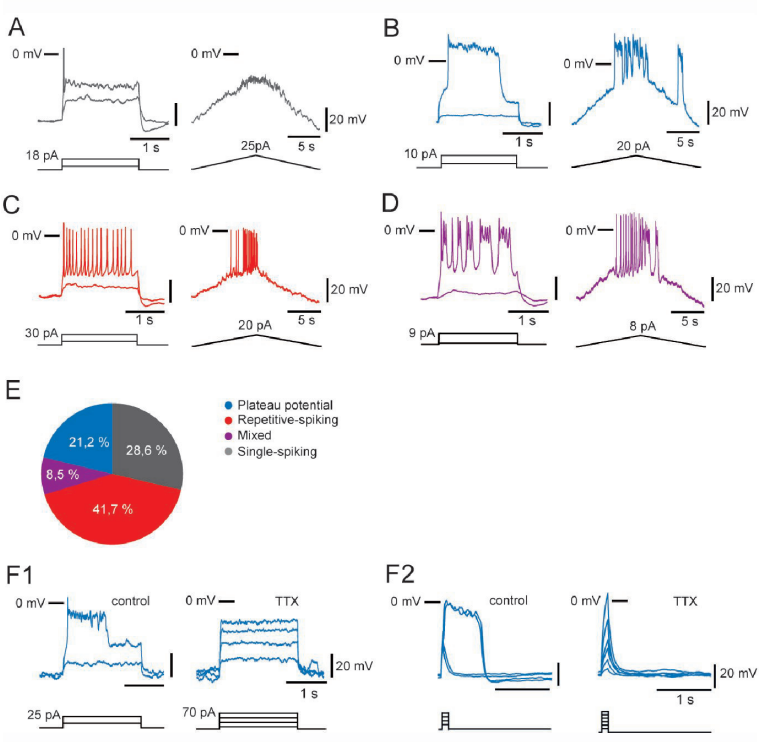


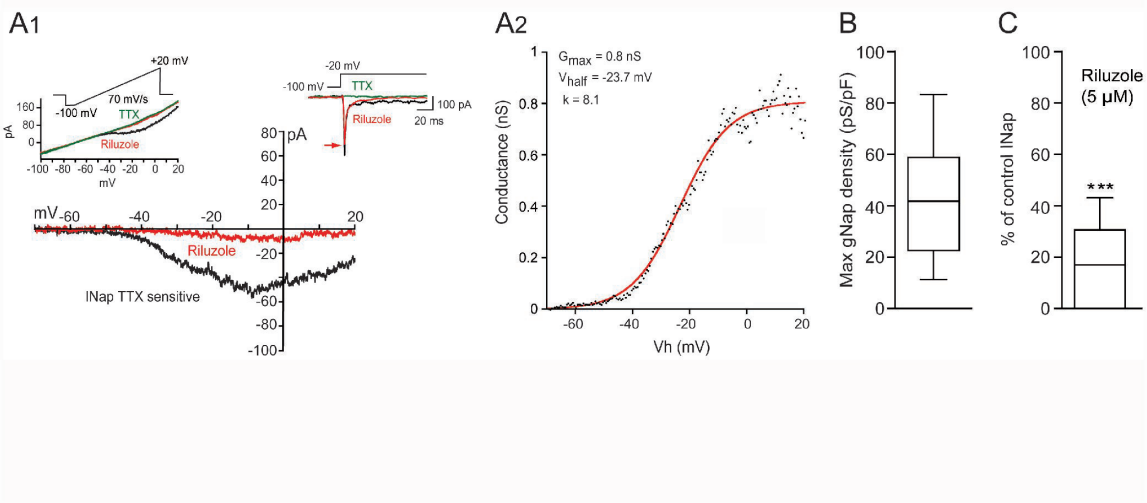


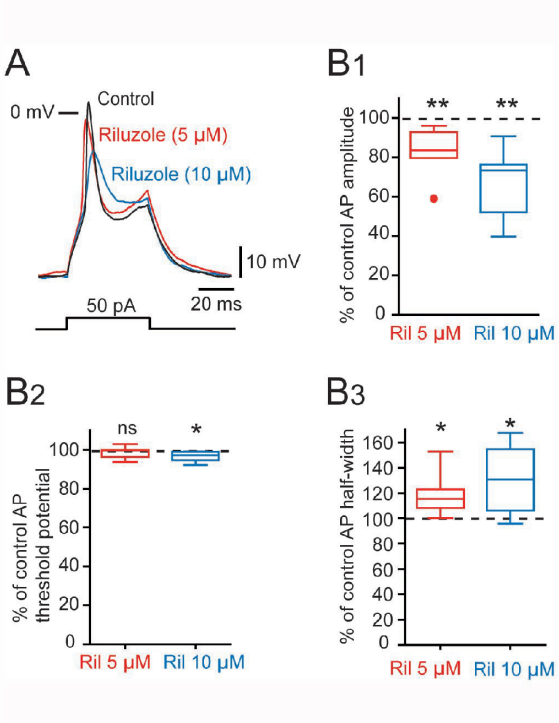


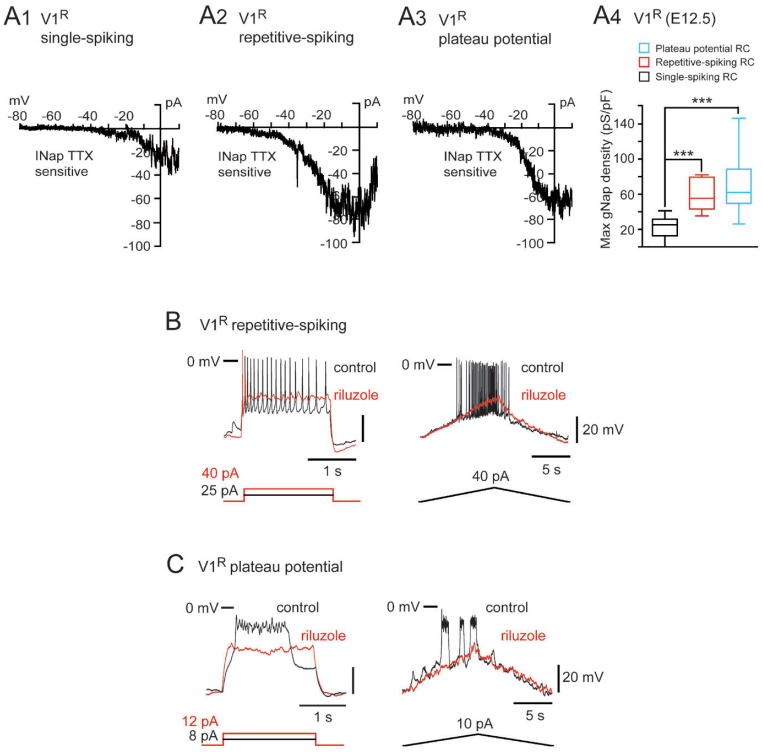












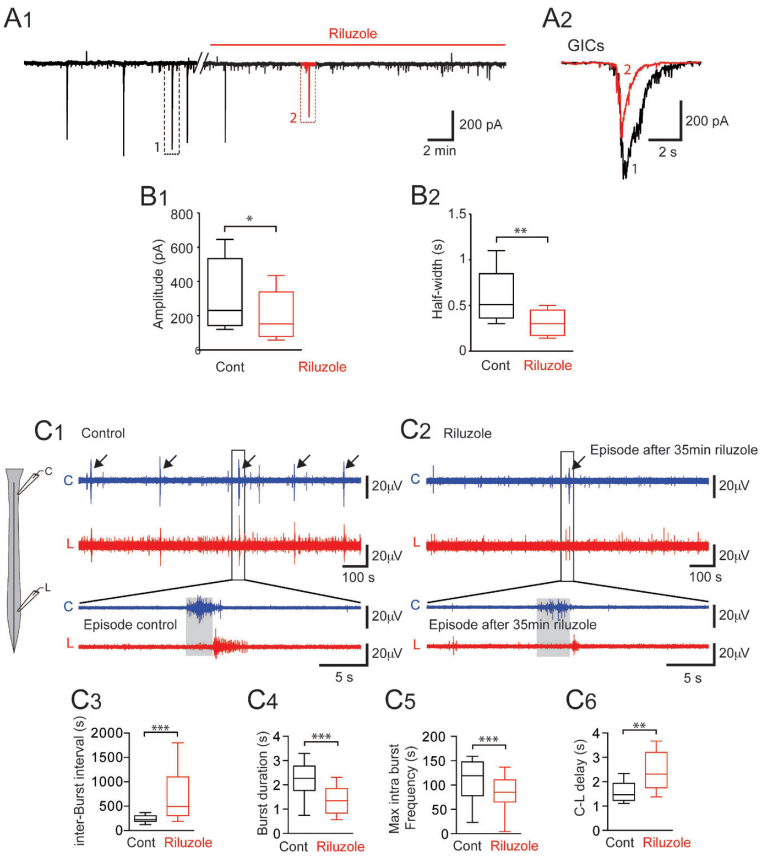


Table 1. Primary antibodies

Primary antibody	Company	Reference	Host/isotype	Dilution
anti-calbindin-D_{28k}	Swant, Switzerland	(Swant Cat# CB38, RRID:AB_2721225)	Rabbit polyclonal	1:1500
anti-FoxD3	provided by C.Birchmeier, MDC Berlin, Germany	Storm R, et al.(2009) Development. ;136:295–305.	Guinea pig polyclonal	1:5000
anti-MafB	Bethyl Laboratories	Bethyl Cat# IHC-00351, RRID:AB_1279487	Rabbit polyclonal	1:1000
anti-GABA	Sigma-Aldrich	Sigma-Aldrich Cat# A0310, RRID:AB_476667	Mouse monoclonal	1:800
Anti-synaptophysin	Synaptic System	Synaptic Systems Cat# 101 011, RRID:AB_887824	Mouse monoclonal	1:1500
Anti-GFP	Aves Labs	Aves Labs Cat# GFP-1020, RRID:AB_10000240)	Chicken polyclonal	1 :1000

	Single-spiking $V1^R$ (SS)	Repetitive-spiking $V1^R$ (RS)	Plateau potential $V1^R$ (PP)
Whole cell capacitance (pF)	13.3 ± 2.8 N = 82	15.3 ± 4.5 N = 60	12.4 ± 3.6 N = 50
Input resistance (MOhm)	1298 ± 682 N = 82	1156 ± 616 N = 60	1254 ± 508 N = 50
Threshold (mV)	-34.1 ± 3.3 N = 46	-33.6 ± 4.9 N = 53	-35.9 ± 6.0 N = 36
peak amplitude (mV)	-6.8 ± 6.1 N = 46	3.2 ± 6.7 N = 53	3.4 ± 10.3 N = 36
half width (ms)	11.2 ± 8.2 N = 46	12.8 ± 6.9 N = 53	705.7 ± 631.7 N = 36
rate of rise (mV/ms)	8.4 ± 3.8 N = 27	13.4 ± 9.4 N = 27	6.2 ± 3.7 N = 19

Table 2: Intrinsic functional properties of $V1^R$.

SS-$V1^R$: single-spiking Renshaw cells, RS-$V1^R$: repetitive-spiking Renshaw cells, PP-$V1^R$: plateau potential Renshaw cells. AP: action potential. Threshold values represent the averaged action potential threshold for single spiking $V1^R$ and repetitive spiking $V1^R$ and the averaged plateau potential threshold for plateau potential $V1^R$. Peak amplitude values represent the averaged peak amplitude of the action potential for single spiking $V1^R$, the averaged peak amplitude of the first action potential in a train for repetitive spiking $V1^R$ and the averaged peak amplitude of plateau potentials for plateau potential $V1^R$. Half width values represent the averaged half width of the action potential for single spiking $V1^R$, the averaged half width of the first action potential in a train for repetitive spiking $V1^R$ and the averaged half width of plateau potentials for plateau potential $V1^R$. Rate of rise values represent the averaged rate of

rise of the action potential for single spiking $V1^R$, the averaged rate of rise of the first action potential in a train for repetitive spiking $V1^R$ and the averaged rate of rise of plateau potentials for plateau potential $V1^R$. Values are expressed as mean ± SD. Input resistance of SS-$V1^R$, input resistance of RS-$V1^R$ and input resistance of PP-$V1^R$ were not significantly different ($P = 0.17$).