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Title: Neuroepithelial progenitors generate and propagate non-neuronal action potentials across the spinal cord

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SUMMARY

1 In the developing central nervous system, electrical signaling is thought to rely
2 exclusively on differentiating neurons as they acquire the ability to generate and propagate
3 action potentials. Accordingly, neuroepithelial progenitors (NEPs), which give rise to all
4 neurons and glial cells during development, have been reported to remain electrically passive.
5 Here, we investigated the physiological properties of NEPs at the onset of spontaneous neural
6 activity (SNA) initiating motor behavior in mouse embryonic spinal cord. Using patch-clamp
7 recordings, we discovered that spinal NEPs exhibit spontaneous membrane depolarizations
8 during episodes of SNA. These rhythmic depolarizations exhibited a ventral-to-dorsal
9 gradient with the highest amplitude located in the floor-plate, the ventral-most part of the
10 neuroepithelium. Paired-recordings revealed that NEPs are coupled via gap-junctions and
11 form an electrical syncytium. Although other NEPs were electrically passive, we discovered
12 that floor-plate NEPs generated large $\text{Na}^+/\text{Ca}^{2+}$ action potentials. Unlike in neurons, floor-
13 plate action potentials relied primarily on the activation of voltage-gated T-type calcium
14 channels (TTCCs). *In situ* hybridization showed that all 3 known subtypes of TTCCs are
15 predominantly expressed in the floor-plate. During SNA, we found that acetylcholine released
16 by motoneurons rhythmically triggers floor-plate action potentials by acting through nicotinic
17 acetylcholine receptors. Finally, by expressing the genetically encoded calcium indicator
18 GCaMP6f in the floor plate, we demonstrated that neuroepithelial action potentials are
19 associated with calcium waves and propagate along the entire length of the spinal cord. Our
20 work reveals a novel physiological mechanism to generate and propagate electrical signals
21 across a neural structure independently from neurons.

INTRODUCTION

1 Spontaneous neural activity (SNA) is a hallmark of developing neural networks and
2 has been shown to regulate various developmental processes such as neuronal differentiation,
3 migration, axon guidance and synapse formation¹⁻⁴. Until now, the electrical signals
4 generated during SNA have been found to originate exclusively from differentiating neurons
5 as they acquire the ability to fire action potentials and form their first functional synapses. By
6 contrast, neuroepithelial progenitors (NEPs) – the stem cells of the central nervous system -
7 have never been reported to actively generate or propagate any form of electrical signals,
8 although they can be passively depolarized and/or exhibit calcium events in response to
9 neurotransmitters released by developing neurons⁴⁻¹¹.

10 SNA was initially described in the embryonic spinal cord where it triggers the first
11 motor rhythmic behavior^{12,13}, necessary for the proper development of the neuro-muscular
12 and musculo-skeletal systems of vertebrates¹⁴⁻¹⁶. Spinal SNA starts at midgestation stages -
13 around the 12th day of embryonic development in mice (E12.5) - and is generated
14 autonomously by the ventral spinal cord, before the establishment of sensory inputs from the
15 periphery and descending inputs from the brain. Therefore, SNA is fully preserved in the
16 isolated fetal spinal cord^{17,18}. Although its underlying molecular and cellular mechanisms
17 have yet to be fully resolved, spinal SNA has been shown to involve spontaneous waves of
18 electrical activity depolarizing cholinergic motoneurons as well as GABAergic and
19 glutamatergic interneurons along the length of the ventral spinal cord¹⁶⁻¹⁸. By contrast, the
20 electrophysiological behavior of ventral NEPs remains poorly characterized and they have
21 been assumed to remain electrically passive, as shown in other brain structures. Previous
22 reports indicate that ventral NEPs can exhibit calcium transients during episodes of SNA¹¹
23 and that the ventral-most NEPs located in the floor-plate display T-type-like voltage-gated
24 calcium currents¹⁹. This suggests that spinal NEPs located in the ventral spinal cord could

- 1 generate active electrical signals during SNA. To investigate this possibility, we used patch-
- 2 clamp, optogenetic stimulation and calcium imaging to elucidate the electrophysiological
- 3 behavior of ventral NEPs during spinal SNA.

RESULTS

1 We first examined the electrical activity of ventral NEPs using patch-clamp recordings
2 on a preparation of whole spinal cord from E12.5 mouse embryos in the open-book
3 configuration^{18,20}. This preparation gave us access to all NEPs lining the central canal while
4 preserving SNA generation (**Figure 1A**). Five distinct bilateral domains of NEPs are
5 classically identified along the central canal of the ventral spinal cord: p0, p1, p2, pMN and
6 p3 domains (**Figure 1A**). A sixth unique domain called the floor-plate is located at the base of
7 the spinal cord, joining both sides of the neuroepithelium at the ventral midline. Patch-clamp
8 recordings first revealed that ventral NEPs exhibit spontaneous and rhythmic depolarizations
9 (**Figure 1B & 1G**) at an interval of 183 ± 38 s (N = 32 cells from 23 embryos), similar to the
10 interval previously reported in cholinergic motoneurons during spinal SNA¹⁸. Strikingly, the
11 amplitude of NEP spontaneous depolarization was not uniform but was inversely correlated to
12 the distance of the recorded NEPs from the ventral midline (**Figure 1C-E**), with the largest
13 depolarization observed in floor-plate NEPs. To determine whether NEP depolarizations were
14 temporally correlated between these different regions, we performed paired recordings
15 between the floor-plate and the neighboring p3 domain. Some of the recorded pairs were also
16 filled with neurobiotin and their molecular identity assessed by post-hoc immunostaining
17 against the transcription factors FOXA2 and NKX2.2 (**Figure 1F**), which are respectively
18 expressed in floor-plate NEPs and in the adjacent p3 neuroepithelial progenitor cells²¹. The
19 average distance between pairs of FP and p3 NEPs filled with neurobiotin was 22 ± 7 μ m (N=
20 6 pairs in 6 embryos). Simultaneous pair recordings demonstrated that rhythmic
21 depolarizations were systematically correlated between FP and p3 NEPs (16/16 events; 5
22 pairs from 5 embryos; **Figure 1G**), although depolarizations in the p3 domain exhibited
23 significantly smaller amplitudes ($\text{Amplitude}_{\text{p3}} = 22.2 \pm 5.5$ mV vs $\text{Amplitude}_{\text{FP}} = 55.3 \pm 6.8$
24 mV; **Figure 1G and 1H**) with a slight delay ($\text{Delay}_{[\text{FP-p3}]} = 61.2 \pm 16.4$ ms; **Figure 1G and**

1 **1D)** when compared to the floor-plate. The results suggested that these rhythmic
2 depolarizations propagated from the floor-plate to other NEP domains through direct
3 electrical coupling. Indeed, neuroepithelial progenitors are known to be extensively coupled
4 by gap-junctions at this developmental stage²². We found that neurobiotin filling via patch
5 pipette often labeled several adjacent cells in the p3 domain (14.7 ± 6.3 cells in p3, N= 6 cells
6 from 6 embryos; **Figure 1F**) as well as in the floor-plate (6.6 ± 2.9 cells in FP, N = 10 cells
7 from 10 embryos; **Figure 1F and 2C**). Depolarizing and hyperpolarizing current steps
8 injected in the floor-plate NEP were also systematically associated with a proportional, albeit
9 much smaller, depolarization or hyperpolarization in the NEP located in the p3 domain (Ratio
10 $_{[p3/FP]} = 4.2 \pm 1.5 \%$, N=14 pairs from 14 embryos), showing that they are electrically coupled
11 (**Figure 1J**). Conversely, injecting currents in the NEP located in the p3 domain led to
12 depolarization of the floor-plate cell (Ratio $_{[FP/p3]} = 4.6 \pm 1.7 \%$, N = 18 pairs from 18
13 embryos). Electrical coupling was lost after application of the connexin blocker β 18-GA
14 ($50\mu\text{M}$, paired Wilcoxon test, $p < 0.05$; Control Ratio $_{[p3/FP]} = 2.9 \pm 0.6 \%$ vs β 18-GA Ratio
15 $_{[p3/FP]} = 0.18 \pm 0.07 \%$, N = 5 pairs from 5 embryos; **Figure 1J and 1K**)²³. Taken together, our
16 results indicate that NEP depolarizations observed during episodes of SNA originate from the
17 floor-plate and propagate dorsally into the rest of the neuroepithelium, most likely through
18 gap-junctions.

19 To understand how floor-plate NEPs could exhibit such large and rhythmic
20 depolarizations, we investigated their electrophysiological properties in more detail. Floor-
21 plate cells are easily discriminated from surrounding NEPs by their location at the midline,
22 their thin apical processes and their higher translucency. Identity of the recorded cells were
23 also confirmed by post-hoc immunostaining against the transcription factors FoxA2 and
24 Nkx2.2. All floor-plate cells filled by neurobiotin were found to be FoxA2⁺ and Nkx2.2⁻ (N=
25 10/10 cells from 10 embryos; **Figure 1F and 2C**). Unlike other NEPs that sequentially

1 generate neurons and glial cells during embryonic development²⁴, floor-plate NEPs display
2 glial features at an early embryonic stage and have only been shown to generate
3 ependymocytes²⁵⁻²⁹. Indeed, we found that all FoxA2⁺/Nkx2.2⁻ cells co-expressed the
4 glutamate astrocyte transporter GLAST, a marker for glial progenitors (**Figure S1A-H**)
5 together with the transcription factor Sox2, a marker for neuroepithelial progenitors (**Figure**
6 **S1I-L**). Floor-plate NEPs had an average membrane resistance of $480 \pm 410 \text{ M}\Omega$, an average
7 membrane capacitance of $18.3 \pm 6.6 \text{ pF}$ and an average resting membrane potential of $-64.8 \pm$
8 9.7 mV (N= 24 cells from 24 embryos). More importantly, we found that floor-plate NEPs
9 had the unique ability to generate action potentials (AP) in response to membrane
10 depolarization (threshold = $-37 \pm 3 \text{ mV}$; amplitude = $43.8 \pm 14 \text{ mV}$; N= 15 cells from 15
11 embryos; **Figure 2A**). Unlike most neuronal APs which only rely on voltage-gated sodium
12 channels, floor-plate APs could only be blocked by a combination of TTA-P2 ($3 \mu\text{M}$), a
13 specific T-type calcium channel blocker, and TTX, a sodium channel blocker (**Figure 2A &**
14 **2B**). By contrast, NEPs recorded in the adjacent p3 domain did not exhibit any voltage-
15 dependent responses (N= 24 cells from 24 embryos). During SNA, floor-plate rhythmic
16 depolarizations (amplitude = $54 \pm 15 \text{ mV}$; N = 22 cells from 22 embryos; **Figure 1G & 2D**)
17 exhibited a complex and stereotypical time course (**Figure 2E**). These spontaneous events
18 comprised a small initial depolarization (amplitude = $6.9 \pm 1.0 \text{ mV}$; half-width = $2.8 \pm 1.0 \text{ s}$)
19 followed by an AP exhibiting a biphasic time course comprising first a slow component
20 (amplitude = $18.9 \pm 5.5 \text{ mV}$; half-width = $274 \pm 57 \text{ ms}$) followed by a fast component
21 (amplitude = $42.4 \pm 7.2 \text{ mV}$; half-width = $11.8 \pm 3.2 \text{ ms}$; **Figure 2E**). Identical events could
22 be evoked using electrical stimulation (32 mA , $200 \mu\text{s}$) placed on the midline, 2 mm rostral to
23 the site of recording (**Figure 2F**). Application of the T-type calcium channel blocker TTA-P2
24 was sufficient to fully block the biphasic action potential, while the small initial
25 depolarization remained unaffected (N=8/8 cells from 8 embryos). To confirm and further

1 characterize the expression of T-type calcium channels in the floor-plate, we performed *in situ*
2 hybridization for the 3 known T-type calcium channels (*cacnalg*, *cacnalh*, *cacnali*). We
3 found that mRNA coding for T-type calcium channels were all highly and predominantly
4 expressed in the floor-plate (**Figure 2G & 2H**).

5 Since SNA is dependent on acetylcholine released from motoneurons^{17,18}, we
6 investigated whether rhythmic floor-plate action potentials were triggered by this
7 neurotransmitter. First, we found that spontaneous rhythmic depolarizations disappeared
8 (N=5/5 cells from 5 embryos; **Figure 3A**) in the presence of the nicotinic acetylcholine
9 receptor (nAChRs) antagonists mecamylamine (50 μ M) and d-Tubocurarine (5 μ M). Evoked
10 floor-plate depolarizations were also completely blocked by the same nAChR antagonists
11 (paired Wilcoxon test, $p < 0.01$; N=12 cells from 12 embryos; **Figure 3B & 3C**) as well as by
12 the nAChR antagonist DH β E (5 μ M; N=4 cells from 4 embryos). By contrast, they remained
13 unaffected by antagonists against GABA_A receptors (Gabazine, 3 μ M) and glutamate
14 ionotropic receptors (DL-APV 200 μ M and CNQX 20 μ M), two other neurotransmitters
15 released by spinal interneurons during SNA^{17,18} (N=6 cells from 6 embryos; **Figure 3B &**
16 **3C**). Conversely, exogenous application of acetylcholine (30 μ M) was sufficient to depolarize
17 floor-plate cells (Amplitude = 14.0 ± 2.9 mV; N=9 cells from 9 embryos) and trigger biphasic
18 action potentials (amplitude = 59 ± 7 mV) in a nAChR dependent manner (paired Wilcoxon
19 test, $p < 0.05$; N=6 cells from 6 embryos; **Figure 3D & 3E**). Application of the sodium channel
20 blocker TTX together with antagonists of GABA, glutamate and glycine ionotropic receptors
21 had no effect on the depolarization induced by acetylcholine, suggesting that acetylcholine
22 acts directly onto nAChR expressed by floor-plate cells (**Figure 3D & 3E**). We found that
23 TTX suppressed the fast component of the biphasic action potential triggered by acetylcholine
24 (paired Wilcoxon test, $p < 0.05$; N=6 cells from 6 embryos; **Figure S2**), demonstrating that it
25 resulted from the activation of voltage-gated sodium channels in floor-plate cells. The slow

1 component was blocked by TTA-P2 indicating that this resulted from the activation of T-type
2 calcium channels (paired Wilcoxon test, $p < 0.05$; $N = 6$ cells from 6 embryos; **Figure S2**). To
3 confirm that ACh released from motoneurons triggers floor-plate action potentials, we
4 performed optogenetic stimulation using a transgenic mouse model (ChAT:ChR2-YFP) where
5 channelrhodopsin 2 is expressed in cholinergic motoneurons (**Figure 3F-G**). Optogenetic
6 stimulation of cholinergic neurons was sufficient to depolarize and trigger an action potential
7 in floor-plate cells, which could be fully blocked by nAChR antagonists (paired Wilcoxon
8 test, $p < 0.05$; $N = 5$ cells from 5 embryos ; **Figure 3H & 3I**). To investigate whether
9 cholinergic axons from motoneurons directly contact the floor-plate, we performed
10 immunostaining against vAChT and FoxA2 in coronal sections from E12 HB9:GFP
11 transgenic mice, where GFP is expressed under the promoter for HB9, a homeodomain
12 transcription factor specific of motoneurons at this developmental stage^{30,31}. We found the
13 presence of a few HB9:GFP⁺/vAChT⁺ axons crossing into the ventral midline (**Figure S3**)
14 where floor-plate basal processes are located. Taken together, our results show that floor-plate
15 action potentials are triggered by the activation of nAChR, most likely in response to
16 acetylcholine released by cholinergic motoneurons during SNA.

17 Finally, we examined whether and how floor-plate activity could propagate between
18 floor-plate cells along the rostro-caudal axis of the spinal cord. We expressed the genetically
19 encoded calcium indicator GCaMP6f in floor-plate cells using a conditional transgenic
20 approach (GCaMP6floxP x GLASTCreERT2; see Methods). This was achieved by taking
21 advantage of the fact that floor-plate cells are the first neuroepithelial cells to express the
22 glutamate aspartate transporter (GLAST) during spinal cord development^{25,26,32} (**Figure 4A**
23 and **Figure S1A-H**). Using this approach, we found that midline floor-plate cells expressing
24 GCaMP6f (**Figure 4A**) generated spontaneous calcium waves at an interval of 176 ± 67 s (N
25 = 31 waves from 10 embryos; **Figure 4B & C** and **Video S1**), similar to the interval observed

1 for spontaneous floor-plate action potentials. Spontaneous floor-plate calcium waves
2 propagated along the length of the spinal cord and could propagate in either direction.
3 However, rostral to caudal waves initiated in the cervical area were more frequent (67%,
4 41/61 waves from 10 embryos) than caudal to rostral ones (33%, 20/61 waves) (**Figure S4**
5 and **Video S2**). Average propagation speed of spontaneous rostro-caudal (2.4 ± 0.9 mm/s) and
6 caudo-rostral floor-plate calcium waves (2.1 ± 1.3 mm/s) were not significantly different
7 (Mann-Whitney test, $p=0.12$) and their speed was consistent with the propagation speed
8 estimated for SNA waves in motoneurons^{17,33}.

9 As observed for floor-plate action potentials, calcium waves could also be consistently
10 triggered using electrical stimulation (32 mA, 200 μ s, **Figure 4G, H, J, K** and **Video S3**).
11 Application of the T-type calcium channel blocker TTA-P2 was sufficient to fully block
12 evoked calcium waves (Mann-Whitney test, $p<0.001$, N = 24 waves in 8 embryos; **Figure**
13 **S5A, S5B & Video S4**), even after increasing the duration of stimulation (32 mA, 1200-1600
14 μ s). The initiation and propagation of evoked calcium waves was also blocked by the
15 application of nAChR antagonists (paired Wilcoxon test, $p<0.001$, N = 6 embryos; **Figure 4G**
16 **& Video S5**), as observed with floor-plate action potentials (**Figure 3B**). However, increasing
17 stimulation duration (32 mA, 1200-1600 μ s) allowed the recovery of floor-plate calcium
18 waves, albeit with a significantly decreased propagation speed of 0.8 ± 0.5 mm/s (paired
19 Wilcoxon test, $p<0.001$, N =31 waves in 6 embryos; **Figure 4G & H, Video S5**). Similarly,
20 patch-clamp experiments showed that after increasing the duration of stimulation in the
21 presence of a cocktail of antagonists to nAChR, GABA_AR, GluR and GlyR, the electrical
22 stimulus triggered a delayed biphasic AP without the small initial cholinergic depolarization
23 (N = 4 cells in 4 embryos; **Figure 4I**). This result suggests that, although the initiation and
24 subsequent propagation of floor-plate APs and calcium waves strongly depend on cholinergic
25 transmission, gap-junction coupling between floor-plate cells could also participate in this

1 propagation (**Figure 2C**). We tested this hypothesis by applying the connexin blocker β 18-
2 GA. We found that it either reduced or blocked the propagation of floor-plate calcium waves
3 (paired Wilcoxon test, $p < 0.05$, $N = 18$ waves in 6 embryos; **Figure 4J & K, Video S5**) and
4 action potentials ($N = 4$ cells in 4 embryos; **Figure 4L**) that were evoked in the presence of
5 nAChR antagonists and other neurotransmitter antagonists. Floor-plate calcium waves were
6 also blocked when applying the connexin blocker β 18-GA alone (**Figure S5C & D**),
7 confirming that connexins are necessary for calcium wave propagation. The effect of β 18-GA
8 could result from the blockade of gap-junction and/or connexin hemichannels³⁴.

9 It has previously been shown that the propagation of slow calcium waves between
10 cortical progenitors occurs via ATP signaling acting through high-affinity and slow-acting
11 metabotropic P2YR⁹. By contrast, we found here that the propagation speed of floor plate
12 cells is at least a hundred times faster and primarily rely on nAChRs, T-type calcium channels
13 and connexins. However, there is evidence that ependymal cells derived from the floor-plate
14 express low-affinity but fast-acting ionotropic P2X7 receptors to ATP³⁵. We found that floor
15 plate cells only responded to ATP at concentrations above 1 mM, suggesting that they only
16 expressed low-affinity P2X7R (**Figure 5A & 5B**). Indeed, ATP responses were blocked by
17 the competitive antagonist A438079, specific to the low-affinity P2X7R³⁶ (**Figure 5C & D**).
18 We thus investigated whether P2X7R could actively participate in the propagation of floor
19 plate calcium waves. We found that A438079 significantly reduced the propagation speed of
20 evoked floor-plate calcium waves (**Figure 5E & F**). Since P2X7R have been found to be
21 predominantly expressed by ependymal cells in the postnatal nervous system^{37,38}, our results
22 are consistent with the ependymal nature of floor plate cells.

23 Altogether, our results show that the floor-plate of the neuroepithelium possesses the
24 intrinsic ability to generate and propagate electrical signals along the embryonic spinal cord

- 1 via the combined activity of nAChRs, voltage-gated T-type calcium channels, voltage-gated
- 2 sodium channels, connexins and P2X7Rs.

DISCUSSION

1 Although it has been shown previously that a subpopulation of glial progenitors
2 expressing the proteoglycan NG2 can be induced to generate TTX-sensitive sodium spikes in
3 the postnatal brain^{39,40}, the neuroepithelial floor-plate is the first example of a non-neuronal
4 structure able to both spontaneously generate and propagate action potentials in the
5 developing central nervous system. Until now, these abilities were considered to be exclusive
6 features of differentiating neurons. Accordingly, the ability to generate and propagate action
7 potentials has been routinely used as a functional assay to confirm the neuronal phenotype of
8 cells during development. In this context, although $\text{Ca}^{2+}/\text{Na}^{+}$ biphasic action potentials
9 relying on T-type calcium channels are not commonly observed in neurons, their previous
10 discovery in neural cells in the *Xenopus* embryonic spinal cord⁴¹ and in the mouse fetal
11 hindbrain⁴² has been interpreted as an early and transient form of excitability in developing
12 neurons. Instead, we found here that $\text{Ca}^{2+}/\text{Na}^{+}$ biphasic action potentials are generated in the
13 mouse spinal cord by gliogenic neuroepithelial progenitors located in the floor-plate and that
14 they propagate dorsally in other neuroepithelial cells also engaged into a gliogenic program at
15 this stage²⁵.

16 It has previously be shown that T-type voltage-gated currents can be elicited in floor
17 plate cells at later embryonic and perinatal stages in rats¹⁹. However, only modest
18 depolarizations (10-15 mV) could be elicited in this study when triggering T-type calcium
19 channels by current injection and it was not reported that floor-plate cells could actually
20 generate action potentials. This discrepancy may be explained by the use of coronal slices
21 and/or the absence of voltage-gated sodium channels in floor-plate cells of rats. Indeed, our
22 experiments suggest that the generation of full-fledged action potentials involves the
23 simultaneous activation of numerous cells along the rostro-caudal axis of the floor-plate, as

1 observed during our calcium imaging experiments. The use of coronal sections may thus have
2 greatly impeded the ability of floor-plate cells to coordinate their activation via gap-junction
3 coupling. The absence of voltage-gated sodium channels would also have impeded the ability
4 of floor-plate cells to generate action potentials in rats. This lack of voltage-gated sodium
5 channels could be the result of the differences in species and/or developmental stages⁴³.

6 We found that spontaneous floor-plate calcium waves are initiated at different rostro-
7 caudal location and can propagate in both directions. 2 thirds of the waves are initiated in the
8 cervical area from where they propagate both caudally into the rest of the spinal cord and
9 rostrally towards the brainstem. This latter observation suggests that floor-plate calcium
10 waves may not be restricted to the spinal cord. The last third of the waves propagate in a
11 caudal-to-rostral direction – both at cervical and thoracic level – originating from a yet to be
12 determined caudal location(s). This proportion of waves with a cervical and caudal origin is
13 similar to that previously reported for waves of muscle contractions observed *in vivo* in mouse
14 embryos at the same developmental stage¹³. This suggest that floor-plate activity is likely to
15 be tightly correlated with the first pattern of motoneuron activity and muscle contraction
16 observed *in vivo*.

17 From an evolutionary point of view, the discovery of an epithelial electrical
18 conduction system in the vertebrate fetal CNS should not come as a complete surprise.
19 Indeed, epithelial and syncytial conduction systems are commonly observed in non-bilaterian
20 animals exhibiting either a “primitive” nervous system, like jellyfish and comb jellies, or in
21 those lacking a nervous system like the glass sponges^{44–47}. It has been proposed that the
22 modern neuron-based nervous systems of bilaterians using axonal/chemical synaptic
23 transmission evolved from such epithelial conduction systems coupled electrically by gap-
24 junctions⁴⁸. This evolution would also have been accompanied by a switch from slow voltage-
25 gated calcium channels - inherited from our protist ancestors and still found in the modern

1 paramecium⁴⁹ - to fast voltage-gated sodium channels⁴⁸. Our discovery of a “primitive”
2 electrical conduction system in the neuroepithelium that gives rise to the “modern” nervous
3 system of vertebrates during fetal development could therefore be interpreted as strong
4 evidence supporting this evolutionary scenario.

5 One may also notice the functional similitude existing between the floor-plate of His
6 and the heart electrical conduction system known as the bundle of His. The bundle of His was
7 discovered by the anatomist and cardiologist Wilhem His, Jr. in 1893⁵⁰, five year after his
8 father had described the floor-plate⁵¹. Both structures act as electrical conduction systems
9 connected by gap junctions able to propagate electrical signals across their respective organs.
10 By showing that these structures not only share the same name but also share similar
11 functional properties, our work reveals that the nervous and cardiac physiologies of
12 vertebrates is more deeply related than previously thought.

13 Further work will be needed to decipher what function(s) are fulfilled by this non-
14 neuronal electrical conduction system. Although the electrical excitability of floor-plate cells
15 could only be a vestigial property inherited from non-bilaterian ancestors, it is more likely
16 playing a significant role in the development of the spinal cord. Indeed, the energetic cost of
17 generating and propagating electrical signals⁵² as well as the redundancy of T-type channel
18 subtypes expressed in the floor-plate point towards having a conserved and significant
19 function. One hypothesis is that floor-plate excitability regulates and coordinates the release
20 of the morphogen Sonic Hedgehog (Shh), required for neural tube patterning, or the
21 expression of axon guidance molecules such as Slit and Netrin. Indeed, recent studies have
22 shown unexpected interactions between early calcium electrical excitability and Shh
23 signalling in the *Xenopus* spinal cord^{53,54}. Moreover, T-type channel mutants have recently
24 been identified in a forward genetic screen for neural tube defects in *Ciona intestinalis*⁵⁵,
25 suggesting that the electrical gradient established by the floor-plate in the ventral

1 neuroepithelium could represent a novel morphogenetic mechanism. An additional and non-
2 exclusive hypothesis is that floor-plate action potentials initiated by acetylcholine released by
3 motoneurons during SNA act as a feedback signal to promote the switch from neurogenesis to
4 gliogenesis in ventral neuroepithelial progenitors^{25,26} since both events occur around the same
5 developmental stage.

6 In conclusion, our discovery of the floor-plate of His acting as a non-neuronal
7 electrical conduction system could profoundly change how we conceive of the origin, extent
8 and role of electrical signals during the development of the central nervous system of
9 vertebrates.

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AUTHOR CONTRIBUTIONS

K.H.A, G.O and H.L.C. performed and analysed the electrophysiological recordings. K.H. K.H.A, G.O, S.C. and B.L.B. performed and analysed the immunohistochemistry experiments. A.L., K.H.A, L.C. and E.H. performed and analysed calcium imaging experiments. K.H.A, J.B. and A.C. performed and analysed optogenetic experiments. NE. C.S., KK.H.A. and E.H. performed and analysed *in situ* hybridization experiments. C.M. and E.B. managed the animal colony, performed animal genotyping and contributed to immunohistochemistry experiments. J-M.M. supervised the project and wrote the manuscript with P.L., C.S. and E.H..

DECLARATION OF INTERESTS

The authors declare no competing interests.

MAIN-TEXT FIGURE LEGENDS

Figure 1. Neuroepithelial progenitors are spontaneously depolarized during SNA following a ventro-dorsal gradient.

A, Location of the ventral neuroepithelial progenitor domains (p2-p0, pMN, p3, floor-plate) in a coronal view and in open-book configuration. Domains were determined by their distance from the midline (p3 = 30-70 μm ; pMN = 70-110 μm ; p0-p2 = 110-200 μm). **B**, Spontaneous rhythmic depolarizations recorded in the pMN domain. **C**, Spontaneous depolarizations recorded separately in the p0-p2, pMN and p3 domains. **D**, Average amplitude of spontaneous depolarizations recorded in different NEP domains. **E**, Drawing illustrating the depolarization gradient observed in the ventral neuroepithelium. **F**, Pair of FP and p3 NEPs filled with neurobiotin and stained against FoxA2 and Nkx2.2. Recorded cells are indicated by yellow arrowheads. **G**, Spontaneous rhythmic depolarizations simultaneously recorded in the FP and a p3 domain and close up of a simultaneous depolarization. **H**, Amplitude of depolarizations recorded simultaneously in the p3 and floor-plate NEPs (N= 16 depolarizations; 5 pairs from 5 embryos). **I**, Delay between the peak of depolarizations in p3 and floor-plate NEPs. **J**, Current injection (top traces) in FP or p3 NEPs show electrical symmetrical electrical coupling (bottom traces) which is lost after application of the connexin blocker β 18-GA. **K**, Effect of β 18-GA on electrical coupling (paired Wilcoxon test; * $p < 0.05$; N= 5 pairs from 5 embryos).

Figure 2. Floor-plate neuroepithelial progenitors generate rhythmic action potentials during spinal SNA.

A, Responses of a FP cell to current steps in control conditions, after application of the T-type calcium channel blocker TTA-P2 (3 μM) and subsequent addition of the sodium channel blocker TTX (1 μM). **B**, Maximal amplitude of the spike triggered in each pharmacological

condition (N = 15 cells from 15 embryos). **C**, FP cell (yellow asterisk) filled with neurobiotin and immuno-stained against FoxA2 and Nkx2.2. **D**, Spontaneous rhythmic depolarization recorded in a FP cells. **E**, Magnified view of a spontaneous depolarization revealing the presence of three distinct components: a small initial depolarization (1) and a biphasic action potential (AP, grey area magnified in insert) composed of a slow (2) and fast component (3). **F**, Depolarization and biphasic AP evoked by electrical stimulation. Application of TTA-P2 (3 μ M) blocked the biphasic AP while the small initial depolarization remained unaffected (N = 8 cells from 8 embryos, paired Wilcoxon text; ** p<0.01). **G**, *In situ* hybridization showing the location of mRNA transcripts for the 3 known sub-types of T-type channels (*cacnalg*, *cacnalh*, *cacnali*) in coronal sections of E12.5 mouse embryos. **H**, *In situ* hybridization showing location of mRNA transcripts for *cacnalg* in open-book configuration. See also Figure S1 and S2.

Figure 3. Floor-plate biphasic action potentials are triggered by the activation of nicotinic acetylcholine receptors.

A, Rhythmic spontaneous floor-plate action potentials are blocked after addition of the nAChR antagonists Mecamylamine (50 μ M) and d-Tubocurarine (5 μ M). **B**, FP action potential evoked by electrical stimulation in control condition, after addition of the nAChR antagonists and after addition of antagonists against ionotropic receptor for GABA (Gabazine 3 μ M) and glutamate (DL-APV 200 μ M and CNQX 20 μ M). **C**, Total depolarization amplitude in control conditions and after addition of nAChR antagonists (N = 12 cells from 12 embryos, paired Wilcoxon text, ** = p<0.01) as well as in presence of antagonists against ionotropic receptor for GABA and glutamate (N = 6 cells from 6 embryos; n.s. = non-significant) **D**, FP action potential can be evoked by local application of 30 μ M ACh, even in the presence of TTX (1 μ M) and antagonists to AMPA/Kainate glutamate receptor (CNQX 10

μM), NMDA glutamate receptor (DL-APV 200 μM), GABA_A receptor (Gabazine 3 μM) and glycine receptor (strychnine 1 μM). Floor-plate action potential evoked by ACh were blocked by the addition of nAChR antagonists. **E**, Slow depolarization amplitude induced by 30 μM Ach (arrows in d) in each condition (N = 6 cells from 6 embryos, paired Wilcoxon text; ** $p < 0.01$, n.s. non-significant). **F**, Coronal section from a ChAT:ChR2-YFP mouse embryo showing the expression of Channelrhodopsin2-YFP fusion protein in cholinergic motoneurons located in the ventro-dorsal horns and labelled with the vesicular acetylcholine transporter vAChT. All cell nuclei were labelled using DAPI. **G**, Action potential triggered in a ChAT:ChR2-YFP⁺ motoneuron in response to a 300 ms blue light stimulus delivered through a optic fiber cannula (470 nm, 400 μm -diameter cannula, output power: 9-18 mW). **H**, FP response recorded in a ChAT:ChR2-YFP⁺ fetal spinal cord showing how a 4 s blue light stimulus (470 nm, 400 μm -diameter cannula, output power: 9-18 mW) could evoke a slow cholinergic depolarization and trigger a biphasic action potential that were blocked by the addition of nAChR antagonists. **I**, Total depolarization amplitude evoked by a blue light stimulus in control conditions and after application of nAChR antagonists (N = 5 cells from 5 embryos; paired Wilcoxon text; * $p < 0.05$). See also Figure S2 and S3.

Figure 4. Floor-plate action potentials are associated with calcium waves and propagate along the length of the spinal cord.

A, GCaMP6 expression in FoxA2⁺/Nkx2.2⁻ cells. **B**, Rhythmic spontaneous calcium events are observed in FP cells expressing GCaMP6. Asterisk indicates the event represented in d. **C**, Intervals between spontaneous calcium events (Mann-whitney test; n.s. non-significant). **D**, Rostro-caudal propagation of calcium waves in FP cells visualized in the thoracic region. Dotted boxes represent the region of interest used to calculate propagation speeds. **E**, Variation of intensity of fluorescence (dF/F) over time in the 3 regions of interest indicated in

E. Dotted lines mark the onset of the calcium signals in region i (left) and iii (right). **F**, Propagation speed of FP calcium waves (Mann-whitney test; n.s. = non-significant). **G**, Color-coded calcium waves evoked by rostral electrical stimulation (32 mA, 200 μ s) in control condition, after application of antagonists to nAChR and subsequent increase of stimulation intensity (32 mA, 1200 μ s). Time after stimulation is coded in distinct colors as indicated in the scale at the bottom. **H**, Propagation speed of evoked FP calcium waves in different pharmacological conditions (Mann-whitney test; *** $p < 0.001$). **I**, Evoked depolarization in control condition, after application of a cocktail of antagonists to nAChR, GABA_AR, iGluR and GlyR and subsequent increase of stimulation intensity. **J**, Color-coded calcium waves evoked by increased electrical stimulation (32 mA, 1200 μ s) in the presence of antagonists to nAChR and after addition of the connexin blocker β 18-GA. **K**, Propagation speed before and after addition of antagonists to nAChR and gap junction blockers (Mann-whitney test; * $p < 0.05$). **L**, Evoked depolarization in the presence of a cocktail of antagonists to nAChR, GABA_AR, iGluR and GlyR and after subsequent addition of the connexin blocker β 18-GA. See also Figure S4, S5 and Video S1-S5.

Figure 5. P2X7R are expressed by floor-plate cells and participate to calcium wave propagation.

A, Responses of a floor-plate cell to different concentration of ATP. **B**, Slow depolarization amplitude (arrows in a) induced by different concentration of ATP (N = 7 cells from 7 embryos, paired Wilcoxon test; n.s. non-significant). **C**, FP response to 3 mM ATP in control conditions, in the presence of a P2X7R antagonist (30 μ M of A438079) and after washout. **D**, Slow depolarization amplitude (arrows in c) induced by 3 mM ATP application in each condition (N = 6 cells from 6 embryos, paired Wilcoxon test; *** $p < 0.001$). **e**, Color-coded calcium waves evoked by rostral electrical stimulation (32 mA, 200 μ s) in control condition,

after application of a P2X7R antagonist (30 μ M of A438079) and after washout. **f**, Propagation speed of evoked FP calcium waves in control conditions, after addition of P2X7R antagonist and washout (Mann-whitney test; * $p < 0.05$, *** $p < 0.001$). See also Figure S5.

1 **STAR METHODS**

2 **Resource availability**

3 *Lead contact*

4 Further information and requests for resources and reagents should be directed to and will be
5 fulfilled by the lead contact, jean-Marie Mangin (jean-marie.mangin@inserm.fr)

6

7 *Materials Availability*

8 This study did not generate new unique reagents.

9

10 *Data and code availability*

- 11 • This study did not generate any unique dataset.
- 12 • This study did not generate any original code.
- 13 • All data needed to evaluate the conclusions are present in this paper and/or the
14 supplemental information. Any additional information required to reanalyze the data
15 reported in this paper is available from the lead contact upon request.

16

17 **Experimental models and subject details**

18 All experiments were performed in accordance with European Community guiding
19 principles on the care and use of animals (86/609/CEE, CE Off J no. L358, 18 December,
20 1986), French decree no. 97/748 of October 19, 1987 (J Off République Française, 20
21 October, 1987, pp. 12245–12248) and recommendations from the CNRS. All efforts were
22 made to minimize the suffering of animals. In order to maximize litter size, experiments were
23 performed on E12.5 mouse embryos obtained by crossing C57BL/6 males with females from
24 a Swiss strain. For optogenetic stimulation of cholinergic motoneurons, we used C57BL/6

1 breeding male mice expressing a BAC transgene containing a Channelrhodopsin2-EYFP
2 sequence driven by a Choline Acetyl Transferase (ChAT) promoter (ChAT-ChR2–EYFP, The
3 Jackson Laboratory, USA, <https://www.jax.org/strain/014546>)⁵⁶. We used C57BL/6 mice
4 expressing a Hb9-GFP transgene³⁰ to visualize motoneuron axons crossing into the floor-
5 plate. For calcium imaging of floor-plate cells, we used C57BL/6 breeding male mice
6 expressing a genetically encoded floxed Calcium indicator GCaMP6f (Ai95, The Jackson
7 Laboratory, USA, <https://www.jax.org/strain/028865>), crossed with Swiss female mice
8 expressing a tamoxifen-inducible Cre recombinase under the control of the glutamate
9 aspartate transporter promoter (GLASTCreERT2, gift from Dr Frank Pfrieder)⁵⁷. Cre
10 recombination was induced by oral gavage of pregnant mice with 5 mg of tamoxifen (Sigma-
11 Aldrich, France, T-5648) at embryonic days E10.5 and E11.5⁵⁸. Tamoxifen was dissolved in
12 corn oil at 37°C for several hours to prepare 20 mg/mL solution⁵⁹.

13

14 **Method details**

15 *In situ RNA hybridization staining*

16 Mouse embryos were fixed in 3.7% paraformaldehyde (PFA) in PBS overnight at 4°C.
17 Tissues were then sectioned at 60–80 µm using a vibratome (Microm, France). In all
18 experiments, sections were performed at the cervico/brachial level. In situ hybridization (ISH)
19 was performed on vibratome sections automatically (InsituPro, Intavis, Germany) using the
20 whole-mount ISH as previously reported⁶⁰. Digoxigenin (DIG)-labeled antisense RNA probes
21 were synthesized using T3 polymerase from the following I.M.A.G.E. Consortium cDNA
22 Clones⁶¹: *cacnalg*, (ID 6410519); *cacnalh* (ID 6820407) and *cacnali* (ID 6825802). RNA
23 labeled probes were detected by an alkaline-phosphatase coupled antibody (Roche
24 Diagnostics), and NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate)
25 were used as a chromogenic substrate for the alkaline phosphatase (Boehringer Mannheim,

1 Germany). Stained sections were digitized and analyzed using Zeiss acquisition software
2 (Zeiss, Germany).

3

4 ***Isolated embryonic spinal cord preparation***

5 E12.5 embryonic mouse spinal cords were dissected as previously described^{18,20}.
6 Briefly, pregnant mice were killed using a lethal dose of CO₂ followed by cervical
7 dislocation. Mice embryos of both sexes were dissected and whole spinal cord were left to
8 recover for at least 30 min at 32°C in artificial cerebrospinal fluid (ACSF) containing 125 mM
9 NaCl, 25mM NaHCO₃, 11 mM Glucose, 3 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, and 1 mM
10 NaH₂PO₄ (pH 7.3; 307 mOsm) continuously bubbled with 95% O₂ – 5% CO₂ gas, before
11 being recorded or imaged. For ChAT-ChR2-EYFP and GCaMP6floxP x GLASTCreERT2
12 transgenic embryos, their tails were cut and observed under a fluorescent stereo-microscope
13 (Leica MZ FLIII) to identify positive embryos for EYFP and GCaMP6, respectively.

14

15 ***Patch-clamp recordings***

16 Isolated spinal cords were placed in open-book configuration in a recording chamber
17 under a nylon holding grid and continuously perfused (2 ml/min) at room temperature with
18 the oxygenated ACSF described above. In contrast to previous studies^{18,20}, the orientation of
19 open-book spinal cord was inverted so that the neuroepithelial layer was facing upward.
20 Whole-cell voltage-clamp recordings of floor-plate cells and other neuroepithelial progenitors
21 were performed under direct visualization using an infrared-sensitive CCD video camera. In
22 addition to their location at the midline, floor-plate cells were distinguished from surrounding
23 neuroepithelial progenitors based on their smaller apical process and their higher translucency
24 when using brightfield microscopy. To confirm their identity, some recorded cells were filled
25 with neurobiotin (1 mg/ml, Vector labs, USA) and revealed in combination with FoxA2 and

1 Nkx2.2 immunostaining. Whole-cell patch-clamp electrodes were pulled from thick-wall
2 borosilicate glass using a Brown-Flaming puller (P97, Sutter Instrument, USA). The tip of the
3 electrode was fire-polished using a microforge (MF-830, Narishige, Japan). Patch-clamp
4 electrodes had tip resistances comprised between 4 and 7 M Ω . Electrodes were filled with an
5 intracellular solution containing either (in mM): 96.4 K⁺-methanesulfonate, 33.6 KCl, 4
6 MgCl₂, 4 Na₂ATP, 0.3 Na₃GTP, 10 EGTA, and 10 HEPES or 100 K⁺-gluconate, 34 KCl, 0.5
7 EGTA, 10 HEPES, 4 MgCl₂, 4 Na₂ATP (pH 7.2; 290 mOsm). In both solutions, the
8 theoretical equilibrium potential for chloride ions (E_{Cl}) was -30 mV, while cation reversal
9 potential (E_{cat}) was 0 mV. Signals were recorded with a low-pass filtered at 4 kHz using a
10 Multiclamp 700B amplifier (Molecular Devices, USA) and digitized at 20 kHz using a Digi-
11 data 1440A interface coupled to pClamp 10.5 software (Molecular Devices, USA) running on
12 a PC computer. Cells exhibiting an average resting membrane potential above -50 mV (I=0
13 pA) were discarded from further analysis.

14

15 *Electrical and optogenetic stimulation*

16 For electrical stimulations experiments, we used bipolar platinum electrodes (FHC,
17 USA) placed 2 mm rostrally to the recording site onto the midline. For optogenetic
18 stimulation of ChAT-ChR2 we used a 400 μ m diameter optical fiber coupled with a 470 LED
19 (Thorlabs, Germany) and centered onto the recording site. Electrical and optogenetic
20 stimulations were performed at a low frequency (0.005 Hz, 1 stimulation every 180 s) in order
21 to evoke a stable cholinergic response.

22

23 *Calcium imaging*

24 Isolated spinal cords were placed in open-book configuration under a nylon holding
25 grid in a recording chamber and continuously perfused (2mL/min) with the same oxygenated

1 ACSF described above. Recombined fluorescent cells were observed under an
2 epifluorescence microscope BX51W1 (Olympus) with a 470-515nm fluorescent excitation
3 light to confirm GCaMP6f expression in the floor-plate. Time-lapse imaging was performed
4 using either an Orca Flash4.0 (Hamamatsu, Japan) or Orca 03G (Hamamatsu, Japan) camera
5 and recorded using the HC Image Live software (Hamamatsu, Japan) at an acquisition
6 frequency of 5 or 10Hz with an exposure time of 200 ms and 100 ms, respectively. Images
7 were encoded using a 256x256 or 512x512 pixel resolution and 16-bit grey scale. Time-lapse
8 images were analyzed using the ImageJ software (National Institute of Health, USA). Signals
9 were either extracted by subtracting each frame from the one following it (Figure 4g & 4i) or
10 by subtracting an average of 10 frames preceding the onset of an event (Figure 4d,
11 Supplementary Figure 3 and Supplementary videos 1-5). To measure the propagation speed,
12 we pinpointed three regions of interest on the midline and measured the delays necessary to
13 reach half-peak between each region of interest and averaged them. Intensity of signals (dF/F)
14 were measured by dividing the peak amplitude of the events by the baseline value in raw
15 images.

16

17 *Pharmacological agents*

18 In most experiments, drugs were applied via the bath perfusion system. Typically, the
19 effect of drugs reached equilibrium within 3 to 5 minutes. For acetylcholine application, we
20 used a 0.5 mm diameter quartz tubing positioned 50 μ m away from the recording area under
21 direct visual control. Typically, response to acetylcholine reached equilibrium a few seconds
22 (6 sec) after solution switch. The quartz tubing was connected using a manifold to six
23 solenoid valves linked with six reservoirs. Solutions were gravity-fed into the quartz tubing.
24 Drug application was controlled using a VC-8 valve controller (Warner Instruments, USA).
25 At least 3 events were recorded in each condition after waiting 5 min for drugs to act. The

1 following pharmacological agents were used: Acetylcholine chloride (30 μ M, Sigma-Aldrich,
2 Germany), TTX (1 μ M, Alomone Labs, Israel), gabazine (3 μ M, Tocris, USA), CNQX
3 Disodium (20 μ M, Tocris, USA), DL-APV (200 μ M, Tocris, USA), Mecamylamine
4 hydrochloride (100 μ M, Tocris, USA), D-tubocurarine chloride (10 μ M, Tocris, USA), 18- β
5 glycyrrhetic acid (50 μ M, Sigma-Aldrich, Germany), TTA-P2 (3 μ M, Alomone, Israel),
6 A438079 (30 μ M, Tocris, USA) and DH β E (5 μ M, Tocris, USA). All drugs were dissolved at
7 final concentration in ACSF.

8

9 *Immunohistochemistry*

10 E12.5 embryos or whole embryonic spinal cord were immersion-fixed in phosphate
11 buffer saline (PBS) with 4% PFA freshly prepared in PBS (pH 7.4) for 1 h at 4°C. Embryos
12 were then rinsed with PBS and cryoprotected in PBS-15% sucrose at 4°C for 24 h and then in
13 PBS-30% sucrose at 4°C for 24 h. Embryos were embedded in OCT medium (VWR, USA)
14 and quickly frozen. Serial sections 20 μ m thick were collected onto slides using a cryostat
15 (Leica, Wetzlar, Germany) and stored at -80°C until immunohistochemical studies. For
16 immunostaining, slides and whole spinal cord were washed in PBS, incubated in NH₄Cl (50
17 mM) diluted in PBS for 20 min and then permeabilized for 30 min in a blocking solution
18 (10% goat serum in PBS) with 0.2% Triton X-100. They were incubated overnight at 4°C
19 with the primary antibodies, which were diluted in the 0.2% Triton X-100 blocking solution.
20 Slides or whole spinal cords were then washed in PBS and incubated for 2h at room
21 temperature in the appropriate secondary antibodies (diluted at 1/1000 in the 0.2% Triton X-
22 100 blocking solution). The following primary antibodies were used: a mouse monoclonal
23 anti-HNF-3 β /FoxA2 (H-4) antibody sc-374376 (1:300, Santa Cruz Biotechnology, USA), a
24 mouse monoclonal anti-Nkx2.2 antibody 74.5A5 (1:600, Developmental Studies Hybridoma
25 Bank, USA), a guinea-pig polyclonal anti-EAAT1/GLAST antibody 250 114 (1:1000,

1 Synaptic System, Germany), a mouse monoclonal anti-Sox2 (E-4) antibody sc-365823 (1:500,
2 Santa Cruz Biotechnology, USA), a rabbit polyclonal anti-vAChT antibody 139 103 (1:1000,
3 Synaptic System, Germany) and a rabbit polyclonal anti-GFP antibody A-11122 (1:1000,
4 Thermo Fisher Scientific, USA). Alexa Fluor 405-, 488-, or 594- and 647-conjugated
5 secondary antibodies (1/1000; Invitrogen, USA) were used to detect mouse monoclonal,
6 guinea pig and rabbit polyclonal primary antibodies. After washing in PBS, slides or whole
7 spinal cords were dried and mounted in Mowiol medium (Millipore, USA).

8

9 *Confocal microscopy*

10 Once mounted, immunostained sections were imaged using a SP5 confocal
11 microscope (Leica, Germany) using a 20X oil-immersion objective with a numerical aperture
12 of 1.25, as well as with a 63X oil-immersion objective with a numerical aperture of 1.32 and a
13 1X digital zoom magnification. Serial optical sections were acquired with a Z-step of 3 μm
14 (20X) and 0.78 μm (63X). Images (1024 X 1024; 8 to 16 bit grayscale) were acquired using
15 Leica software LAS-AF and analyzed using ImageJ (National Institutes of Health, USA).
16 Colocalization with neurobiotin staining was assessed in three axes using a single confocal
17 slice and x and y orthogonal views of the stack (ImageJ 1.5). To quantify the number of cells
18 filled by neurobiotin after patch-clamp recording in the floor-plate and/or p3 domain,
19 neurobiotin staining was combined with antibody staining against FoxA2, Nkx2.2 and DAPI.
20 FoxA2 and Nkx2.2 are respectively labelling the nucleus of NEPs in the floor-plate and the p3
21 domain. No neurobiotin staining was found to colocalize with a DAPI stained nucleus without
22 being also positive for either FoxA2 or Nkx2.2. DAPI is not shown for clarity in merged
23 confocal images.

24

25 **Quantification and statistical analysis**

1 Each protocol was triplicated in each cell (patch-clamp experiments) and embryos (calcium
2 imaging experiments). Statistical comparisons for electrophysiological recordings and
3 calcium imaging parameters were conducted using GraphPad Prism 8 (GraphPad Software,
4 USA) by performing non-parametric Wilcoxon-Mann-Whitney U tests (calcium imaging
5 experiments) and Wilcoxon signed rank test for paired samples (patch-clamp experiments).
6 We determined that statistical significance was obtained when the one-tailed p-value was
7 <0.05.

8

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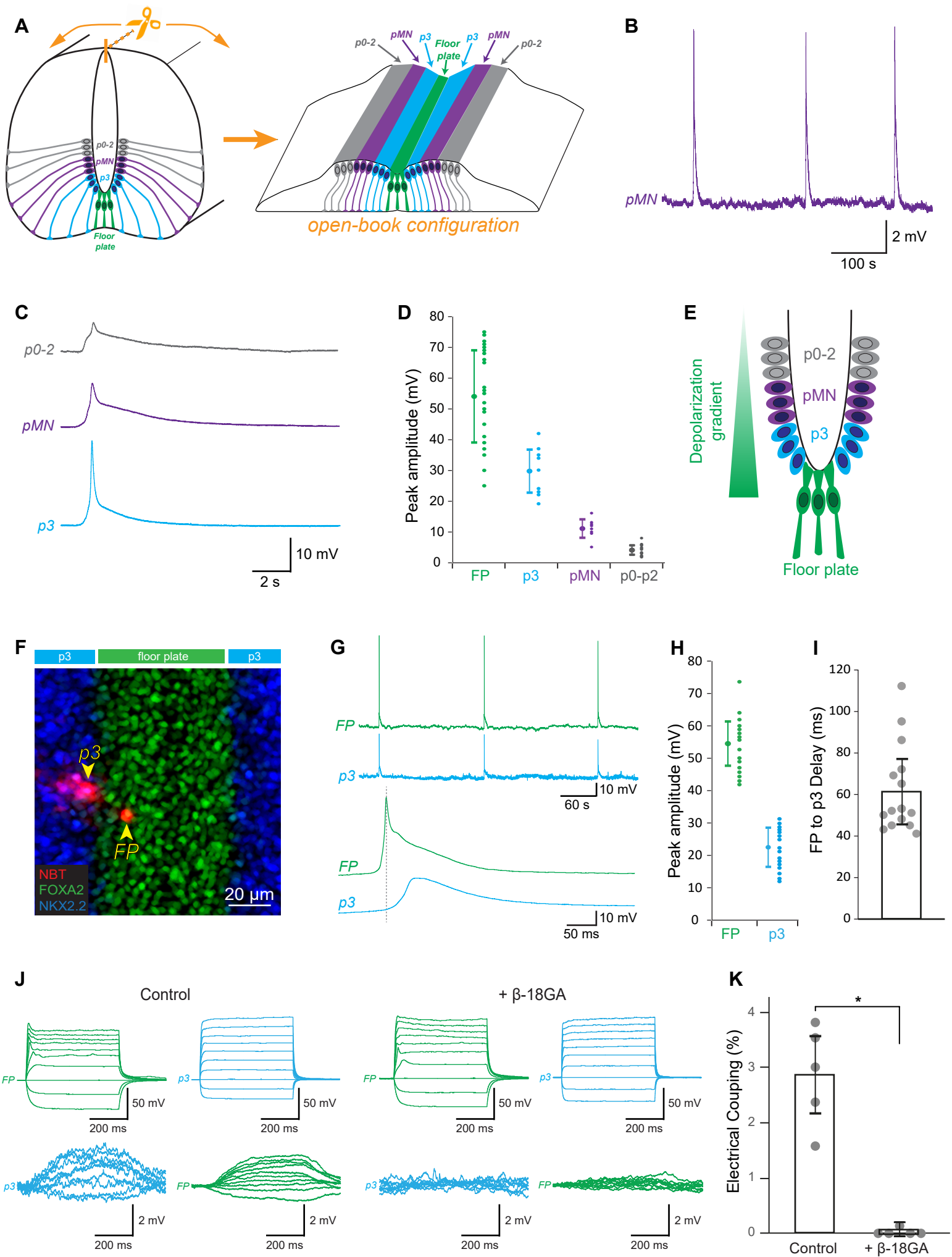
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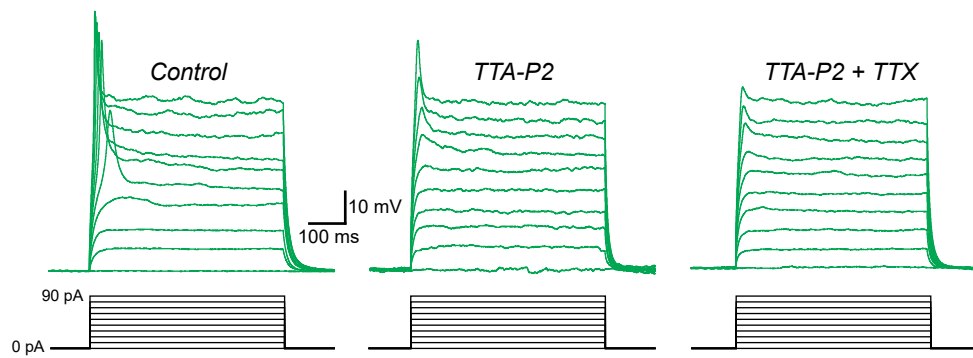
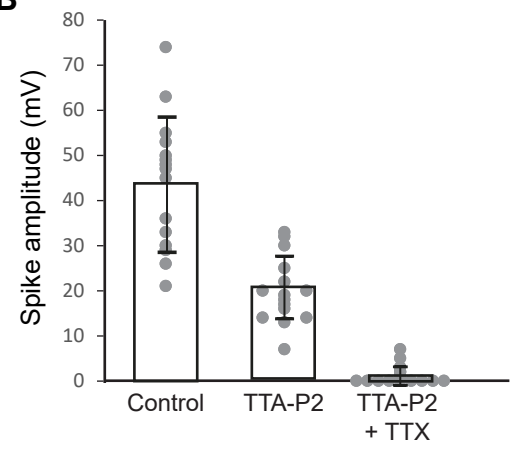
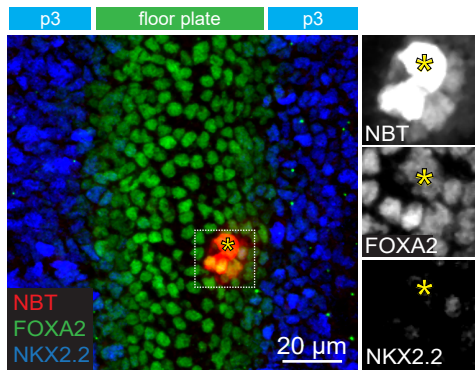
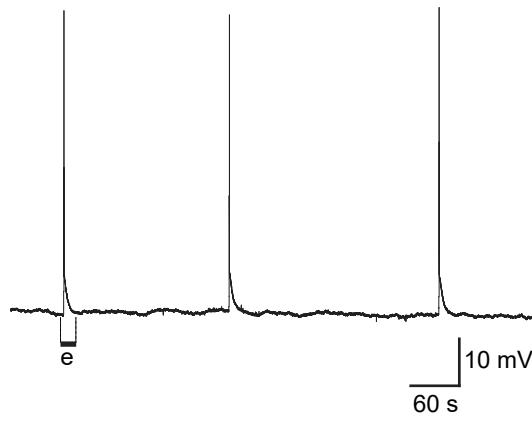
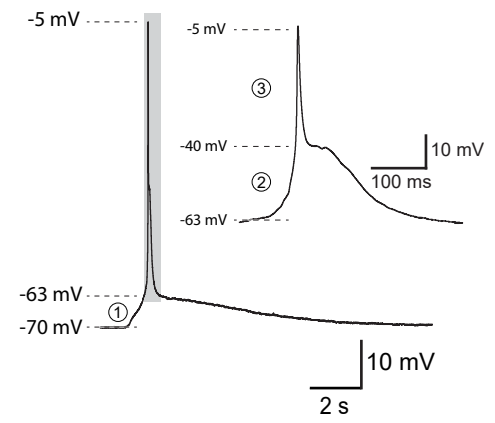
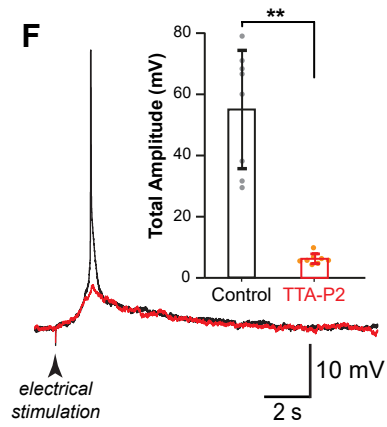
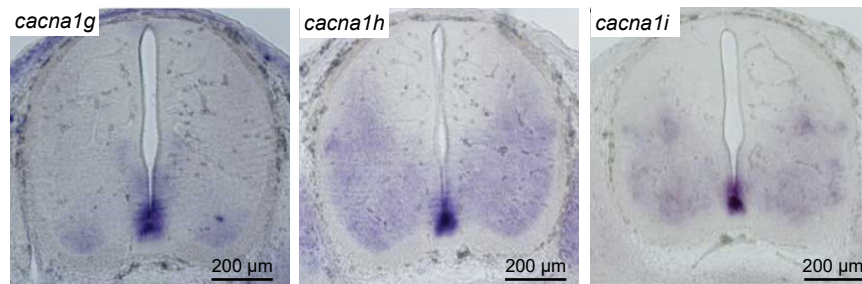
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