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► To cite this version:

Tony Barbay, Emilie Pecchi, Myriam Ducrocq, Nathalie Rouach, Frédéric Brocard, et al.. Astrocytes regulate locomotion by orchestrating neuronal rhythmicity in the spinal network via potassium clearance. 2022. hal-03807834

HAL Id: hal-03807834

<https://hal.science/hal-03807834v1>

Preprint submitted on 10 Oct 2022

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1 **Astrocytes regulate locomotion**
2 **by orchestrating neuronal rhythmicity in the spinal network**
3 **via potassium clearance**
4

5 by

6
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36 Number of figures: 7

37 Supplementary information: 7 figures

38
39 Running Title: Astrocytic modulation of spinal rhythmicity
40

41 Keywords: Locomotion, Spinal cord, Astrocytes, Neuronal Oscillations, Potassium uptake,
42 Kir4.1.
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1 **SUMMARY**

2 Neuronal rhythmogenesis in the spinal cord is correlated with variations in extracellular K^+ levels
3 ($[K^+]_e$). Astrocytes play important role in $[K^+]_e$ homeostasis and compute neuronal information. Yet it
4 is unclear how neuronal oscillations are regulated by astrocytic K^+ homeostasis. Here we identify the
5 astrocytic inward-rectifying K^+ channel Kir4.1 (a.k.a. *Kcnj10*) as a key molecular player for neuronal
6 rhythmicity in the spinal central pattern generator (CPG). By combining two-photon calcium imaging
7 with electrophysiology, immunohistochemistry and genetic tools, we report that astrocytes display
8 Ca^{2+} transients before and during oscillations of neighbouring neurons. Inhibition of astrocytic Ca^{2+}
9 transients with BAPTA decreases the barium-sensitive Kir4.1 current responsible of K^+ clearance.
10 Finally, we show in mice that Kir4.1 knockdown in astrocytes progressively prevents neuronal
11 oscillations and alters the locomotor pattern resulting in lower motor performances in challenging
12 tasks. These data identify astroglial Kir4.1 channels as key regulators of neuronal rhythmogenesis in
13 the CPG driving locomotion.

14

15 **SIGNIFICANCE STATEMENT**

16 Despite decades of research, the cellular mechanisms responsible of the synchronized rhythmic
17 oscillations driving locomotion remain elusive. To gain insight into the function of the spinal
18 locomotor network, numerous studies have characterized diverse classes of locomotor-related
19 neurons to determine their role in generating rhythmic movements during locomotion. In contrast,
20 studies investigating non-neuronal components of the spinal cord are sparse. Our study represents a
21 significant breakthrough by identifying astrocytic K^+ uptake as a key regulator of neuronal
22 rhythmicity synchronization and locomotor pattern at the cellular, microcircuit and system levels.
23 These data provide mechanistic insights into the neuroglial dialogue at play during rhythmogenesis
24 and point to a novel astroglial target for restoring normal neuronal network excitability in brain
25 disorders and neurodegenerative diseases.

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1 INTRODUCTION

2 Mammalian locomotion relies on repeated sequences of muscle contractions triggered by a
3 rhythmic central pattern generator (CPG) network, which is mainly located in the ventromedial part
4 of the lumbar spinal cord (Grillner and El Manira, 2020; Kiehn, 2016). Activation of the spinal CPG
5 network is triggered by descending inputs releasing neuromodulators such as glutamate, serotonin
6 and dopamine. Several populations of CPG interneurons - V0, V2a and Hb9 –display conditional
7 rhythmic oscillations in response to neuromodulators (Grillner and El Manira, 2020; Kiehn, 2016). No
8 single population of ventromedial interneurons appears to act alone or to orchestrate the entire CPG
9 pattern. The spinal rhythmogenesis emerges from the synchronization of individual neurons with
10 intrinsic bursting properties (Brocard et al., 2010). Despite decades of research, the functional
11 organization of the locomotor CPG remains still unclear.

12
13 In the spinal locomotor CPG, physiological variation in extracellular K^+ ($[K^+]_e$) coincides with the
14 emergence of the oscillatory pattern of intrinsic bursting neurons (Brocard et al., 2013), and thus
15 powers up the motor output (Bos et al., 2018; Bracci, 1998). At rest, $[K^+]_e$ is kept close to 3 mM in
16 serum levels (Kofuji and Newman, 2004; Verkhratsky et al., 2018). The maintenance of K^+
17 homeostasis is one of the crucial supportive functions mediated by astrocytes (Ben Haim et al.,
18 2015; Sibille et al., 2015). In addition, to display a high resting K^+ conductance that facilitates the
19 uptake of neuron released K^+ , astrocytes have a hyperpolarized resting membrane potential (RMP).
20 Both parameters rely on the activity of the weakly inwardly rectifying Kir4.1 K^+ channel, which in the
21 nervous system is expressed exclusively in glial cells, with the highest expression in astrocytes (Kelley
22 et al., 2018). Astrocytic Kir4.1 channels have been described in many CNS regions, including the
23 spinal cord (Olsen et al., 2006; Ransom and Sontheimer, 1995). Studies using genetic,
24 pharmacological and modeling approaches in the brain networks identified Kir4.1 as the main
25 astrocytic inwardly rectifying K^+ channel regulating $[K^+]_e$ and thus influencing neuronal excitability
26 (Nwaobi et al., 2016; Sibille et al., 2015).

27 Astrocytes display ramified processes enwrapping synapses, placing them in an ideal position to (i)
28 sense synaptic activity (Pاناتier et al., 2011) and (ii) adjust network activity by releasing
29 gliotransmitters (Araque et al., 2014; Savtchouk and Volterra, 2018). Rhythmic oscillations are
30 strongly affected in the brain networks when gliotransmission is genetically or pharmacologically
31 modified (Lee et al., 2014; Sheikhabaei et al., 2018). In the spinal motor network, the locomotor
32 oscillatory rhythm is correlated with enhanced calcium (Ca^{2+}) transients in astrocytes (Broadhead
33 and Miles, 2020), which likely triggers ATP release (Witts et al., 2015), modulating excitatory
34 synaptic transmission (Carlsen and Perrier, 2014). Thus, growing evidence point to the contribution
35 of gliotransmission in modulating neuronal rhythmogenesis (Montalant et al., 2021). Astrocytes can
36 also respond to neurotransmitters and neuromodulators (Paukert et al., 2014; Rosa et al., 2015) by
37 modifying $[K^+]_e$, which greatly impact brain oscillations (Bellot-Saez et al., 2017; Ding et al., 2016;
38 Sibille et al., 2015). However, the mechanisms underlying the contribution of astrocytic K^+
39 homeostasis to rhythmogenesis remain incompletely understood.

40
41 Here we investigated whether astrocyte K^+ uptake modulates neuronal oscillations patterns in the
42 spinal CPG network. We demonstrate that most of the ventromedial astrocytes are active during
43 neuronal oscillations, and that the inwardly-rectifying Kir4.1 channels are crucial for maintaining
44 neuronal oscillations, which ultimately influence the locomotor activity. This study places astrocytes
45 as central elements of the spinal cord locomotor CPG.

46 RESULTS

47 **Astrocytes respond to changes in extracellular ions underlying neuronal oscillations in the spinal**
48 **CPG.** Astrocytes from the ventromedial part of upper lumbar segments (L1–L2), the main locus of

1 the locomotor CPG (El Manira, 2014; Kiehn, 2016), were recorded from transgenic mice expressing
2 GFP under the control of the astrocytic *Aldh1L1* promoter (Tsai et al., 2012) (Fig 1A). This spinal
3 region contains interneurons endowed with two types of inherent membrane oscillations at a
4 frequency range similar to stepping rhythms: (i) one is TTX-sensitive dependent on the persistent
5 sodium current (INaP) (Tazerart et al., 2008) and triggered by a rise in $[K^+]_e$ with a concomitant
6 decrease in extracellular Ca^{2+} levels ($[Ca^{2+}]_e$) (Brocard et al., 2013), (ii) the other is TTX-insensitive
7 dependent on the Ca^{2+} currents and triggered by a neuromodulator cocktail composed of 5-HT,
8 dopamine and NMDA, in presence of the voltage-dependent sodium channels blocker, tetrodotoxin
9 (TTX) (Masino et al., 2012; Wilson, 2005; Ziskind-Conhaim et al., 2008). This neuromodulator cocktail
10 mimics the effect of descending inputs from the hindbrain for inducing a locomotor rhythmicity
11 (Grillner and El Manira, 2020). In recording conditions required to generate bursting cells, all
12 astrocytes displayed a reversible depolarisation of their membrane potentials: (i) when $[K^+]_e$ was
13 increased to 6mM and $[Ca^{2+}]_e$ decreased to 0.9mM (Fig.1 B-C) or (ii) when the neuromodulator
14 cocktail was applied in presence of TTX (Fig. 1 D-E). We observed that in absence of neuronal
15 activity, neuromodulators increased $[K^+]_o$ to ~ 6 mM (Fig. S1). To explore the temporal dynamics of
16 the astrocytic response compared to neuronal oscillations, we performed dual recordings of a
17 ventromedial Hb9 GFP+ interneuron which is known to display the two types of bursting properties
18 (Tazerart et al., 2008; Ziskind-Conhaim et al., 2008) and an adjacent astrocyte from the upper lumbar
19 segments (L1-L2) (Fig. 1F). In 13 pairs, astrocytic depolarization in response to the TTX-sensitive
20 $[K^+]_e/[Ca^{2+}]_e$ variations (Fig. 1 G) or the TTX-insensitive neuromodulator cocktail (Fig. 1H) either
21 preceded (69%) or followed (31%) the onset of the neuronal rhythmic bursting of the adjacent GFP+
22 pacemaker interneurons (Fig. 1I), suggesting that astrocytes play an active role in spinal
23 rhythmogenesis.

24

25 **Enhanced astrocytic Ca^{2+} transients occur during neuronal oscillations in the ventral spinal cord.** To
26 better locate and characterize the dialogue between neurons and astrocytes during spinal
27 oscillations, we performed two-photon Ca^{2+} imaging of neuronal and astrocyte signals in the
28 locomotor CPG area. Mice were injected at birth with adeno-associated virus serotype 9 (AAV9) (Fig.
29 S2A) designed to express GCaMP6f in astrocytic processes under the *gfaABC1D* promoter and
30 *jRGECO1a* in neurons under the *Syn.NES* promoter (Fig. S2B). We simultaneously imaged the
31 chromophores in lumbar slices (L1-L2) of post-natal mice (day 13-16) and first confirmed that
32 neuronal signals were prevented by TTX, in contrast to the signals recorded astrocyte processes that
33 remain intact (Fig. S2C-D).

34 We next explored the spatio-temporal dynamics of the astrocytic transients and compared it to the
35 neuronal transients. In response to changes in extracellular K^+ and Ca^{2+} levels or bath application of
36 the neuromodulator cocktail in presence of TTX, neurons displayed Ca^{2+} oscillatory transients ($0.16 \pm$
37 0.03 Hz and 0.15 ± 0.01 Hz, respectively) (Fig. 2A-B), which correlated with enhanced astrocytic Ca^{2+}
38 transients in processes (Fig. 2A and 2C). Interestingly, we distinguished distinct subsets of astrocytic
39 Ca^{2+} signals. Some astrocytes showed increased Ca^{2+} transients before the onset of the neuronal Ca^{2+}
40 oscillations, in contrast to others, which displayed Ca^{2+} transients during neuronal oscillations (Fig.
41 2A). Quantification showed that the occurrence of Ca^{2+} transients preceded the neuronal oscillations
42 in $\sim 64\%$ of astrocytes, whereas in $\sim 36\%$ of them the Ca^{2+} transients occurred during neuronal
43 oscillations (Fig. 2D). These data confirmed our electrophysiological dual recordings (Fig. 1I). We also
44 observed that most of the astrocytes ($\sim 70\%$) showing Ca^{2+} signal increases in response to ionic
45 variations or perfusion of the neuromodulator cocktail were located in the ventromedial part of the
46 spinal cord (Fig. 2E). Altogether, these data suggest that ventromedial astrocytes are functionally
47 coupled with neuronal rhythmicity in the spinal CPG area.

48

49 **The astrocytic Ca^{2+} transients regulate the Kir4.1-mediated inwardly rectifying K^+ current.** Because
50 astrocytic Ca^{2+} signals modulate $[K^+]_e$ in hippocampus (Wang et al., 2012a) and cerebellum (Wang et
51 al., 2012b), we then investigated whether the increase in astrocytic Ca^{2+} transients related to

1 neuronal oscillations in the spinal CPG area (Fig. 2) stimulates K^+ uptake. We first recorded the
2 passive membrane properties of the ventromedial GFP+ astrocytes from upper lumbar slices (Fig.
3 3A-B) and then isolated the Ba^{2+} -sensitive Kir4.1 current underlying K^+ uptake in standard artificial
4 cerebro-spinal fluid (aCSF) (Fig. 3C-D). We then switched to oscillatory aCSF by increasing $[K^+]_e$ to
5 6mM and reducing $[Ca^{2+}]_e$ to 0.9mM, which leads to neuronal bursting (Fig. 1G and Fig. 2A-B) and
6 intracellular astrocytic Ca^{2+} transients (Fig. 2A and 2C). In this condition, we observed that
7 preventing the astrocytic Ca^{2+} transients with BAPTA (30mM) inside the patch pipette significantly
8 reduced the barium (Ba^{2+})-sensitive current driven by Kir4.1 channels (Fig. 3 E-F). These data strongly
9 suggest that the increase in intracellular Ca^{2+} from ventromedial astrocytes observed during
10 neuronal oscillations modulates K^+ uptake via Kir4.1 channels.

11
12 **Kir4.1 channels are expressed in astrocytes from the spinal locomotor CPG region.** We next
13 investigated whether Kir4.1 channels are expressed in the spinal locomotor CPG. Using Hb9-eGFP
14 mice which express GFP+ interneurons with intrinsic bursting properties (Tazerart et al., 2008;
15 Ziskind-Conhaim et al., 2008), we found that ventromedial GFP+ interneurons were wrapped by
16 Kir4.1 labeling, which is diffusely expressed in the grey matter of the upper lumbar segments (Fig.
17 S3A). To confirm that Kir4.1 channels in the locomotor CPG area are exclusively expressed in glial
18 cells with a highest expression in astrocytes, we used the *Aldh1L1-eGFP* astrocyte-specific reporter
19 mice (Cahoy et al., 2008). We examined co-expression of *Aldh1L1-eGFP* with immunolabeling of
20 Kir4.1 channels and neuronal marker NeuN in lumbar slices. We observed that 100% of the GFP+
21 astrocytes expressed Kir4.1 channels contrary to NeuN+ cells, which lacked Kir4.1 staining (Fig. S3B-
22 C).

23
24 **The Ba^{2+} -sensitive inwardly rectifying K^+ (Kir4.1) current is crucial for maintaining neuronal**
25 **oscillations and locomotor-like activity in the CPG.** We next studied the role of Kir4.1 channels in
26 the lumbar rhythmogenesis. We first induced rhythmic oscillations in the GFP+ ventromedial
27 interneurons from Hb9-eGFP mice in response to $[K^+]_e/[Ca^{2+}]_e$ variations (Fig. 4A) or bath perfusion
28 of the neuromodulator cocktail in presence of TTX (Fig. 4B). In both conditions, pharmacological
29 blockade of Kir4.1 channels with Ba^{2+} (100 μ M) caused a shift in the spiking pattern from bursting to
30 tonic firing in ~70% of the TTX-sensitive neuronal oscillations (Fig. 4C) or resulted in a cessation of
31 bursting activity (~85%) of the TTX-insensitive neuronal oscillations (Fig. 4D). Interestingly, we
32 noticed that before totally preventing the neuronal oscillations, Ba^{2+} progressively decreased the
33 rhythmic bursting frequency accompanied with an increase in burst amplitude and duration in both
34 conditions (Fig. S4). We next confirmed the Ba^{2+} effect on preventing neuronal oscillations at a larger
35 scale by two-photon Ca^{2+} imaging of cell populations. We observed a progressive loss of Ca^{2+}
36 oscillations in the vast majority of neurons in response to Ba^{2+} in presence of the oscillatory aCSF
37 (n=25/28) or the neuromodulator cocktail (n=7/9) (Fig. 4E-F). Bath application of Ba^{2+} also induced a
38 significant rise in amplitude of the astrocytic Ca^{2+} signal in both conditions (Fig. 4G).

39 To confirm the functional role of the inwardly rectifying K^+ (Kir4.1) current in the integrated
40 rhythmogenesis, we investigated the contribution of Kir4.1 in the locomotor rhythm-generating
41 network by using *ex vivo* whole-mount spinal cords. Since rostral lumbar segments (L1–L2) have a
42 more powerful rhythmogenic capacity than the caudal ones, we recorded locomotor-like activities
43 from the contralateral L2 ventral roots (Fig. 5A) in response to ionic variations (Fig. 5B) or bath
44 application of a neuromodulator cocktail composed of N-methyl-DL aspartate (NMA) and 5
45 hydroxytryptamine (5-HT) (Fig. 5C). In both conditions, Ba^{2+} (100 μ M) slowed down the locomotor
46 rhythm by increasing the locomotor burst duration and decreasing the burst frequency without
47 apparent effect on burst amplitude (Fig 5B-D). We also observed a decrease in the cross-correlogram
48 coefficient in presence of Ba^{2+} related to a decreased rhythmic alternation (Fig 5E-F) confirming the
49 role of Kir4.1 channels in modulating rhythmogenesis at the network level.

50

1 **Loss-of-function of Kir4.1 decreases the probability for ventromedial CPG interneurons to oscillate**
2 To further explore the functional role of Kir4.1 channels in spinal rhythmicity, we performed their
3 targeted loss-of function by using a short hairpin RNAs (shRNAs). We injected intrathecally at birth at
4 T13–L1 level an AAV9 virus encoding the Kir4.1-shRNA with a eGFP reporter (Fig. 6A-B). The viral
5 transfection led to a decrease of ~40% in Kir4.1 membrane protein expression in the lumbar spinal
6 cord (Fig. 6C). Concomitantly, a strong expression of eGFP in the ventromedial part of the spinal cord
7 was observed from T1 to L5-S1 (Fig. 6D, Fig. S5). We first examined the effect of Kir4.1-shRNA on
8 glial and neuronal electrophysiological properties. In the virally transfected GFP+ astrocytes (Fig.
9 S6A-B), we observed in whole-cell configuration a more depolarized RMP (Fig. S6C), a higher input
10 resistance (Fig. S6C), a marked change in the current-voltage (I-V) relationship and a reduced Ba²⁺-
11 sensitive current in mice transduced with Kir4.1-ShRNA compared to Ctrl-ShRNA mice (Fig. S6D-I).
12 We also observed that the RMPs of ventromedial interneurons surrounded by the virally transfected
13 GFP+ astrocytes (Fig. 6E) from mice transduced with Kir4.1-ShRNA were overall more depolarized
14 than the RMPs of interneurons from mice infected with Ctrl-ShRNA (Fig. 6E-F), suggesting that
15 knockdown of Kir4.1 in astrocytes had a widespread impact on the excitability of neighbouring
16 neurons.
17 We then tested the ability of the ventromedial interneurons to oscillate after the viral transfection of
18 the spinal cord. We found a significant lower proportion of rhythmic bursting in ventromedial
19 interneurons surrounded by GFP+ astrocytes in mice transduced with Kir4.1-ShRNA compared to
20 Ctrl-ShRNA in response to both ionic variations (~24% vs 80%) (Fig. 6G and 6I) or bath perfusion of
21 the neuromodulator cocktail in presence of TTX (~ 23% vs 75 %) (Fig. 6H and 6J). Altogether, these
22 data demonstrate the pivotal role of astrocytic Kir4.1 channels in the oscillatory pattern of
23 ventromedial interneurons from the CPG.

24
25 **Loss-of-function of Kir4.1 slows down the *in vivo* locomotor pattern and decreases the locomotor**
26 **performances.** We then tested whether the knockdown of Kir4.1 in the spinal cord has a functional
27 impact on the locomotor behaviors. We thus injected intrathecally at birth an AAV9 encoding either
28 the Kir4.1-shRNA or the Ctrl-shRNA. From 12 to 18 days after the viral injection, we performed a set
29 of behavioural locomotor tests on freely moving mice (Fig. 7A). We first used footprint analysis to
30 evaluate the locomotor pattern (Fig. 7B). Mice transduced with Kir4.1-shRNA displayed an increase
31 in both swing and stand phases compared to Ctrl-ShRNA mice (Fig. 7C-D) related to a slower speed
32 of locomotion and a decrease in regularity index without any modification of the base of support
33 (Fig. 7D). We next tested whether Kir4.1 channels also modulate locomotor behaviours in more
34 challenging tasks. We thus assayed motor coordination using the rotarod and swimming forced test
35 without practice sessions to avoid any compensatory learning mechanisms. Consistently, mice
36 transduced with Kir4.1-shRNA failed to adapt to accelerated speed (Fig. 7E) and displayed a lower
37 swimming performance (Fig. 7F). Importantly, we did not observe any modification of the
38 motoneuronal soma size in mice transduced with Kir4.1-ShRNA compared to Ctrl-ShRNA mice (Fig.
39 S7). In sum, the Kir4.1-mediated inwardly rectifying K⁺ current appears to play a key role for
40 locomotor coordination and the maintenance of the locomotor pattern in freely moving mice.

41 42 **DISCUSSION**

43 This study demonstrates that astrocytic regulation of extracellular K⁺ plays a critical role in
44 maintenance of neuronal rhythmicity in the spinal cord motor circuitry. We report that spinal
45 astrocytes from neonatal mice display Ca²⁺ signals before and during rhythmic neuronal oscillations.
46 Inhibiting astrocytic Ca²⁺ transients with BAPTA decreases the Ba²⁺-sensitive inwardly rectifying K⁺
47 current underlying K⁺ uptake. The Kir4.1 channels mediating the K⁺ current are expressed in
48 astrocytes enwrapping the ventromedial interneurons from the locomotor CPG. We also
49 demonstrated that knockdown of Kir4.1 decreases the ability of the ventromedial interneurons to
50 oscillate. This perturbation results in an alteration of the locomotor pattern and in lower locomotor
51 performances in challenging tasks.

1 **Functional coupling of astrocytic Ca^{2+} signals and neuronal oscillations.** Recent studies have
2 highlighted unexpected contribution of astrocytic Ca^{2+} signals in regulating rhythmic behaviours
3 including respiration (Sheikhhahaei et al., 2018), mastication (Morquette et al. 2015) and locomotion
4 (Broadhead and Miles, 2020). However, it is still incompletely understood whether the increased
5 astrocytic Ca^{2+} transient is a prerequisite or a consequence of neuronal oscillations. Here, we reveal
6 that ventromedial astrocytes depolarize and display an increase in Ca^{2+} signals both before and in
7 response to ions and neuromodulator changes related to neuronal oscillations. Our two-photon Ca^{2+}
8 imaging data reveal a functional coupling between spinal cord astrocytes and CPG interneurons. The
9 simultaneous imaging of neuronal and astrocytic activity led to the characterization of distinct
10 subsets of active astrocytes in the spinal CPG area. In one subset, the response precedes neuronal
11 oscillation. This active role of astrocytes in rhythmicity has been suggested in the trigeminal
12 sensorimotor circuit (Morquette et al., 2015) or in cortical areas where the increase in astrocytic Ca^{2+}
13 events precedes the switch to the slow-oscillation state (Poskanzer and Yuste, 2016). In another
14 group, the responses are concomitant to neuronal bursting. In line with this, in *Drosophila* astrocyte
15 Ca^{2+} transients are driven by rhythmic firing of the octopaminergic neurons (Ma et al., 2016). A
16 recent study also demonstrated such a dynamics in fish radial astroglia (analogs of the mammalian
17 astrocyte), where the noradrenergic neuronal activity precedes the Ca^{2+} wave observed in radial
18 astroglia (Mu et al., 2019). However, we cannot totally rule out that one astrocyte may belong to
19 both groups. Altogether, our data highlight a bi-directional coupling between astrocytic Ca^{2+} signal
20 and neuronal oscillations.

21
22 **The Ca^{2+} -dependent K^+ uptake in spinal astrocytes.** Astrocytic Ca^{2+} transients are often associated to
23 gliotransmission (Araque et al. 2014). Here we propose that an alternative mechanism to the
24 classical gliotransmission operates for spinal rhythmogenesis. Previous works demonstrated that
25 GPCR-mediated Ca^{2+} signalling in astrocytes is linked to an increase of K^+ uptake in hippocampus
26 (Wang et al., 2012a) and cerebellum (Wang et al., 2012b). This astrocytic Ca^{2+} transients stimulate
27 the Na^+ , K^+ ATPase pump resulting in a transient decrease in $[\text{K}^+]_e$. In our study, we provide for the
28 first time evidence that a blockade of the Ca^{2+} transients from ventromedial lumbar astrocytes
29 significantly reduces the Ba^{2+} -sensitive current mediated by Kir4.1. This result is in line with
30 observations showing that menthol-induced Ca^{2+} transients in astrocytes increases the Ba^{2+} -sensitive
31 Kir4.1 current from a U251 cell line (Ratto et al., 2020). Because (i) the locomotor-like activity is
32 associated with $[\text{K}^+]_e$ homeostasis (Brocard et al., 2013), and (ii) the astrocytic K^+ uptake is activated
33 by intracellular Ca^{2+} transients (Wang et al., 2012a), we assume that the Ca^{2+} dependent astrocytic K^+
34 homeostasis plays a key role in the spinal rhythmicity.

35
36 **Astrocytic-dependent K^+ regulation of the spinal rhythmogenesis.** The maintenance of K^+
37 homeostasis by astrocytes is crucial in modulating neuronal excitability (Dallérac et al., 2013;
38 Neprasova et al., 2007). However, whether spinal astrocytes may exert a “ionostatic control” of
39 neuronal bursting, as suggested by Ding et al. 2016, is still unclear. Here, we evaluated the
40 consequence of blocking the astrocytic inwardly rectifying K^+ (Kir4.1) channels on neuronal
41 oscillations in the locomotor CPG. Blocking the Kir4.1-mediated astrocytic K^+ uptake with Ba^{2+} (100
42 μM) (Cui et al., 2018; Djukic et al., 2007; Ransom and Sontheimer, 1995) decreases the frequency of
43 neuronal oscillations until progressively preventing them. In line with this effect, injection of Kir4.1-
44 ShRNA resulting in the decrease in Kir4.1 astrocytic membrane expression also significantly reduces
45 the probability of driving neuronal oscillations. These results highlight the key role of astrocytic K^+
46 uptake in maintaining the neuronal oscillatory pattern. Although the astrocytic Kir4.1 channels are
47 not homogeneously expressed among and within brain structures (Higashi et al. 2001), we showed a
48 strong Kir4.1 expression in astrocytes enwrapping the ventromedial bursting interneurons and
49 lamina IX motoneurons. This strategic distribution of Kir4.1 channels surrounding the bursting spinal
50 neurons may be viewed as a powerful mechanism to rapidly modulate network rhythmicity.
51 Moreover, since glutamate tunes the CPG network by exerting a speed control of locomotor-like

1 activity (Talpalar et al., 2013) and that $[K^+]_e$ provides the driving force for glutamate uptake (Nwaobi
2 et al., 2016; Verkhatsky and Nedergaard, 2018) the effective K^+ uptake from the extracellular space
3 through Kir4.1 is thus crucial for tuning the neuron oscillations. In line with this, previous works
4 highlighted the importance of $[K^+]_e$ for rhythmogenesis in hippocampal (Jensen et al. 1994) or
5 cortical (Bellot-Saez et al., 2018) networks. Furthermore, the increase in $[K^+]_e$ induced by
6 neuromodulators may promote the shift between neuronal oscillatory states in cortical slices
7 electrically silenced by TTX (Ding et al., 2016). A computational work also demonstrated that
8 extracellular K^+ dynamics can cause transition between fast tonic spiking and slow bursting in
9 neocortical networks (Frohlich et al., 2006) in the same range than the spinal locomotor network.

10 **The ventromedial astrocytes regulate the locomotor pattern in vivo.** A dense literature pointed out
11 that during locomotion, mice display enhanced Ca^{2+} signals in astrocytes of the cerebellum
12 (Nimmerjahn et al., 2009; Paukert et al., 2014), visual cortex (Paukert et al., 2014), or somatosensory
13 cortex (Bojarskaite et al., 2020; Dombeck et al., 2007). On the other hand, silencing astrocyte Ca^{2+}
14 activity in brainstem with optogenetics or chemogenetics can modulate the rhythmic respiratory
15 behaviour (Gourine et al., 2010; Sheikhabahaei et al., 2018). In this study, we used genetic tools with
16 ShRNA to knockdowns Kir4.1 in the locomotor CPG and demonstrate that the astrocytic regulation of
17 K^+ homeostasis plays a key role in controlling the locomotor speed and challenging locomotor tasks
18 *in vivo*. Mice transduced with Kir4.1-ShRNA showed striking locomotor deficits compared to Ctrl-
19 ShRNA mice. Kir4.1 had been reported to contribute to the maintenance of the soma size of large α -
20 motoneurons and peak strength in adult mice (Kelley et al., 2018). We demonstrated that the
21 lowered locomotor performances from Kir4.1-ShRNA mice do not result from changes in
22 motoneuronal morphological features. Indeed, we did not observe any modification of the
23 motoneuronal soma size in mice transduced with Kir4.1-ShRNA compared to Ctrl-ShRNA mice. Tong
24 et al. 2014 observed that the Kir4.1 expression decrease in the striatal network of a mouse model of
25 Huntington disease, results in altered locomotor pattern (Tong et al., 2014). We did not observe any
26 Kir4.1-ShRNA expression in brain or brainstem structures involved in the locomotor control. This
27 result is in line with the restricted diffusion to the thoraco-lumbar segments of the AAV9-ShRNA
28 injected intrathecally at birth in the lumbar segments (Bos et al., 2021). Thus we assume that the
29 behavioural consequence of Kir4.1 knockdown on locomotor performances is due to Kir4.1 decrease
30 in the spinal locomotor networks.

31 Overall this study defines the contours of a new concept for the spinal locomotor CPG. We propose
32 that the astrocytic Ca^{2+} transients which occurs during the neuronal oscillations likely maintains
33 extracellular K^+ homeostasis, which in turn adjusts the locomotor pattern (Graphical abstract).
34 Further studies will be needed to decipher the importance for spinal rhythmicity of the astrocytic
35 syncytium mediating K^+ buffering in the locomotor CPG.

36

37

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5 **EXPERIMENTAL PROCEDURES**

6 Further details and an outline of resources used in this work can be found in the Supplemental
7 Procedures.

8

9 **Mice.** Mice from the 1st to 3rd postnatal week of either sex were used in this study. Animals from
10 different litters were used for each experiment. All animal care and use were conformed to the
11 French regulations (Décret 2010-118) and approved by the local ethics committee (Comité d’Ethique
12 en Neurosciences INT-Marseille, CE71 NbA1301404, authorization Nb 2018110819197361). See the
13 [Supplemental Experimental Procedures](#) for more details.

14

15 **Ex vivo Models.** Slice preparations were used for whole-cell recordings and two-photon Ca²⁺ imaging
16 experiments, whereas whole spinal cord preparations were used for fictive locomotion experiments.
17 Preparation procedures are detailed in the [Supplemental Experimental Procedures](#).

18

19 **Intracellular Recordings.** For *ex vivo* experiments, whole-cell patch-clamp recordings were made
20 from L1-L2 ventromedial GFP+ interneurons (*Hb9:eGFP* mice) or GFP+ astrocytes (*Aldh1L1:eGFP*
21 mice) from lumbar slices. Procedures of intracellular recordings are detailed in the [Supplemental](#)
22 [Experimental Procedures](#).

23

24 **Extracellular Recordings.** Motor outputs were recorded using glass suction electrodes placed in
25 contact with right and left lumbar L2 ventral roots in response to bath application of a
26 monoaminergic cocktail. See the [Supplemental Experimental Procedures](#) for more details.

27

28 **Two-photon Ca²⁺ imaging.** Two-photon excitation of Gcamp6f and jRGECO1a was simultaneously
29 evoked with a laser tuned to 960 nm. Imaging was done at 30.5202 Hz. The microscope was
30 equipped with two detection channels for fluorescence imaging. See the [Supplemental Experimental](#)
31 [Procedures](#) for more details.

32 **shRNA constructs.** Specific shRNA sequence designed to knockdown *Kir4.1* transcript was
33 incorporated into an adeno-associated viral (AAV) vector (serotype 9), which features a H1 promoter
34 to drive shRNA expression and a CAG promoter to drive eGFP expression for identification of
35 transduced cells (Cui et al. 2018). See the [Supplemental Experimental Procedures](#) for more details.

36

37 **Intrathecal vector delivery.** A minimally-invasive technique was used to micro-inject adeno-
38 associated viral (AAV) vectors into the T13-L1 intervertebral space. A total volume of 2 µL /animal
39 was injected. See the [Supplemental Experimental Procedures](#) for more details.

40

41 **Kir4.1 protein quantification.** Kir4.1 expression was analyzed using the 12-230 kDa separation
42 module (SM-W004 ProteinSimple) on an automated capillary western blotting system (‘Jess’
43 ProteinSimple). See the [Supplemental Experimental Procedures](#) for more details.

44

1 **Immunohistochemistry.** Transverse spinal cord sections at the lumbar L1-L2 level were processed
2 for immunohistochemistry using antibodies against Kir4.1, NeuN. Tissue processing and staining are
3 detailed in the [Supplemental Experimental Procedures](#).
4

5 **Behavioral tests.** Assessment of motor behaviour were performed on neonatal mice with three
6 different tests: walking, rotarod and swimming. See the [Supplemental Experimental Procedures](#) for
7 more details.

8 **Statistical Analysis.** Group measurements were expressed as means \pm S.E.M. All statistical analyses
9 are indicated in Figure legends. The level of significance was set at $p < 0.05$. Statistical analyses were
10 performed using Graphpad Prism 9 software. See the [Supplemental Experimental Procedures](#) for
11 more details.

12

13 **ACKNOWLEDGMENTS**

14 This work was mainly funded by the CNRS and “Fonds d’Investissement de l’INT jeunes chercheuses,
15 jeunes chercheurs” (FI_INT_JCJC_2019) (to R.B.) and a small part by the Agence National de la
16 Recherche Scientifique (CalpaSCI, ANR-16-CE16-0004) (to F.B.). We thank Ivo Vanzetta, Pascal Weber
17 and Sebastien Roux for their technical advices in imaging sessions, Anne Duhoux for animal care,
18 Cécile Brocard for genotyping and Jérémy Verneuil for providing the Matlab script. We also
19 gratefully acknowledge Eduardo Gascon, Florence Jaouen, Catherine Lepolard and Ana Borges-
20 Correia from Neuro-Vir platform of the Neuro-Bio-Tools facility (Institut de Neurosciences de la
21 Timone, UMR 7289, Marseille, France) for their advices, support and assistance in the design and
22 production of vectors used in this work. We also thank Geneviève Rougon for her critical insights on
23 the manuscript.
24

25 **AUTHOR CONTRIBUTIONS**

26 Research design, T.B., E.P. and R.B.; Methodology, T.B., E.P., N.R. and R.B.; Investigation and data
27 analysis, T.B., E.P., M.D., and R.B.; Writing, R.B.; Funding Acquisition, R.B. and F.B.; Supervision, R.B.

28

29 **DECLARATION OF INTERESTS**

30 The authors declare no conflict of interest.

31

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FIGURE LEGENDS

8 **Figure 1. Astrocytes respond to variations in $[K^+]_e$ and $[Ca^{2+}]_e$ related to neuronal oscillations in the**
9 **spinal CPG. A.** Confocal image of endogenous GFP from a lumbar slice of a P8 *Aldh1L1-eGFP* mouse. The
10 White inset represents the patch-clamp recording area of the ventromedial GFP+ astrocytes. The
11 recording glass microelectrode is in white. CC stands for central canal, GM for grey matter and WM
12 for white matter. **B.** Representative voltage trace of a GFP+ astrocyte in response to $[K^+]_e$ rise to
13 6mM and $[Ca^{2+}]_e$ decrease to 0.9mM. **C.** Quantification of the effect of the $[K^+]_e$ and $[Ca^{2+}]_e$ variations
14 on the astrocytic membrane potential (n = 22 astrocytes from 16 mice). **D.** Representative voltage
15 trace of a GFP+ astrocyte in response to bath application of a neuromodulator cocktail (dopamine,
16 serotonin, and NMDA) in presence of TTX. **E.** Quantification of the effect of the neuromodulator
17 cocktail on the astrocytic membrane potential (n = 8 astrocytes from 4 mice). **F.** Confocal image of
18 the endogenous GFP from the ventromedial part of a lumbar slice of a P12 Hb9-eGFP mouse. The
19 green and orange glass microelectrodes represent the dual recording of a GFP+ interneuron and an
20 adjacent astrocyte, respectively. CC for central canal. **G-H.** The $[K^+]_e$ and $[Ca^{2+}]_e$ variations (G, light
21 blue) or the bath application of the neuromodulator cocktail in presence of TTX (H, light blue)
22 induces bursting in Hb9 GFP+ interneurons (green traces) preceded by a depolarization of the
23 neighbouring astrocyte (orange traces). The black inset in H represents the TTX-insensitive neuronal
24 oscillations. **I.** Proportion of the astrocytic membrane potential depolarization preceding (light
25 orange) or not preceding (dark orange) the onset of neuronal bursting (n=13 pairs from 8 mice). **P
26 < 0.01; ***P < 0.001 (two-tailed Wilcoxon paired test for C and E). Mean \pm SEM. For detailed P
27 values, see Source data file.

28

29 **Figure 2. Increase in astrocytic Ca^{2+} transients is correlated to neuronal oscillations A. Top.**
30 Examples of neuronal jRGECO1a Ca^{2+} transients ($\Delta F/F_0$) oscillating in response to K^+ rise and Ca^{2+}
31 decrease (left) or in response to the neuromodulator cocktail in presence of TTX (right). *Middle,*
32 *bottom.* Examples of the increase of the astrocytic Gcamp6f Ca^{2+} signals ($\Delta F/F_0$) before (middle) or
33 during (bottom) the neuronal oscillations (top) from the same field of view. **B.** Quantification of the
34 mean amplitude (left), duration and frequency (right) of the neuronal jRGECO1a Ca^{2+} transients in
35 response to K^+ rise and Ca^{2+} decrease (empty blue circles) or in response to monoaminergic cocktail
36 in presence of TTX (filled blue circles) (n= 9 and 24 dendritic ROIs from 2 and 3 mice in the ionic
37 variation condition and in the TTX+cocktail condition, respectively). **C.** Quantification of the mean
38 amplitude (left), duration (middle) and frequency (right) of the astrocytic Gcamp6f Ca^{2+} signals in
39 response to K^+ rise and Ca^{2+} decrease (empty light blue circles) or in response to monoaminergic
40 cocktail in presence of TTX (filled light blue circles) (n= 86 and 165 astrocytic ROIs from 2 and 3 mice
41 in the ionic variation condition and in the TTX+cocktail condition, respectively). **D.** Proportion of
42 astrocytic ROIs which display Ca^{2+} signals increase in amplitude and/or frequency before the onset of
43 neuronal oscillations. **E.** Proportion of astrocytic ROIs which display Ca^{2+} signals increase above
44 (dorsal) or below (ventral) the central canal (CC) in response to oscillatory conditions (ionic
45 variations and neuromodulator cocktail). *P < 0.05, **P < 0.01, ***P < 0.001 (two-tailed Wilcoxon
46 paired test for C). Mean \pm SEM. For detailed P values, see Source data file.

47 **Figure 3. Spinal astrocytes display a Ba^{2+} -sensitive Kir4.1 current modulated by intracellular Ca^{2+} .**
48 **A. Left.** DIC image of a lumbar slice from a P8 *Aldh1L1-eGFP* mouse superimposed with the

1 fluorescent GFP signal. The black stars indicate the GFP+ astrocytes. The recording glass pipette is
2 represented in black. *Right*. Representative membrane potentials (top) of the targeted GFP+
3 astrocyte (left image) in response to incremental current pulses (500ms, $\Delta = 50\text{pA}$). **B**. Resting
4 membrane potential (RMP) (left) and input resistance (right) of lumbar GFP+ astrocytes recorded in
5 standard aCSF ($[\text{K}^+]_e 3\text{mM}$ and $[\text{Ca}^{2+}]_e 1.2\text{mM}$) ($n = 28$ astrocytes from $n = 15$ mice) **C**. Representative
6 membrane currents (top) of one GFP+ astrocyte clamped at the RMP in response to incremental
7 voltages pulses (bottom, 2.5s, -140 to -40mV, $\Delta = 10\text{mV}$) before (left) or during (middle) application
8 of Ba^{2+} (100 μM) in standard aCSF. **D**. The averaged I/V relationship of the Ba^{2+} -sensitive currents of
9 GFP+ astrocytes ($n = 17$ astrocytes from 9 mice). **E**. Representative Ba^{2+} -sensitive currents of two
10 astrocytes clamped at the RMP in response to incremental voltages pulses (2.5s, -140 to +0mV, $\Delta =$
11 10mV) without (blue) or with (black) intracellular perfusion of BAPTA (30 mM) in oscillatory aCSF
12 ($[\text{K}^+]_e 6\text{mM}$ and $[\text{Ca}^{2+}]_e 0.9\text{mM}$). **F**. *Left*. The averaged I/V curves from lumbar astrocytes without
13 (blue circles) or with (black circles) intracellular BAPTA perfusion in oscillatory aCSF. *Right*. Average
14 effect of intracellular perfusion of BAPTA on the peak amplitude of the astrocytic Kir4.1 current at -
15 140mV (G). ($n = 8$ and 7 astrocytes from 3 and 4 mice for control and BAPT*a*i, respectively). $**P < 0.01$
16 (two-tailed Mann–Whitney test for F). Mean \pm SEM. For detailed P values, see Source data file.

17 **Figure 4. K^+ homeostasis maintenance through Kir4.1 channels is crucial for neuronal oscillations.**
18 **A-B**. Representative voltage traces of a lumbar GFP+ ventromedial interneuron from a P6 Hb9-eGFP
19 mouse before (left) and during (middle) ionic variations ($[\text{K}^+]_e = 6\text{mM}$, $[\text{Ca}^{2+}]_e = 0.9\text{mM}$) on which Ba^{2+}
20 (100 μM) is added (right) (A) or in presence of TTX (left) and following application of the
21 neuromodulator cocktail (middle) on which Ba^{2+} is added (right) (B). **C-D**. Quantification of the
22 proportion of GFP+ interneurons oscillating in response to ionic variations (C, left) or to the
23 neuromodulator cocktail (D, left) and following bath application of Ba^{2+} for more than 5 minutes (C-
24 D, right). **E**. *Top*. Examples of neuronal jRGECO1a Ca^{2+} signals ($\Delta\text{F}/\text{Fo}$) before (blue) and during bath
25 application of Ba^{2+} (grey) in presence of high $[\text{K}^+]_e$ and low $[\text{Ca}^{2+}]_e$ (left) or in presence of the
26 neuromodulator cocktail + TTX (right). *Bottom*. Examples of astrocytic Gcamp6f Ca^{2+} signals ($\Delta\text{F}/\text{FO}$)
27 before (blue) or during (grey) application of Ba^{2+} in presence of high $[\text{K}^+]_e$ and low $[\text{Ca}^{2+}]_e$ (left) or in
28 presence of TTX and the neuromodulator cocktail (right). **F**. Proportion of neuronal ROIs still
29 oscillating or not in response to Ba^{2+} application (>5min) in presence of high $[\text{K}^+]_e$ and low $[\text{Ca}^{2+}]_e$
30 (left) or in presence of TTX and the neuromodulator cocktail (right). **G**. Effect of Ba^{2+} application
31 (empty and filled grey circles) on the mean amplitude (left), duration (middle) and frequency (right)
32 of the astrocytic Gcamp6f Ca^{2+} signals in presence of K^+ rise and Ca^{2+} decrease (empty light blue
33 circles) or in presence of the neuromodulator cocktail + TTX (filled light blue circles). Each circle
34 represents one astrocytic ROI ($n = 24$ ROIs from 2 mice in the ionic variation condition and $n = 157$
35 ROIs from 3 mice in the TTX+cocktail condition) ns, no significance, $*P < 0.05$, $***P < 0.001$ (two-
36 tailed Wilcoxon test for G). Mean \pm SEM. For detailed P values, see Source data file.

37 **Figure 5. Modulation of astrocytic Kir4.1 channels alters the locomotor-like activity pattern.** **A**.
38 Schematic representation of the whole-mount spinal cord (ventral side up) with two contralateral
39 recording glass electrodes. VRL2L stands for ventral root lumbar segment 2 left. VRL2R stands for
40 ventral root lumbar segment 2 right. **B-C**. Extracellular recordings of alternating rhythmic activities of
41 L2 segment ventral roots (integrated signals) in response to $[\text{K}^+]_e$ rise and $[\text{Ca}^{2+}]_e$ decrease (B, left) or
42 in response to the neuromodulator cocktail (C, left) and in the presence (B-C, right) of Ba^{2+} . **D**.
43 Quantification of the effect of Ba^{2+} (grey circles) on the amplitude (left), duration (middle), and
44 frequency (right) of the rhythmic bursts recorded at the ventral roots (L2 segments) in ionic variation
45 condition (light blue empty circles) or in presence of the neuromodulator cocktail (light blue filled
46 circles). Each circle represents one individual animal ($n = 6$ mice in high $[\text{K}^+]_e$ and low $[\text{Ca}^{2+}]_e$

1 condition and $n = 7$ mice in neuromodulator cocktail condition). **E.** Graphical representation of the
2 cross-correlation coefficient between cocktail-induced rhythmic activities of contralateral ventral
3 roots of the L2 segment before (blue trace) or after (red trace) Ba^{2+} application. **F.** Quantification of
4 the mean cross-correlation coefficients between the rhythmic activities of the contralateral ventral
5 roots of the L2 segment before (blue) and after (grey) Ba^{2+} application ($n = 6$ mice in high $[K^+]_e$ and
6 low $[Ca^{2+}]_e$ condition and $n = 7$ mice in neuromodulator cocktail condition). ns, no significance, $*P <$
7 0.05 (two-tailed Wilcoxon paired test for D and F). Mean \pm SEM. For detailed P values, see Source
8 data file.

9 **Figure 6. Targeted decrease in Kir4.1 channels reduces the occurrence of neuronal oscillations. A.**
10 Schematics of the AAV vector engineered to overexpress shRNA or dominant-negative Kir4.1. H1,
11 human H1 promoter; CAG, CMV early enhancer/chicken *Actb* promoter. **B.** Schematic representation
12 of the experimental design. WT for wild-type, INs for interneurons and P for postnatal day. **C.** Left:
13 Kir4.1 pseudo-gel images from Capillary Western Blot of lumbar segments from P14 intrathecally
14 injected at birth with an adeno-associated virus (AAV9) encoding either a scramble shRNA ($n = 5$
15 mice) or a Kir4.1-targeting shRNA ($n = 5$ mice). One mouse per lane. Right: group mean
16 quantification of the ~ 168 kDa band normalized to scramble-injected controls. **D.** Representative
17 image of GFP in lumbar slices from P10 mice after intrathecal injection at birth of an adeno-
18 associated virus (AAV9) encoding a Kir4.1-targeting shRNA (Kir4.1-ShRNA). **E.** DIC image of the
19 ventromedial part of a lumbar slice and the corresponding one-photon GFP signal (right) of a P10
20 mouse injected with AAV9-Ctrl-ShRNA (left) or AAV9-Kir4.1-ShRNA (right). CC means central canal.
21 Scale bars, $20\mu m$. **F.** Quantification of the mean RMP (left), input resistance (middle) and cocktail-
22 evoked membrane depolarisation (right) of ventromedial interneurons from wild-type mice injected
23 with AAV9 encoding for Ctrl-ShRNA (left, $n = 18$ neurons from 4 mice) or Kir4.1-ShRNA (right, $n = 30$
24 neurons from 5 mice). **G-H.** Representative voltage traces of one interneuron of a mouse injected
25 with AAV9-Ctrl-ShRNA (left) or AAV9-Kir4.1-ShRNA (right) in response to ionic changes (E) or in
26 response to the neuromodulator cocktail in presence of TTX (F). **I-J.** Proportion of oscillatory
27 interneurons (blue) or not (grey) from mice injected at birth with an AAV9 encoding either a
28 Luciferase shRNA (Ctrl-ShRNA, $n = 4$ mice) (left) or a Kir4.1-targeting shRNA (Kir4.1-ShRNA, $n = 7$
29 mice) (right) in presence of high $[K^+]_e$ and low $[Ca^{2+}]_e$ (H) or in presence of the neuromodulator
30 cocktail + TTX (I). ns, no significance, $**P < 0.01$, $***P < 0.001$ (two-tailed Mann-Whitney test for C
31 and F, two-tailed Fisher test for I and J). Mean \pm SEM. For detailed P values, see Source data file.
32
33

34 **Figure 7. Astrocytic Kir4.1 channels influence the locomotor behaviour. A.** Schematic
35 representation of the experimental design. WT for wild-type and P for postnatal day. **B. Top.** Bottom
36 view of P18 mice injected at birth with AAV9 encoding either for the Ctrl-ShRNA (left) or the Kir4.1-
37 ShRNA (right) freely walking on a glass platform (Catwalk). **Bottom.** Side view of the same P18 mice.
38 **C.** Representative footfall diagrams during CatWalk locomotion of a Ctrl-ShRNA P18 mouse (blue,
39 top) or Kir4.1-ShRNA P18 mice (green, bottom). The stance phase is indicated by horizontal bars and
40 the swing phase by open spaces. RF for Right Forelimb, RH for Right Hindlimb, LF for Left Forelimb,
41 LH for Left Hindlimb. **D.** Comparative quantification of the normalized step cycle phases (stance,
42 swing) (left), regularity index (middle left), body speed (middle right) and hind limb base of support
43 (right) between Ctrl-ShRNA mice (blue, $n = 14$ mice) and Kir4.1-ShRNA mice (green, $n = 14$ mice).
44 Each circle represents an individual mouse. **E.** Latency to fall from a rod rotating at accelerated
45 speed (4–40 rpm) during 300s of wild-type mice transduced either with the Ctrl-ShRNA (blue, $n = 14$
46 mice) or with Kir4.1-ShRNA (green, $n = 14$ mice). **F. Left:** Heatmaps illustrate the swimming activity of
47 two wild-type mice transduced either with the Ctrl-ShRNA (top) or with the Kir4.1-shRNA (bottom).
48 Scale bar, 10 cm. **Middle:** Comparison of the mean swimming distance (middle left) and the mean
49 velocity (middle right) between Ctrl-shRNA (blue, $n = 9$ mice) and Kir4.1-shRNA (green, $n = 9$ mice)

1 mice. Each circle represents one individual mouse. *Right*: Percentage of mobility and immobility for
2 Ctrl-shRNA mice (blue, n = 9 mice) and Kir4.1-shRNA mice (green, n = 9 mice) during the swimming
3 forced test. ns, no significance, *P < 0.05, **P < 0.01, ***P < 0.001 (Mann–Whitney test for D, E right
4 and F, Two-way ANOVA with Sidak’s multiple comparisons test for E left). Mean ± SEM. For detailed
5 P values, see Source data file.

6

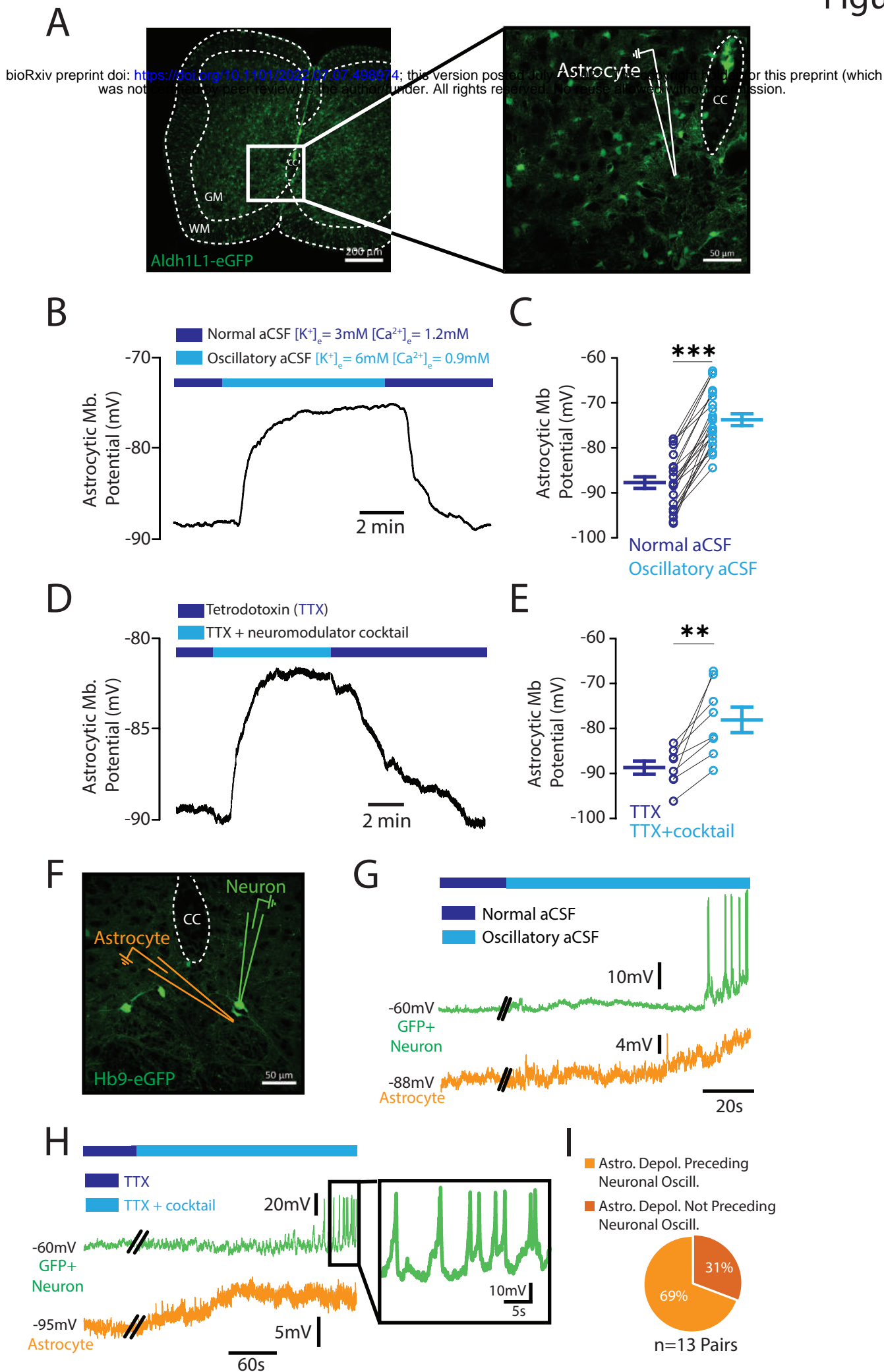


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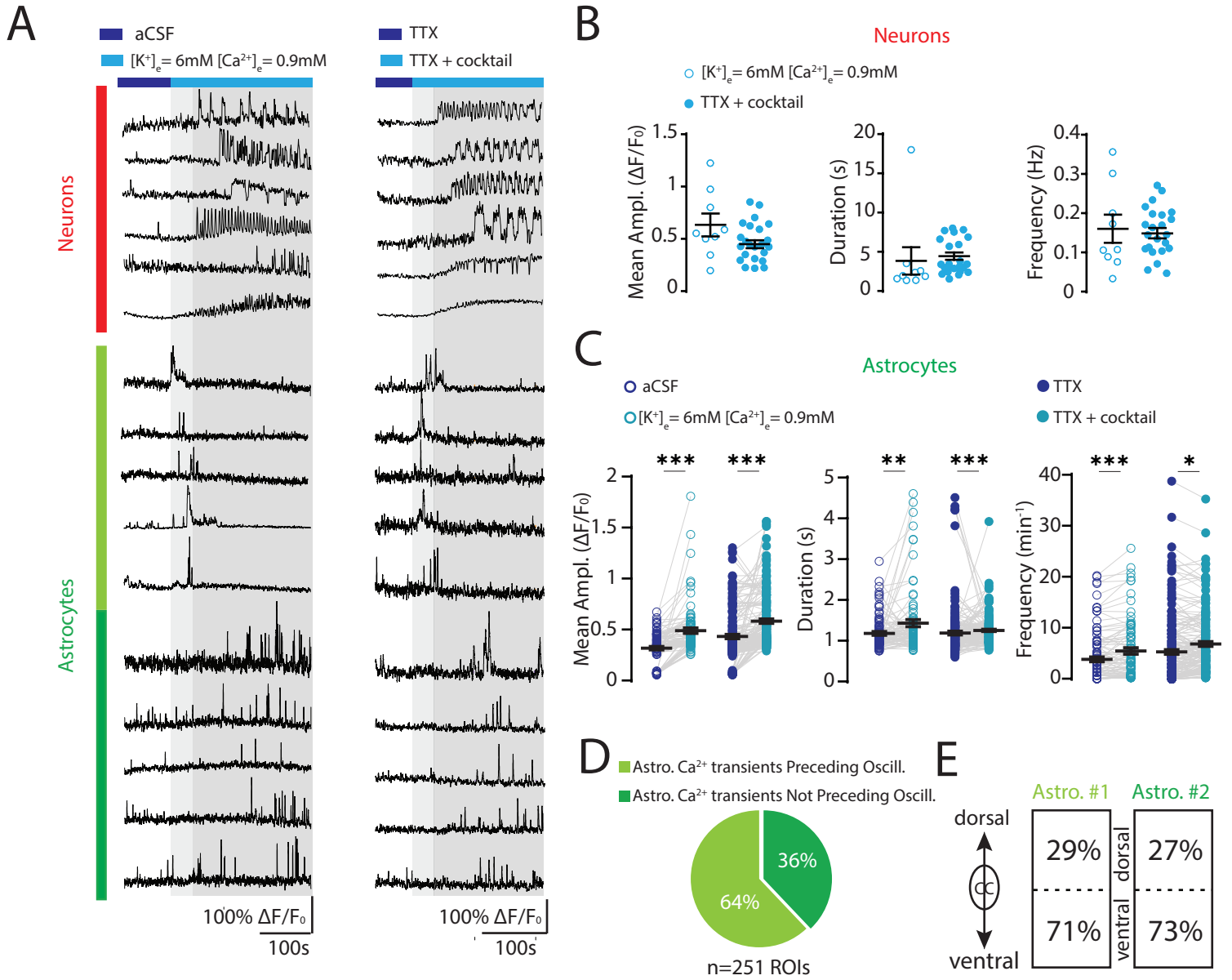


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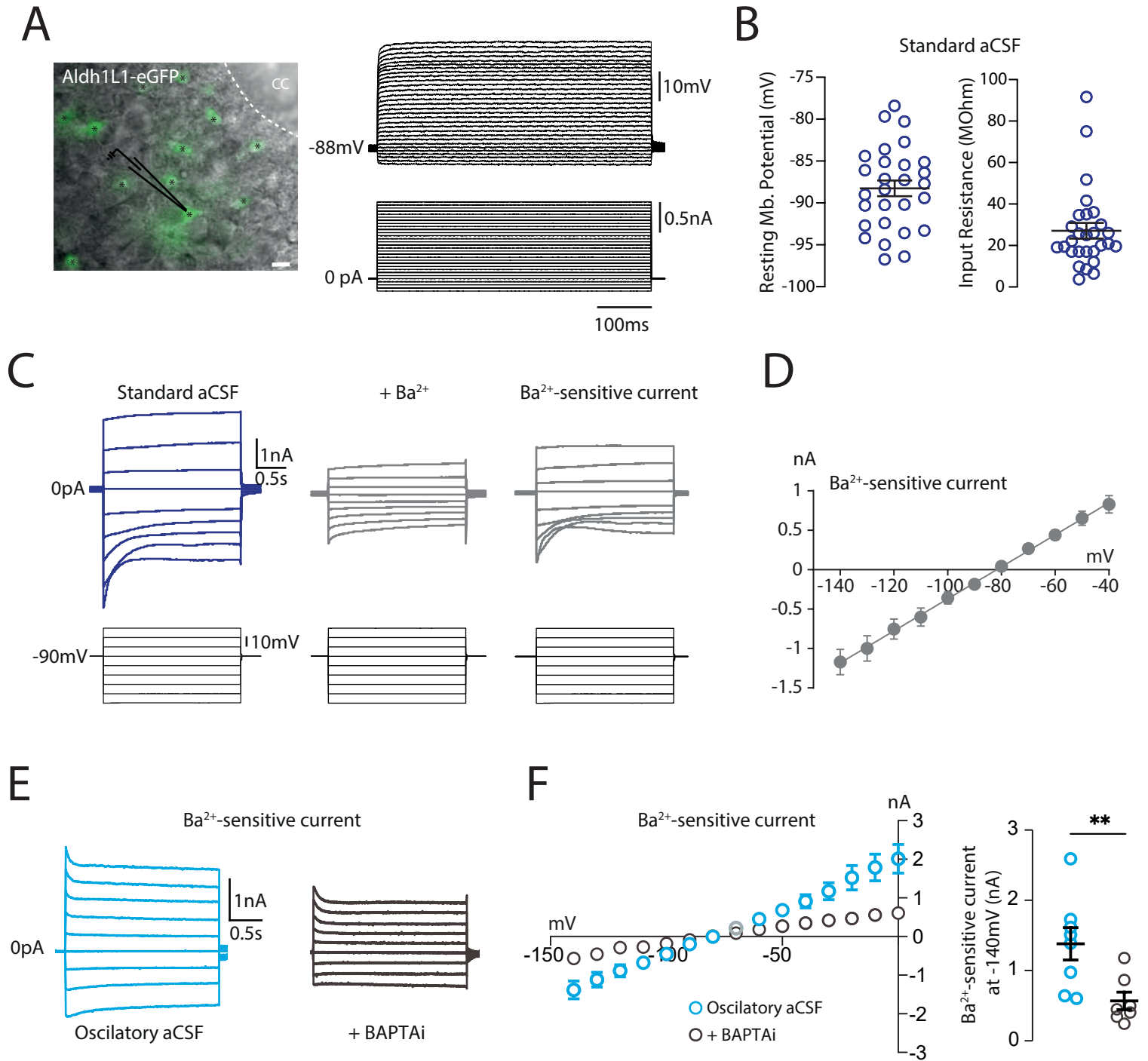
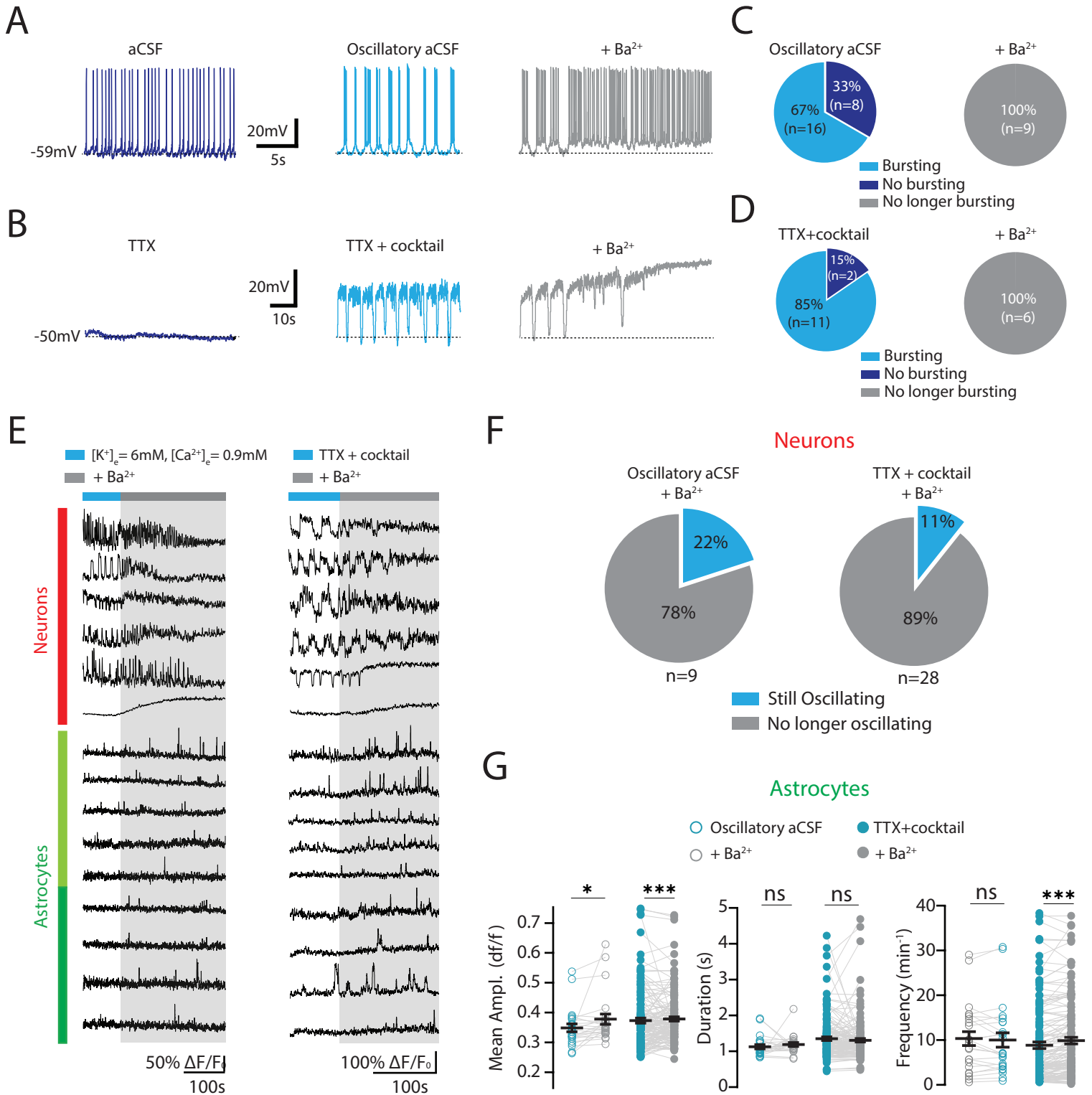
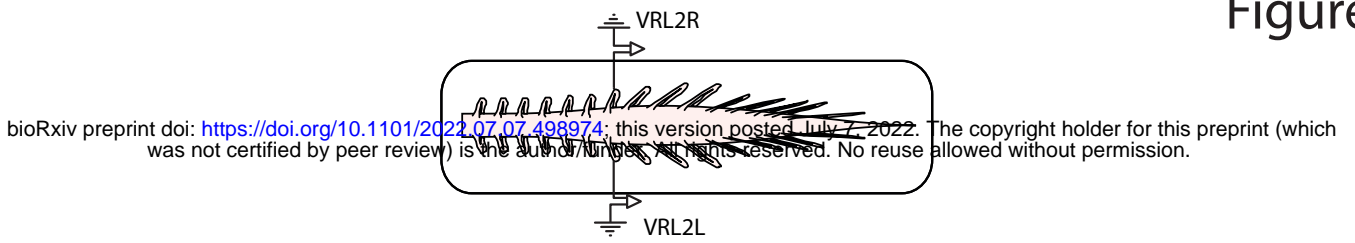


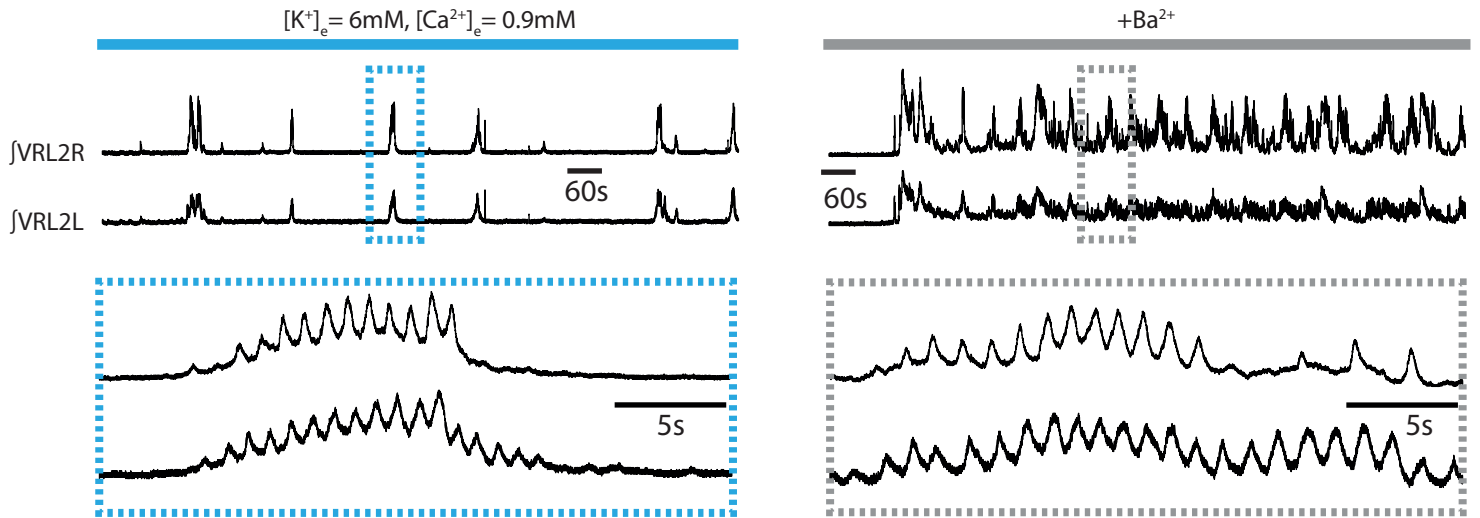
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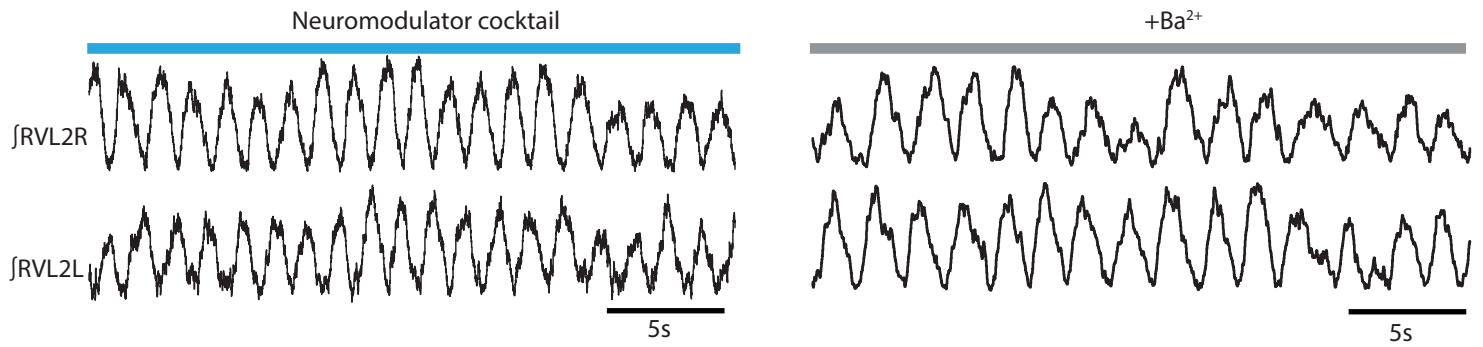
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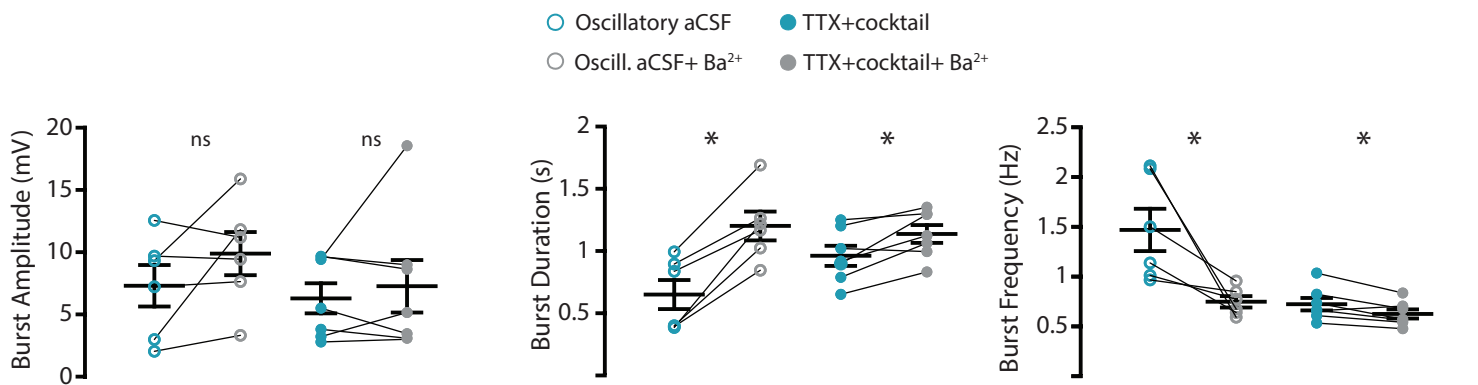
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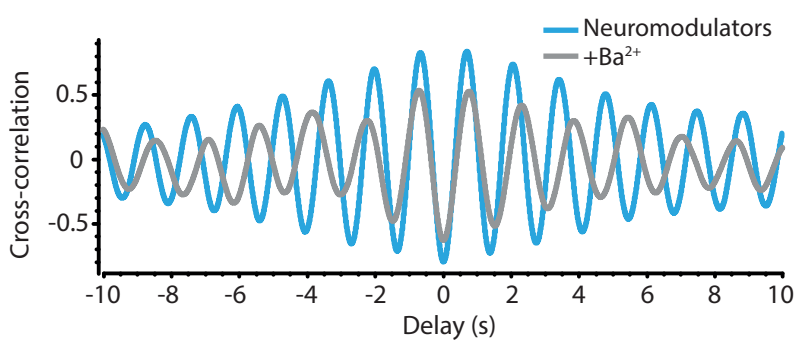
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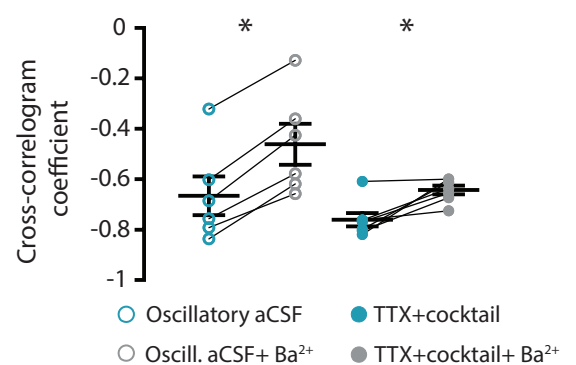
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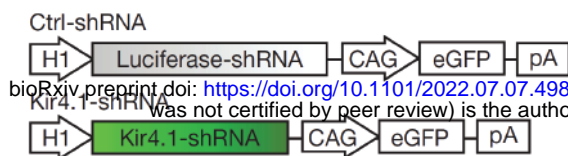
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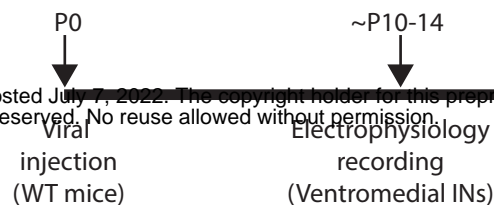


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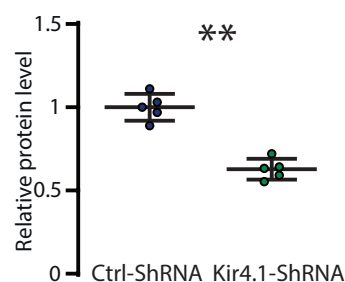
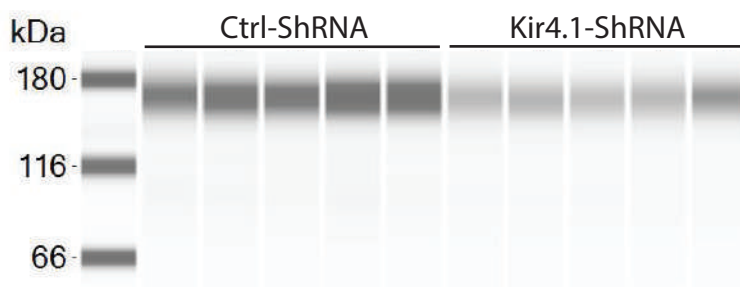


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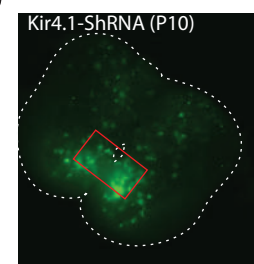
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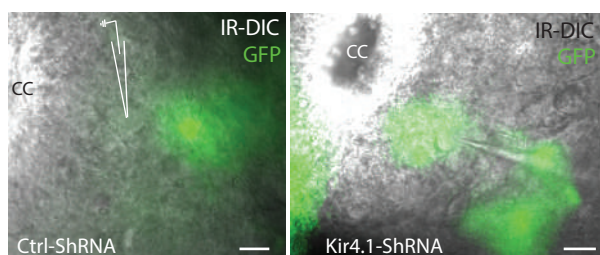
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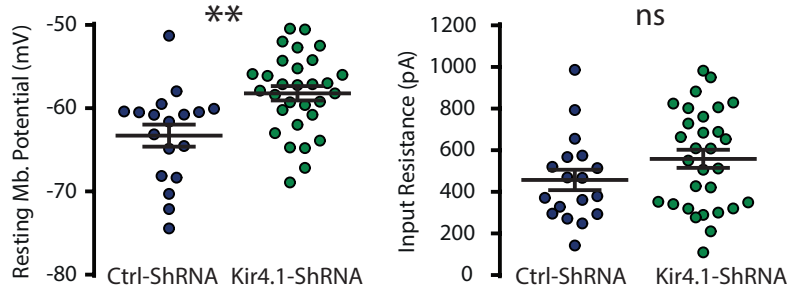
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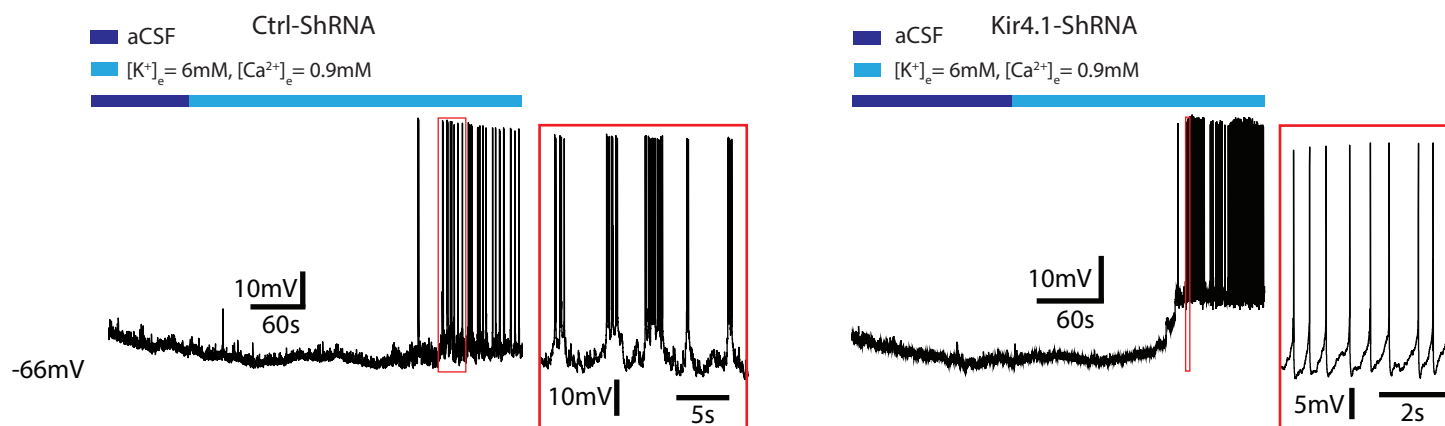
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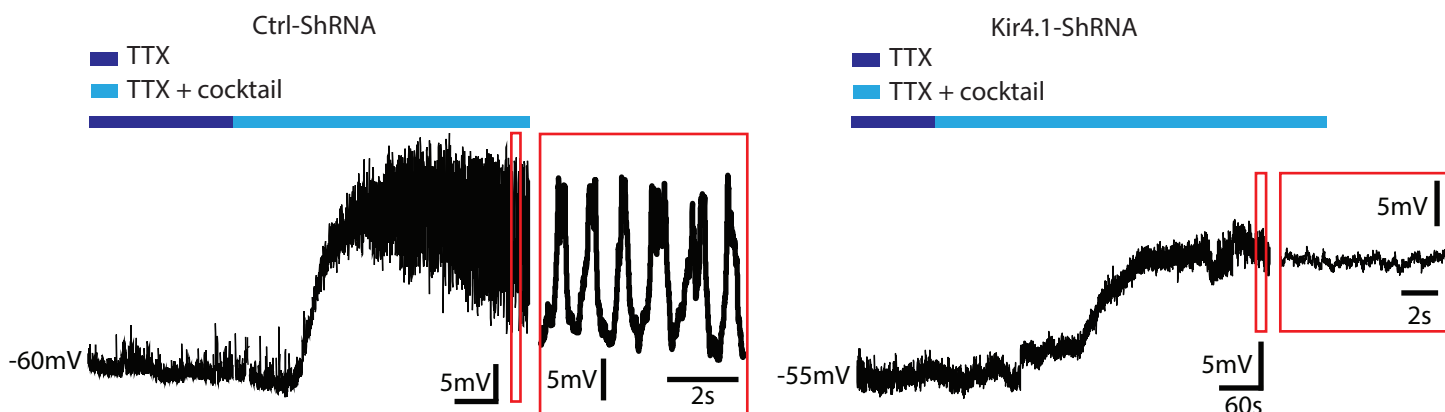
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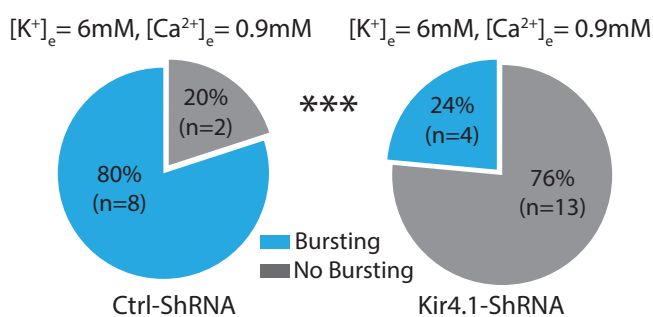
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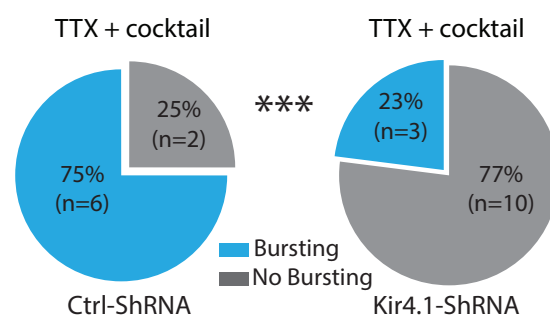
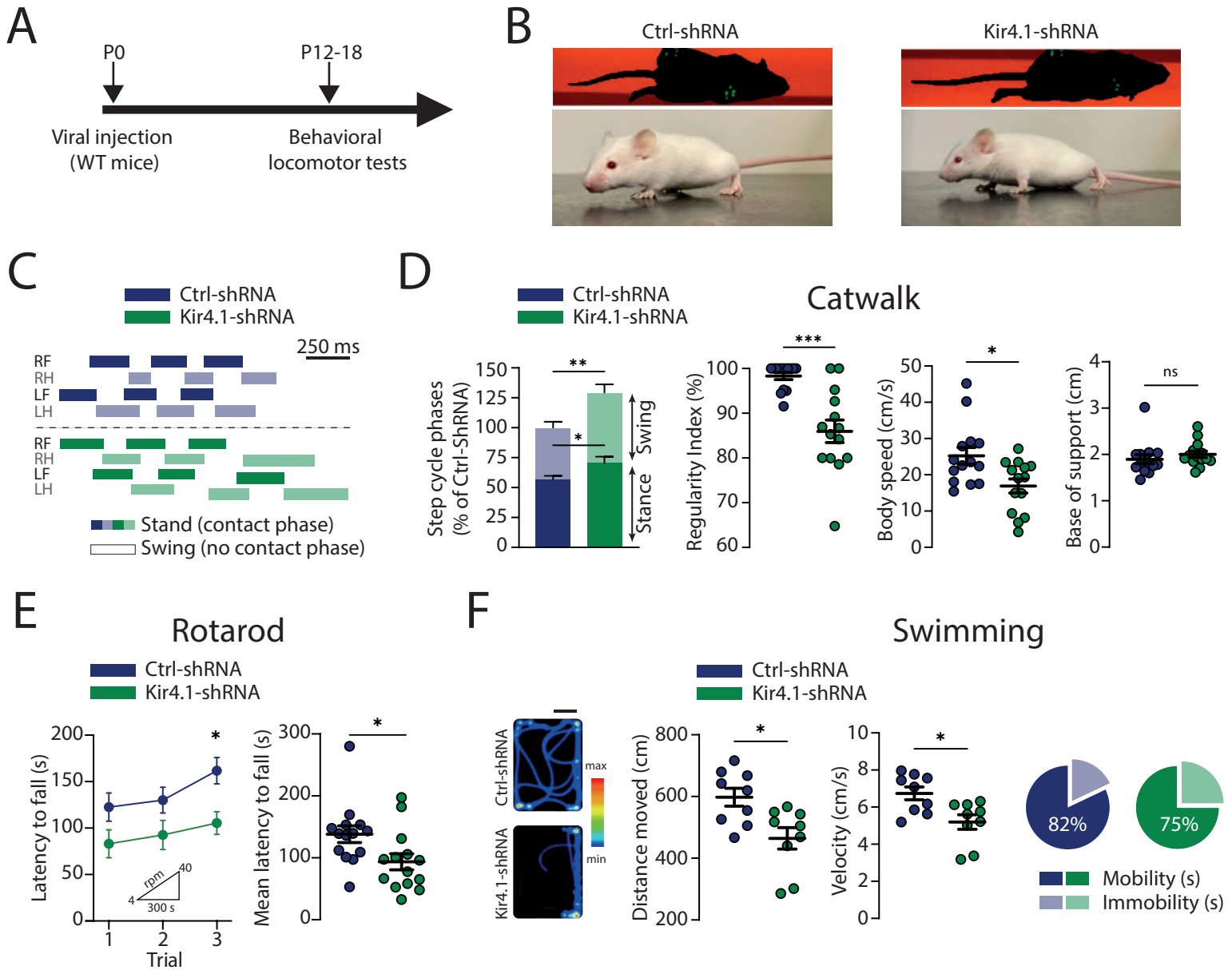
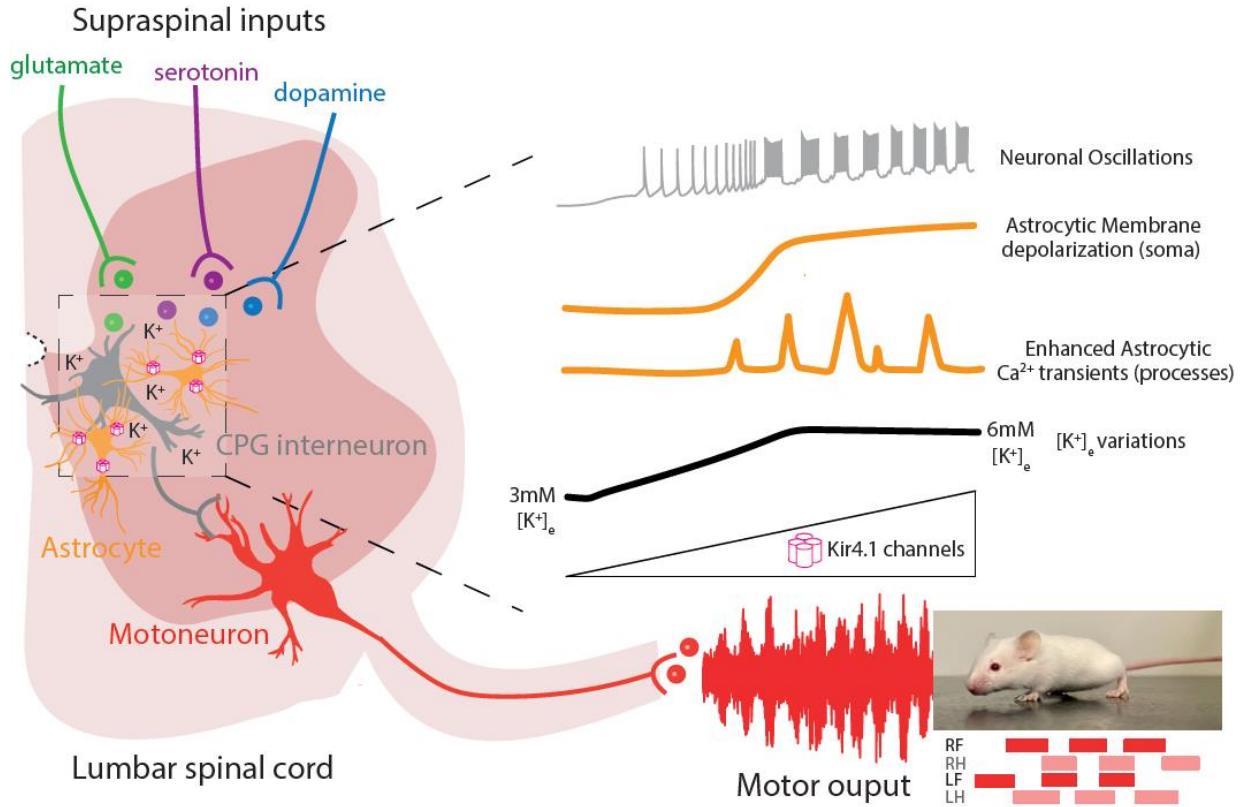


Figure 7





Graphical Abstract. Overview of the astrocytic K⁺ uptake mechanisms influencing the locomotor pattern. Schematic relationship between astrocytes and neurons in the spinal locomotor central pattern generator (CPG) network. K⁺ for potassium, RF for right front limb, LF for left front limb, RH for right hind limb, LH for left hind limb.