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Intraspinal Sensory Neurons Provide Powerful Inhibition to Motor Circuits Ensuring Postural Control during Locomotion

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1 Title

2 Intraspinal sensory neurons provide powerful inhibition to motor circuits ensuring
3 postural control during locomotion

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15 Summary

16 In the vertebrate spinal cord, cerebrospinal fluid-contacting neurons (CSF-cNs) are
17 GABAergic neurons whose functions are only beginning to unfold. Recent evidence
18 indicates that CSF-cNs detect local spinal bending and relay this mechanosensory
19 feedback information to motor circuits. Yet many CSF-cN targets remain
20 unknown. Using optogenetics, patterned illumination and *in vivo* electrophysiology,
21 we show here that CSF-cNs provide somatic inhibition onto fast motor neurons and
22 excitatory sensory interneurons involved in the escape circuit. Ventral CSF-cNs
23 respond to spinal bending, including a longitudinal component, and induce large
24 inhibitory postsynaptic currents (IPSCs) sufficient to silence spiking of their targets.
25 Upon repetitive stimulation, these IPSCs promptly depress enabling the
26 mechanosensory response to the first bend to be the most effective. When CSF-cNs

27 are silenced, postural control is compromised resulting in rollovers during escapes.
28 Altogether our data demonstrates how GABAergic sensory neurons provide
29 powerful inhibitory feedback onto the escape circuit to maintain balance during
30 active locomotion.

31 **Keywords**

32 spinal cord, connectome, CSF-cN, GABAergic sensory neuron, zebrafish, optogenetics,
33 sensory-motor feedback, posture, escape behavior

34 **Running title**

35 Ventral CSF-contacting neurons detect longitudinal contraction of the spinal cord
36 and locally project onto elements of the escape circuit to control posture

37 **Introduction**

38 Cerebrospinal fluid-contacting neurons (CSF-cNs) were first identified nearly a
39 century ago and are highly conserved in the spinal cord, having been described in
40 over 200 vertebrate species [1, 2]. Despite being a central element of the vertebrate
41 spinal cord, the precise cellular connectivity and function of CSF-cNs is only recently
42 beginning to be described [3-7]. CSF-cNs exhibit an apical dendritic extension
43 bearing microvilli situated in the lumen of the central canal. These cells express the
44 transient receptor potential channel TRPP3 (or Polycystic Kidney Disease 2-Like 1,
45 PKD2L1) [8, 9], allowing them to respond to variations in pH and osmolarity in the
46 CSF [4, 8]. Based on their anatomy, these cells have been proposed to detect flow
47 or content of the CSF [10, 11].

48 Recently we demonstrated that dorsal CSF-cNs on either side of the central canal are
49 activated by curvature of the spinal cord selectively on the side of bending in larval
50 zebrafish [7]. We showed evidence that CSF-cNs modulate stereotyped behaviors in
51 intact zebrafish thought to be driven by locomotor central pattern generators (CPGs),
52 both for slow locomotion [5] as well as for fast locomotion during acoustic escapes
53 [7]. However, precise cellular connections by which CSF-cNs modulate fast

54 locomotion have not been previously investigated. Escapes in fish are a stereotyped
55 movement program that is typically triggered by the sequential activation of sensory
56 neurons, leading to recruitment of the Mauthner cell in the hindbrain [12, 13] and
57 finally the activation of spinal neurons including primary motor neurons. This induces
58 a large C-bend on one side of the animal that is coincident with recruitment of
59 commissural inhibitory glycinergic interneurons to silence motor output on the other
60 side [14, 15]. The neurons that underlie locomotion are known to reside in the
61 ventral spinal cord where CSF-cNs send most of their projections [3, 5, 11]. This
62 places CSF-cNs in an optimal position to modulate the spinal escape circuit. To
63 establish the postsynaptic targets of CSF-cNs within the spinal cord, we combined
64 whole cell patch clamp recordings of putative targets with 2D light patterning and
65 ChannelRhodopsin (ChR2) mediated activation of CSF-cNs in the zebrafish larva. We
66 took advantage of transgenic lines labeling specific classes of spinal neurons in order
67 to target the recordings to given cell types, whose identity was later confirmed by
68 cell filling and morphological reconstruction.

69 Here we provide evidence that ventral CSF-cNs are recruited during spontaneous
70 contraction of the animal involving a longitudinal bend. We show that these CSF-cNs
71 innervate multiple components of the escape circuit, namely, a subset of primary
72 motor neurons as well as a class of glutamatergic interneurons involved in sensory
73 motor gating. We found that this connectivity with key elements of the escape circuit
74 is specific since CSF-cNs did not project onto either glycinergic premotor
75 interneurons or mechanosensory neurons involved in the escape response. Ventral
76 CSF-cNs provide somatic, perisomatic and axon initial segment innervation onto
77 primary motor neurons, reminiscent of basket-cell synapses. The innervation of the
78 motor neuron pool by CSF-cNs is selective for caudal primary motor neurons
79 referred to as CaP, which are involved in fast locomotion and postural control [16,
80 17]. The innervation of sensory interneurons is restricted to the initial segment and
81 soma, with occasional axo-dendritic contacts. On both of these CSF-cN targets,
82 ventral CSF-cNs induce a remarkably large and reliable inhibitory postsynaptic
83 current (IPSC) with similar properties. Stimulus trains at moderate frequencies (10-

84 20Hz) rapidly induce short-term depression of the postsynaptic response. Spatially-
85 restricted photoactivation of single CSF-cNs indicates that multiple CSF-cNs converge
86 onto a given target. The convergence of inputs onto single primary motor neurons
87 from ventral CSF-cNs provides strong GABAergic inhibition capable of efficiently
88 silencing motor output. Furthermore, we show behaviorally that silencing CSF-cN
89 output with botulinum toxin results in a defect in postural control during acoustically
90 induced escapes responses.

91 Our findings demonstrate that an intraspinal GABAergic system actively senses spinal
92 cord curvature during locomotion and constitutes a local sensory-motor loop that
93 modulates posture during rapid movement.

94

95 **Results**

96 **Dorsal projections from ventral CSF-cNs innervate primary motor neurons**

97 In order to identify CSF-cN targets, we carefully investigated the morphology of their
98 axonal projections. While a large density of CSF-cN axons project within the ventral
99 portion of the spinal cord [5], some of the ventral CSF-cNs extend axonal projections
100 dorsally, encircling large cell bodies (Figure 1A-C, 1E). This structure contained
101 multiple large varicosities (Figure 1A, 1B) associated with putative presynaptic
102 boutons labeled by Synaptophysin-GFP (Figure 1C). The position of these presynaptic
103 structures suggested innervation of dorsal primary motor neurons (pMNs), which are
104 recruited during escapes and fast swimming in zebrafish larvae [17]. We screened
105 different transgenic cell lines labeling specific cell types in the zebrafish spinal cord
106 and identified the anatomical contact of CSF-cNs to caudally-located dorsal primary
107 motor neurons (Figure 1E), referred to as CaP [18].

108 **Selective connectivity onto primary motor neurons involved in fast locomotion** 109 **and postural control**

110 To test the functional connectivity of CSF-cNs to primary motor neurons, we optically

111 activated CSF-cNs expressing ChR2 while recording from the cell body surrounded
112 by presynaptic boutons (Figure 1D). Cells whose soma were encircled by the CSF-cN
113 basket structure correspond to CaP primary motor neurons as shown by their
114 characteristic morphology after dye filling (Figure 1E), their input resistance, and their
115 sustained firing of action potentials at high frequency (Figure 1F). The morphology of
116 the axonal projection suggests that individual CSF-cNs innervate multiple CaP motor
117 neurons along the rostro-caudal axis (Figure 1E). In our conditions, a 5 ms light pulse
118 typically induces a single spike in CSF-cNs expressing ChR2-mCherry (see [5]).
119 Following the optical activation of CSF-cNs, we recorded large IPSCs in CaP motor
120 neurons occurring without failure (34 out of 34 CaP motor neurons recorded). These
121 IPSCs were abolished by bath application of the GABA_A receptor antagonist gabazine
122 (Figure 1G, 1H). The light-induced current-voltage relationship showed that the
123 IPSCs reversed around - 53 mV, close to the calculated reversal potential of chloride
124 in our conditions ($E_{Cl} = - 51$ mV, Figure 1I, 1J). The timing and kinetics of the light-
125 induced IPSCs were consistent with monosynaptic currents mediated by GABA_A
126 receptors (Figure 1K-N). These data indicate that CaP motor neurons are one major
127 target of CSF-cNs.

128 **Other motor neurons are minimally innervated by CSF-cNs**

129 Given the significant innervation pattern observed for CaP motor neurons, we
130 proceeded to determine whether other motor neurons (both primary and secondary)
131 receive synaptic input from CSF-cNs. Targeted whole cell recordings of primary and
132 secondary motor neurons were performed in *Tg(parg^{mnet2}-GFP)* transgenic fish
133 (Figure 2A). As shown previously, CaP motor neurons were distinguished based on
134 soma location within the segment and the characteristic basket-like synaptic contacts
135 from CSF-cNs (Figure 1A, 1B, 1E and Figure 2A, CaP motor neurons indicated by "C"
136 in magnified images). Responses for non-CaP primary motor neurons (Figure 2A
137 magnified boxes, indicated by arrows) following ChR2-mediated activation of CSF-
138 cNs fell into three classes (Figure 2B1-B3). Only one non-CaP primary motor neuron
139 out of 17 recorded showed IPSCs comparable to responses observed in CaP motor
140 neuron recordings (Figure 2B1, lower panel). In 11 of 17 non-CaP primary motor

141 neurons the postsynaptic responses were very small (< 5 pA, Figure 2B2, lower
142 panel) and in the remaining 5 non-CaP primary motor neurons no IPSCs were
143 observed (Figure 2B3, lower panel). The majority of IPSCs observed in non-CaP
144 primary motor neurons were of small amplitude (< 5 pA, Figure 2C). All but two of
145 the events greater than 10 pA were observed in trials from a single neuron (Figure
146 B1), suggesting that CSF-cN innervation of primary motor neurons is overwhelmingly
147 restricted to CaP motor neurons. Secondary motor neurons were also tested for
148 CSF-cN connectivity and were targeted based on fluorescence, ventral location and
149 small soma size in the *Tg(parg^{mnnet2}-GFP)* transgenic line (Figure 2A magnified boxes,
150 indicated by arrowheads). Secondary motor neurons showed typical bursting action
151 potential firing patterns (see example in Figure 2D), however CSF-cN activation with
152 5 ms blue light pulses never produced IPSCs in secondary motor neurons in 10 of 10
153 cells recorded (Figure 2E, three secondary motor neuron examples shown). CSF-cNs
154 therefore form very specific contacts within the motor pool onto CaP motor neurons.

155 **Optogenetic-mediated mapping reveals connectivity onto sensory interneurons**

156 We noted that some of the CSF-cN axons project to the dorsal spinal cord,
157 suggesting they target other spinal neurons. We hypothesized that they might target
158 sensory interneurons in this population and tested a subtype of glutamatergic
159 interneuron (called CoPA), known to be involved in sensory-motor gating and
160 recruitment of motor neurons in the contralateral spinal cord [19, 20]. By selectively
161 labeling CoPA interneurons in the *Tg(tbx16-GFP)* line [21], we observed that some
162 CSF-cN varicosities were located on the CoPA soma (Figure 3A1, 3A2) and axon
163 initial segment (Figure 3A1-A4). Interestingly, we noted that the morphology of CSF-
164 cN axons suggests that an individual CSF-cN in contact with CaP (forming the
165 basket-like synapse) may also diverge onto the adjacent CoPA dendrite (Figure 3A3,
166 3A4). We performed targeted whole cell patch clamp recordings (Figure 3B) and
167 simultaneous photostimulation of CSF-cNs and found evidence of monosynaptic
168 connections onto CoPA interneurons (Figure 3C). CoPA IPSCs were large and did not
169 fail (8 out of 8 cells, Figure 3C). The IPSCs recorded in CoPA showed properties
170 typical of GABA_A mediated currents, similar to the IPSCs recorded in CaP motor

171 neurons (Figure 3D-G). However, IPSC amplitudes tended to be larger for CoPA
172 sensory interneurons than those observed in CaP motor neurons (Figure 3H).

173 **Convergence of inputs from multiple CSF-cNs onto individual targets**

174 We took advantage of a 2D light patterning approach [3, 22] to activate specific
175 ChR2-expressing cells within the zebrafish spinal cord in order to test the
176 connectivity of individual CSF-cNs onto CaP and CoPA targets (Figure 4). We used a
177 custom-built illumination setup based on a Digital Mirror Device (Figure 4A) to
178 pattern the stimulation light to spatially restricted targets (Figure 4B, 4C). The light
179 stimulation was effective in triggering an IPSC only when it was directed onto the
180 soma or occasionally on the initial segment of CSF-cNs (Figure 4D) but not on the
181 rest of the axonal projection, including the varicosities within the basket structure
182 surrounding the soma of the recorded cell (Figure 4D). The amplitude of IPSCs
183 tended to decrease as a function of distance between the presynaptic CSF-cN and its
184 target, with connections emanating from CSF-cNs less than three segments away
185 from the target producing the largest responses (Figure 4E). Our data also shows
186 that multiple CSF-cNs often innervate the same target neuron, either the CaP motor
187 neuron (Figure 4D, 4F and 4G) or CoPA interneuron (Figure 4H), indicating a high
188 degree of convergence from CSF-cNs onto their targets.

189 **Neither commissural glycinergic neurons nor mechanosensory neurons involved** 190 **in the escape circuit receive inputs from CSF-cNs**

191 We next sought to address whether the functional connectivity of CSF-cNs was
192 specific to the glutamatergic interneurons and motor neurons of the escape circuit or
193 whether they exert a distributed modulation impacting all elements of the escape
194 pathway. We tested whether CSF-cNs project on the contralaterally-projecting
195 glycinergic neurons, referred to as CoLo cells, involved in silencing activity on the
196 contralateral side during the initial tail bends of the escape response ([15], Figure 5A-
197 C). Targeted patch clamp recordings of CoLos using the *Tg(Tol-056-GFP)* transgenic
198 line ([15], Figure 5A) showed no light-induced IPSCs in 13 out of 13 CoLos recorded
199 (Figure 5B, 5C three examples shown). We also tested the connectivity onto

200 mechanosensory Rohon-Beard neurons that are well upstream of the escape circuit.
201 Anatomical analysis of Rohon-Beard neurons and CSF-cNs in the *Tg(p2rx3.2:GFP ;*
202 *pkd2l1:gal4 ; UAS:ChR2-mCherry)* triple transgenic larvae showed no overlap of CSF-
203 cN axons onto the Rohon-Beard soma or axons ([23], Figure 5D). Whole cell
204 recordings of Rohon-Beard neurons were performed to rule out functional
205 connectivity to CSF-cNs (Figure 5E, 5F). IPSCs in Rohon-Beard neurons were never
206 observed following ChR2-mediated activation of CSF-cNs with 5 ms light pulses
207 (Figure 5F, n = 10, three examples shown). Taken together our data establishes a
208 map of CSF-cN innervation onto specific elements of the escape circuit. CSF-cNs
209 create extensive synaptic contacts specifically onto CaP primary motor neurons and
210 CoPA glutamatergic sensory interneurons with minimal projections onto other
211 primary motor neurons, and an exclusion of projections on secondary motor
212 neurons, CoLo glycinergic commissural interneurons and the Rohon-Beard
213 mechanosensory neurons.

214 **CSF-cN synapses onto targets of the escape circuit show strong short term** 215 **depression**

216 Common features of CSF-cN mediated IPSCs recorded from primary motor and
217 sensory interneurons include their high reliability and large amplitude (Figure 6A1-
218 A3). Since CSF-cNs are recruited by spinal curvature during active locomotion [7],
219 we tested whether these synapses showed short term plasticity when stimulated at
220 higher frequencies corresponding to larval swimming (10-20Hz, Figure 6C, 6D). While
221 1 Hz stimulation induced moderate short-term depression following 10 light pulses
222 (Figure 6B1-B3), raising the stimulation frequency to 10 and 20 Hz led to an
223 incremental increase in short term synaptic depression (Figure 6C1-C3, 6D1-D3).

224 **A single CSF-cN action potential leads to prompt silencing of spiking in CSF-cN** 225 **targets within the escape circuit**

226 Although we showed that CSF-cN firing causes a large and reliable chloride
227 conductance in CaP motor neurons and CoPA sensory interneurons, the impact of
228 this modulation on the output of the CPG, namely motor neuron activity, was

229 unclear. It has been suggested that an immature chloride gradient in the larval spinal
230 cord could lead GABAergic input to be depolarizing in postsynaptic neurons [24]. We
231 therefore tested how CSF-cNs modulated the spiking of their motor neuron targets
232 by recording CaP motor neurons in cell-attached mode to preserve the chloride
233 gradient in the postsynaptic neuron. A large voltage step induced high frequency
234 firing in CaP motor neurons in this configuration (Figure 7A, 7B). A 5 ms light pulse
235 (producing a single large IPSC) was sufficient to transiently silence the spiking of CaP
236 motor neurons (Figure 7B, 7C). Quantification of the maximum interspike interval
237 (ISI) for control trials and trials where a 5 ms light pulse activated CSF-cNs showed a
238 significant increase after the light pulse in all cells tested (ISI control = $9.10 \text{ ms} \pm$
239 3.04 ms , ISI light $26.69 \text{ ms} \pm 10.55 \text{ ms}$, $n = 4$), confirming the inhibitory nature of the
240 GABAergic IPSCs from CSF-cNs onto their targets (Figure 7D).

241 **CSF-cNs are mechanosensory cells that control balance during fast locomotion**

242 We monitored CSF-cN activity using the calcium genetically-encoded indicator
243 GCaMP3 combined with the position marker mCherry in unparalyzed larvae, which
244 were mounted on their side and embedded in agarose. In these conditions, we
245 found that ventral CSF-cNs are recruited during spontaneous longitudinal
246 contractions (Figure S1 and Movie S1). Imaging and functional mapping experiments
247 suggest that only ventral CSF-cNs, not dorsal CSF-cNs, innervate CaP primary motor
248 neurons involved in postural control (Figure 1E and Figure 4D, 4F and 4G). From
249 these results we hypothesized that ventral CSF-cNs could act as a mechanosensory
250 system detecting longitudinal spinal bending and subsequently provide inhibitory
251 tone to CaP motor neurons. We tested this hypothesis by analyzing the behavior of
252 animals in which CSF-cN synapses were silenced by botulinum toxin [7]. We
253 reanalyzed the dataset from Böhm, et al. and rollover events were scored by a
254 blinded observer. We determined a roll ratio for each fish (number of trials the fish
255 rolled/the number of trials the fish responded to the acoustic stimulus) and found
256 that rollovers occurred twice as often in animals expressing botulinum toxin in CSF-
257 cNs compared to control siblings (Figure 7E, 7F and Movies S2 and S3). This result
258 indicates that CSF-cNs contribute to maintaining balance during active locomotion.

259 **Discussion**

260 **Selective inhibition from GABAergic sensory neurons onto sensory interneurons**
261 **and motor neurons of the escape circuit**

262 Our work demonstrates a strong and selective connection from CSF-cNs onto
263 primary motor neurons and glutamatergic sensory interneurons (CaP and CoPA
264 respectively). This connectivity appears specific within the escape circuit of the
265 zebrafish spinal cord, as CSF-cNs avoid synaptic contacts to secondary motor
266 neurons, mechanosensory neurons and glycinergic premotor interneurons which are
267 involved in escapes. CSF-cN input to motor neurons is mainly limited to the primary
268 motor neuron, CaP, while other primary motor neurons generally receive little to no
269 synaptic input. The specificity of the CSF-cN synapse onto CaP motor neurons
270 suggests that these motor neurons may play a specialized role that differs from
271 other primary motor neurons. CaP motor neurons are the first motor neurons to
272 extend from the spinal cord to the skeletal muscle in the developing embryo [18].
273 Primary motor neurons (CaP, MiP, and the two RoPs) innervate distinct territories of
274 axial, fast skeletal muscle fibers. Of the primary motor neurons, CaP innervates the
275 largest field of fast skeletal muscle, covering approximately 2/3rds of the ventral
276 fibers. The differential activation of primary motor neurons is thought to induce
277 body torque and therefore a change in vertical trajectory [16]. Beyond their
278 importance in fast locomotion and the escape response, CaP motor neurons most
279 likely play a role in maintaining postural control. In this study, we observe that CSF-
280 cNs project selectively onto CaP motor neurons, and that the silencing of CSF-cNs
281 leads to a balance defect causing larvae to tip and roll over during acoustically
282 induced escape responses. This observation suggests that inhibition to CaP motor
283 neurons by CSF-cNs plays a critical role in the control of posture during fast
284 swimming. Yet, we cannot exclude that other putative targets of CSF-cNs contribute
285 to this effect as well.

286 **Physiology of CSF-cN synapses onto their targets within the escape circuit**

287 The somatic and axonic innervation of CSF-cNs onto CaP motor neurons and CoPA

288 interneurons is enhanced by the convergence of inputs from multiple CSF-cNs onto
289 one target neuron. This convergence is reminiscent of the projection from basket
290 cells onto pyramidal neurons [25-28], and is associated with large reliable IPSCs. The
291 CSF-cN mediated inhibition from a single spike is efficient enough to transiently
292 silence postsynaptic targets within the escape circuit. At higher stimulation
293 frequencies, synapses of CSF-cNs onto their targets rapidly depress. In direct
294 recordings from CSF-cNs in the cell-attached configuration, optogenetically-mediated
295 activation of CSF-cNs has been confirmed up to 25Hz without action potential
296 failures. We therefore believe that the observed plasticity most likely reflects a
297 presynaptic mechanism consistent with other high release probability synapses that
298 undergo short-term depression rather than failure to optogenetically elicit spiking in
299 CSF-cNs. Remarkably, the short-term depression occurs at frequencies that closely
300 match the naturally occurring tail beat frequencies of zebrafish larvae. A result of this
301 property is that within this range of CSF-cN firing frequencies, the first IPSC is the
302 most effective at modulating the spiking of motor and sensory interneuron targets.
303 This feature suggests a homeostatic function for the feedback inhibition provided by
304 CSF-cNs: large motor neurons triggering the massive muscle contractions during the
305 C-bend also recruit GABAergic sensory neurons that rapidly silence them.

306 The physiology of CSF-cN synapses onto elements of the fast escape circuit shown
307 here is remarkably different from their modulation of the slow swimming circuit [5].
308 The connections from CSF-cNs onto MCoD glutamatergic premotor interneurons
309 produce small amplitude IPSCs that are subject to failures and facilitate during
310 repetitive stimulation [5]. In contrast, the projections of CSF-cNs onto both CaP and
311 CoPA targets within the escape circuit are large, show no failure and rapidly depress
312 over time. During repetitive contractions when the animal swims at high speed, this
313 GABAergic sensory-motor pathway may therefore promptly silence motor neurons
314 and interneurons involved in the initial phase of the escape, enabling a tight control
315 on spike timing of motor neurons and a rapid transition from fast to slow swimming
316 frequencies [29].

317

318 **Relevance to physiology and postural control**

319 As indicated by anatomy [3, 5, 30-33], we demonstrate, using physiology and
320 optogenetics, that the GABAergic sensory feedback provided on the escape circuit is
321 local and intraspinal, never reaching targets more than five segments away in the
322 larval stage. This GABAergic pathway can therefore locally tune the excitability of
323 components of the escape in the spinal cord, without affecting the activity of
324 reticulospinal neurons in the hindbrain.

325

326 Fast escapes in zebrafish larvae are highly regulated both in terms of lateral
327 displacement as well as vertical elevation [34], so that larvae do not perform spiral
328 trajectories as seen in *Xenopus* tadpoles [35]. Control of posture most certainly
329 involves visual and vestibular feedback relayed by reticulospinal neurons down the
330 spinal cord in order to optimally activate primary motor neurons [36]. Here we
331 describe a local sensory-motor pathway for regulating posture situated within the
332 spinal cord, a concept which had to our knowledge has only been described in birds
333 where balance is stabilized by the vestibular organ during flight and by the
334 lumbosacral system during walking [37]. By genetically targeting the optimized
335 botulinum toxin to selectively block synaptic release from CSF-cNs, we observed a
336 balance defect in BoTx fish compared to control siblings not expressing the toxin.
337 Animals with CSF-cN neurotransmission silenced were twice as likely as their wild
338 type siblings to tip and roll over during an acoustically induced escape response. In
339 addition, we show that ventral CSF-cNs project onto CaP motor neurons and are
340 physiologically activated during longitudinal contractions, which is not the case in
341 differential left or right bending of the tail [7]. These results point to an asymmetrical
342 proprioceptive function for CSF-cNs, whereby dorsal CSF-cNs respond to left or right
343 horizontal bending while ventral CSF-cNs respond to longitudinal bending of the
344 spinal cord. CSF-cNs would therefore provide mechano-sensory feedback during
345 locomotion to inhibit motor output through the specific connectivity to the CaP
346 motor neuron and excitatory interneurons such as CoPA and MCoDs. CSF-cNs
347 therefore may constitute a mechanosensory system within the spinal cord, which

348 provides important proprioceptive feedback to coordinate locomotion and balance.
349 The CSF-cNs may themselves be modulated by reticulospinal neurons or
350 vestibulospinal pathways involved in the control of posture. This will be the focus of
351 future investigations as descending inputs were severed by decapitation prior to
352 testing intraspinal CSF-cN connectivity in this study.

353

354 **Experimental Procedures**

355 *Animal care and transgenics used*

356 Animal handling and procedures were validated by the Institut du Cerveau et de la
357 Moelle épinière (ICM, Paris) and the French National Ethics Committee (Comité
358 National de Réflexion Éthique sur l'Expérimentation Animale- Ce5/2011/056) in
359 agreement with the European Union legislation. Adults were reared at a maximal
360 density of 8 animals per liter in a 14/10 (light/dark) cycle environment. Fish were fed
361 live artemia twice a day and feeding regime was supplemented with solid extracts
362 matching the developmental stage (ZM Systems, UK). Larvae were raised at 28.5°C
363 with a 14/10 (light/dark) light cycle. Experiments were performed at room
364 temperature (22-25°C) on 3 to 7 dpf larvae. All transgenic lines used here are
365 detailed in Suppl. Table S1. We injected the UAS:synaptophysin-GFP [38] DNA
366 construct at 60 ng/μl into *Tg(pkd2l1:gal4;UAS:Chr2-mCherry)* single cell-stage
367 embryos.

368 *Electrophysiology*

369 3-7dpf zebrafish larvae were decapitated and pinned to a Sylgard coated recording
370 chamber (Sylgard 184, Dow Corning, Midland, MI, USA) through the notochord with
371 electrolytically sharpened tungsten pins. The skin was removed and the specimen
372 was bathed briefly in a 10% formamide solution and subsequently washed in bath
373 recording solution to eliminate spontaneous muscle twitching. The dura was
374 exposed by suctioning away dorsal muscle fibers with a glass pipette. Typically 3-7
375 segments of dorsal muscle were removed. Recording electrodes were fashioned
376 from capillary glass (1.5 mm O.D., 1.1 ID, WPI, Sarasota, FL, USA) with a horizontal

377 puller (P1000, Sutter Instruments, Novato, CA). Electrode resistances were 10-16
378 M Ω . Positive pressure (65mm Hg) was applied to the recording electrode via a
379 pneumatic transducer (Fluke Biomedical DPM1B, Everett, WA). Once the electrode
380 was driven through the dura in order to approach neurons targeted for patch
381 experiments, the positive pressure was reduced to 35mm Hg [39]. Cells were chosen
382 based on their soma location matching the axonal projections of CSF-cNs expressing
383 ChR2-mCherry and the expression of GFP in the transgenic lines used (Table S1).
384 External bath recording solution contained the following (in mM), 134 NaCl, 2.9 KCl,
385 2.1 CaCl₂-H₂O, 1.2 MgCl₂, 10 Glucose, 10 HEPES with pH adjusted to 7.4, and
386 osmolarity to 290 mOsm. Spinal neuron internal solution contained the following (in
387 mM), 115 K-Gluconate, 15 KCl, 2 MgCl₂, 0.5 EGTA, 4 Mg-ATP, 10 HEPES pH 7.2, 290
388 mOsm. All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless
389 otherwise noted. Patch electrodes contained 40 μ M Alexa Fluor 488 or 594 hydrazide
390 (Life Technologies Ltd., Paisley, UK). Physiological recordings were made with an
391 Axopatch 700B amplifier and digitized with a Digidata 1440A (Molecular Devices,
392 Fremont, CA, USA). pClamp software was used to acquire electrophysiological data
393 at a sampling rate of 50 kHz and low pass filtered at 2.2 kHz. Data were analyzed
394 with Clampfit (Molecular Devices, Fremont, CA, USA), Igor Pro 6.34 (WaveMetrics,
395 Lake Oswego, OR), Excel 2010 (Microsoft, Redmond, WA, USA), and Matlab
396 (Mathworks, Natick, MA, USA). Summary data are presented as average \pm SEM.

397 *Confocal Imaging*

398 For imaging, larvae were prepared as described for physiological
399 recordings. Confocal images were acquired with an Evolve 10 MHz Digital
400 Monochrome Camera EM-CCD camera (Photometrics, Tucson, AZ, USA) using a
401 Yokogawa X1 spinning disk (Yokogawa, Tokyo, Japan) mounted to an upright
402 widefield microscope (Axio Examiner Z1, Zeiss, Germany) equipped with 20X, 40X,
403 63X water dipping objectives. Laser lines used here were a 50mW 488nm laser for
404 imaging GFP and a 50 mW 561 nm laser for imaging mCherry. Z stacks were taken
405 at 0.5 μ m step size. Data was acquired using SlideBook 6 image acquisition software
406 (3i, Denver, CO, USA). Images were assembled with ImageJ (NIH, Bethesda, MD),

407 Adobe Photoshop and Illustrator CS6 (Adobe Systems Incorporated, San Jose, CA).

408 *2D Light patterning using a DMD*

409 To generate the patterned illumination we used a DLP discovery kit including a 0.7"
410 digital mirror device and software API (Vialux, Germany). The DMD was imaged via a
411 telescope (f = 80 mm and f = 40 mm, Thorlabs, Newton, NJ, USA) onto the back
412 focal plane of the epifluorescence light path of an upright widefield microscope (Axio
413 Examiner D1, Zeiss, Germany). The DMD light path was combined with the
414 epifluorescence light source via a 30% reflection 70% transmission beamsplitter (AHF,
415 Germany) to allow the patterned and epifluorescence illumination through the same
416 path. As a light source for the patterned illumination we used an ultra-high power
417 white Light Emitting Device (LED, Prizmatix, Israel). The LED was coupled into the
418 light path of the Digital Mirror Device (DMD) via a total internal reflection (TIR) prism
419 (Lida optical and electronic, China). For fluorescent imaging and target cell selection
420 the microscope was equipped with an EM-CCD camera (ImageEM, Hamamatsu,
421 Japan). Integrated software control of the DMD and the camera was done via custom
422 scripts in LabView (National Instruments, Austin, TX) and Matlab (Mathworks, Natick,
423 MA). The Matlab code was partly based on Zhu et al., 2012 [40].

424 *Behavior setup and analysis*

425 Behavior setup was previously described [7]. Each larva was subjected to five trials
426 and rolling behavior was assessed for each trial. The roll ratio was calculated as the
427 number of trials the animal rolled during an escape divided by the number of trials
428 the animal attempted an escape. Roll ratio for BoTxBLC-GFP+ fish and control
429 siblings are presented as average \pm SEM. Larvae were screened for GFP fluorescence
430 to establish BoTx positive and BoTx negative siblings prior to data acquisition and
431 the experimenter was blinded to genotype prior to assessment of the rolling
432 behavior.

433

434 **Author Contributions**

435 Conceptualization, J.M.H., C.S. and C.W.; Methodology, J.M.H., U.L.B., A.P., P.B.T., C.S.
436 and C.W.; Software, U.L.B.; Formal Analysis, J.M.H. and C.W.; Investigation, J.M.H.;
437 Resources, M.N. and C.W.; Writing, J.M.H. and C.W. who received inputs from all
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440

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451

452 **References**

- 453 1. Kolmer, W. (1921). Das "Sagittalorgan" der Wirbeltiere. In *Z Anat Entwicklungs*, pp.
454 652–717.
- 455 2. Agduhr, E. (1922). Über ein Zentrales Sinnesorgan beiden Vertebraten. In *Z Anat*
456 *Entwicklungs*, pp. 223–360.
- 457 3. Wyart, C., Del Bene, F., Warp, E., Scott, E.K., Trauner, D., Baier, H., and Isacoff, E.Y.
458 (2009). Optogenetic dissection of a behavioural module in the vertebrate spinal cord.
459 *Nature* 461, 407-410.

- 460 4. Orts-Del'immagine, A., Wanaverbecq, N., Tardivel, C., Tillement, V., Dallaporta, M.,
461 and Trouslard, J. (2012). Properties of subependymal cerebrospinal fluid contacting
462 neurones in the dorsal vagal complex of the mouse brainstem. *J. Physiol.* 590, 3719-
463 3741.
- 464 5. Fidelin, K., Djenoune, L., Stokes, C., Prendergast, A., Gomez, J., Baradel, A., Del
465 Bene, F., and Wyart, C. (2015). State-Dependent Modulation of Locomotion by
466 GABAergic Spinal Sensory Neurons. *Curr. Biol.* 25, 3035-47.
- 467 6. Jalalvand, E., Robertson, B., Wallen, P., and Grillner, S. (2016). Ciliated neurons
468 lining the central canal sense both fluid movement and pH through ASIC3. *Nature*
469 *Communications* 7, 10002.
- 470 7. Böhm, U.L., Prendergast, A., Djenoune, L., Nunes Figueiredo, S., Gomez, J., Stokes,
471 C., Kaiser, S., Suster, M., Kawakami, K., Charpentier, M., Concordet, J.P., Rio, J.P., Del
472 Bene, F., and Wyart, C. (2016). CSF-contacting neurons regulate locomotion by
473 relaying mechanical stimuli to spinal circuits. *Nature Communications* 7, 10866.
- 474 8. Huang, A.L., Chen, X., Hoon, M.A., Chandrashekar, J., Guo, W., Trankner, D., Ryba,
475 N.J., and Zuker, C.S. (2006). The cells and logic for mammalian sour taste detection.
476 *Nature* 442, 934-938.
- 477 9. Djenoune, L., Khabou, H., Joubert, F., Quan, F.B., Nunes Figueiredo, S., Bodineau,
478 L., Del Bene, F., Burckle, C., Tostivint, H., and Wyart, C. (2014). Investigation of spinal
479 cerebrospinal fluid-contacting neurons expressing PKD2L1: evidence for a conserved
480 system from fish to primates. *Front. Neuroanat.* 8, 26.
- 481 10. Vigh, B., and Vigh-Teichmann, I. (1998). Actual problems of the cerebrospinal
482 fluid-contacting neurons. *Microsc. Res. Tech.* 41, 57-83.
- 483 11. Stoeckel, M.E., Uhl-Bronner, S., Hugel, S., Veinante, P., Klein, M.J., Mutterer, J.,
484 Freund-Mercier, M.J., and Schlichter, R. (2003). Cerebrospinal fluid-contacting neurons
485 in the rat spinal cord, a gamma-aminobutyric acidergic system expressing the P2X2
486 subunit of purinergic receptors, PSA-NCAM, and GAP-43 immunoreactivities: light

- 487 and electron microscopic study. *J. Comp. Neurol.* 457, 159-174.
- 488 12. Fetcho, J.R. (1991). Spinal network of the Mauthner cell. *Brain Behav. Evol.* 37,
489 298-316.
- 490 13. Lacoste, A.M., Schoppik, D., Robson, D.N., Haesemeyer, M., Portugues, R., Li, J.M.,
491 Randlett, O., Wee, C.L., Engert, F., and Schier, A.F. (2015). A convergent and essential
492 interneuron pathway for Mauthner-cell-mediated escapes. *Curr. Biol.* 25, 1526-1534.
- 493 14. Fetcho, J.R., and Faber, D.S. (1988). Identification of motoneurons and
494 interneurons in the spinal network for escapes initiated by the mauthner cell in
495 goldfish. *J. Neurosci.* 8, 4192-4213.
- 496 15. Satou, C., Kimura, Y., Kohashi, T., Horikawa, K., Takeda, H., Oda, Y., and
497 Higashijima, S. (2009). Functional role of a specialized class of spinal commissural
498 inhibitory neurons during fast escapes in zebrafish. *J. Neurosci.* 29, 6780-6793.
- 499 16. Bagnall, M.W., and McLean, D.L. (2014). Modular organization of axial
500 microcircuits in zebrafish. *Science* 343, 197-200.
- 501 17. Menelaou, E., and McLean, D.L. (2012). A gradient in endogenous rhythmicity
502 and oscillatory drive matches recruitment order in an axial motor pool. *J. Neurosci.*
503 32, 10925-10939.
- 504 18. Myers, P.Z., Eisen, J.S., and Westerfield, M. (1986). Development and axonal
505 outgrowth of identified motoneurons in the zebrafish. *J. Neurosci.* 6, 2278-2289.
- 506 19. Pietri, T., Manalo, E., Ryan, J., Saint-Amant, L., and Washbourne, P. (2009).
507 Glutamate drives the touch response through a rostral loop in the spinal cord of
508 zebrafish embryos. *Dev. Neurobiol.* 69, 780-795.
- 509 20. Knogler, L.D., and Drapeau, P. (2014). Sensory gating of an embryonic zebrafish
510 interneuron during spontaneous motor behaviors. *Front. Neural Circuits* 8, 121.
- 511 21. Wells, S., Nornes, S., and Lardelli, M. (2011). Transgenic zebrafish recapitulating

512 *tbx16* gene early developmental expression. PLoS One 6, e21559.

513 22. Warp, E., Agarwal, G., Wyart, C., Friedmann, D., Oldfield, C.S., Conner, A., Del
514 Bene, F., Arrenberg, A.B., Baier, H., and Isacoff, E.Y. (2012). Emergence of patterned
515 activity in the developing zebrafish spinal cord. *Curr. Biol.* 22, 93-102.

516 23. Kucenas, S., Soto, F., Cox, J.A., and Voigt, M.M. (2006). Selective labeling of
517 central and peripheral sensory neurons in the developing zebrafish using P2X(3)
518 receptor subunit transgenes. *Neuroscience* 138, 641-652.

519 24. Brustein, E., and Drapeau, P. (2005). Serotonergic modulation of chloride
520 homeostasis during maturation of the locomotor network in zebrafish. *J. Neurosci.*
521 25, 10607-10616.

522 25. Freund, T.F., and Buzsaki, G. (1996). Interneurons of the hippocampus.
523 *Hippocampus* 6, 347-470.

524 26. Jonas, P., Bischofberger, J., Fricker, D., and Miles, R. (2004). Interneuron Diversity
525 series: Fast in, fast out--temporal and spatial signal processing in hippocampal
526 interneurons. *Trends in Neurosciences* 27, 30-40.

527 27. Freund, T.F., and Katona, I. (2007). Perisomatic inhibition. *Neuron* 56, 33-42.

528 28. Huang, Z.J., Di Cristo, G., and Ango, F. (2007). Development of GABA innervation
529 in the cerebral and cerebellar cortices. *Nature Reviews Neuroscience* 8, 673-686.

530 29. Mirat, O., Sternberg, J.R., Severi, K.E., and Wyart, C. (2013). ZebraZoom: an
531 automated program for high-throughput behavioral analysis and categorization.
532 *Frontiers in Neural Circuits* 7, 107.

533 30. Dale, N., Roberts, A., Ottersen, O.P., and Storm-Mathisen, J. (1987a). The
534 development of a population of spinal cord neurons and their axonal projections
535 revealed by GABA immunocytochemistry in frog embryos. *Proc. R. Soc. Lond. B. Biol.*
536 *Sci.* 232, 205-215.

- 537 31. Dale, N., Roberts, A., Ottersen, O.P., and Storm-Mathisen, J. (1987b). The
538 morphology and distribution of 'Kolmer-Agduhr cells', a class of cerebrospinal-fluid-
539 contacting neurons revealed in the frog embryo spinal cord by GABA
540 immunocytochemistry. *Proc. R. Soc. Lond. B. Biol. Sci.* 232, 193-203.
- 541 32. Christenson, J., Alford, S., Grillner, S., and Hokfelt, T. (1991). Co-localized GABA
542 and somatostatin use different ionic mechanisms to hyperpolarize target neurons in
543 the lamprey spinal cord. *Neurosci. Lett.* 134, 93-97.
- 544 33. Jalalvand, E., Robertson, B., Wallen, P., Hill, R.H., and Grillner, S. (2014). Laterally
545 projecting cerebrospinal fluid-contacting cells in the lamprey spinal cord are of two
546 distinct types. *J. Comp. Neurol.* 522, 1753-1768.
- 547 34. Nair, A., Azatian, G., and McHenry, M.J. (2015). The kinematics of directional
548 control in the fast start of zebrafish larvae. *The Journal of Experimental Biology* 218,
549 3996-4004.
- 550 35. Roberts, A., Hill, N.A., and Hicks, R. (2000). Simple mechanisms organise
551 orientation of escape swimming in embryos and hatchling tadpoles of *Xenopus*
552 *laevis*. *The Journal of Experimental Biology* 203, 1869-1885.
- 553 36. Deliagina, T.G., Beloozerova, I.N., Orlovsky, G.N., and Zelenin, P.V. (2014).
554 Contribution of supraspinal systems to generation of automatic postural responses.
555 *Frontiers in Integrative Neuroscience* 8, 76.
- 556 37. Necker, R. (2006). Specializations in the lumbosacral vertebral canal and spinal
557 cord of birds: evidence of a function as a sense organ which is involved in the
558 control of walking. *Journal of Comparative Physiology A, Neuroethology, sensory,*
559 *neural, and behavioral physiology* 192, 439-448.39.
- 560 38. Meyer, M.P., and Smith, S.J. (2006). Evidence from in vivo imaging that
561 synaptogenesis guides the growth and branching of axonal arbors by two distinct
562 mechanisms. *J. Neurosci.* 26, 3604-3614.

563 39. Wen, H., and Brehm, P. (2005). Paired motor neuron-muscle recordings in
564 zebrafish test the receptor blockade model for shaping synaptic current. *J. Neurosci.*
565 25, 8104-8111.

566 40. Zhu, P., Fajardo, O., Shum, J., Zhang Scharer, Y.P., and Friedrich, R.W. (2012).
567 High-resolution optical control of spatiotemporal neuronal activity patterns in
568 zebrafish using a digital micromirror device. *Nat. Protoc.* 7, 1410-1425.

569

570 **Figure Legends**

571 **Figure 1 | CSF-cNs specifically innervate the CaP primary motor neuron**

572 (A) Z projection stack showing a single ventral CSF-cN in a *Tg(pkd2l1:gal4;UAS:ChR2-*
573 *mCherry)* double transgenic larva at 3 days post fertilization (dpf). Top right: dorsal,
574 ventral, rostral and caudal orientation indicated by the cross. Central canal location
575 indicated by blue dashed lines.

576 (B) CSF-cN axons and varicosities in the 3dpf *Tg(pkd2l1:gal4;UAS:ChR2-mCherry)*
577 transgenic larva surround a dorsal cell body.

578 (C) Labeling of putative presynaptic boutons originating from a single CSF-cN (arrow)
579 expressing Synaptophysin-GFP in a 4 dpf *Tg(pkd2l1:gal4;UAS:ChR2-mCherry)*
580 transgenic larva after injection of the construct *UAS:Synaptophysin-GFP*.

581 (D) Schematic of the experimental paradigm used for ChR2-mediated mapping of
582 connectivity illustrates: top, a CSF-cN expressing ChR2-mCherry (green) illuminated
583 by a short pulse of light and the whole cell patch clamp recording of the target
584 neuron with a pipette containing the Alexa dye to confirm the nature of the cell
585 type; bottom: a 5 ms light pulse is sufficient to induce a single spike reliably in CSF-
586 cNs (see [5]) and a subsequent IPSC recorded in the target neuron.

587 (E) Z projection stack showing a CaP motor neuron filled with the Alexa dye
588 (magenta) innervated by a single CSF-cN (green) in a 4 dpf

589 *Tg(pkd2l1:gal4;UAS:ChR2-mCherry)* transgenic larva with sparse expression of
590 ChR2. Boxed region shows a widefield image with the CaP cell body contacted by
591 the axonal projection of the labeled CSF-cN. Arrows indicate the dorsal projections
592 that surround the soma of the CaP motor neuron recorded and filled as well as
593 another putative CaP motor neuron in the adjacent caudal segment.

594 (F) Current clamp recording of a typical CaP motor neuron showing phasic action
595 potential firing in response to current injection (steps of 20 pA from -50 pA to +90
596 pA).

597 (G) Voltage clamp recording from a CaP motor neuron ($V_m = -65$ mV) showing
598 evoked IPSCs following 5 ms light pulses before (black trace, average of 10 trials)
599 and after 10 μ M gabazine treatment (red trace, average of 10 trials).

600 (H) Summary data showing the IPSCs are abolished by gabazine. Each experiment
601 (grey circle) is the average of ten trials before (Ctl) and after gabazine (Gbz)
602 treatment (mean amplitude of control IPSC = 36.2 ± 24.9 pA, mean amplitude of
603 gabazine IPSC = 5.4 ± 4.1 pA, $n = 5$, $p < 0.0001$).

604 (I, J) Voltage steps and corresponding I-V curve indicate that the IPSCs (within the
605 red dashed box) reverse at -53 mV (red trace in (I) indicates -50 mV), close to the
606 reversal potential of chloride ($E_{Cl} = -51$ mV) in our recording conditions ($n = 6$ cells).

607 (K-N) Distribution of IPSC delay (H, mean = 6.86 ± 0.09 ms), 20-80% rise time (I,
608 mean = 0.89 ± 0.19 ms), current amplitude (J, mean = 55.19 ± 2.34 pA
609 corresponding to a conductance of 3.94 nS) and time decay (K, mean $\tau = 18.04 \pm$
610 0.42 ms) ($n = 34$ cells, 271 trials).

611 Scale bars are 10 μ m in 1A-1C and 1E.

612 **Figure 2 | Motor neurons other than CaP receive limited CSF-cN input**

613 (A) Motor neurons and CSF-cNs labeled in the *Tg(parg^{mmet2}-GFP;*
614 *pkd2l1:gal4;UAS:ChR2-mCherry)* transgenic line throughout the rostro-caudal axis

615 (12 axial segments) at 3 dpf. Boxes with magnified images highlight extensive
616 innervation of large dorsal CaP primary motor neurons (labeled C). However, other
617 (non-CaP) primary motor neurons (indicated by arrows) and secondary motor
618 neurons (indicated by arrowheads) do not exhibit the same extensive perisomatic
619 innervation. Scale bars are 20 μm (2A, top) and 10 μm (2A, magnified boxes).

620 (B1-B3) Examples of whole cell recordings from non-CaP primary motor neurons
621 showing three types of post-synaptic responses observed. (B1) Upper panel, current
622 clamp recording of a primary motor neuron showing a single action potential in
623 response to current injection (steps of 40 pA from -50 pA to +70 pA). Lower panel,
624 voltage clamp recording from the same primary motor neuron ($V_m = -65$ mV)
625 showing evoked IPSCs following 5 ms light pulses (black trace is the average of 10
626 trials shown in grey). (B2) Upper panel, current clamp recording of a primary motor
627 neuron showing tonic action potentials in response to current injection (steps of 40
628 pA from -50 pA to +150 pA). Lower panel, voltage clamp recording from the same
629 primary motor neuron ($V_m = -65$ mV) showing small evoked IPSCs following 5 ms
630 light pulses (black trace is the average of 10 trials shown in grey). (B3) Upper panel,
631 current clamp recording of a primary motor neuron showing a single action potential
632 in response to current injection (steps of 40 pA from -30 pA to +130 pA). Lower
633 panel, voltage clamp recording from the same primary motor neuron ($V_m = -65$ mV)
634 showing no IPSCs following 5 ms light pulses (black trace is the average of 10 trials
635 shown in grey).

636 (C) Histogram of IPSC current amplitudes from non-CaP primary motor neurons
637 (mean = 1.78 ± 0.42 pA, $n = 17$ cells, 170 trials).

638 (D) Current clamp recording of a secondary motor neuron showing bursts of action
639 potentials in response to current injection (steps of 20 pA from -30 pA to +30 pA).

640 (E) Example voltage clamp recordings from secondary motor neurons ($V_m = -65$ mV)
641 showing no IPSCs following 5 ms light pulses (black trace is the average of 10 trials
642 shown in grey). IPSCs in secondary motor neurons were never observed following
643 CSF-cN stimulation ($n = 10$).

644 **Figure 3 | CSF-cNs innervate CoPA glutamatergic sensory interneurons**

645 (A1-A4) Z projection stack showing CoPA sensory interneurons expressing GFP
646 (magenta) innervated by CSF-cNs (green) in *Tg(pkd2l1:gal4;UAS:ChR2-mCherry;*
647 *tbx16:GFP)* transgenic larvae. Note the varicosities (arrows) from CSF-cNs onto CoPA
648 soma (A1, A2), initial segment (A2, A3) and sometimes dendrites (A3, A4). Scale bars
649 are 10 μm .

650 (B) Current clamp recording of a typical CoPA interneuron showing sparse action
651 potential firing in response to current injections (steps are 20 pA from -50 pA to
652 +150 pA).

653 (C) Voltage clamp recording from a CoPA interneuron ($V_m = -65$ mV) showing an
654 evoked IPSC following a 5 ms light pulse (black trace, average of 10 trials).

655 (D-G) Distribution of IPSC delay (D, mean = 7.73 ± 0.15 ms), 20-80% rise time (E,
656 mean = 0.88 ± 0.08 ms), amplitude (F, mean = 146.83 ± 16.75 pA corresponding to
657 a conductance of 10.49 nS) and time decay τ (G, mean = 18.16 ± 1.22 ms) (n = 8
658 cells, 64 trials).

659 (H) Cumulative probability plot of IPSC amplitudes for CaP motor neurons (blue, n =
660 271) and CoPA interneurons (red, n = 64).

661 **Figure 4 | 2D Light patterning at the single cell resolution reveals convergence of**
662 **inputs from CSF-cNs onto their targets**

663 (A) Schematic of the Digital Mirror Device (DMD) setup showing the light path. A
664 beamsplitter (1) was attached to the epi-port of an upright microscope in order to
665 combine light from the epifluorescence light source via fiber optic (2) and the light
666 from the DMD. The patterned light from the DMD was relayed into the
667 epifluorescence light path via a telescope (3). A white Light Emitting Device (LED) (4)
668 providing the light for patterned illumination was directed via a total internal
669 reflection prism (5) to the DMD (6).

670

671 (B, C) Physiological responses to either full field illumination (B) or a small spot on an
672 individual CSF-cN (C). Top: Fluorescent image of multiple CSF-cNs from the
673 *Tg(pkd2l1:gal4;UAS:ChR2-mCherry)* transgenic line with all (B) or a subset of the
674 central mirrors activated (C). Scale bar, 20 μ M Bottom: IPSCs following a 5 ms light
675 pulse from either full field (B) or patterned illumination (C). In cases where only one
676 CSF-cN is connected to the target, the IPSC amplitude evoked by the spot
677 recapitulates the IPSC amplitude evoked by the full field illumination. Scale bars are
678 50 ms and 20 pA.

679
680 (D) Example experiment investigating the connectivity from CSF-cNs (green) to a CaP
681 motor neuron (magenta) showing multiple CSF-cNs projecting onto the postsynaptic
682 target. Light was patterned in rectangles (indicated in yellow), which were
683 sequentially illuminated along the rostro-caudal axis during voltage clamp recording
684 of the target neuron. Right: voltage clamp traces resulting from the light activation
685 of the corresponding rectangular region. IPSCs are observed when the light is
686 patterned onto a subset of CSF-cNs (yellow stars). White dashed lines indicate
687 segment boundaries. Scale bars are 50 μ m for the image and 10 ms and 50 pA for
688 the electrophysiological traces.

689 (E) Quantification of the IPSC amplitude for CaP (blue circles) and CoPA (red circles)
690 as a function of the number of segments between a CSF-cN and its target. Mean
691 IPSC amplitude for CaP and CoPA combined are plotted for each segment (white
692 boxes).

693 (F) Convergence of CSF-cNs onto a CaP motor neuron. Image of CSF-cNs expressing
694 ChR2-mCherry (green) and the target CaP motor neuron filled with Alexa dye
695 (magenta). IPSCs in response to either full field or patterned illumination show that
696 cells "4" and "6" converge on the CaP motor neuron target.

697 (G, H) Examples of identified connections from CSF-cNs onto CaP motor neurons (G)
698 and CoPA neurons (H) in three different larvae. Arrows indicate patched target cell
699 body. Yellow circles show connected CSF-cNs. Scale bars are 50 μ m.

700
701 **Figure 5 | CSF-cN local innervation onto the escape circuit is restricted to**
702 **excitatory interneurons and motor neurons**

703 (A) Z projection stack of CoLo glycinergic premotor interneurons expressing GFP
704 (magenta) and CSF-cNs (green) in a *Tg(pkd2l1:gal4;UAS:ChR2-mCherry ; Tol-056-GFP)*
705 transgenic larva at 3 dpf. Arrows indicate CoLo cell bodies. Scale bar is 10 μm .

706 (B) Current clamp recording showing the typical firing pattern of a CoLo neuron with
707 a single weak action potential in response to current injection (steps of 20 pA from
708 -30 pA to +370 pA).

709 (C). CSF-cN stimulation elicited by a 5 ms light pulse fails to induce a IPSCs in CoLos.
710 Example voltage clamp recordings from three CoLos ($V_m = -65$ mV) showing no
711 IPSCs following 5 ms light pulses (black trace is the average of 10 trials shown in
712 grey). IPSCs in CoLo were never observed following CSF-cN stimulation ($n = 13$
713 cells).

714 (D) Z projection stack showing Rohon-Beard neurons expressing GFP (magenta) and
715 CSF-cNs (green) in a *Tg(pkd2l1:gal4;UAS:ChR2-mCherry ; p2rx3.2 :GFP)* transgenic
716 larva at 3 dpf. Note the axonal projections of CSF-cNs do not reach Rohon-Beard
717 somas or axons. Scale bar is 10 μm .

718 (E) Current clamp recording showing the typical firing pattern of a Rohon-Beard
719 neuron with a single weak action potential in response to current injection (steps of
720 20 pA from -30 pA to +170 pA).

721 (F) CSF-cN stimulation elicited by a 5 ms light pulse fails to induce an IPSC in
722 Rohon-Beard neurons. Example voltage clamp recordings from three Rohon-Beard
723 neurons ($V_m = -65$ mV) showing no IPSCs following 5 ms light pulses (black trace is
724 the average of 10 trials shown in grey). IPSCs in Rohon-Beard neurons were never
725 observed following CSF-cN stimulation ($n = 10$ cells).

726

727 **Figure 6 | Stimulation of CSF-cNs at moderate frequencies results in short-term**
728 **synaptic depression in CaP motor neurons and CoPA interneurons**

729 (A1, A2) Typical examples of IPSCs (grey) recorded from CaP (A) and CoPA (B) at 0.2
730 Hz. Average of 10 trials in black. Note the absence of failure and the large IPSC
731 amplitude.

732 (A3) Stimulation at 0.2 Hz induced moderate short term depression in CaP (blue
733 circles, t-test for the difference between the 1st and the 10th light pulse $p = 0.036$, n
734 $= 7$) and no depression for CoPA (red triangles, $p = 0.48$, $n = 5$).

735 (B1, B2) Typical examples of IPSCs recorded from CaP (B1) and CoPA (B2) at 1 Hz.
736 Trace is an average of 10 trials. Note the absence of failure of the IPSC.

737 (B3) Trains of stimuli at 1 Hz induce small but significant short term depression (23%
738 for CaP, blue circles, $p = 0.037$, $n = 7$ and 41% for CoPA, red triangles, $p = 0.030$, n
739 $= 5$).

740 (C1, C2) Typical examples of IPSCs recorded from CaP (C1) and CoPA (C2) at 10 Hz.
741 Trace is an average of 10 trials. Note the absence of failure and the promptly
742 decreasing amplitude of the IPSC.

743 (C3) Trains of stimuli at 10 Hz induce large, significant short term depression (57%
744 for CaP, blue circles, $p = 0.00002$, $n = 7$ and 75% for CoPA, red triangles, $p =$
745 0.00009 , $n = 5$).

746 (D1, D2) Typical examples of IPSCs recorded from CaP (D1) and CoPA (D2) at 20 Hz.
747 Trace is an average of 10 trials. Note the absence of failure and the promptly
748 decreasing amplitude of the IPSC.

749 (D3) Trains of stimuli at 20 Hz induce large, significant short term depression (68%
750 for CaP, blue circles, $p = 0.000008$, $n = 7$ and 81% for CoPA, red triangles, $p =$
751 0.00005 , $n = 5$).

752

753 **Figure 7 | Silencing CSF-cNs induces a defect in balance during fast swimming**

754 (A) A voltage step (+180 mV for 100 ms from a holding potential of -65 mV) in cell-
755 attached mode leads to high frequency CaP motor neuron spiking in the control
756 condition.

757 (B) Typical trial showing that in a *Tg(pkd2l1:gal4 ; UAS:ChR2-mCherry)* transgenic
758 larva a 5 ms light pulse applied during the voltage step leads to the prompt
759 silencing of the CaP motor neuron for approximately 20 ms.

760 (C) Raster plot of CaP spiking without (top trace) and with (bottom 10 traces)
761 optogenetic stimulation of CSF-cNs. Repetition of 10 sequential trials confirms the
762 robust effect of silencing CaP firing. The duration of silencing tended to increase
763 during sequential trials.

764 (D) Maximum interspike interval (ISI) was quantified for 5 spikes prior to the light
765 pulse and 5 spikes following the light pulse. All cells showed silencing following the
766 5 ms light pulse illustrated by an increase in ISI (9.10 ms \pm 3.04 ms before and 26.69
767 ms \pm 10.55 ms after light pulse, n = 4, paired t-test: p = 0.02).

768 (E) Sample sequence of images acquired with a high speed camera during
769 acoustically evoked escaped responses for *Tg(pkd2l1:gal4 ; UAS:BotxBLC-GFP)*
770 transgenic larvae and control siblings. Magnified images (5 panels on the left)
771 demonstrate the rolling phenotype when CSF-cNs are genetically targeted with
772 BotxBLC-GFP+ to silence GABA release (scale bars are 1 mm). Z-stack of the entire
773 escape response sequence for BotxBLC-GFP+ and control siblings (panels on right,
774 scale bars are 2mm).

775 (F) Calculated roll ratio for *Tg(pkd2l1:gal4 ; UAS:BotxBLC-GFP)* transgenic larvae and
776 control siblings (n = 148 fish for each genotype). BotxBLC-GFP+ fish were
777 significantly more likely to tip over and roll during the escape response than the
778 control siblings (p<0.001).

779

Figure 1

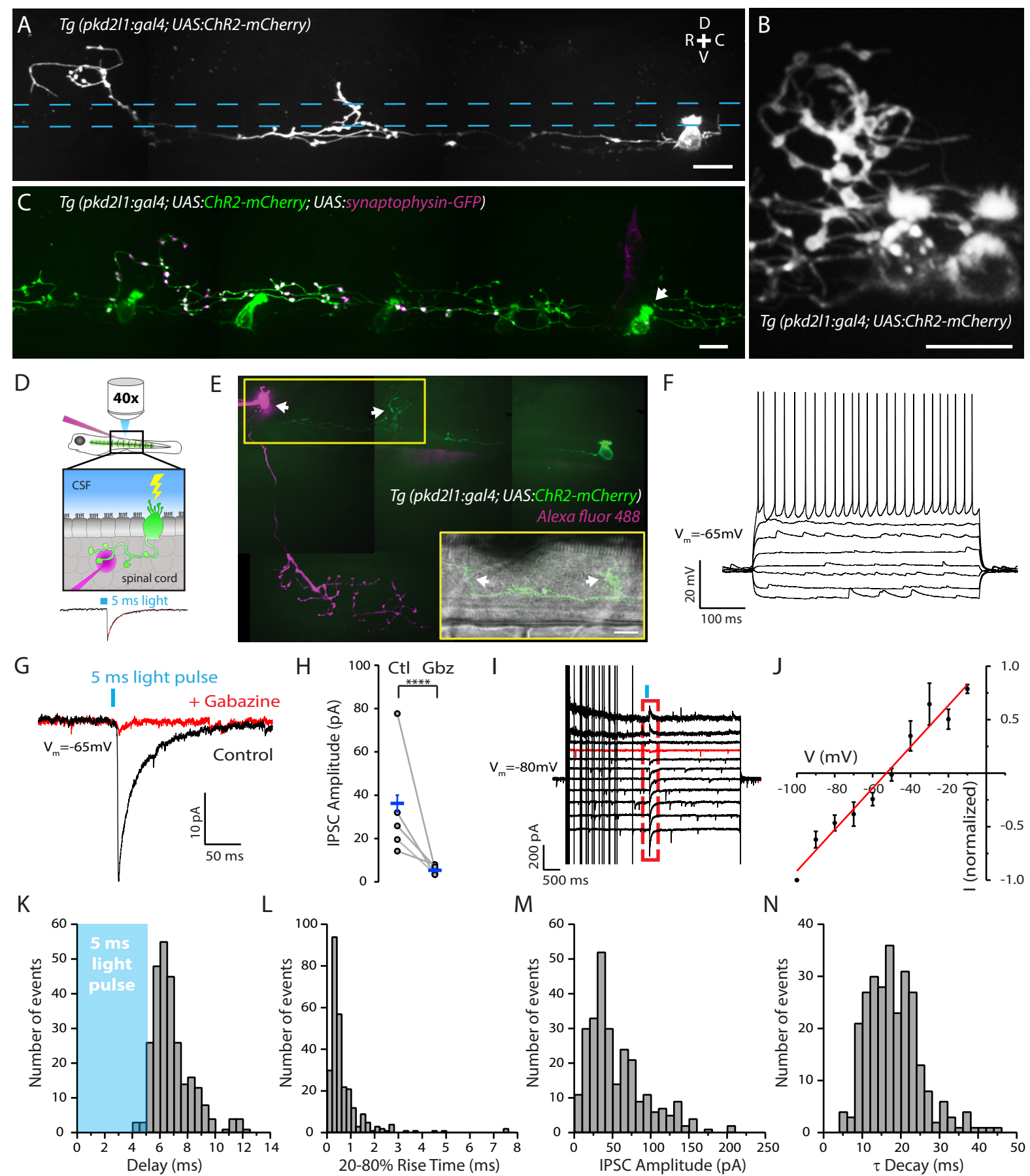


Figure 2

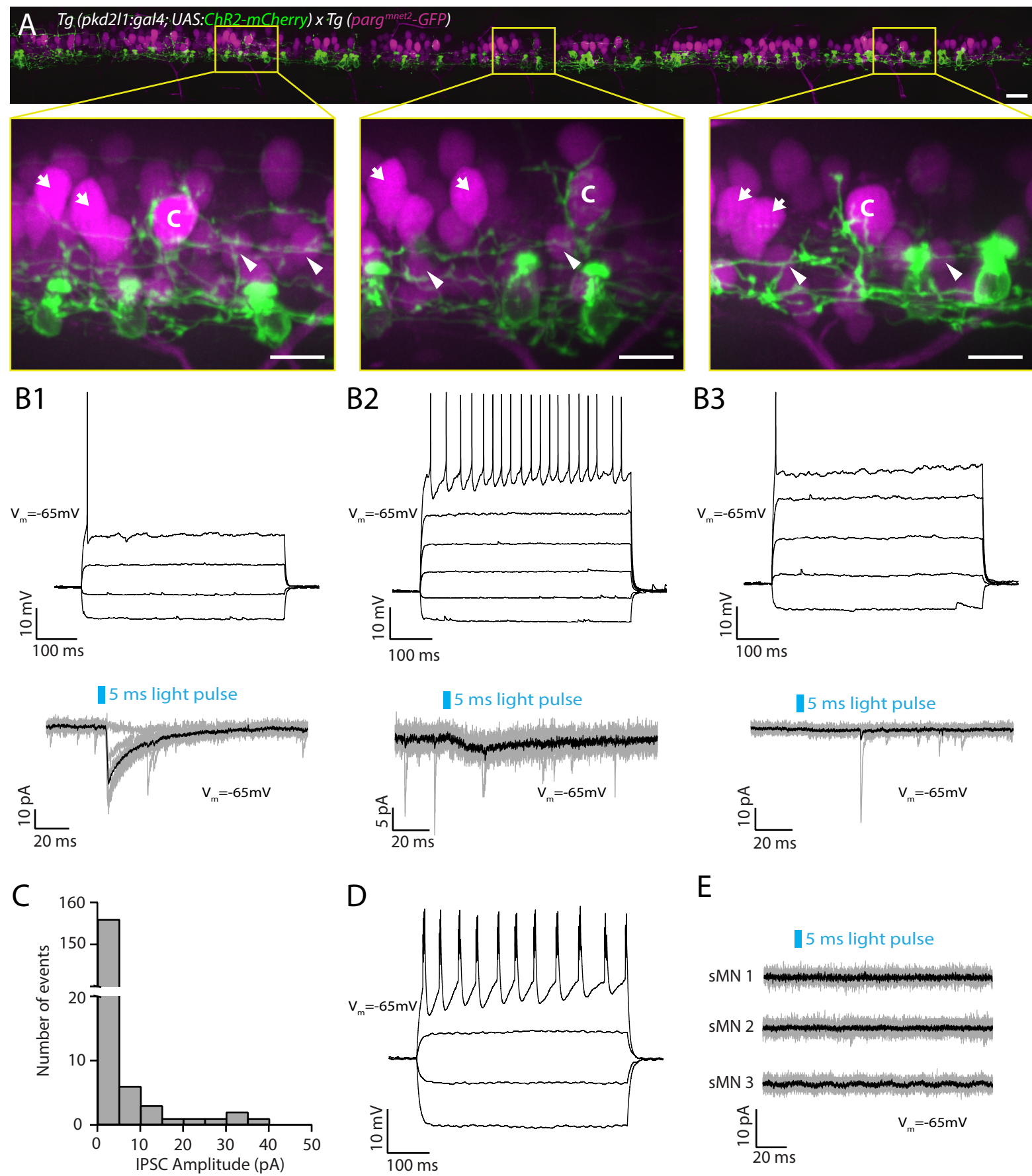


Figure 3

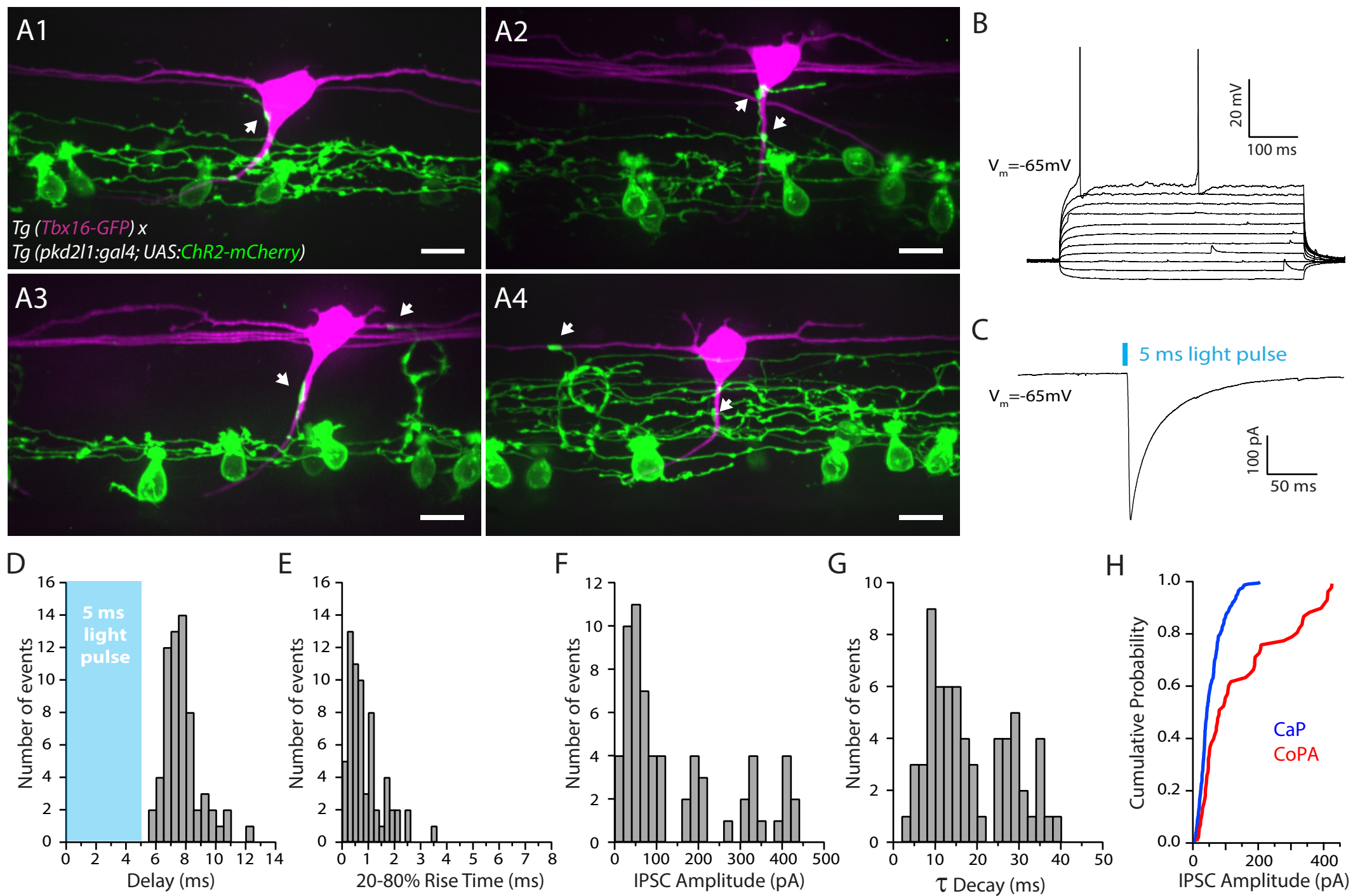


Figure 4

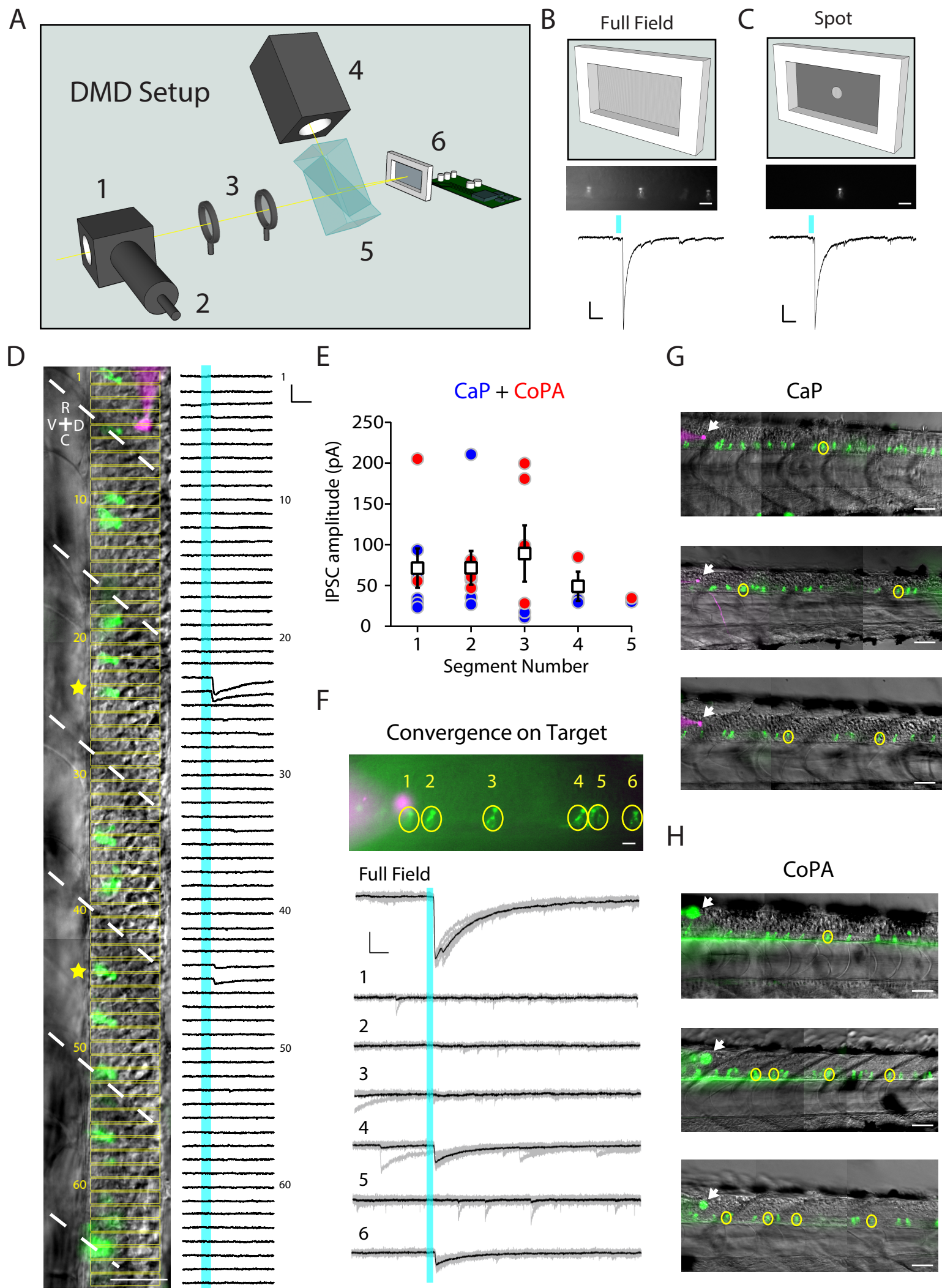


Figure 5

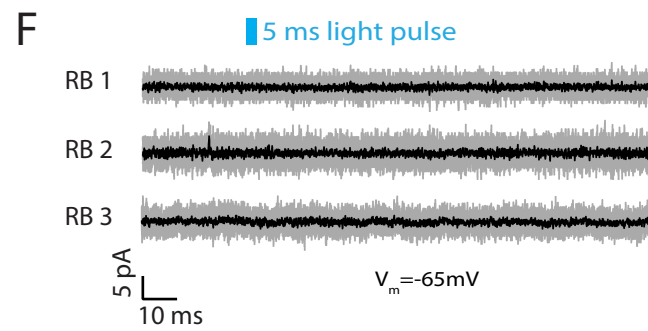
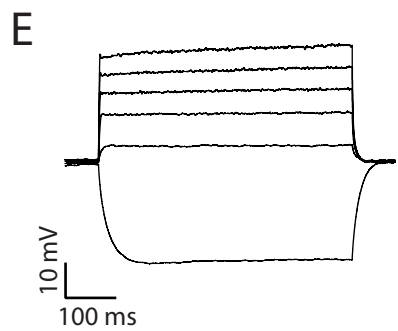
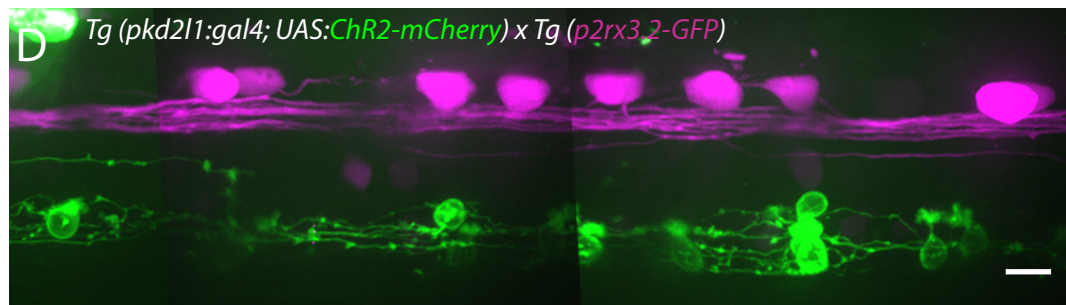
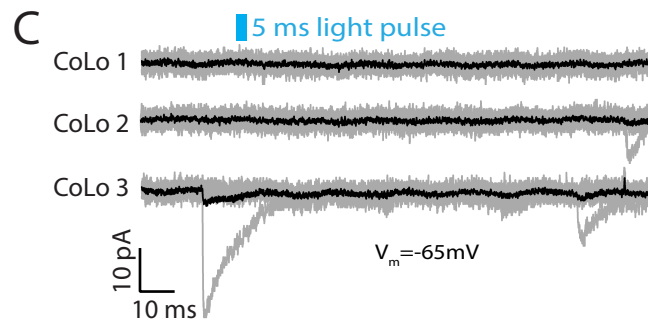
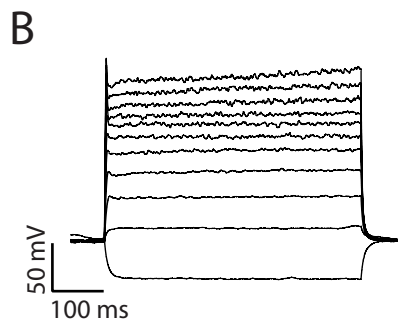
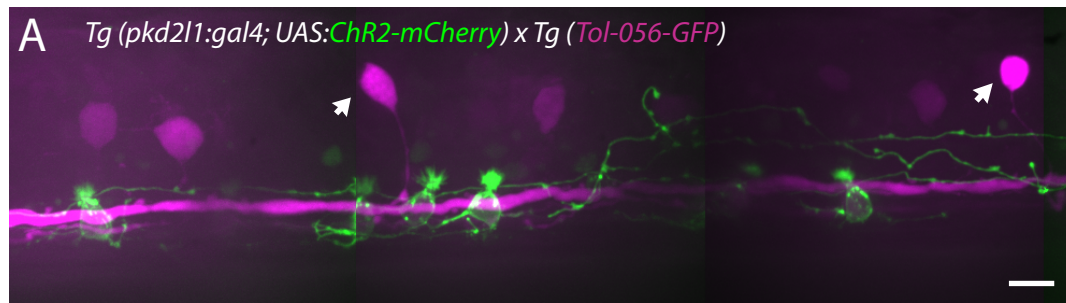


Figure 6

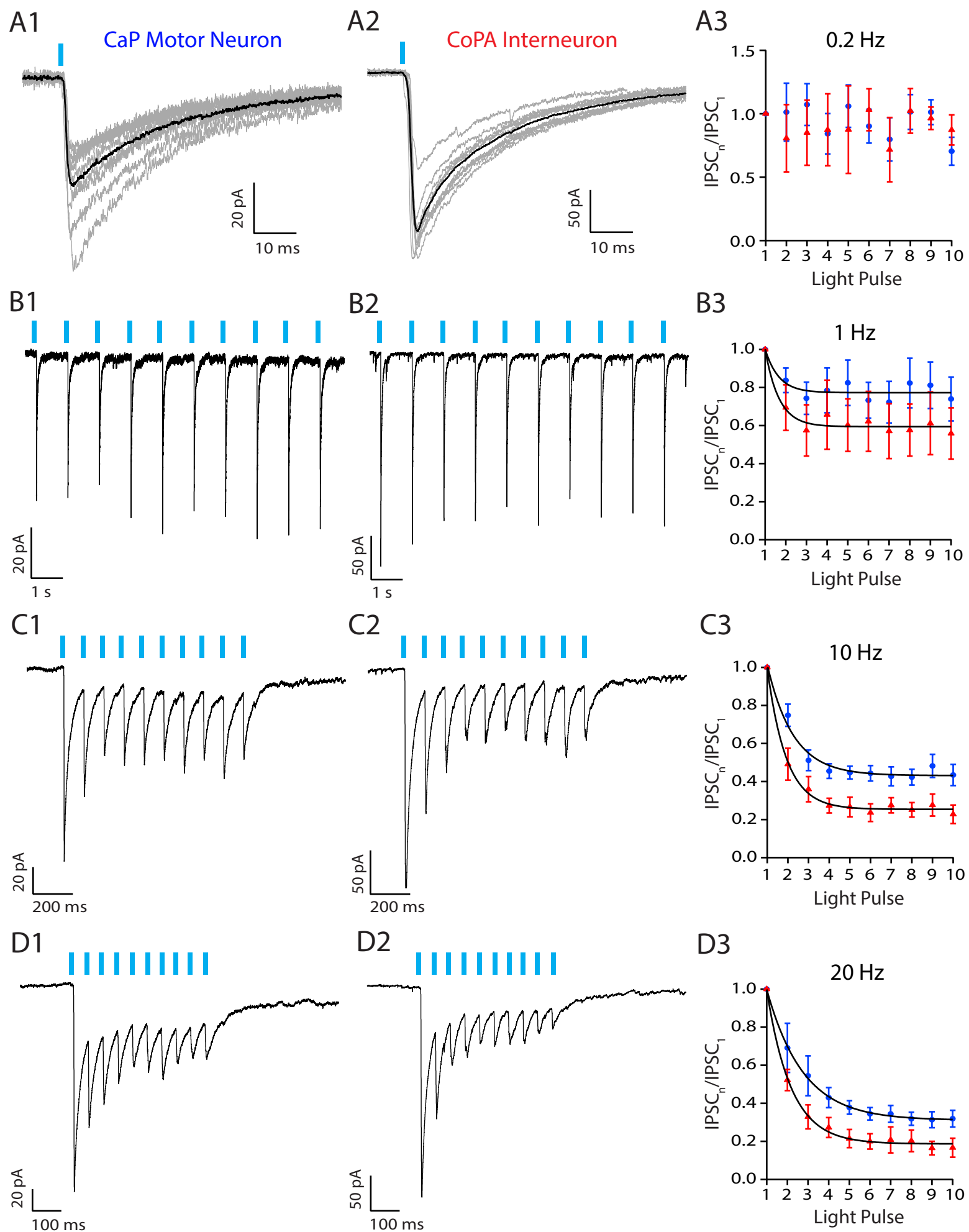
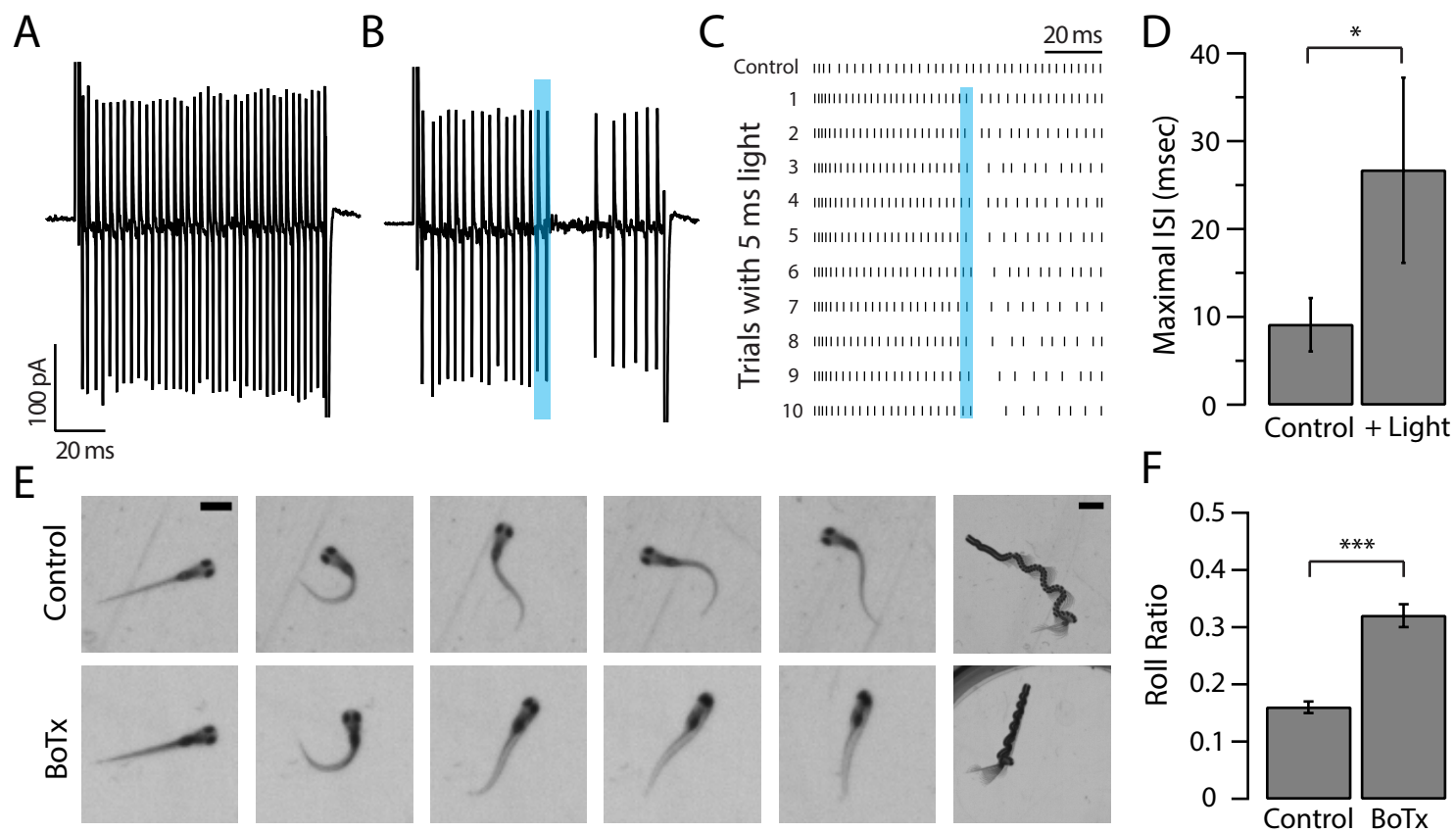


Figure 7



Supplemental information Hubbard et al.

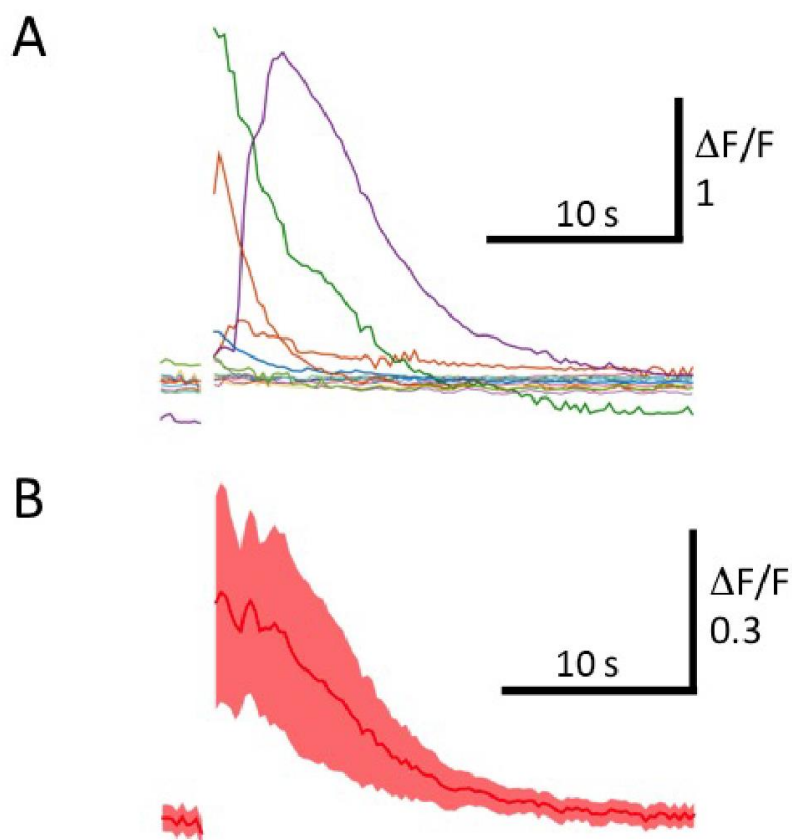


Figure S1. Related to Figure 7. Calcium response of ventral CSF-cNs to longitudinal contractions.

(A) Single traces of Ca²⁺ activity in ventral CSF-cNs in response to spontaneous contractions of 5-7 dpf *Tg(pkd211:gal4; UAS:GCaMP3; UAS:mCherry)* larvae embedded on their side. Visible is the strong response of several cells due to the muscle contraction. N = 13 cells from 3 fish. (B) Average response of the data presented in A. Shaded area designates the S.E.M.

Movie S1. Related to Figure 7. Spontaneous muscle contractions activate CSF-cNs.

Example movie of calcium signals from GCaMP3 fluorescence in CSF-cNs during spontaneous muscle contractions. Images were acquired at 4 Hz and the movie is compressed to 60 frames per second (fps).

Movie S2. Related to Figure 7. WT escape response.

Example movie of an acoustically-induced escape response in a control animal. The larva swims upright without rolling over in most cases. Images were acquired at 650 Hz and the movie is shown at 15 fps. Scale bar is 2 mm.

Movie S3. Related to Figure 7. BoTx escape response.

Example movie of an acoustically induced escape response in a BoTxBLC+ animal. The larva is initially upright and rolls over during the escape. Note that the swim bladder is initially down, but our camera captures an inverted image of the animal. Images were acquired at 650 Hz and the movie is shown at 15 fps. Scale bar is 2 mm.

Table S1. Summary of stable transgenic lines used in this study

Table 1 : Transgenic lines			
Name	Other Name	Labelling in the spinal cord	Original publication
<i>Tg(pkd2l1:gal4)icm10</i>	-	CSF-cNs	Fidelin et al., 2015 [S1]
<i>Tg(UAS:ChR2-mCherry)</i>	<i>Tg(UAS:ChR2H134R-mCherry)</i>	-	Schoonheim et al., 2010 [S2]
<i>Tg(parg:GFP)mnet2</i>	-	Motor Neurons	Balciunas et al., 2004 [S3]
<i>Tg(Tol56:GFP)</i>	Tol56	CoLo	Satou et al., 2009 [S4]
<i>Tg(tbx16:GFP)812C</i>	812C	CoPA	Wells et al., 2011 [S5]
<i>Tg(p2x3.2::eGFP^{GR})</i>	<i>Tg(p2rx3.2^{GR})</i>	Rohon Beard neurons	Kucenas et al., 2006 [S6]
<i>Tg(UAS:BoTxBLC-GFP)</i>	<i>Tg(UAS:BoTxBLC-GFP)</i>	-	Auer et al., eLife 2015 [S7] ; Böhm et al., Nature Communications 2016 [S8] ; Sternberg et al., <i>in press.</i> [S9]
<i>Tg(UAS :GCaMP3)</i>	<i>Tg(UAS :GCaMP3)</i>	-	Warp et al., Curr. Biol. [S10]

Supplemental Experimental Procedures

Iv vivo imaging of calcium

5-7 dpf *Tg(pkd2l1:gal4; UAS:GCaMP3; UAS:mCherry)* were laterally embedded in 1.5% low melting agarose. Images were acquired at 4Hz on a widefield microscope (Axio Examiner D1, Zeiss, Germany). To correct the motion artifact due to muscle contraction, the calcium signal from GCaMP3 fluorescence and the control signal from mCherry fluorescence were recorded simultaneously on two cameras (ImageEM, Hamamatsu, Japan and Stingray F145B, Allied Vision, Germany) using a dual excitation/emission filter set (GFP/DsRed-A-000, Semrock, USA). Images were processed as described in Böhm and Prendergast *et al.*, 2016 and the fluorescence ratio plotted.

Supplemental References

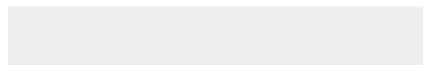
S1. Fidelin, K., Djenoune, L., Stokes, C., Prendergast, A., Gomez, J., Baradel, A., Del Bene, F., Wyart, C. (2015). State-Dependent Modulation of Locomotion by GABAergic Spinal Sensory Neurons. *Curr. Biol.* 25, 3035-47.

- S2. Schoonheim, P. J., Arrenberg, A. B., Del Bene, F., and Baier, H. (2010). Optogenetic localization and genetic perturbation of saccade-generating neurons in zebrafish. *J. Neurosci.* 30, 7111-7120.
- S3. Balciunas, D., Davidson, A.E., Sivasubbu, S., Hermanson, S.B., Welle, Z., and Ekker, S.C. (2004). Enhancer trapping in zebrafish using the Sleeping Beauty transposon. *BMC genomics* 5, 62.
- S4. Satou, C., Kimura, Y., Kohashi, T., Horikawa, K., Takeda, H., Oda, Y., and Higashijima, S. (2009). Functional role of a specialized class of spinal commissural inhibitory neurons during fast escapes in zebrafish. *J. Neurosci.* 29, 6780-6793.
- S5. Wells, S., Nornes, S., and Lardelli, M. (2011). Transgenic zebrafish recapitulating *tbx16* gene early developmental expression. *PLoS One* 6, e21559.
- S6. Kucenas, S., Soto, F., Cox, J.A., and Voigt, M.M. (2006). Selective labeling of central and peripheral sensory neurons in the developing zebrafish using P2X(3) receptor subunit transgenes. *Neuroscience* 138, 641-652.
- S7. Auer, T.O., Xiao, T., Bercier, V., Gebhardt, C., Duroure, K., Condordet, J-P., Wyart, C., Suster, M., Kawakami, K., Wittbrodt, J., Baier, H., and Del Bene, F. (2015). Deletion of a kinesin 1 motor unmasks a mechanism of homeostatic branching control by neurotrophin-3. *eLife* 4, e05061.
- S8. Böhm, U.L., Prendergast, A., Djenoune, L., Nunes Figueiredo, S., Gomez, J., Stokes, C., Kaiser, S., Suster, M., Kawakami, K., Charpentier, M., et al. (2016). CSF-contacting neurons regulate locomotion by relaying mechanical stimuli to spinal circuits. *Nature communications* 7, 10866.
- S9. Sternberg, J.R., Severi, K.E., Fidelin, K., Gomez, J., Ihara, H., Alcheikh, Y., Hubbard, J.M., Kawakami, K., Suster, M., Wyart, C. (2016). Optimization of a neurotoxin to investigate the contribution of excitatory interneurons to speed modulation *in vivo*. *Curr Biol.* (*in press*).
- S10. Warp, E., Agarwal, G., Wyart, C., Friedmann, D., Oldfield, C.S., Conner, A., Del Bene, F., Arrenberg, A.B., Baier, H., Isacoff, E.Y. (2012). Emergence of patterned activity in the developing zebrafish spinal cord. *Curr. Biol.* 22: 93-102.



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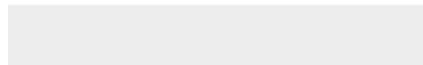
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