

1    **Spinal V2b neurons reveal a role for ipsilateral inhibition in speed control**

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10 **Abstract**

11 The spinal cord contains a diverse array of interneurons that govern motor output. Traditionally,  
12 models of spinal circuits have emphasized the role of inhibition in enforcing reciprocal  
13 alternation between left and right sides or flexors and extensors. However, recent work has  
14 shown that inhibition also increases coincident with excitation during contraction. Here, using  
15 larval zebrafish, we investigate the V2b (Gata3+) class of neurons, which contribute to flexor-  
16 extensor alternation but are otherwise poorly understood. Using newly generated transgenic lines  
17 we define two stable subclasses with distinct neurotransmitter and morphological properties.  
18 These two V2b subclasses make direct synapses onto motor neurons with differential targeting to  
19 slower and faster circuits. *In vivo*, optogenetic suppression of V2b activity leads to increases in  
20 locomotor speed. We conclude that V2b neurons exert speed-specific influence over axial motor  
21 circuits throughout the rostrocaudal axis. Together, these results indicate a new role for  
22 ipsilateral inhibition in speed control.

23 **Introduction**

24 Rhythmic, coordinated body movements require selective recruitment of motor neurons by spinal  
25 and supraspinal premotor circuits. Most vertebrates locomote via alternating left-right  
26 contractions that travel from rostral to caudal; tetrapods additionally alternate between flexors  
27 and extensors to regulate limb movements. Due in part to the technical challenges in identifying  
28 and manipulating specific classes of neurons in the spinal cord, the underlying circuitry of  
29 locomotion remains only poorly worked out.

30

31 Spinal premotor neurons are broadly divided into five superclasses arising from distinct  
32 progenitor domains (dI6, V0, V1, V2, V3) [1]. Within these superclasses, cardinal neuron classes  
33 have been identified based on transcription factor expression and neurotransmitter identity (e.g.,  
34 V2a / Chx10 / excitatory; V2b / Gata3 / inhibitory). Recently, it has become clear that many of  
35 these classes can be further subdivided into anywhere from 2 to 50 subclasses, based on  
36 anatomical and genetic distinctions, with as-yet unclear implications for circuit connectivity and  
37 function [2-6].

38

39 Traditionally, patterned locomotion has been modeled as an alternation between excitation and  
40 inhibition, which dominate motor neurons during contraction and extension portions of the cycle,  
41 respectively [7, 8]. Recently, however, evidence from both fish and turtles has challenged the  
42 notion that inhibition is minimal during the contraction of the cycle, i.e. in-phase with excitation.  
43 Instead, inhibitory conductances appear to be significant both in- and out-of-phase [9-13],  
44 suggesting that simultaneous recruitment of excitation and inhibition during the contraction is  
45 important for regulating motor neuron firing [14].

46

47 In-phase inhibition is thought to derive from two spinal interneuron classes, the V1 and V2b  
48 populations. The V1 population includes Renshaw cells [15, 16], which provide recurrent  
49 inhibition onto motor neurons with potentially significant shunting effects [17]. To date, most  
50 analysis of drive from V2b neurons has focused on the shared contributions of V1s and V2bs to  
51 reciprocal inhibition governing flexor/extensor alternation in limbed animals [18-20]. However,  
52 this does not shed light on potential functions of ipsilateral inhibition in gain control for  
53 regulation of motor neuron firing *during* contraction, as opposed to suppression of motor neuron  
54 firing during extension.

55

56 In-phase inhibition increases in amplitude for faster locomotor movements [10] suggesting a  
57 potential role in speed control. Here we investigated whether V2b neurons could indeed provide  
58 direct inhibition to motor neurons for speed control, taking advantage of the speed-dependent  
59 organization of zebrafish motor circuits [21-23]. V2b neurons are good candidates for in-phase  
60 gain control because they are exclusively inhibitory in mouse and zebrafish [24] with ipsilateral,  
61 descending axons within the spinal cord [18, 25]. They arise from a final progenitor division that  
62 produces pairs of V2a and V2b neurons [26]. Given the role of V2a neurons in triggering motor  
63 output [27, 28], particularly through speed-specific circuits for titrating levels of motor excitation  
64 [4, 29-31], it seems plausible that their sister V2b neurons exert an opposing, inhibitory role in  
65 speed control. However, the V2b class has not been well characterized at anatomical or  
66 neurochemical levels outside of very early development.

67

68 Here, we define two subclasses of V2b neurons in larval zebrafish based on differential  
69 transmitter expression and anatomy, and further show that these neurons directly inhibit axial  
70 motor neurons in speed-specific circuits. Optogenetic suppression of V2b activity elicits faster  
71 locomotion, consistent with a new role for ipsilateral inhibition in speed control.

72

### 73 **Results**

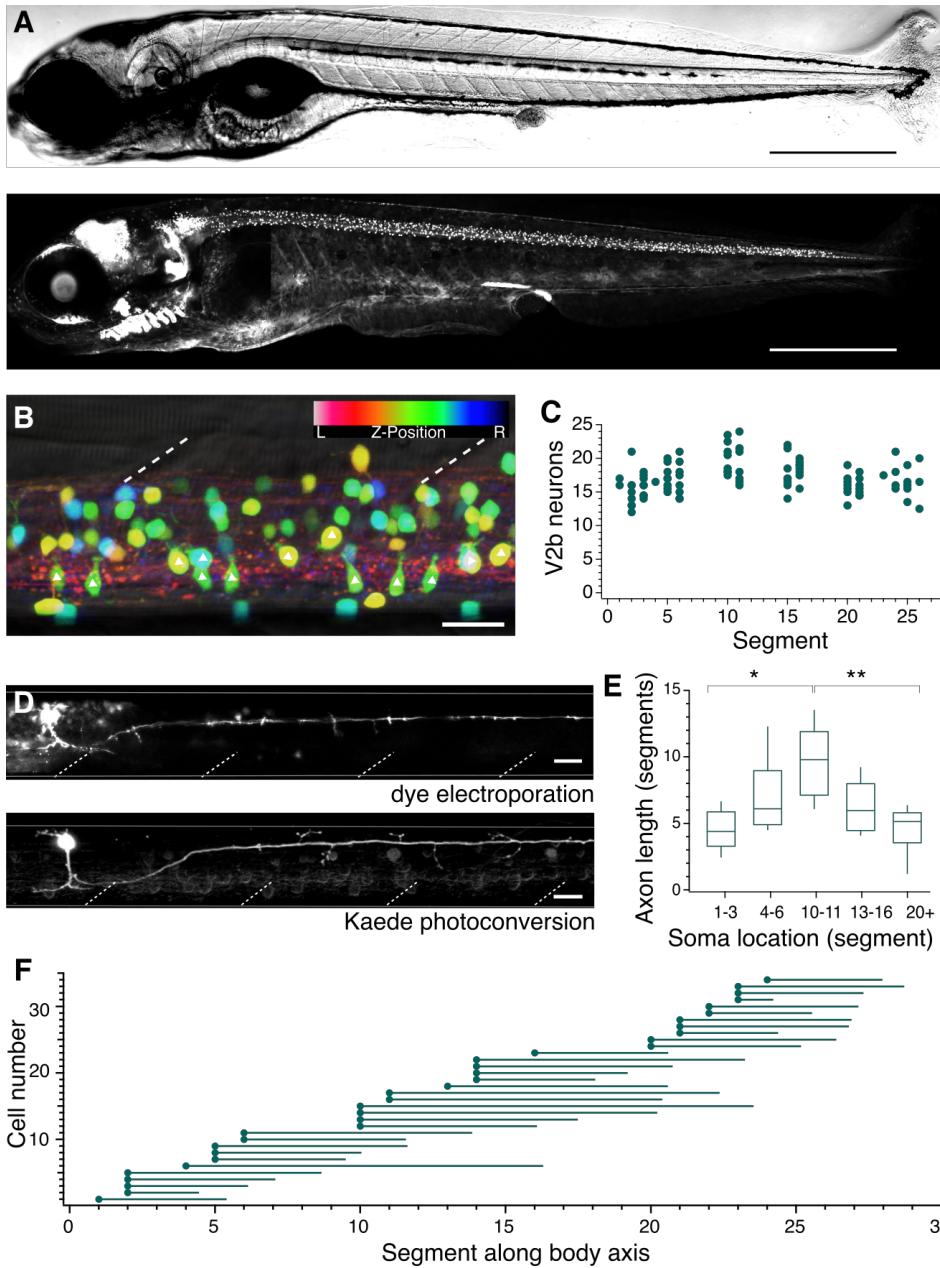
#### 74 *Gata3 transgenic lines label V2b neurons*

75 V2b neural identity is, in part, conferred by the developmental expression of the transcription  
76 factor Gata3 [19, 32]. To provide transgenic labeling of the V2b population, we generated two  
77 *gata3* transgenic lines, *Tg(gata3:loxP-DsRed-loxP:GFP)* and *Tg(gata3:Gal4)* from bacterial  
78 artificial chromosomes (BAC) insertion transgenesis. Both lines label V2b neurons throughout  
79 the rostrocaudal extent of the larval zebrafish spinal cord (Fig. 1A; *Tg(gata3:loxP-DsRed-*  
80 *loxP:GFP)* line shown). *Gata3*-driven fluorescent proteins are also broadly expressed in the  
81 brain, hindbrain, and assorted non-nervous system soft tissue including the pronephric duct [33].

82

83 In a typical spinal segment, V2b soma position ranged from medial to lateral, as visualized with  
84 a color depth code (Fig. 1B). Gata3 is expressed in not only V2b neurons but also the  
85 mechanosensory cerebrospinal fluid contacting neurons (CSF-cN)[34]. CSF-cNs have a distinct  
86 anatomy including large soma size, ventral position, and stereotyped extension into the central  
87 canal (triangles, Fig. 1B), permitting straightforward exclusion from further V2b analysis. On  
88 average, each hemisegment contained  $17.2 \pm 2.5$  (mean  $\pm$  SD) V2b neuron somata with  
89 relatively little variation from rostral to caudal segments (Fig. 1C).

90



91

92 Figure 1. V2b neurons are found throughout the rostral-caudal axis of zebrafish spinal cord.  
93 (A) Transmitted DIC image (top) and confocal image (bottom) of a 5dpf *Tg(gata3:loxP-dsRed-loxP:GFP)*  
94 animal. Scale bars = 0.5 mm.

95 (B) Lateral view of a midbody spinal cord segment, false color depth-coded from left to right; dashed lines  
96 mark muscle segments. In this and all subsequent figures, rostral is to the left and dorsal is to the top. Triangles  
97 mark CSF-cN neurons. Scale bar = 20  $\mu$ m.

98 (C) V2b cell counts per hemisegment quantified along the rostrocaudal body axis,  $n = 7$  fish.

99 (D) Example cell morphology using two techniques to label single V2b axons: single-cell dye electroporation  
100 (E) and Kaede photoconversion. Scale bar = 20  $\mu$ m.

101 (F) Midbody V2b neurons extend axons through more segments than V2b neurons in other rostrocaudal  
102 locations. \* $p < 0.01$ ; \*\* $p < 0.001$ , ANOVA and Tukey's test.

103 (F) Ball and stick plots indicate soma position and axon extension along the body axis for 35 V2b neurons.

104

105

106 *V2b axons extend throughout the spinal cord*

107 In V2b axons To visualize V2b axonal trajectories within the spinal cord, we labeled individual  
108 neurons via either single cell dye-electroporation or Kaede photoconversion in a *Tg(gata3:Gal4,*  
109 *UAS:Kaede*) line (Fig. 1D) [35]. No difference in axon length or trajectory was observed  
110 between the two methods. In all 59 neurons, the axon descended caudally and ipsilaterally, with  
111 an extent ranging from 2 - 15 segments. V2b axons originated on the ventral aspect of the soma  
112 and projected laterally into the white matter. Putative en passant boutons were seen as swellings  
113 distributed along the axon. Most V2b axons projected short collaterals into the soma-dense  
114 medial spinal cord along the axon extent. V2b dendrites extended from the main axon branch  
115 near the soma, (Fig. 1D), similar to identified mixed processes in V2a neurons [4]. However, in  
116 contrast to V2a neurons[4], no V2b neurons extended rostral axons beyond the segment of  
117 origin.

118

119 Single-cell Kaede photoconversions made at different positions along the rostrocaudal extent of  
120 the spinal cord revealed that axonal projections were longest for V2b somata located in the  
121 midbody range (Figs. 1E and 1F). Overall, these data reveal that zebrafish V2b neurons  
122 exclusively innervate areas ipsilateral and caudal to the soma, with the greatest territory of  
123 axonal coverage originating from mid-body neurons with long axons.

124 *In situ hybridization validates transgenic animal lines*

125 To validate the transgenic lines used in this work, we performed two-color fluorescent in-situ  
126 hybridization on each line, examining whether the fluorescent reporter expression matched with

127

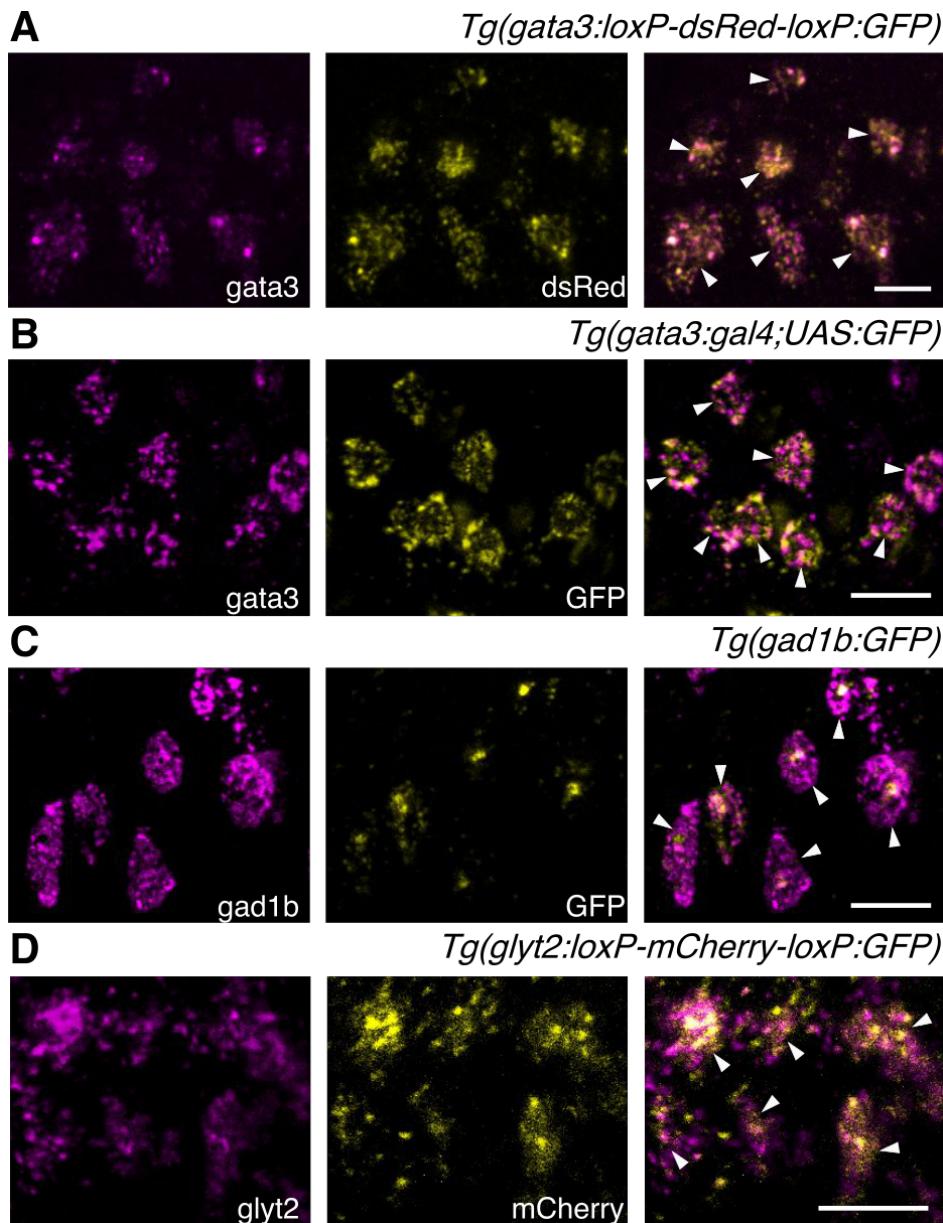
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129

	Completeness (%)	sd	Accuracy (%)	sd
<i>Tg(gata3:loxP-DsRed-loxP:GFP)</i>	96.57	5.34	95.75	6.45
<i>Tg(gata3:gal4;UAS:GFP)</i>	84.36	10.79	89.64	5.92
<i>Tg(gad1b:GFP)</i>	93.33	6.67	88.77	12.69
<i>Tg(glyt2:loxP-mCherry-loxP:GFP)</i>	86.51	7.26	92.21	2.70

130 Table 1. Summary of in-situ hybridization transgenic line validation, including completeness and  
131 accuracy.

132



133

134 Figure 2. Two-color fluorescent in-situ hybridization validates transgenic line expression patterns.

135 Confocal images (z-projection of ~5  $\mu$ m) showing fluorescent in-situ hybridization for endogenous RNA

136 (magenta, left), transgenic fluorophore (yellow, middle) and overlaid two-color image (right). White

137 arrowheads indicate colocalization. (A) *Tg(gata3:loxP-dsRed-loxP:GFP)*; (B) *Tg(gata3:gal4;UAS:GFP)*;

138 (C) *Tg(gad1b:GFP)*; (D) *Tg(glyt2:loxP-mCherry-loxP:GFP)*. Scale bars = 10  $\mu$ m.

139

140 RNA expression of the targeted gene. We evaluated *completeness* of label, i.e. the percentage of  
141 neurons expressing the endogenous gene that also express the fluorescent reporter, and *accuracy*  
142 of label, i.e. the percentage of fluorescent reporter-expressing neurons that express the targeted  
143 endogenous gene. These metrics were evaluated for *Tg(gata3:loxP-DsRed-loxP:GFP)*,  
144 *Tg(gata3:Gal4,UAS:GFP)*, *Tg(gad1b:GFP)*, and *Tg(glyt2:loxP-mCherry-loxP:GFP)*, n = 4-6  
145 animals for each line. An example of each is provided in Figure 2. Results for the completeness  
146 and accuracy of transgenic lines are reported in Table 1. All lines were sufficiently complete and  
147 accurate for use in further quantitative analyses.

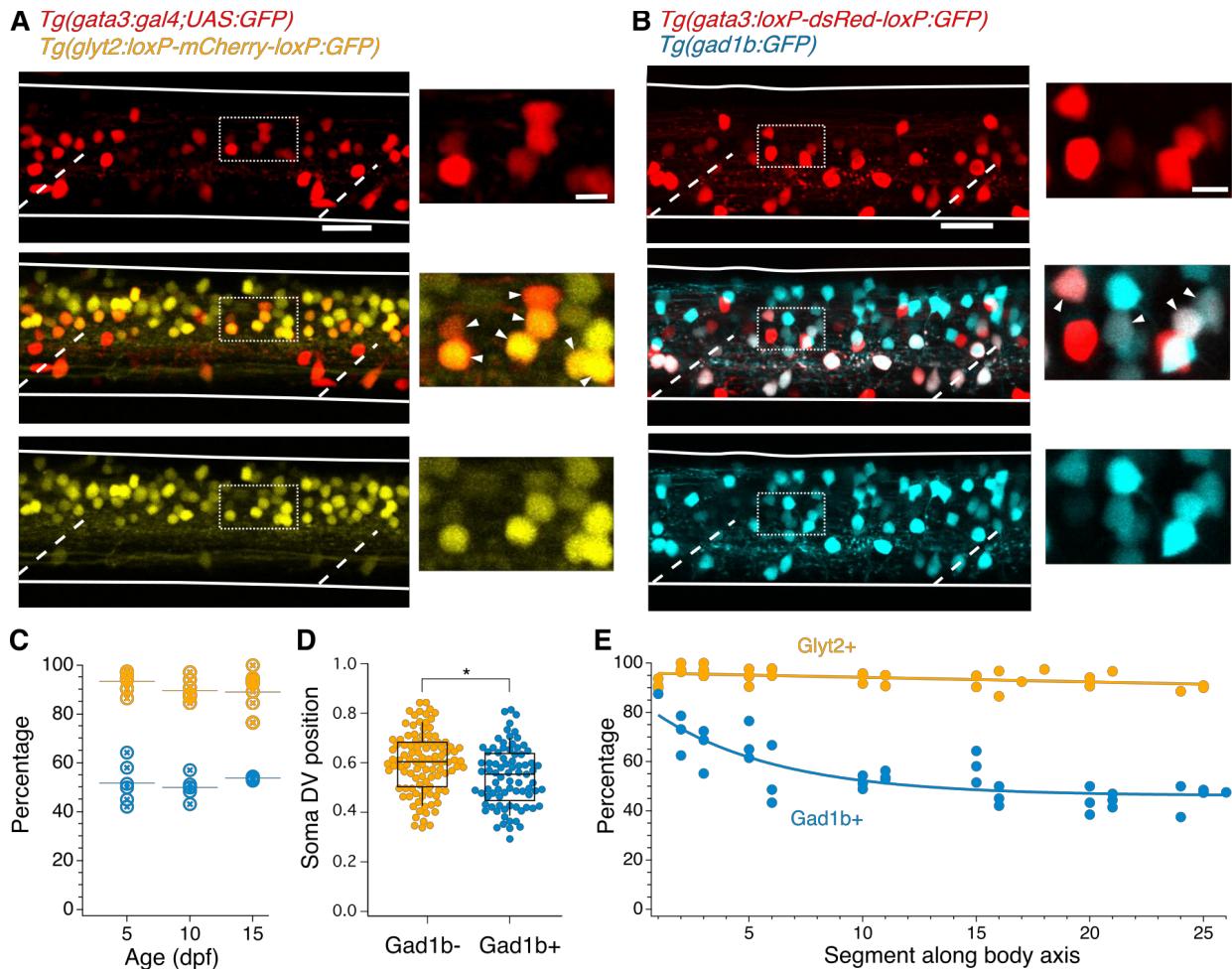
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#### 149 *Neurotransmitter expression defines subpopulations of V2b neurons*

150 Previous work has established that V2b neurons in embryonic zebrafish, as identified by Gata3  
151 RNA expression, are exclusively inhibitory and predominantly GABAergic [24]. However, some  
152 spinal neurons are known to switch inhibitory neurotransmitters at early developmental stages  
153 [36, 37]. To resolve the neurotransmitter profile of V2b neurons in larvae, Gata3+ neurons were  
154 evaluated for coexpression with transgenic markers for *glyt2*, a glycine transport protein, and  
155 *gad1b*, a GABA synthesis enzyme. Nearly all larval V2b neurons expressed Glyt2 in 5 dpf larvae  
156 (Fig. 3A), in contrast to embryonic stages. Furthermore, Gad1b is expressed in approximately  
157 half of the V2b population (Fig. 3B).

158

159 Inhibitory neurotransmitter switching is posited to occur at early developmental stages in  
160 zebrafish [36]. Therefore, we examined whether the variation of neurotransmitter expression in  
161 V2b neurons at 5 dpf represented a transient developmental stage or a stable pattern of  
162 expression. We assessed coexpression of the neurotransmitter markers in midbody V2b neurons  
163



164

165 Figure 3. V2b neurons express the inhibitory neurotransmitter markers Glyt2 and Gad1b.

166 (A) Lateral z-projection of a spinal cord hemisegment in a *Tg(gata3:gal4;UAS:GFP;glyt2:loxP-mCherry-167 loxP:GFP)* (gata3, top; glyt2, bottom) double transgenic animal with composite image (middle). Dashed lines 168 indicate muscle segments and solid lines indicate the spinal cord dorsal and ventral boundaries. Magnified 169 inset, from dashed box, showing soma-level colocalization is shown to the right. Soma colocalization indicated 170 with white arrowheads. Scale bar = 20  $\mu$ m; inset 5  $\mu$ m.

171 (B) *Tg(gata3:loxP-dsRed-loxP:GFP;gad1b:GFP)* (gata3, top; gad1b, bottom) and dual-color composite image 172 (middle). Magnified inset, from dashed box, is shown to the right. Soma colocalization indicated with white 173 arrowheads. Scale bar = 20  $\mu$ m; inset 5  $\mu$ m.

174 (C) Percentage of V2b neurons co-expressing GlyT2 or Gad1b is stable from ages 5-15 dpf, as measured in 175 body segments 15-16. N = 6 animals at each time point.

176 (D) V2b soma position for Gad+ and Gad- neurons differs slightly in the dorsoventral axis, \*p < 0.01, 177 Student's t-test.

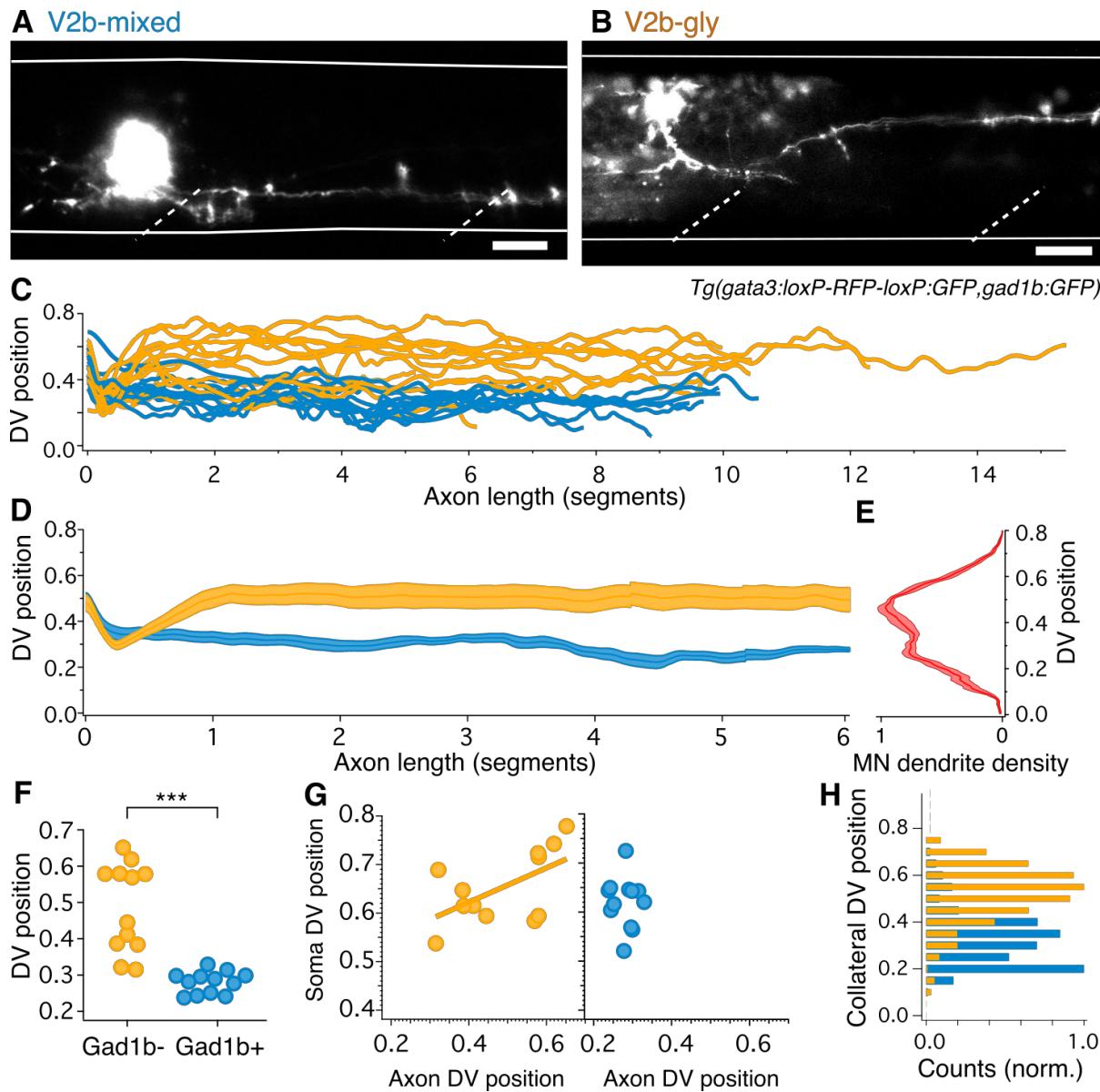
178 (E) Percentage of V2b neurons co-expressing GlyT2 or Gad1b along the rostrocaudal body axis.

179

180 at 5, 10, and 15 dpf, after which V2b neurons are not reliably labeled by transgenic lines (data  
181 not shown). Gad1b and Glyt2 expression in V2b neurons remains unchanged across these ages,  
182 with ~52% of neurons expressing Gad1b and ~91% expressing GlyT2 (Fig. 3C).

183  
184 Are GABAergic and non-GABAergic neurons distributed similarly throughout the neuraxis? By  
185 plotting dorsal-ventral (DV) position relative to spinal boundaries, we found that on average,  
186 GABAergic V2b somata are located slightly ventral to non-GABAergic V2b somata, but that  
187 both populations span the same DV range (Fig. 3D). Therefore, soma position is not predictive of  
188 neurotransmitter expression. In the rostrocaudal axis, the percentage of GABAergic V2b cells is  
189 highest (~80%) in rostral segments, then decreases to ~50% by midbody and throughout the rest  
190 of the spinal cord. In contrast, Glyt2 robustly colabels with V2b cells throughout the entire spinal  
191 cord (Fig. 3E). These data indicate that the Gad1b+ and Gad1b- populations comprise distinct  
192 and persistent subclasses. V2b neurons expressing both Glyt2 and Gad1b will be referred to as  
193 V2b-mixed, in reference to their mixed neurotransmitter expression, whereas V2b neurons that  
194 solely express Glyt2 will be referred to as V2b-gly.

195  
196 *Axonal morphology varies by subpopulation identity*  
197 The classic axonal morphology of zebrafish VeLD neurons is ventral, with little change in DV  
198 position from the onset [24]. However, some V2b neuron fills exhibited axons with much more  
199 dorsal trajectories (e.g. Fig. 1D). To resolve whether these represent different subclasses, we  
200 investigated axonal morphology of identified V2b-mixed and V2b-gly neurons using single-cell  
201 dye electroporation in the double transgenic line *Tg(gata3:loxP-DsRed-loxP:GFP; gad1b:GFP)*,  
202



204 Figure 4. V2b-gly and V2b-mixed V2b neurons have distinct axon morphology and innervation territories.  
205 (A) Examples of a V2b-mixed (*Tg(gad1b:GFP)*+) and a (B) V2b-gly (*Tg(gad1b:GFP)*-) single-cell dye fill.  
206 Dashed lines indicate muscle segments and solid lines indicate the spinal cord dorsal and ventral boundaries.  
207 Scale bars = 20  $\mu$ m.  
208 (C) Axon traces for V2b neurons, aligned at the segment of origin, relative to the spinal cord dorsoventral  
209 boundaries (V2b-mixed, cyan, n = 12; V2b-gly, orange, n = 12). All axons were exclusively descending.  
210 (D) Mean and SEM of V2b-gly and V2b-mixed axon trajectories.  
211 (E) Motor neuron dendrite fluorescence intensity, measured in *Tg(mnx:GFP)*, relative to the same dorsoventral  
212 landmarks.  
213 (F) Mean axon position for each traced axon. \*\*\*p < 0.0001, Student's t-test.  
214 (G) Average axon position of V2b-mixed (cyan, left) and V2b-gly (orange, right) relative to soma position for  
215 each neuron. A correlation between soma position and axon position is observed for V2b-gly but not V2b-  
216 mixed neurons. V2b-gly:  $r^2 = 0.33$ , p < 0.05, V2b-mixed:  $r^2 = 0.0059$ , p = n.s.  
217 (H) Axon collaterals of V2b-gly neurons also innervate more dorsal spinal cord territory than V2b-mixed  
218 axons.

219

220 in which expression of GFP (Gad1b) differentiates between the mixed and glycinergic  
221 subclasses.

222

223 Although both V2b-gly and V2b-mixed neurons extend axons caudally and ipsilaterally,  
224 consistent with data in Fig. 1, the DV position of their axons was different. GABAergic V2b-  
225 mixed neurons projected axons ventrally along the spinal cord, with an average axon location  
226 found between 0.24 - 0.33 DV (example, Fig. 4A). In contrast, axons from V2b-gly neurons  
227 typically make an initial ventral dip but then turn more dorsally, ranging from 0.31 - 0.65 in the  
228 DV axis (example, Fig. 4B). Traces from all filled neurons are shown in Fig. 4C, and averaged  
229 trajectories in Fig. 4D. Somata were filled in segments ranging from 14 to 18; the traces are  
230 shown aligned at the soma for ease of visualization.

231

232 Other features of anatomy also varied between the two subtypes. The axon DV position of V2b-  
233 gly neurons is positively correlated to the soma DV position, i.e. a more dorsal soma projects a  
234 more dorsally positioned axon (Fig. 4G). However, this trend is not realized for V2b-mixed cells,  
235 which project axons ventrally to a narrow spinal cord region regardless of soma position.

236 Putative en passant boutons were found in both cell populations. Most filled axons (22/24)  
237 extended vertical collaterals from the main axon. The number of collaterals per axon did not  
238 significantly vary between populations (V2b-mixed, median = 3, range 0-5; V2b-gly, median =  
239 5, range 0-23, Mann-Whitney Wilcoxon test  $p = 0.056$ ). However, collaterals of V2b-mixed and  
240 V2b-gly axons cover largely distinct DV regions of the spinal cord (Fig. 4H).

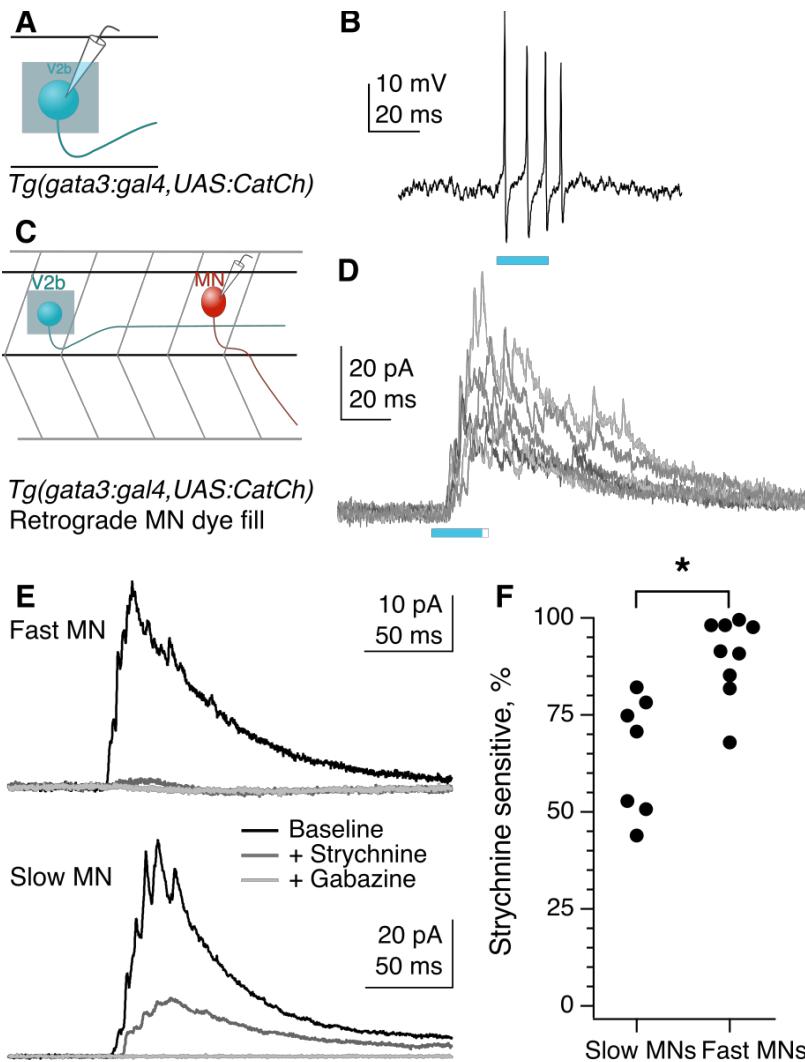
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242 What is the significance of differential DV axon trajectories between V2b-gly and V2b-mixed  
243 subclasses? Previous work has shown that motor neurons active during fast movements are  
244 located more dorsally within the spinal cord, whereas those for slower movements are located  
245 more ventrally [21]. Therefore, we compared population averages of the V2b-gly and V2b-mixed  
246 axons (Fig. 4D) to a plot of motor neuron dendritic territory (Fig. 4E; see Methods) shown in  
247 Figure 4D. Notably, V2b axon position of the two classes overlaps with two peaks in the motor  
248 neuron density profile. Consequently, we next investigated the direct influence of V2b neurons  
249 on motor neurons.

250

251 *V2b subpopulations provide differential inputs to fast and slow circuits*  
252 Anatomical evidence indicates that V2b neurons make contact onto limb motor neurons where  
253 they are partially responsible for enforcing flexor/extensor alternation [18, 19]. However, to date  
254 there are no physiological recordings of synaptic connections from V2bs to other neurons in any  
255 species. We first validated that optogenetic stimulation in the *Tg(gata3:Gal4; UAS:CatCh)* line  
256 was sufficient to elicit action potentials in V2b neurons (Figs. 5A and 5B). We then targeted  
257 spinal motor neurons for *in vivo* recording in *Tg(gata3:Gal4, UAS:CatCh)* larvae at 4-6 dpf (Fig.  
258 5C). Optogenetic activation of V2b neurons with a 20-50 ms pulse of light delivered 3-7  
259 segments rostral to the recording site elicited robust IPSCs in motor

260



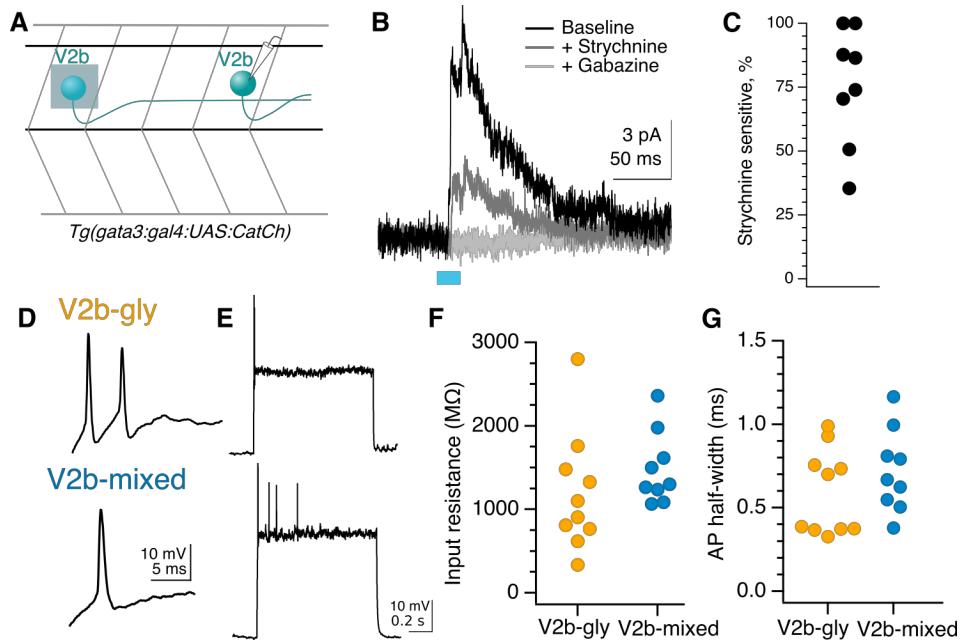
261  
262 Figure 5. Fast motor neurons receive predominantly glycinergic V2b inputs, whereas V2b synaptic inputs to  
263 slow motor neurons are mediated by both GABA and glycine receptors.  
264 (A) Schematic of recording to validate CatCh expression in V2b neurons.  
265 (B) Cell-attached recording from a V2b neuron expressing CatCh during a 20 ms illumination epoch. Note that  
266 evoked action potentials outlast the duration of illumination, presumably due to membrane depolarization  
267 and/or Ca influx.  
268 (C) Schematic illustrating whole-cell recordings from motor neurons paired with optogenetic stimulation of  
269 V2b neurons.  
270 (D) Six overlaid sweeps showing ISPCs barrages recorded in a motor neuron in response to optogenetic  
271 activation of V2b neurons. Blue bar represents the light stimulus. All recordings were carried out in the  
272 presence of NBQX.  
273 (E) Average IPSC responses to light stimulation in fast (top) and slow (bottom) motor neurons, as identified by  
274 soma location and input resistance. Response during baseline (black), after application of strychnine (dark  
275 grey), and after additional application of gabazine (light grey). In all cases, the IPSC was entirely abolished by  
276 the combination of strychnine and gabazine.  
277 (F) Percentage peak current reduction by strychnine in fast and slow motor neurons. \*p < 0.01.  
278

279 neurons (Fig. 5D). Synaptic conductance amplitudes exhibited a median of 139 pS (25-75%  
280 range, 97-174 pS). Although the *Tg(gata3:Gal4)* line labels CSF-cNs in addition to V2bs (Fig.  
281 1B), CSF-cNs exhibit short ascending axons that do not contact motor neurons other than the  
282 CaP [38]. To validate that V2b neurons are providing these inhibitory inputs, we used a digital  
283 micromirror device to deliver targeted squares of light stimuli (~20  $\mu$ m x 20  $\mu$ m) to dorsal spinal  
284 cord areas containing V2b but not CSF-cN somata. These localized stimuli still elicited reliable  
285 IPSCs in both primary and secondary motor neurons (Figs. S1B and S1C). As a second control,  
286 recordings were also made in a subset of animals with strong CatCh expression in CSF-cNs and  
287 negligible expression in V2b neurons (see Methods and Fig. S1D). In these recordings, even full-  
288 field light stimulation evoked only minimal IPSCs in motor neurons (Fig. S1E). Together these  
289 results indicate that the optogenetically elicited inhibitory inputs arise from monosynaptic V2b to  
290 motor neuron connections.

291

292 The striking difference in dorsal-ventral targeting of V2b-gly and V2b-mixed axonal trajectories  
293 (Fig. 4D) suggests a potential relationship with the well-described dorsal-ventral distribution of  
294 motor neurons based on size and speed at recruitment. Large motor neurons with low input  
295 resistance are located dorsally within the motor pool and are recruited for the fastest speeds of  
296 swimming, whereas more ventrally located motor neuron somata exhibit higher input resistance  
297 and are recruited during slower movements [21, 22]. Accordingly, we tested whether the  
298 glycinergic and GABAergic components of the IPSC differed between fast and slow motor  
299 neurons. Bath application of strychnine to block glycine receptors abolished a median of 91% of  
300 the V2b-evoked IPSC in fast motor neurons, but only 71% of the V2b-evoked IPSC in slow  
301 motor neurons (Fig 6F, C;  $p = 0.003$ , Wilcoxon Rank test). The GABA<sub>A</sub> receptor antagonist

302



303

304 Figure 6. Rostral V2b neurons inhibit more caudal V2b neurons, providing circuit disinhibition; V2b-gly and  
305 V2b mixed populations are physiologically indistinguishable.

306 (A) Example action potential magnified from (B) responses to step depolarizations in both classes of V2b  
307 neurons. Most recorded neurons in both groups could not sustain action potentials across a step.

308 (C) Input resistance measured via hyperpolarizing test pulse. N = 10 Gad- (orange), 9 Gad+ (cyan).

309 (D) Action potential peak half-widths are not significantly different between the two groups.

310 (E) Experimental schematic for V2b-to-V2b connectivity recordings.

311 (F) Evoked IPSCs recorded in an example V2b neuron in response to optogenetic stimulation of more rostral  
312 V2b neurons, black, and the response after the successive addition of glycine and GABA<sub>A</sub> receptor  
313 antagonists, dark grey and light grey traces respectively. The blue bar represents the duration of optogenetic  
314 stimulation.

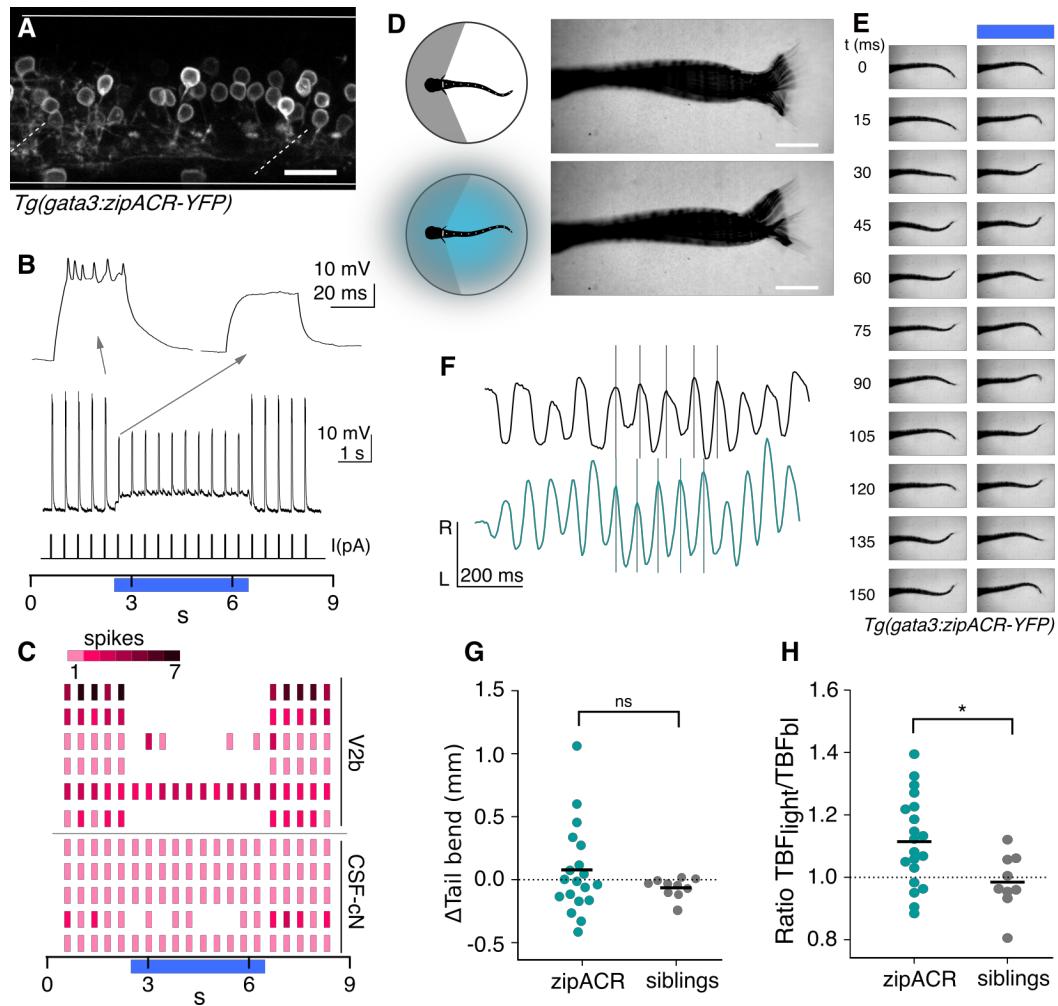
315 (G) Percentage peak current sensitivity to strychnine.

316

317 gabazine (SR-95531) eliminated the remaining IPSC in all cases. Therefore V2b-mediated  
318 inhibition onto fast motor neurons is carried out predominantly by glycinergic synapses, whereas  
319 V2b inhibition onto slow motor neurons is carried by mixed glycinergic/GABAergic  
320 transmission. These results are consistent with the idea that V2b-gly preferentially inhibit more  
321 dorsally located fast motor neurons, whereas V2b-mixed inhibit the more ventrally located slow  
322 motor neurons.

323  
324 Some spinal premotor neurons form synaptic connections within their own populations,  
325 suggestive of speed- or state-related “gears” [39]. Optogenetic activation of rostrally-located V2b  
326 neurons evoked IPSCs in 11/21 (52%) mid-body V2b neurons (Figs. 6A and 6B). The median  
327 conductance of individual IPSCs was 158 pS (25-75% range, 84-158 pS). Application of  
328 strychnine blocked a median of 80% of the V2b-evoked IPSC, while the remainder was  
329 abolished by gabazine (Figs. 6B and 6C). Thus, some V2b neurons inhibit other members of the  
330 V2b pool forming a disinhibitory pathway.

331  
332 *V2b cell physiology does not distinguish between subtypes*  
333 Intrinsic physiological characteristics, including input resistance and spiking properties, can be  
334 used to subdivide some spinal interneuron populations into distinct subpopulations [2, 6, 40]. We  
335 examined whether the V2b-gly and V2b-mixed subgroups exhibited differences in intrinsic  
336 physiology by targeting whole-cell recordings to these neurons. V2b neurons were silent at rest,  
337 in contrast to CSF-cNs which exhibited spontaneous spiking (data not shown). Spikes were  
338 elicited by depolarizing current steps, (Figs. 6D and 6E) which usually led to one or a few spikes,  
339 with only 3/10 V2b-gly and 3/9 V2b-mixed neurons able to sustain spiking across the step. There  
340



341

342

Figure 7. Optogenetic suppression of V2b activity leads to increased locomotor speeds.

343 (A) Z-projection of *Tg(gata3:zipACR-YFP)* over one full segment of spinal cord showing expression in V2b  
 344 but not CSF-cN somata. CSF-cN apical extinctions show minimal YFP expression. See also Fig. S2. Scale bar  
 345 = 20  $\mu$ m.

346 (B) A whole cell recording during repeated current steps (20 ms duration) is shown for an example V2b neuron  
 347 in a *Tg(gata3:zipACR-YFP)* animal. Blue bar indicates period of optical stimulation. An expanded view of  
 348 current steps before and during optical stimulation are shown above with arrows. Recordings indicate that  
 349 current steps normally elicit bursts of action potentials, but coincident optogenetic suppression prevents  
 350 spiking, yielding only subthreshold depolarizations.

351 (C) Raster plot of action potentials for 6 V2b cells and 5 CSF-cN cells summarizes optogenetic suppression  
 352 across cell types. Color value represents number of spikes elicited during each current step. 5/6 V2b neurons  
 353 were mostly or entirely suppressed, whereas only 1/5 CSF-cN were affected.

354 (D) Schematic of behavioral recording depicting the NMDA-induced tail movements of spinalized head-  
 355 embedded animals without and with optogenetic stimulation. Image overlay of 100 ms of tail movements  
 356 without and with light stimulation in a *Tg(gata3:zipACR-YFP)* animal show striking similarities in tail  
 357 displacement during swim. Scale bar = 0.5 mm.

358 (E) Time point images of tail movements in the same animal at baseline (left) and with optogenetic stimulus  
 359 (right) demonstrating the difference in timing of tail movements during V2b suppression.

360 (F) Tracked left-right tail position during recordings with (teal) and without (black) optical stimulation for the  
 361 same *Tg(gata3:zipACR-YFP)* animal. Lines for each recording are aligned to consecutive peaks in the baseline  
 362 trace to illustrate the phase advance and increased tail beat frequency during optogenetic stimulation (teal).

363 (G) Average change in tail bend between stimulation and control recordings during swim movements for each  
364 animal, ns p = 0.14.

365 (H) Ratio of average TBF during stimulation to baseline TBF for each animal, cohort averages shown with  
366 black dash. N = 20 *Tg(gata3:zipACR-YFP)* and N = 9 siblings. \*p < 0.01.

367

368 was no difference in input resistance (Fig. 6F) or spike shape (Fig. 6G) between the V2b-gly and  
369 V2b-mixed neurons. Therefore, the two V2b subpopulations are indistinguishable at the level of  
370 intrinsic physiology despite their differences in axon trajectory.

371

372 *Optogenetic V2b suppression increases tail beat frequency*

373 What are the functional consequences of V2b inhibition onto motor neurons (Fig. 5)? To better  
374 understand this role, we carried out high-speed behavioral recordings during optogenetic  
375 inactivation of V2b neurons with a light-gated Cl<sup>-</sup> channel, ZipACR [41]. To eliminate  
376 contributions from Gata3 expressing neurons in the brain, we used a spinally transected  
377 preparation. Tail movements were induced pharmacologically with application of N-methyl-d-  
378 aspartate (NMDA, 200 μM) [42]. NMDA induces tail movements with episodic, left-right  
379 alternations that mimic the natural beat-and-glide swims of 5 dpf larvae [42-44].

380

381 Spinal CSF-cNs are labeled in BAC-generated Gata3 transgenic lines (Fig. 1A). However, a  
382 CRISPR-generated *Tg(gata3:zipACR-YFP)* knock-in shows robust expression of the fluorescent  
383 ZipACR protein in V2b neurons but only sparse, dim expression in CSF-cNs (Fig 7A). Within  
384 CSF-cNs expression was observed exclusively in the apical extension into the central canal but  
385 not the soma (Figs. 7A and S2). We first validated the efficacy of the ZipACR construct in  
386 suppressing V2b firing under high-intensity light (Fig. S3A, n = 4). Under lower-intensity light  
387 conditions, identical to those of the behavioral recordings, action potentials were completely  
388 suppressed in 4 out of 6 V2b cells and partially suppressed in 1 additional cell (Figs. 7B and C),

389 in *Tg(gata3:zipACR-YFP; gata3:loxP-DsRed-loxP:GFP)* animals. In contrast, identical  
390 stimulation partially suppressed spiking in only 1 of 5 CSF-cNs (Figs. 7C and S3C). Therefore,  
391 we used these light stimulation parameters, under which V2b neurons are mostly if not entirely  
392 suppressed whereas CSF-cNs are not substantially affected, to carry out behavioral experiments  
393 assessing the effects of suppressing V2b neurons on locomotion.

394

395 Animals were head-embedded with a free tail and high-speed (200 Hz) recordings were acquired  
396 to capture fictive locomotion. Swim dynamics were recorded and evaluated both with and  
397 without optogenetic stimulation (Figs. 7D and 7E). Kinematic analysis of the high-speed video  
398 was performed with code adopted from [45]. The total tail displacement and quantity of tail  
399 movements did not significantly differ during optogenetic stimulation (Figs. 7D and 7G).

400 Strikingly, tail beat frequency (TBF), a metric of locomotor speed, increased in  
401 *Tg(gata3:zipACR-YFP)+* animals during light stimulation but not in their ZipACR negative  
402 clutchmates (Figs. 7E, 7F, and 7H, paired t-test  $p = 0.0025$ ). The average TBF change was 1.4  
403 Hz and was robustly observed in animals from three clutches. In contrast, elimination or genetic  
404 silencing of CSF-cN causes the opposite effect, a decrease in TBF [46]. Therefore, any modest  
405 changes in CSF-cN activity in our experiments (Fig. 7C) would if anything serve to counteract  
406 the effects of V2b silencing. We conclude that suppression of V2b neurons increases TBF, and  
407 consequently the normal role of V2b-mediated inhibition is to serve as a brake on locomotor  
408 frequency.

409

410

411 **Discussion**

412 In this study we demonstrated that V2b neurons exert direct control over axial musculature in the  
413 larval zebrafish. The V2b population comprises two stable subclasses, defined by  
414 neurotransmitter identity: one subclass is exclusively glycinergic and the other mixed  
415 glycinergic/GABAergic. These distinct V2b-gly and V2b-mixed subclasses preferentially inhibit  
416 fast and slow motor neurons, respectively, analogous to the speed-dependent connectivity found  
417 in a diverse range of zebrafish spinal interneurons [23, 31, 47, 48]. Moreover, we found that the  
418 suppression of V2b activity led to an increase in locomotor speeds. Together, these results  
419 indicate that inhibition from V2b neurons is not restricted to enforcing agonist-antagonist muscle  
420 coordination but also influences locomotor speed through in-phase modulation of axial motor  
421 neurons.

422

423 *V2b conservation across species*

424 We demonstrated that V2b neurons are inhibitory and extend axons ipsilaterally and caudally in  
425 zebrafish (Figs. 1, 3) similar to V2b neurons in mice [18, 25]. Gata3+ V2b neurons are widely  
426 present in vertebrates but have also been identified in the nerve cord of a marine annelid  
427 indicating an ancestral persistence in motor circuitry [32, 49]. V2b neurotransmitter profiles  
428 appear to vary throughout development and across species. Gata3-expressing cells in the  
429 embryonic 24 hours post fertilization zebrafish are predominantly GABAergic with a smaller  
430 subset expressing or co-expressing glycine [24], an inversion of our finding that V2b cells in 5-  
431 15 dpf zebrafish are all glycinergic with approximately half co-expressing GABA. Early in  
432 development, murine V2b neurons broadly co-express GABA and glycine [19]. By P0 in mouse,  
433 however, nearly all V2bs are glycinergic and ~25% are GABAergic [25], which is broadly

434 similar to our results. In zebrafish, these two subclasses persist out to 15 dpf which implies that  
435 they are distinct identities.

436

437 Consistent with the idea that V2b-gly and V2b-mixed are distinct identities, the two subclasses  
438 exhibit different axon trajectories in the DV axis, perhaps indicating responsiveness to different  
439 axon guidance cues. In mouse, V2b subpopulations have not been directly shown. However, the  
440 differential expression of the transcription factors Gata2/Gata3/BhlhB5 in non-overlapping  
441 neural subsets may imply their presence [50]. More broadly, our finding of subclasses in the V2b  
442 population is parallel to previously identified genetically and anatomically distinct subclasses  
443 within the V0, V1 and V2a populations in the mouse and zebrafish [2, 4-6, 51].

444

445 *Do V2b-gly and V2b-mixed populations match existing zebrafish neural classes?*

446 Historically, zebrafish spinal neurons have been classified by anatomy [52]. V2b (Gata3+)  
447 neurons are thought to correlate to ventral longitudinal (VeLD) neurons, an anatomically defined  
448 cell class with a characteristic longitudinal ventral-positioned axon [24, 53]. How do the  
449 subpopulations we have described here relate to the VeLD population? Based on the ventral axon  
450 morphology and GABA co-expression, the V2b-mixed subtype represents a matured version of  
451 the embryonic VeLD neurons. In contrast, V2b-gly neurons are distinct in morphology and  
452 neurotransmitter profile from VeLDs, indicating either that they have not been characterized in  
453 embryonic stages or that they develop at a later time.

454

455 In mice, the V2 progenitor domain gives rise to a third class of neurons called V2c, which  
456 express Sox1 and only transiently Gata3 in very early development (prior to E12.5) before later

457 downregulation [54]. It is unclear whether zebrafish have a homologous V2c population  
458 although Sox1a/b is present in the 24 hpf spinal cord and notably also colabels with Gata3 [55].  
459 A possible V2c homolog, referred to as V2s, has been identified as a Sox1a/b+ glycinergic cell  
460 type deriving from the V2 domain with long, ipsilateral, caudally projecting axons [56] similar to  
461 the V2b-gly neurons described here. However, Gata3 expression was not investigated in V2s  
462 neurons, leaving it unclear whether V2s neurons are in fact V2b-gly [56]. Given the persistent,  
463 distinguishing expression of Gata3 in both V2b-gly and V2b-mixed subtypes, our data are  
464 consistent with the designation of two subclasses within V2b, not a V2c homolog or additional  
465 V2s class. Further detailed investigation of Sox1a/b gene expression in these neurons will be  
466 required to clearly separate these classes.

467

468 *Speed specific inputs to motor circuits*

469 Locomotion at faster versus slower speeds engages different sets of spinal interneurons, both  
470 within a genetically defined population [23, 29, 31, 47] and across populations [21, 57]. Given  
471 the observation that slow motor neurons likely receive more input from V2b-mixed neurons  
472 whereas fast motor neurons receive largely V2b-gly input, it would be of interest to explore  
473 whether the V2b subpopulations are recruited at different speeds of locomotion. Furthermore, the  
474 intra-V2b connectivity suggests a possible “gear shift” within the V2bs, with the V2b-gly and  
475 V2b-mixed populations potentially inhibiting each other to enforce a given speed of swim. One  
476 caveat in interpretation of these results is that despite their different somatic positions, fast and  
477 slow motor neurons have overlapping dendritic fields [48]. Therefore, it is possible that V2b-  
478 mixed neurons make synapses onto all motor neurons, and differential receptor expression is  
479 responsible for their IPSC pharmacology (Fig. 5); meanwhile, V2b-gly might be responsible for

480 other functions, such as suppression of dorsal horn sensory interneurons [58]. Paired recordings  
481 or higher resolution anatomical experiments will be required to distinguish these possibilities.

482

483 *V2b suppression increases tail movement speed*

484 One role of ipsilateral inhibition is to mediate flexor-extensor alternations via Ia reciprocal  
485 inhibition from V2b and V1 populations [18, 19]. Ipsilaterally descending propriospinal neurons  
486 may also stabilize left-right alternation, although specific ablation of inhibitory ipsilaterally  
487 descending neurons has not been tested [8, 59]. Our work establishes that selective suppression  
488 of V2b activity increases tail beat frequency (Fig. 7). In contrast, genetic ablation of V1 neurons  
489 in mouse dramatically reduced fictive step speed [60]. Similarly, perturbation of in-phase  
490 inhibition led to slower crawling speeds in larval Drosophila and reduced rhythmic motor drive  
491 in Xenopus [13, 61]. From this we surmise that ipsilateral inhibition from V1 and V2b shape  
492 distinct features of locomotion. V1 neurons may act to terminate motor neuron burst cycles while  
493 V2b neurons may limit overall speed, much like a brake.

494

495 The behavioral outcome of V2b inactivation is modest at slow speeds. However, V2a influence  
496 over motor circuits strengthens for increasing speeds [21, 47]. Thus, it is possible that the effect  
497 of silencing V2b neurons will be larger at faster locomotor speeds, particularly given that in-  
498 phase inhibition increases for faster movements [10, 11, 62]. It is unclear from current  
499 experiments whether the V2b-associated speed increase is due to the loss of on-cycle V2b  
500 inhibition onto motor neurons or through inputs to the premotor circuitry, for example through  
501 V2b-V2b interconnectivity (Fig. 6). More selective optogenetic manipulations will be useful to  
502 separate out these effects.

503

504 *Overall role of V2b in motor circuits*

505 What is the functional role of V2b-mediated ipsilateral inhibition onto motor circuits? Three  
506 broad categories of V2b function occur. First, as discussed above, enforcing flexor-extensor and  
507 potentially forelimb-hindlimb alternation in limbed animals. Second, as supported in this work,  
508 V2b neurons may serve to titrate motor neuron spiking differentially across varying speeds of  
509 movement. Measuring inhibitory conductances in motor neurons *in vivo* has revealed,  
510 surprisingly, that inhibition in-phase with excitation actually increases for increasingly strong  
511 movements [10, 11, 13, 62], rather than diminishing to allow more powerful contractions. In this  
512 context, shunting ipsilateral inhibition might serve to enforce tight temporal control over spike  
513 timing via shortening membrane time constants. Alternatively or in addition, ipsilateral  
514 inhibition might function as a form of gain control to prevent saturation, analogous to somatic  
515 inhibition in hippocampus and cortex [11, 63, 64].

516

517 Thirdly, ipsilateral inhibition may act to isolate movements in certain behaviors that engage  
518 dedicated premotor circuitry, e.g. through the selective inhibition of interneurons during  
519 scratching versus swimming in turtle [65, 66]. Some V2b neurons, by virtue of their direct  
520 connections with ipsilateral motor circuits, could form part of the locomotor “switch” from one  
521 behavior to another. A thorough investigation of V2b-gly and V2b-mixed recruitment during  
522 natural behaviors, such as speed transitions, turning, or balance, will allow us to better  
523 understand the similar or distinct ways that V2b subclasses influence locomotion.

524

525

526 **Methods**

527 *Animal care*

528 Adult zebrafish (*Danio rerio*) were maintained at 28.5°C with a 14:10 light:dark cycle in the  
529 Washington University Zebrafish Facility following standard care procedures. Larval zebrafish,  
530 4-7 days post fertilization (dpf), were kept in petri dishes in system water or housed with system  
531 water flow. Animals older than 7 dpf were fed rotifers daily. All procedures described in this  
532 work adhere to NIH guidelines and received approval by the Washington University Institutional  
533 Animal Care and Use Committee.

534

535 *Line generation*

536 The *Tg(gata3:gal4)* and *Tg(gata3:LoxP-dsRed-LoxP:GFP)* lines were generated via the bacterial  
537 artificial chromosome (BAC) transgenic technique [67], using BAC zK257H17. The Gal4 and  
538 LRL-GFP constructs are described in Kimura et al. [28] and Satou et al. [68], respectively. The  
539 *Tg(glyt2:LoxP-mCherry-LoxP:GFP)* line was generated with CRISPR/Cas9 genome targeting  
540 methods utilizing the short guide RNA, donor plasmid, and methods described in Kimura et al.  
541 [69]. *Tg(gata3:zipACR-YFP)* animals were generated with CRISPR/Cas9 techniques using a  
542 gata3 short guide, TAG GTG CGA GCA TTG AGC TGA C. The donor Mbait-hs-zipACR-YFP  
543 plasmid was made by subcloning ZipACR [41], obtained from Addgene, into a Mbait-hs-GFP  
544 plasmid with Gibson Assembly cloning methods. A UAS:Catch [70] construct containing tol2  
545 transposons was microinjected along with tol2-transposase RNA into one-cell *Tg(gata3:gal4)*  
546 embryos to generate the *Tg(gata3:gal4;UAS:Catch)* line.

547

548 *Single-cell photoconversion*

549 Fluorescent protein photoconversion was performed on anesthetized and embedded 5 dpf  
550 *Tg(gata3:gal4; UAS:kaede)* animals using an Olympus FV1200 microscope. Single-plane  
551 confocal images were continuously acquired to monitor conversion progress while 500 ms bursts  
552 of 405 nm light (100% intensity) were applied to an ROI ~1/10<sup>th</sup> the size of the targeted soma to  
553 elicit photoconversion. Animals were removed from agarose and allowed to recover in system  
554 water for 1-3 hours. After recovery, fish were anesthetized, embedded, and imaged as above.  
555 Tiled image stacks were acquired over an area ranging from the most rostral processes to the  
556 most caudal with a minimum of 10% area overlap between adjacent fields of view to aid the  
557 image stitching process.

558

559 *Single-cell dye electroporation*

560 *Tg(gata3:LoxP-dsRed-LoxP:GFP; gad1b:GFP)* animals (5-6 dpf) were anesthetized in 0.02%  
561 MS-222 and three electroetched tungsten pins were placed through the notochord securing the  
562 animal to a Sylgard-lined 10 mm well dish. Forceps and an electroetched dissecting tool were  
563 used to remove skin and one segment of muscle fiber to expose the spinal cord. A pipette  
564 electrode filled with 10% Alexa Fluor 647 anionic dextran 10,000 MW (Invitrogen) in internal  
565 recording solution, was positioned to contact the soma of the transgenic-labeled target neuron.  
566 Dye was electroporated into the cell via one or more 500 ms, 100 Hz pulse trains (1 ms pulse  
567 width) at 2-5 V (A-M systems Isolated Pulse Stimulator Model 2100). Confocal imaging was  
568 performed as described above, after > 20 min for dye filling.

569

570 *Fluorescent hybridization chain reaction (HCR)*

571 Animals were fixed at 5 dpf in 4% paraformaldehyde and *in situ* hybridization was performed  
572 according to the HCR v3.0 protocol [71] with noted modifications. Preparation, dehydration and  
573 rehydration steps 1 through 14 were replaced with steps 2.1.1 through 2.2.8 with a Heat Induced  
574 Antigen Retrieval (HIAR) option in place of Proteinase K treatment [72, 73]. *In situ* probes were  
575 designed and distributed by Molecular Technologies (Beckman Institute, Caltech) to target  
576 gata3, gad1b, glyt2 (slc6a5), DsRed, mCherry, and GFP. Samples were kept in 4x saline-sodium  
577 citrate solution at 4°C prior to imaging. Samples were mounted in Vectashield (Vector  
578 Laboratories) or low-melting point agarose (Complex SeaPlaque Agarose, 1.2% in system water)  
579 and positioned laterally on a microscope slides with #1.5 coverslip glass.

580

581 *Confocal imaging*

582 5-7 dpf larvae were anesthetized in 0.02% MS-222 and embedded in low-melting point agarose  
583 in a 10 mm FluoroDish (WPI). Images were acquired on an Olympus FV1200 Confocal  
584 microscope equipped with high sensitivity GaAsP detectors (filter cubes FV12-MHY and  
585 FV12-MHYR), and a XLUMPLFLN-W 20x/0.95 NA water immersion objective. A transmitted  
586 light image was obtained along with laser scanning fluorescent images. Sequential scanning was  
587 used for multi-wavelength images. Z-steps in 3D image stacks range from 0.8-1.4 microns.  
588 Fluorescent *in situ* hybridization samples were imaged with an UPLSAPO-S 30x/1.05 NA and  
589 silicone immersion media. Spectral images were collected for *Tg(gata3:zipACR-YFP;*  
590 *gata3:loxP-DsRed-loxP:GFP)* animals to distinguish between expression patterns of overlapping  
591 fluorophores. Samples were excited with a 515 nm laser. Emission was collected with a PMT  
592 detector from 10 nm wide spectral windows across the emission range 525-625 nm for each z-  
593 plane. Spectral deconvolution was performed with Olympus Fluoview software.

594

595 *Electrophysiology*

596 Whole-cell patch-clamp recordings were targeted to V2bs or motor neurons in *Tg(gata3:gal4;*  
597 *UAS:CatCh*), *Tg(gata3:zipACR-YFP)*, doubly-transgenic *Tg(gata3:LoxP-dsRed-LoxP:GFP;*  
598 *gad1b:GFP*) or *Tg(gata3:zipACR-YFP; gata3:loxP-DsRed-loxP:GFP)* larvae at 4-6 dpf. Larvae  
599 were immobilized with 0.1%  $\alpha$ -bungarotoxin and fixed to a Sylgard lined petri dish with custom-  
600 sharpened tungsten pins. One muscle segment overlaying the spinal cord was removed at the  
601 mid-body level (segments 9-13). The larva was then transferred to a microscope (Scientifica  
602 SliceScope Pro or Nikon Eclipse) equipped with infrared differential interference contrast optics,  
603 epifluorescence, and immersion objectives (Olympus: 40X, 0.8 NA; Nikon: 60X, 1.0 NA). The  
604 bath solution consisted of (in mM): 134 NaCl, 2.9 KCl, 1.2 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, 2.1  
605 CaCl<sub>2</sub>. Osmolarity was adjusted to ~295 mOsm and pH to 7.5.

606

607 Patch pipettes (5-15 M $\Omega$ ) were filled with internal solution for current clamp composed of (in  
608 mM): 125 K gluconate, 2 MgCl<sub>2</sub>, 4 KCl, 10 HEPES, 10 EGTA, 4 Na<sub>2</sub>ATP, 0.05-0.1 Alexa Fluor  
609 647 hydrazide; for voltage clamp, 122 cesium methanesulfonate, 1 tetraethylammonium-Cl, 3  
610 MgCl<sub>2</sub>, 1 QX-314 Cl, 10 HEPES, 10 EGTA, 4 Na<sub>2</sub>ATP and 0.05 - 0.1 Alexa Fluor 568 or 647  
611 hydrazide. Osmolarity was adjusted to ~285 mOsm and KOH or CsOH, respectively was used to  
612 bring the pH to 7.5. Recordings were made in whole-cell configuration using a Multiclamp  
613 700B, filtered at 10 kHz (current clamp) or 2 kHz (voltage clamp) and digitized at 20 kHz with a  
614 Digidata 1440 or 1550 (Axon Instruments) and acquired with WinWCP (J. Dempster, University  
615 of Strathclyde).

616

617 The *Tg(gata3:gal4; UAS:CatCh)* line labels both V2b and Kolmer-Agduhr / cerebrospinal fluid-  
618 contacting neurons (CSF-cNs). To ensure that evoked IPSCs derived from presynaptic V2bs  
619 rather than CSF-cNs, epifluorescent illumination was targeted 3-7 segments rostral to the  
620 recorded segment. A Polygon400 Digital Micromirror Device (Mightex) was used to provide  
621 patterned illumination in indicated recordings. Previous studies found that CSF-cNs have short  
622 ascending axons and do not contact any motor neurons besides the caudal primary (CaP) [38].  
623 CatCh expression in the *Tg(gata3:Gal4; UAS:CatCh)* line is variegated, with some animals  
624 showing strong CatCh expression throughout both CSF-cNs and V2bs, and others showing good  
625 expression in CSF-cNs and minimal expression in V2b cells. Additional control experiments  
626 were performed in animals with minimal V2b label to demonstrate the absence of contribution of  
627 CSF-cN synapses in these experiments.

628

629 Motor neurons were identified by axon fill that extended into the musculature and/or by  
630 retrograde dye labeling from the muscle. For retrograde labeling, 4 dpf larvae were anesthetized  
631 (0.02% MS-222) and laid flat on an agarose plate. A Narishige micromanipulator in conjunction  
632 with a microinjection pump (WPI, Pneumatic Picopump) was used to deliver small volumes of  
633 dye (Alexa Fluor 568 dextran, 3000 MW) via glass pipette into the muscle. Fish recovered in  
634 regular system water and were subsequently used for recordings at 5-6 dpf.

635

636 Data were imported into Igor Pro using NeuroMatic [74]. Spike threshold was defined as 10 V/s,  
637 and custom code was written to determine spike width and afterhyperpolarization of the initial  
638 spike elicited by pulse steps. Input resistance was calculated by an average of small

639 hyperpolarizing pulses. To isolate IPSCs, 10  $\mu$ m NBQX was present in the bath and neurons  
640 were voltage clamped at the EPSC reversal potential.

641  
642 Motor neurons at the dorsal extent of the distribution ( $> 50\%$  of distance from bottom of spinal  
643 cord to top) exhibited lower input resistances (mean  $\pm$  SD:  $287 \pm 75$  M $\Omega$ ) and were considered  
644 “fast” and the remainder, which exhibited higher input resistances ( $885 \pm 367$  M $\Omega$ ) considered  
645 “slow” [21]. These groups mostly correspond to primary and secondary motor neurons, but some  
646 dorsally located bifurcating secondaries may be included in the fast group [22].

647  
648 Optogenetic validation of ZipACR in V2b and CSF-cN was performed on *Tg(gata3:zipACR-YFP)* and *Tg(gata3:zipACR-YFP; gata3:loxP-DsRed-loxP:GFP)* animals. Light stimulation was  
649 provided with high intensity illumination, 5-10% intensity with a 40X (0.8 NA) water-immersion  
650 objective, and low intensity illumination which is identical to the conditions of behavioral  
651 recordings, 100% intensity with a 4X (0.1 NA) air objective.

653  
654 *Image analysis*  
655 Image analysis was performed with ImageJ (FIJI) [75]. Igor Pro 6 was utilized for data analysis  
656 and statistics unless otherwise noted. V2b cell counts and neurotransmitter coexpression was  
657 quantified manually by two researchers (R.C. and M.J.); no significant differences in  
658 quantification were detected. Gata3+ V2b cells were identified and marked (ImageJ Cell  
659 Counter) relative to spinal cord and segment boundaries, giving total V2b/segment quantities.  
660 Subsequently, each cell was evaluated for expression of fluorescent proteins marking gad1b or  
661 glyt2.

662

663 Transgenic line validation was performed with *in situ* hybridization and quantified by two  
664 researchers (M.B. and R.C.) with no significant discrepancy in results. A ~3-5  $\mu$ m z-stack  
665 projection was made in a cell-dense area of spinal cord spanning two to three segments for each  
666 animal. ROIs of neurons were drawn in one channel before checking whether there was  
667 colocalization in the other channel. Samples were quantified twice: once for completeness  
668 (percentage of endogenous RNA positive neurons also expressing the transgene) and once for  
669 accuracy (percentage of transgene labeled neurons positive for endogenous RNA). 3-7 animals  
670 were evaluated in each line.

671

672 For axon tracing, stitched projection images were made with the Pairwise stitching [76] ImageJ  
673 plugin. The overlap of the fused image was smoothed with linear blending and was registered  
674 based on the fill channel or the average of all channels. Photoconversion cell fill images  
675 underwent an extra processing step in which the bleached green channel was subtracted from the  
676 photoconverted red channel. The Simple Neurite Tracer plugin [77] was used to trace the axon  
677 projection and branching relative to marked spinal cord boundaries. Axon lengths are reported as  
678 the number of segments transversed.

679

680 Motor neuron dendrites were quantified from confocal z-stack images of *Tg(mnx:GFP)* 5 dpf  
681 animals. Images were cropped to a single hemisegment. The Weka Trainable Segmentation  
682 plugin [78] was used to segment the motor neuron image into three classifiers; soma, axons  
683 exiting the spinal cord, and dendrites. Classification was based on Hessian training features.  
684 Training was performed iteratively for each image. The binary segmented images were applied

685 to mask all non-dendrite fluorescence (n = 4 hemisegments/animal; n = 3 animals.) Fluorescence  
686 was maximum intensity projected in the z-dimension, collapsed along the horizontal plane and  
687 normalized to give an estimate of motor neuron dendrite density in the dorsoventral plane of the  
688 spinal cord.

689

690 *Optogenetic stimulation and behavior*

691 5 dpf *Tg(gata3:zipACR-YFP)* animals and clutchmates were anesthetized in 0.02% MS-222 and  
692 placed on an agar plate under a dissecting microscope. A complete spinal cord transection was  
693 made with Vannas spring microscissors, plus a sharpened pin if necessary, between spinal cord  
694 segments 2 and 5. Tail blood flow was monitored post-transection and throughout the  
695 preparation; animals with significantly reduced blood flow were euthanized and not used for  
696 recordings. After transection the animal briefly recovered in extracellular solution and then was  
697 embedded in a dorsal up position in 1.2% low melting point agarose. Solidified agarose  
698 surrounding the tail caudal to the transection was removed with a dissection scalpel. 200  $\mu$ M  
699 NMDA (Sigma Aldrich) in extracellular solution was added to the dish. Recordings were  
700 initiated after tail movement began, typically 2-10 min later.

701

702 Behavior experiments were performed with a Scientifica SliceScope upright microscope  
703 equipped with a Fastec HiSpec1 camera and an Olympus Plan N 4x/0.10 objective. Image  
704 collection was made with Fastec acquisition software. Images were acquired at 200 Hz for 5  
705 seconds. Optical stimulation was made with 100% intensity full field epi-illumination from a  
706 CoolLED pe300ultra source routed through a GFP filter cube (Chroma 49002). Recordings with

707 optical stimulation were alternated with recordings without stimulation; n = 6 - 17 recordings for  
708 each animal.

709  
710 Analysis was run in MATLAB R2017a with custom code adapted from Severi et al. [45]. The  
711 caudal edge of the transection and the tail periphery were manually selected as tail boundaries  
712 and 10 points for tracking were evenly distributed along the body. The caudal-most tail point was  
713 used to calculate tail speed (mm/s) at each frame of the recordings. A tail speed threshold of 0.5  
714 mm/s was used to distinguish true movement from tail drift. Tail movement amplitude was  
715 calculated as the maximum tail displacement in the initial second of each recording. Tail beat  
716 frequency was computed from left-to-right tail oscillations during manually identified movement  
717 bouts; 6-30 consecutive peaks were averaged for each recording.

718  
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732

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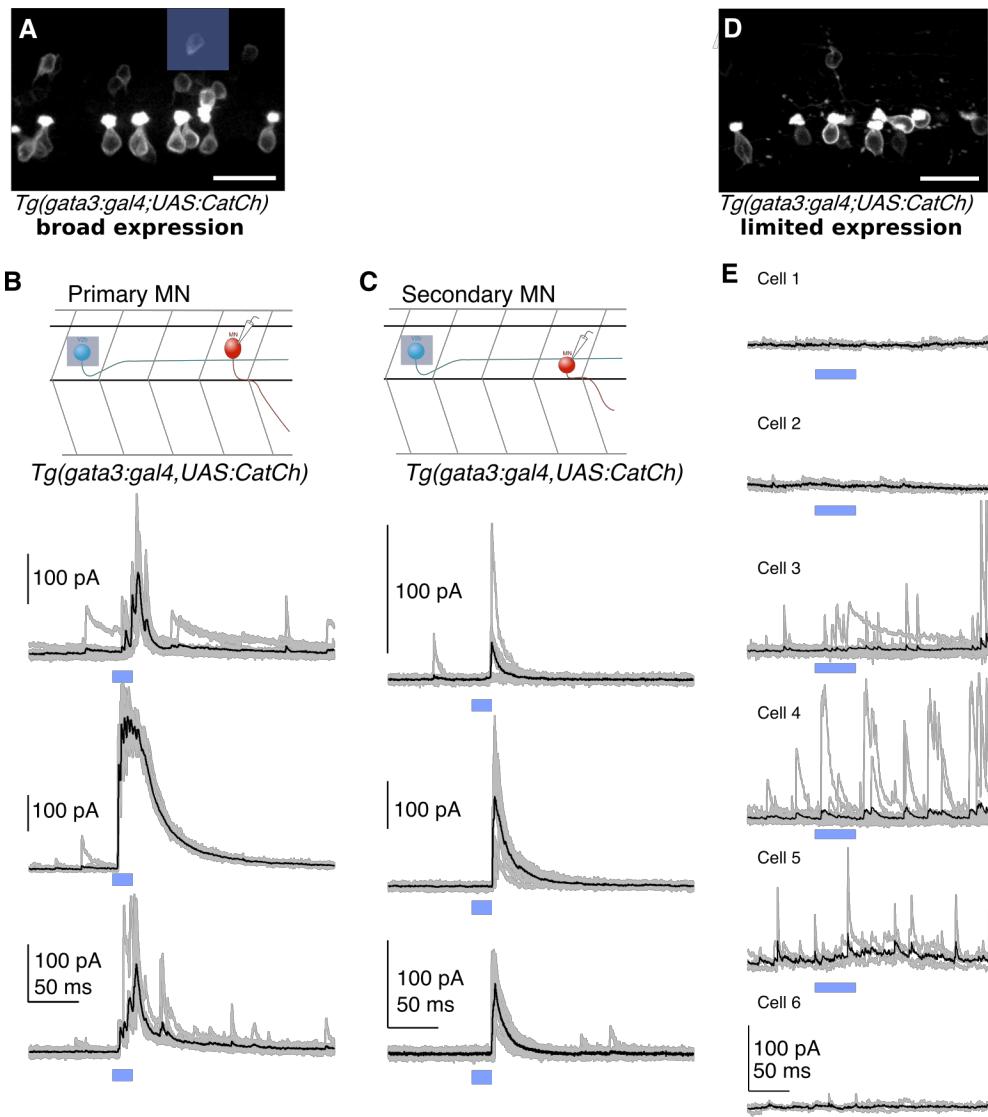
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927 Supplementary Figures

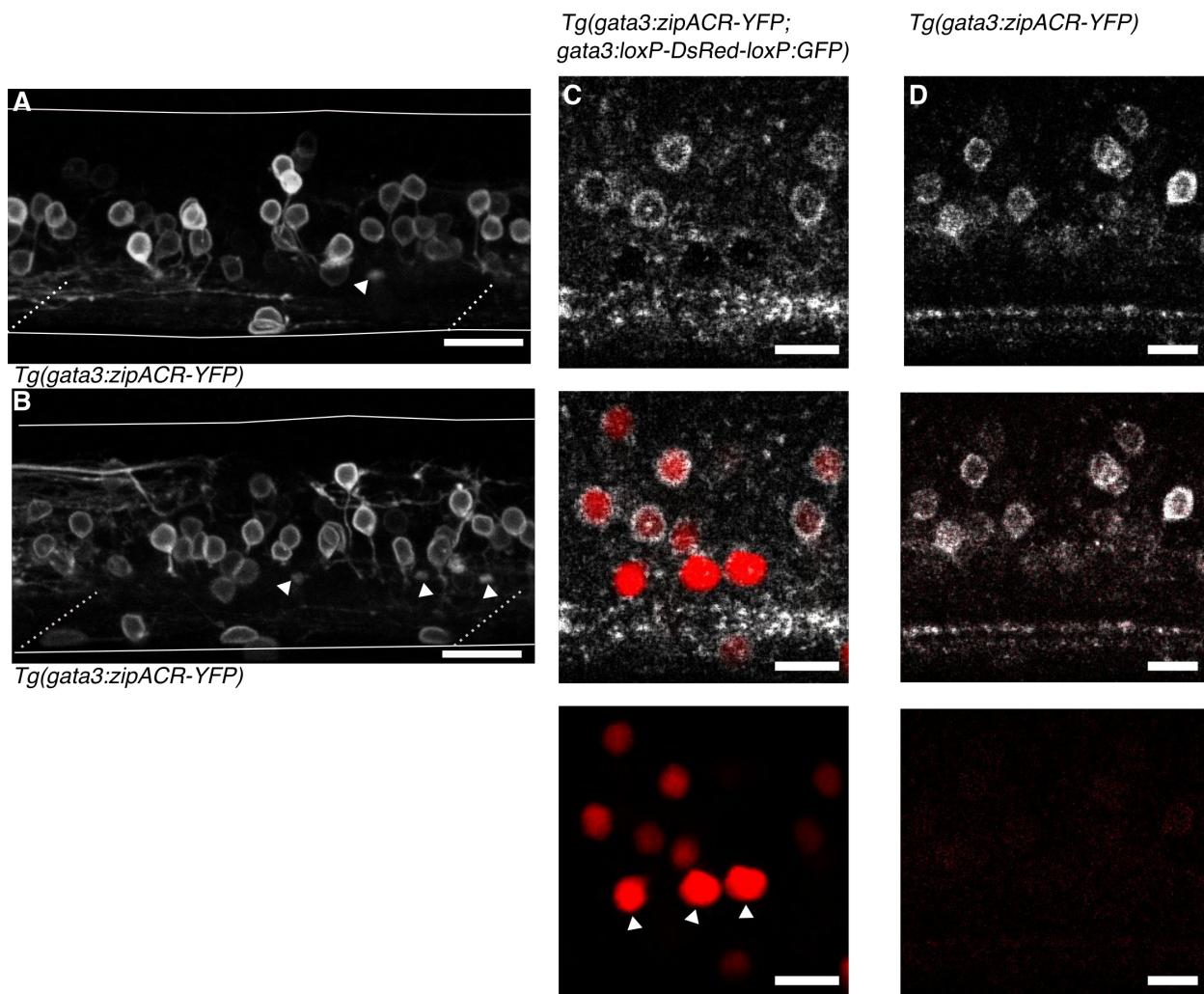


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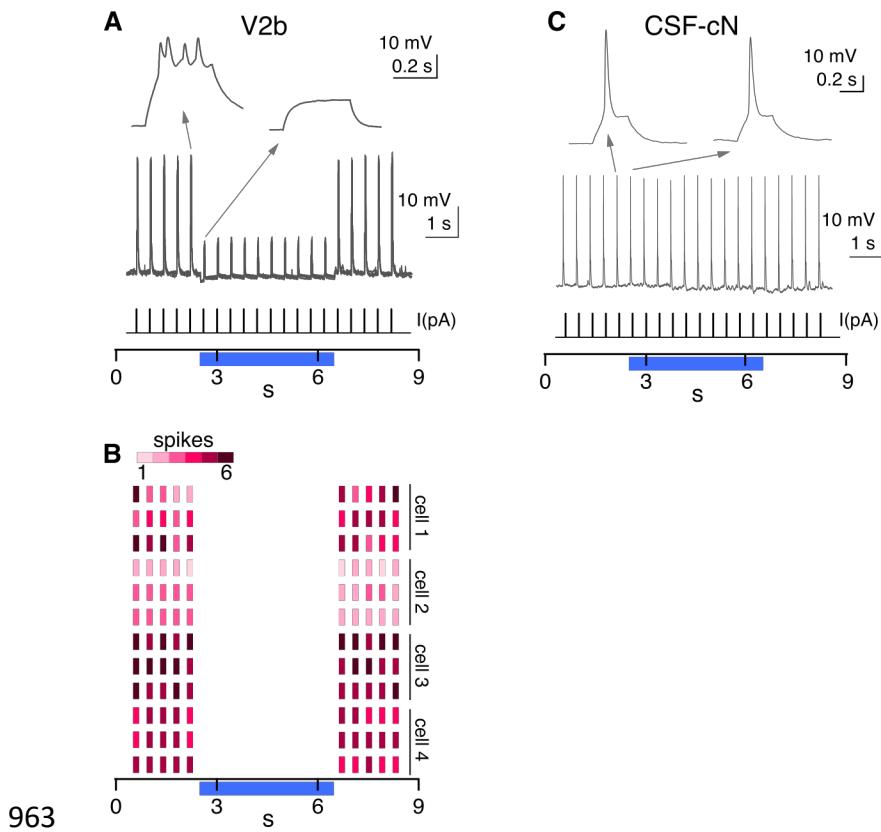
929 Figure S1. Optogenetically evoked IPSCs originate from V2b neurons, not CSF-cNs.  
930 (A) Confocal projection of a hemisegment of spinal cord in a *Tg(gata3:Gal4; UAS:CatCh)* animal with  
931 broad CatCh expression. A blue square in the right panel shows an example 20  $\mu$ m x 20  $\mu$ m DMD  
932 stimulation pattern in which 1-2 V2b cells are targeted. Scale bar = 20  $\mu$ m.  
933 (B) Schematic and whole cell recordings from primary motor neurons in animals with broad CatCh  
934 expression, such as in (A). Shorter stimulation times (20 ms) and targeted illumination, as schematized in  
935 (A), reliably elicit IPSCs in primary motor neurons.  
936 (C) Schematic and recordings from secondary (slow) motor neurons similar to (D). Light stimulation  
937 reliably evoked current responses in secondary motor neurons.  
938 (D) Confocal image showing limited CatCh expression in a hemisegment of spinal cord in  
939 *Tg(gata3:Gal4; UAS:CatCh)* animals. CatCh is widely expressed in CSF-cN in both animals (A and D),  
940 but from animal to animal, there were variations in the intensity of CatCh expression in V2b neurons.  
941 Scale bar = 20  $\mu$ m.

942 (E) Whole cell recordings of primary motor neurons in animals with low V2b CatCh expression.  
943 Individual traces are shown in grey and averages in black. Blue bar represents the optical stimulation  
944 timing. Longer stimulation times (50 ms) and full field illumination were used to maximize IPSC  
945 responses in the recorded cell. Motor neurons receive few light-triggered currents in animals with low  
946 CatCh expression in V2b neurons.  
947 Together this demonstrates that synaptic currents in Figs. 5 and 6 originate from V2b and not CSF-cN  
948 neurons.  
949

950



952 Figure S2. Anatomy of *Tg(gata3:zipACR-YFP)* expression.  
953 (A) and (B) depict additional images of *Tg(gata3:zipACR-YFP)* expression in the full mediolateral extent  
954 of the spinal cord in one segment, white triangles mark putative CSF-cN appical extensions into the  
955 central canal, notably CSF-cN soma are not labeled with YFP. Scale bars = 20  $\mu$ m.  
956 (C) Spectrally deconvolved images of *Tg(gata3:zipACR-YFP; gata3:loxP-DsRed-loxP:GFP)*, see  
957 Methods. White (top) shows YFP emission and red (bottom) shows DsRed emission. Dorsal CSF-cN are  
958 noted with white triangles. CSF-cN somata are distinctly labeled with DsRed (BAC generated line) but  
959 not YFP (CRISPR generated line). Scale bar = 10  $\mu$ m.  
960 (D) Example spectral deconvolution of *Tg(gata3:zipACR-YFP)* showing negligible DsRed emission in  
961 control sample. Scale bar = 10  $\mu$ m.  
962



964 Figure S3. Additional recordings in *Tg(gata3:zipACR-YFP)* animals.  
965 (A) A whole cell recording during repeated current steps (20 ms duration) is shown for an example V2b  
966 neuron in a *Tg(gata3:zipACR-YFP)* animal, high intensity light stimulation is provided and indicated by  
967 the blue bar. An expanded view of current steps before and during optical stimulation are shown above  
968 with arrows.  
969 (B) Raster plot of action potentials for 3 trials of 4 V2b cells demonstrates the robust suppression of  
970 spiking in V2b with high intensity light. Color value represents number of spikes elicited during each  
971 current step.  
972 (C) A whole cell recording is shown for an example CSF-cN neuron in a *Tg(gata3:zipACR-YFP)* animal,  
973 low intensity light stimulation, similar to Fig. 7, is provided and indicated by the blue bar. An expanded  
974 view of current steps before and during optical stimulation are shown above with arrows.