

1 **Spinal cholinergic interneurons differentially control motoneuron excitability and alter**
2 **the locomotor network operational range**

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22 **Summary**

23

24 While cholinergic neuromodulation is important for locomotor circuit operation, the specific
25 neuronal mechanisms that acetylcholine employs to regulate and fine-tune the speed of
26 locomotion are largely unknown. Here, we show that cholinergic interneurons are present in
27 the zebrafish spinal cord and differentially control the excitability of distinct classes of
28 motoneurons (slow, intermediate and fast) in a muscarinic dependent manner. Moreover, we
29 reveal that m2-type muscarinic acetylcholine receptors (mAChRs) are present in fast and
30 intermediate motoneurons, but not in the slow motoneurons, and that their activation decreases
31 neuronal firing. We also provide evidence that this configuration of motoneuron muscarinic
32 receptors serves as the main intrinsic plasticity mechanism to alter the operational range of
33 motoneuron modules. These unexpected findings provide new insights into the functional
34 flexibility of motoneurons and how they execute locomotion at different speeds.

35 **Introduction**

36 Neural networks in the spinal cord are responsible for the generation and execution of
37 movements (Goulding, 2009; Grillner, 2003; Grillner and Jessell, 2009; Kiehn, 2006). These
38 spinal cord networks are organized in distinct functional microcircuit modules (Ampatzis et al.,
39 2014; Bagnall and McLean, 2014) with defined operational ranges, whose sequential activation
40 increases the speed of locomotion (Ampatzis et al., 2014). The activity of the spinal neuronal
41 microcircuits is regulated by a range of neuromodulatory systems (Miles and Sillar, 2011) that
42 permit motoneurons to adjust their final motor output. One such prominent neuromodulatory
43 system is the cholinergic system (Miles et al., 2007; Zagoraiou et al., 2009). In rodents,
44 cholinergic neuromodulation increases motoneuron excitability (Miles et al., 2007; Deardorff
45 et al., 2014) in a task-dependent manner (Zagoraiou et al., 2009), which is exclusively mediated
46 by cholinergic V0c interneurons (Zagoraiou et al., 2009). Whether cholinergic interneurons are
47 present in the zebrafish spinal cord, and how they regulate the activity of the distinct
48 motoneuron modules (slow, intermediate and fast) during locomotion is, however, unknown.

49 In mammals, acetylcholine release increases motoneuron excitability (Chevallier et al., 2006;
50 Hornby et al., 2002; Ireland et al., 2012; Miles et al., 2007). Although m2 type metabotropic
51 muscarinic acetylcholine receptor (m2-mAChR) activation is reported to mediate mammalian
52 motoneuron hyperexcitability (Deardorff et al., 2014; Miles et al., 2007; Zagoraiou et al., 2009),
53 numerous studies also demonstrate m2-mAChR-mediated inhibitory actions, in several
54 neuronal populations (Brown, 2010; Felder, 1995; Hosey, 1992), including motoneurons
55 (Kurihara et al., 1993). Moreover, m2-mAChRs are found to be predominantly expressed in
56 large motoneurons (Welton et al., 1999). This suggests that only a subset of motoneurons are
57 sensitive to cholinergic modulation via the m2-mAChRs. In order to resolve the question of
58 whether m2 receptors are present in all motoneuron pools (slow, intermediate and fast), and

59 determine how activation of the m2-mAChRs influences the motoneuron functionality, we used
60 the accessible neuro-muscular configuration of the adult zebrafish (Ampatzis et al., 2013).

61 Using a combination of anatomical, electrophysiological, pharmacological, *ex vivo* and *in vivo*
62 behavioral approaches in adult zebrafish, we uncover a previously unidentified population of
63 spinal cord cholinergic interneurons that is analogous to mammalian V0c interneurons.
64 Furthermore, we provide the first functional evidence that cholinergic modulation acts as a
65 plasticity mechanism to differentially regulate the excitability and operational range of separate
66 motoneuron modules through activation of distinct mAChR subtypes.

67 **Results**

68 **Zebrafish spinal cholinergic system organization**

69 We first examined the distribution pattern of cholinergic terminals (VChT⁺) in the adult
70 zebrafish spinal cord. Multiple cholinergic terminals were detected in the dorsal horn, neuropil
71 and motor column (Figure 1A). Using the accessible neuro-muscular configuration of the adult
72 zebrafish, we dissected distinct functional motoneuron pools (Ampatzis et al., 2013). All
73 retrogradely traced secondary motoneuron types (slow, intermediate and fast) received
74 abundant cholinergic innervation (Figure 1B). To determine whether this innervation differs
75 among functionally different pools of secondary motoneurons, we analyzed the number of
76 cholinergic terminals (VChT⁺) in close proximity to motoneuron cell bodies (see Materials
77 and Methods). Fast motoneurons receive a significantly higher number of putative cholinergic
78 inputs than the intermediate and slow motoneurons (One-way ANOVA, $F_{2,64} = 16.69$, $p <$
79 0.0001 , $n = 67$ neurons; Figure 1C). Furthermore, our analysis showed that these cholinergic
80 synapses on motoneurons do not display the typical morphology of the mammalian “c-boutons”
81 (Zagoraïou et al., 2009; Herron and Miles, 2012; Skup et al., 2012; Deardorff et al., 2014). To
82 investigate the origin of this input, we retrogradely labeled the brain neurons descending to

83 spinal cord (fluorescent Dextran tracer; see Material and Methods) and subsequently
84 immunolabeled cholinergic neurons (Choline Acetyltransferase (ChAT) immunoreactivity; n =
85 8 brains). Our analysis revealed that none of these neurons were cholinergic as shown by the
86 co-localization experiments (Supplementary file 1A-H). Moreover, few retrogradely labeled
87 neurons in the initial part of the spinal cord were found to be cholinergic (ChAT⁺)
88 (Supplementary file 1I).

89 Having established that spinal cord cholinergic innervation is not provided by brain descending
90 neurons, we hypothesized that spinal cord cholinergic interneurons account for the strong
91 cholinergic input to motoneurons. This hypothesis is supported by the morphology of zebrafish
92 motoneurons, which lack axonal collaterals (Ampatzis et al., 2013), that are recurrent axons
93 that branch off from the main axon. In mammals, motoneuron collaterals form synaptic contacts
94 with the Renshaw cells and other motoneurons (Eccles et al., 1954; Cullheim et al., 1977).
95 Renshaw cells have not been reported in the zebrafish spinal circuits, and zebrafish
96 motoneuron-motoneuron communication is mediated through dendro-dendritic electrical gap
97 junctions (Song et al., 2016). We tested our hypothesis using two complementary approaches.
98 First, we employed the *Islet1:GFP* zebrafish line, in which all motoneurons express green
99 fluorescent protein (GFP; Uemura et al., 2005) and labeled both motoneurons and cholinergic
100 interneurons through ChAT immunoreactivity (Figure 1D). We identified a significant fraction
101 ($38.17 \pm 2.523\%$, Figure 1E), of small to medium size non-motoneuron cholinergic neurons
102 (ChAT⁺Islet1⁻, $40.13 \pm 1.038 \mu\text{m}^2$; Figure 1F), which were distributed throughout the motor
103 column (Figure 1G). In comparison, identified motoneurons were larger (ChAT⁺Islet1⁺, 69.04
104 $\pm 4.729 \mu\text{m}^2$; Figure 1F). To further confirm that all the ChAT⁺Islet1⁻ are indeed interneurons
105 and not motoneurons which had lost the GFP expression in the *Islet1* line, we injected a
106 retrograde tracer into zebrafish spinal cord ventral roots (see Material and Methods; Figure 1H)
107 and combined it with ChAT immunoreactivity (Figure 1I). Given that all zebrafish motoneuron

108 axons exiting from the same ventral root correspond to the spinal hemisegment, this approach
109 enabled us to reveal all the spinal motoneurons (axial and fin; Figure 1H). We observed that
110 $37.37 \pm 3.75\%$ (Figure 1J) of the cholinergic neurons are not motoneurons, similar to findings
111 in the *Islet1* zebrafish transgenic line.

112 Finally, to assess whether spinal cholinergic interneurons innervate targets located in rostral or
113 caudal spinal cord segments, we identified cholinergic interneurons (into segment 15) as
114 ascending or descending, after injection of a retrograde tracer (dextran) into segments 12 or 18,
115 respectively, in an *Islet1:GFP* line (n=14 zebrafish; Figure 1J,K). 24h after the tracer injection
116 we processed the tissue for ChAT immunostaining. We observed that 7.56% of ChAT⁺Islet1⁻
117 neurons were labelled as descending neurons (Supplementary file 1J, L, M), and 13.22% of
118 ChAT⁺Islet1⁻ neurons were determined as ascending neurons (Supplementary file 1K-M),
119 indicating that 20.78% of the cholinergic interneurons project to other spinal cord segments.
120 The significant difference between the soma size of the ascending and descending cholinergic
121 interneurons suggest that they form two non-overlapping neuronal subpopulations
122 (Supplementary file 1N). Overall, our data suggest that zebrafish spinal motoneurons receive
123 unevenly distributed cholinergic input from a local spinal source, represented by cholinergic
124 interneurons.

125 **V0c interneurons are present in zebrafish spinal cord**

126 Most (~75%) ChAT interneurons (ChAT⁺Islet1⁻) were found in the dorsal part of the motor
127 column close to the central canal (Figure 1G), where the mammalian V0c interneurons are also
128 positioned (Zagoraïou et al., 2009). Previously, it was assumed that V0c interneurons do not
129 exist in zebrafish (Satou et al., 2012). To test if the observed zebrafish spinal cholinergic
130 interneurons correspond to the mammalian V0c interneurons, we combined *in situ*
131 hybridization for *Pitx2*, a paired-like homeodomain transcription factor that marks V0c

132 interneurons in mammals (Figure 2A; Zagoraïou et al. 2009), with ChAT immunolabeling
133 (Figure 2B). Our analysis revealed that 28.36 ± 1.83 % of the ChAT neurons were also *Pitx2*
134 positive (*Pitx2*⁺; Figure 2C). Although the *Pitx2*⁺ neurons were distributed throughout the
135 motor column, ~80% were located around the central canal (Figure 2D), suggesting that an
136 equivalent of mammalian V0c interneurons do exist in zebrafish. Moreover, we performed
137 whole cell patch clamp recordings from several unidentified neurons located around the central
138 canal. We found that 3 neurons out of 20 were cholinergic and not motoneurons, and they
139 displayed intrinsic electrical properties (i.e. prominent after-hyperpolarization, rheobase, lack
140 of neuronal sag) comparable to the mammalian V0c interneurons (Zagoraïou et al. 2009; Figure
141 2E). Although these interneurons do not receive rhythmic synaptic inputs during locomotion,
142 they receive strong synaptic potentials at speeds < 6 Hz (paired t test, $t = 5.133$ $df = 40$, $p <$
143 0.0001 , $n = 3$ neurons; Figure 2F).

144 It was apparent that the cholinergic interneurons are not active during regular swimming (<
145 6Hz), so we next assessed when these cholinergic interneurons operate, contributing to
146 locomotor network functionality. To address this issue, we subjected adult zebrafish to forced
147 fast swimming test (80% of the critical speed (U_{crit}), see Material and Methods) for 2 h,
148 followed by functional anatomical analysis of *c-fos* expression as an index of neuronal activity.
149 Analysis of *c-fos* intensity in all ChAT⁺Islet1⁻ neurons revealed that more neurons are active
150 (54%) during fast swimming, compared to the control, as indicated by a significant increase in
151 *c-fos* expression (unpaired t test, $t = 2.89$ $df = 60$, $p = 0.0054$; Figure 2G-J). Our observations
152 are in agreement with previous analysis performed in the cat spinal cord, which showed that
153 cholinergic neurons close to the central canal were functionally active during prolonged fictive
154 locomotion (Huang et al., 2000). Collectively, our results demonstrate that the vast majority of
155 the zebrafish cholinergic interneurons (80%) are analogues to mammalian V0c interneurons
156 and their activity is related to prolonged fast locomotion.

157 **Muscarinic receptors differentially alter motoneuron excitability**

158 In mammals, acetylcholine increases the excitability of spinal motoneurons through the
159 activation of muscarinic receptors (Miles et al., 2007). To investigate whether zebrafish
160 motoneuron excitability is responsive to activation of mAChRs we obtained whole cell current
161 clamp recordings from different classes (slow, intermediate and fast) of axial secondary
162 motoneurons. Since the fast and intermediate motoneurons discharge single action potentials
163 (APs), whereas slow motoneurons fire in bursts of APs (Gabriel et al., 2011; Ampatzis et al.,
164 2013), the excitability of motoneurons was assessed by examining their response (number of
165 single APs or number of bursts) to steps of supra-threshold depolarizing current pulses (500
166 ms, increments of 10% from rheobase), before and after the application of muscarine, a non-
167 selective mAChR agonist. In the presence of muscarine (15 μ M), the firing rate of both slow
168 (one-way ANOVA repeated measures, $F_{1,319,5.277} = 16$, $p = 0.021$, $n = 5$ out of 5) and intermediate
169 (one-way ANOVA repeated measures, $F_{1,269,6.344} = 24.36$, $p = 0.0079$, $n = 6$ out of 6 neurons)
170 motoneurons substantially increased (Figure 3A-C, F). This was accompanied by an increase
171 of the input resistance (slow MNs: one-way ANOVA repeated measures, $F_{1,059,4.237} = 13.34$, $p =$
172 0.0227 ; intermediate MNs: one-way ANOVA repeated measures, $F_{1,493,7.466} = 12.64$, $p = 0.0258$;
173 Figure 3E) and changes in other intrinsic biophysical properties (Supplementary file 2), without
174 significantly altering the resting membrane potential (Figure 3D). In contrast, the fast
175 motoneurons showed a decrease in excitability, resulting in fewer action potentials (one-way
176 ANOVA repeated measures, $F_{1,986,15.89} = 38.05$, $p < 0.0001$, $n = 9$ out of 9 neurons; Figure
177 3A,B,F) associated with a significant decrease of the input resistance (one-way ANOVA
178 repeated measures, $F_{1,12,8.958} = 4.462$, $p = 0.0141$, Figure 3E). Moreover, alterations of the
179 electrical properties of fast motoneurons were similar to those observed in the intermediate
180 motoneurons (Supplementary file 2). The observed changes in intrinsic biophysical properties
181 of the motoneurons are consistent with previously reported alterations of mammalian

182 motoneurons in response to muscarine (Ireland et al., 2012).

183

184 **Motoneurons possess different muscarinic receptor subtypes**

185 We speculated that the differential motoneuron excitability we observed might arise from the
186 presence of different mAChR subtypes in the motoneurons. To test if m2-mAChRs alone
187 mediate the changes in motoneuron excitability, we applied a mixture of muscarine (15 μ M)
188 and methoctramine (10 μ M), a selective m2-mAChR antagonist (Hulme et al., 1990). We
189 recorded an increase in excitability of fast (paired t test, $t = 3.641$, $p = 0.0219$, $n = 5$ out of 5
190 neurons; Figure 4A-C), intermediate (paired t test, $t = 4.323$, $p = 0.0228$, $n = 4$ out of 4 neurons)
191 and slow (paired t test, $t = 4.00$, $p = 0.0161$, $n = 3$ out of 3 neurons) motoneurons (Figure 4A-
192 C). To assess whether m2-mAChRs are present in all classes of motoneurons, we applied
193 oxotremorine-M (Oxo-M; 20 μ M), an m2-mAChR preferential agonist (Murakami et al., 1996).
194 In response, the fast (paired t test, $t = 10.00$, $p = 0.0005$, $n = 5$ out of 5 neurons) and intermediate
195 (paired t test, $t = 6.668$, $p = 0.0026$, $n = 5$ out of 5 neurons) motoneurons exhibited reduced
196 firing (Figure 4D-F), whereas, slow motoneuron firing was unaffected (paired t test, $t = 1.00$, p
197 $= 0.391$, $n = 4$ out of 4 neurons; Figure 4D-F), suggesting that they do not express the m2-
198 mAChR subtype. Moreover, we confirmed the presence of m2-mAChRs in motoneurons
199 anatomically. Immunoreactivity for the m2-mAChRs was strong in fast motoneurons and
200 modest in intermediate motoneurons (One-way ANOVA, $F_{2,35} = 51.32$, $p < 0.0001$, $n = 38$
201 neurons; Figure 4G, H). In contrast, slow motoneurons were weakly labeled for the m2-
202 mAChR, supporting previous reports (Welton et al., 1999; Figure 4G, H). These results indicate
203 that multiple mAChR subtypes are expressed in a motoneuron type-dependent manner, which
204 functions to differentially mediate the actions of acetylcholine.

205

206 **Activation of motoneuron muscarinic receptors controls fast locomotion**

207 Given that motoneuron excitability can retrogradely influence the upstream locomotor network
208 function (presynaptic release, generation of action potentials, swimming duration and
209 frequency) through gap junctions (Song et al., 2016), we tested the functional significance of
210 motoneuron mAChR activation during locomotion. For this, we used the adult zebrafish *ex vivo*
211 preparation (Ampatzis et al., 2013; Ampatzis et al., 2014; Gabriel et al., 2011; Song et al.,
212 2016). Bath application of muscarine facilitated fictive locomotor activity and increased the
213 highest reached swimming frequency by $26.8 \pm 5.8 \%$ (paired t test, $t = 6.675$, $p < 0.0001$, $n =$
214 12 ; Figure 5A-B). It should be noted that the amplitude of the swimming membrane potential
215 oscillations during locomotion is related to swimming frequency (Gabriel et al., 2011). In the
216 presence of muscarine, the correlation between oscillation amplitude and frequency was
217 decreased in the fast motoneurons (Figure 5C) and enhanced in the intermediate motoneurons
218 that were recruited above 7 Hz (Figure 5D). Furthermore, we investigated the number of APs
219 in the slow and intermediate motoneurons during ongoing fictive locomotion at 5 Hz (Figure
220 5E). At this swimming speed, we found that only the intermediate motoneurons discharge more
221 action potentials in the presence of muscarine, compared to controls (paired t test, $t = 3.43$, $p =$
222 0.0018 ; Figure 5E). Overall, our findings demonstrate that activation of spinal muscarinic
223 receptors facilitates the recruitment of the intermediate motoneuron-interneuron module while
224 hindering the recruitment of the fast locomotor module, as seen through the motoneuron
225 activation.

226 Finally, we sought to better understand the *in vivo* behavioral functions of the activation of
227 mAChRs. Therefore, we subjected zebrafish to a critical speed test (Supplementary file 3; see
228 Supplemental Experimental Procedures). Critical speed (U_{crit}) is a measure of the highest
229 sustainable swimming speed that a fish can reach (Brett, 1964). Intraperitoneal administration
230 of muscarine ($50 \mu\text{M}$, 525 ng/g BW ; Supplementary file 3A) significantly reduced the highest
231 sustainable swimming speed (unpaired t test, $t = 2.65$, $p = 0.014$, $n = 23$ zebrafish; Figure 5F).

232 In contrast, methoctramine treated animals (40 μ M, 2.2mg/g BW; Supplementary file 3)
233 increased the uppermost locomotor speed (unpaired t test, $t = 2.12$, $p = 0.047$, $n = 20$ zebrafish;
234 Figure 5G). Similarly, exogenous activation of all muscarinic receptors except the m2-
235 mAChRs, achieved by co-administration of muscarine (50 μ M) and methoctramine (40 μ M),
236 increased the maximum locomotor speed obtained (unpaired t test, $t = 2.156$, $p = 0.046$, $n = 18$
237 zebrafish; Figure 5H).

238 To test the effect of our pharmacological treatments on locomotion under normal conditions,
239 animals were subjected to an open field test (Supplementary file 3, see Material and Methods).
240 We observed that similar treatment did not affect the regular locomotor behavior (distance
241 traveled, average velocity and maximum velocity; Figure 5I-K), suggesting that the differences
242 in critical speed observed here are due to the effect on the spinal locomotor circuit. These data
243 show that stimulation of m2-mAChRs causes a reduction in the maximum swimming speed to
244 $76.5 \pm 6.7 \%$ of the of the U_{crit} , corresponding to the zebrafish optimum speed (Palstra et al.,
245 2010), where swimming relies only on the activity of the slow and intermediate neuro-muscular
246 system, and not on the fast. Our analysis cannot rule out the possibility of an additional direct
247 effect of mAChR activation in the premotor neurons, however, as any alteration in motoneuron
248 excitability will retrogradely affect the excitability and pre-synaptic release of glutamate from
249 V2a interneurons (Song et al., 2016), which will influence premotor network functionality.
250 Taken together, our results suggest that cholinergic modulation acts both on motoneurons and
251 the premotor network to modify the speed of locomotion mainly through the engagement of the
252 fast motoneuron-interneuron module.

253 **Discussion**

254 Our findings suggest that motoneurons receive cholinergic input exclusively from spinal
255 interneurons, with the vast majority of them being analogous to mammalian V0c interneurons.

256 Acetylcholine shifts motoneuron excitability through the parallel activation of different
257 mAChRs subtypes (Figure 6A). Moreover, activation of this pathway selectively alters the
258 operational range of different motoneuron modules during locomotion (Figure 6B). Overall,
259 this work provides novel insights into how intraspinal acetylcholine release can modify the
260 functionality of the spinal circuitry, which requires synaptic specificity and temporal precision
261 to generate locomotion at different speeds (Ampatzis et al., 2013; Ampatzis et al., 2014).

262
263 One of our major findings is establishing the existence of cholinergic interneurons in the
264 zebrafish spinal cord and identifying them as the exclusive source of the cholinergic spinal
265 input. Although earlier studies showed the existence of cholinergic interneurons in several
266 vertebrate species (Thiriet et al., 1992; Anadón et al., 2000; González et al., 2002; Miles et al.,
267 2007; Quinlan and Buchanan, 2008; Zagoraiou et al., 2009), and moreover, the zebrafish
268 cholinergic system is well characterized (Clemente et al., 2004; Mueller et al., 2004), the
269 presence of zebrafish spinal cord cholinergic interneurons has not been previously reported.
270 While a number of Chat⁺Islet1⁻ neurons were identified earlier (Stil and Drapeau, 2016), it has
271 been proposed that these small cholinergic neurons correspond to motoneurons, rather than the
272 ones that innervate the axial muscles (Muller et al., 2004). Previous attempts to localize *Pitx2*
273 expression with *Dbx1* in order to reveal the existence of V0c interneurons in zebrafish spinal
274 cord were ineffective (Satou et al., 2012). In the current study we used a different approach, by
275 combining *Pitx2* expression with ChAT immunostaining, to achieve this goal. We revealed that
276 most cholinergic interneurons share comparable anatomical distribution, molecular identity and
277 electrical properties with mammalian V0c interneurons. Our analysis cannot rule out the
278 possibility that more cholinergic interneuron subtypes are present in zebrafish spinal cord.
279 However, most of zebrafish cholinergic interneurons (~80%) express *Pitx2*, thus we considered
280 them to be similar to mammalian V0c interneurons. Since V0c interneurons have been shown

281 to be the exclusive source of cholinergic c-bouton input to mammalian motoneurons (Zagoraïou
282 et al. 2009), our data directly implicate the activity of these cholinergic interneurons in the
283 cholinergic modulation of motoneurons in the zebrafish.

284 The overall activation of the muscarinic receptors has been shown to increase motoneuron
285 excitability (Alaburda et al., 2002; Chevallier et al., 2006; Hornby et al., 2002; Ireland et al.,
286 2012; Miles et al., 2007) via activation of the m2-mAChR subtype (Miles et al., 2007). On the
287 other hand, numerous studies have shown that activity of the m2-mAChRs is associated with
288 inhibitory actions in the nervous system (Brown, 2010; Felder, 1995; Hosey, 1992; Kurihara et
289 al., 1993). Our findings challenge previous conclusions that activation of motoneuron m2-
290 mAChR is primarily required to increase motoneuron excitability. By pharmacologically
291 manipulating the m2-mAChRs in different motoneuron pools, we found that m2-mAChR
292 activation reduces motoneuron excitability and other receptor subtypes can account for the
293 observed rise in motoneuron firing. In line with this idea, various subtypes of mAChRs have
294 been characterized in the spinal cord neurons (Brown, 2010; Höglund and Baghdoyan, 1997;
295 Zhang et al., 2005; Jordan et al., 2014) and it is also well documented that many nerve cells
296 contain more than one subtype of muscarinic receptor (Finkel et al., 2014; Hassall et al. 1993;
297 Jordan et al., 2014). The exact muscarinic receptor subtype responsible for the elevated
298 excitability of adult zebrafish motoneurons remains to be identified. However, it has been
299 suggested that the excitatory effects of muscarinic agonists on neonatal rat motoneurons are
300 mediated through the m3-mAChRs (Kurihara et al., 1993). In support of our observations,
301 Jordan et al. (2014) recently demonstrated that m2-mAChRs and m3-mAChRs are both
302 involved in the cholinergic modulation of locomotion in mammals. They showed that
303 application of the m2-mAChR antagonist methoctramine, increased the locomotor rhythm,
304 whereas application of m3-mAChR antagonist reduced, and finally blocked, the locomotor
305 activity (Jordan et al., 2014). Our results show that different muscarinic receptor subtypes in

306 the membrane of motoneurons can play a unique role in the regulation of neural activity
307 involved in the control of locomotion.

308

309 Given the emerging evidence regarding the importance and functional repertoire of spinal
310 cholinergic input in the control of motor behaviors, it is not surprising that cholinergic synapses
311 have been implicated in spinal cord injury (Ichiyama et al., 2011; Skup et al., 2012; Jordan et
312 al., 2014) and motor disease (Herron and Miles, 2012; Pullen and Athanasiou, 2009; Saxena et
313 al., 2013). However, no causal link has yet been found between alterations in the number and
314 size of cholinergic synapses, acetylcholine release and muscarinic receptor activation.
315 Therefore, uncovering the mechanisms by which acetylcholine modifies the activity of spinal
316 neurons is of significant biological and medical interest. We find that methoctramine treated
317 animals are able to operate at higher swimming speeds and, in addition, activation of m2-
318 mAChRs reduces the excitability of the motoneurons, and potentially of other spinal cord
319 neurons, and their ability to produce the appropriate muscle force. In support of our findings,
320 Jordan et al., (2014) revealed that application of methoctramine increased the locomotor
321 frequency in mammals. Moreover, application of cholinergic receptor antagonists following
322 spinal cord injury was found to advance locomotor activity, suggesting that adaptive alterations
323 of the spinal cholinergic system obstruct the generation and execution of locomotion (Jordan et
324 al., 2014). In a mouse model of amyotrophic lateral sclerosis (ALS), methoctramine treatment
325 counteracts the loss of muscle force during the pre-symptomatic stages of the disease (Saxena
326 et al., 2013), and is likely to be mediated through the blocking of m2-mAChRs, increasing the
327 excitability of spinal neurons participating in the generation of movement. In conclusion, our
328 results provide a novel contribution to existing knowledge, and further understanding of the
329 causal relationship between activation of muscarinic receptors and locomotion, highlighting
330 their potential as targets for innovative therapeutic strategies.

331

332 A general question about the cholinergic control of locomotion is made addressable in our
333 work: how does the muscarinic acetylcholine receptor configuration gate the execution of
334 locomotor behavior at different speeds? It has been proposed that the cholinergic modulation
335 of motoneurons is activity dependent (Zagoraïou et al., 2009). In favor of this idea, our work
336 revealed that pharmacological manipulations of spinal circuit muscarinic receptors *ex vivo* and
337 *in vivo* only affect the locomotor performance of zebrafish at higher swimming speeds, which
338 are primarily associated with the engagement and function of the fast motoneuron-interneuron
339 module. Thus, the mechanisms we identify here suggest an additional level of control during
340 high-energy-demand locomotion, such as fast swimming.

341 **Materials and Methods**

342 **Animals**

343 All animals were raised and kept in a core facility at the Karolinska Institute according to
344 established procedures. Adult zebrafish (*Danio rerio*; 8-10 weeks old; length, 17-19 mm;
345 weight, 0.025-0.045 g) wild type (AB/Tübingen) and Tg(*Islet1:GFP*) lines were used in this
346 study. All experimental protocols were approved by the local Animal Research Ethical
347 Committee, Stockholm and were performed in accordance with EU guidelines.

348

349 **Motoneuron and descending/ascending neuron labeling**

350 Zebrafish (n = 30) of either sex were anaesthetized in 0.03% tricaine methane sulfonate (MS-
351 222, Sigma-Aldrich). Retrograde labeling of axial motoneurons was performed using dye
352 injections with tetramethylrhodamine-dextran (3000 MW; ThermoFisher, D3307) in specific
353 muscle fiber types (slow, intermediate or fast). In addition, retrograde labeling of all
354 motoneurons was performed using similar procedure to spinal cord ventral roots. To label the

355 neurons descending from the brain to the spinal cord the tracer was injected using dye-soaked
356 pins in the spinal cord at approximately the level of the 6-8th vertebra. Finally, for the
357 investigation of descending and ascending cholinergic interneurons the tracer was injected in
358 the spinal segment 18 (n = 7 zebrafish) or 12 (n = 7 zebrafish) respectively. Afterwards all the
359 animals were kept for at least 24h to allow the retrograde transport of the tracer. We evaluated
360 the number of ChAT⁺Istlet1⁻ interneurons that were positive to the tracer on segment 15 of the
361 adult zebrafish spinal cord.

362

363 **Immunohistochemistry**

364 All animals were deeply anesthetized with 0.1% MS-222. We then dissected the spinal cords
365 and/or the brains and fixed them in 4% paraformaldehyde (PFA) in phosphate buffer saline
366 (PBS) (0.01M; pH = 7.4) at 4°C for 2-14h. We performed immunolabelings in both whole
367 mount and cryosections. For cryosections, the tissues were removed carefully and cryoprotected
368 overnight in 30% (w/v) sucrose in PBS at 4°C, embedded in OCT Cryomount (Histolab),
369 rapidly frozen in dry-ice-cooled isopentane (2-methylbutane; Sigma) at approximately -35°C,
370 and stored at -80°C until use. Transverse coronal plane cryosections (thickness 25 µm) of the
371 tissue were collected and processed for immunohistochemistry. The tissue was washed three
372 times for 5 min in PBS. Nonspecific protein binding sites were blocked with 4% normal donkey
373 serum with 1% bovine serum albumin (BSA; Sigma) and 0.5% Triton X-100 (Sigma) in PBS
374 for 30 min at room temperature (RT). Primary antibodies were diluted in 1% of blocking
375 solution and applied for 24-90 hours at 4 °C. For primary antibodies, we used goat anti-ChAT
376 (1:200; Millipore, AB144P, RRID: AB_2079751), rabbit anti-GFP (1:700; ThermoFisher
377 Scientific, A-11122, RRID: AB_221569), guinea pig anti-VAcHT (1:1000; Millipore, AB1588,
378 RRID: AB_11214110), rabbit anti-cfos (1:200; Sigma, F7799) and rabbit anti-m2 mAChRs
379 (1:700-1:2000; Alomone, AMR-002, RRID: AB_2039995). After thorough buffer rinses the

380 tissues were then incubated with the appropriate secondary antibodies diluted 1:500 in 0.5%
381 Triton X-100 (Sigma) in PBS overnight at 4 °C. We used Alexa Fluor–conjugated secondary
382 antibodies anti-goat 488 (ThermoFisher Scientific, A11055, RRID: AB_142672), anti-goat 568
383 (ThermoFisher Scientific, A11057, RRID: AB_142581), anti-rabbit 488 (ThermoFisher
384 Scientific, A21206, RRID: AB_141708) and anti-guinea pig 488 (ThermoFisher Scientific,
385 A11073, RRID: AB_142018). Finally, the tissues were thoroughly rinsed in PBS and cover-
386 slipped with fluorescent hard medium (VectorLabs; H-1400).

387

388 **In situ hybridization**

389 Adult zebrafish wild type (AB/Tübingen) spinal cords were fixed, cryoprotected and
390 cryosectioned (thickness of 12 µm) as described before (see *Immunohistochemistry* section).
391 Coronal transverse section then processed for fluorescent *in situ* hybridization using the
392 RNAscope Technology (Advance Cell Diagnostics), according to manufacturer’s instructions.
393 Target probe for zebrafish specific *Pitx2* gene (Advance Cell Diagnostics, 432161-C3) was
394 used. After the detection of *Pitx2* mRNA, tissues were processed for ChAT immunodetection
395 as described before (*Immunohistochemistry section*). To investigate how many of cholinergic
396 neurons are *Pitx2*, 3 sections per animal (n = 4 zebrafish) corresponding to 12-14th spinal cord
397 segments were analyzed.

398

399 ***c-fos* functional anatomy**

400 Adult zebrafish (*Islet1:GFP*; n = 8) were used for *c-fos* experiments. Animals were subjected
401 to forced swim test. We used two different experimental protocols to investigate the activity of
402 cholinergic interneurons. Animals were subjected to prolonged swimming (2 h) at 80% of the
403 critical speed (U_{crit}). Control animals were kept under regular tank swimming conditions.
404 Immediately after the test all animals were fixed and processed for triple immunolabeling,

405 against *c-fos*, the ChAT and the GFP (see Supplemental Experimental Procedures, section
406 Immunohistochemistry) in 25 μm thick cryosections. For the *c-fos* experiments, analysis was
407 performed in neurons (n=62) from sections corresponding to segments 14-17. The intensity of
408 the *c-fos* activity was evaluated in the obtained confocal pictures using ImageJ.

409 **Microscopy and image analysis**

410 Imaging was carried out in a laser scanning confocal microscope (LSM 510 Meta, Zeiss).
411 Cholinergic inputs on different motoneuron types were counted on single plan confocal images.
412 Counting was performed in non-overlapping fields of spinal cord sections, in spinal cord
413 segments 14-17. We defined the putative cholinergic inputs as number of large ($>0.5 \mu\text{m}$ of
414 diameter) VAcHT-positive putatively cholinergic synapses, apposing the somata of
415 motoneurons. The evaluation included 10 slow, 27 intermediate and 30 fast positive
416 motoneurons collected from 4 adult zebrafish spinal cords. For the quantification of m2-
417 mAChRs, all images were captured at identical exposure times in order to ensure the same
418 illumination level. The intensity of m2-mAChRs immunoreactivity was evaluated in the
419 obtained confocal pictures using the ImageJ image analysis software. The relative position of
420 the somata of the neurons within spinal cord, was calculated in whole mount preparations, using
421 the lateral, dorsal, and ventral edges of spinal cord as well as the central canal as landmarks.
422 Analysis of all spinal cord neurons was performed between segments 14-17. The relative
423 position was calculated using ImageJ. Examination of the descending neurons was performed
424 from a series of coronal brain section, throughout the brain, without discarding any section from
425 the analysis. The nomenclature used for the brain areas of descending neurons was based on
426 the topological zebrafish brain atlas (Wullimann et al., 1996). All figures and graphs were
427 prepared with Adobe Photoshop and Adobe Illustrator (Adobe Systems Inc., San Jose, CA). All
428 double-labeled images were converted to magenta-green immunofluorescence to make this work
429 more accessible to the red-green color-blind readers.

430 ***Ex vivo* preparation and electrophysiology**

431 The dissection procedure has been described previously (Gabriel et al., 2011; Ampatzis et al.,
432 2013; Ampatzis et al., 2014; Song et al., 2016). The preparations (n = 47) were then transferred
433 to a recording chamber, placed lateral side up, and fixed with Vaseline. The chamber was
434 continuously perfused with extracellular solution contained: 134 mM NaCl, 2.9 mM KCl, 2.1
435 mM CaCl₂, 1.2 mM MgCl₂, 10 mM HEPES, and 10 mM glucose, pH 7.8, adjusted with NaOH,
436 and an osmolarity of 290 mOsm. All experiments were performed at an ambient temperature
437 of 20–22°C. For whole-cell intracellular recordings, electrodes (resistance, 9–13 MΩ) were
438 pulled from borosilicate glass (outer diameter, 1.5 mm; inner diameter, 0.87 mm; Hilgenberg)
439 on a vertical puller (PC-10 model, Narishige) and filled with intracellular solution containing
440 the following: 120 mM K-gluconate, 5 mM KCl, 10 mM HEPES, 4 mM Mg₂ATP, 0.3 mM
441 Na₄GTP, 10 mM Na-phosphocreatine, pH 7.4, adjusted with KOH, and osmolarity of 275
442 mOsm. Dextran-labeled MNs were visualized using a fluorescence microscope (Axioskop FS
443 Plus, Zeiss) equipped with IR-differential interference contrast optics and a CCD camera with
444 frame grabber (Hamamatsu) and were then targeted specifically. Intracellular patch-clamp
445 electrodes were advanced in the exposed portion of the spinal cord through the meninges using
446 a motorized micromanipulator (Luigs & Neumann) while applying constant positive pressure.
447 Intracellular signals were amplified with a MultiClamp 700B intracellular amplifier (Molecular
448 Devices) and low-pass filtered at 10 kHz. In current-clamp recordings, no bias current was
449 injected. Only motoneurons that had stable membrane potentials at or below –48 mV fired
450 action potentials to suprathreshold depolarizations and showed minimal changes in series
451 resistance (<5%) were included in this study. In some experiments neurons were passively filled
452 with 0.25% neurobiotin tracer (Vector Labs) for post hoc analysis of their neurochemical
453 identity. Spinal cords with neurobiotin-filled neurons were dissected out and transferred in 4%
454 PFA solution at 4 °C for 3 h. The tissue was then washed extensively with PBS, and incubated

455 in streptavidin conjugated to Alexa Fluor 555 (1:500; Invitrogen, SP-1120) and were used for
456 detection of ChAT immunoreactivity following the protocol described above. The following
457 drugs were added to the physiological solution: non-selective muscarinic receptor agonist
458 muscarine (15 μ M; Sigma, M104), m2-type selective muscarinic receptor antagonist
459 methoctramine (10 μ M; Sigma, M105) and m2-type preferential muscarinic receptor agonist
460 oxotremorine-M (20 μ M; Sigma, O100). All drugs were dissolved as stock solutions in distilled
461 water. For all the electrophysiological recordings data analysis was performed using Spike2
462 (version 7, Cambridge Electronic Design) or Clampfit (Molecular Devices) software. The
463 action potential voltage threshold of motoneurons was determined from the measured
464 membrane potential at which the dV/dt exceeded 10 mV/msec.

465

466 ***In vivo* swimming behavior**

467 The swimming ability of zebrafish was tested using the open field test and the critical speed
468 (U_{crit}) test. U_{crit} is a measure of the highest sustainable swimming speed achievable by a fish.
469 All zebrafish ($n = 80$) selected for the test displayed similar body length sizes and body weights.
470 Animals were first anaesthetized in 0.03% tricaine methane sulfonate (MS-222, Sigma-Aldrich)
471 in fish water and injected intraperitoneally (volume: 2 μ l) with saline, muscarine (50 μ M; 525
472 ng / g body weight) or/and methoctramine (40 μ M; 2230 ng / g body weight; Figure S3). Treated
473 animals were placed in the swim tunnel (5 L; Loligo systems, Denmark) to recover and
474 acclimated at a low water flow speed (4.5 cm/sec) for 7 min. After, fish were given the U_{crit} test,
475 subjecting the animals to time intervals of a certain flow velocity (increments of 4.5 cm/sec in
476 5 min steps) until the fish could not swim against the water current (fatigued; Figure S3).
477 Fatigue was determined when fish stopped swimming and was forced against the rear net of the
478 tunnel for more than 5 sec. Critical speed was then calculated using the following equation
479 (Brett, 1964):

480

$$481 \quad U_{\text{crit}} = U_{\text{fatigue}} + [U_{\text{step}} \times (t_{\text{fatigue}} / t_{\text{step}})],$$

482

483 *Where:* U_{fatigue} = the highest flow velocity where fish swam the whole interval, U_{step} = velocity
484 increment, t_{fatigue} = time elapsed at final velocity that fish swam in the last interval, t_{step} = time
485 increment that is the duration of one interval. The critical speed was normalized to body length
486 (BL) of the experimental animals and is given as BL/sec.

487

488 For the open field test, treated animals were placed in small dishes (diameter: 8 cm) and allowed
489 to swim freely, while their swimming was recorded for 4 min. Analysis of 2 min swimming
490 behavior was performed after optimization and implementation of wrMTrck, a freely available
491 ImageJ plugin. The average velocity and maximum velocity was normalized to body length
492 (BL) of the experimental animals and is given as BL/sec.

493

494 **Statistics**

495 The significance of differences between the means in experimental groups and conditions was
496 analyzed using the *One-way* ANOVA followed by *post hoc* Tukey test and the two-tailed
497 Student's *t*-test (paired or unpaired), using Prism (GraphPad Software Inc.) Differences were
498 considered to be significant if $p < 0.05$. Data presented here are given as mean \pm SEM.

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506 **References**

- 507 Alaburda A, Perrier J-F, Hounsgaard J. 2002. An M-like outward current regulates the
508 excitability of spinal motoneurons in the adult turtle. *J. Physiol. (Lond.)* **540**:875–881.
509
- 510 Ampatzis K, Ausborn J, Manira El A. 2013. Pattern of innervation and recruitment of different
511 classes of motoneurons in adult zebrafish. *J. Neurosci.* **33**: 10875–10886.
512
- 513 Ampatzis K, Song J, Ausborn J, Manira El A. 2014. Separate microcircuit modules of distinct
514 v2a interneurons and motoneurons control the speed of locomotion. *Neuron* **83**: 934–943.
515
- 516 Anadón R, Molist P, Rodríguez-Moldes I, López JM, Quintela I, Cerviño MC, Barja P,
517 González A. 2000. Distribution of choline acetyltransferase immunoreactivity in the brain of
518 an elasmobranch, the lesser spotted dogfish (*Scyliorhinus canicula*). *J. Comp. Neurol.* **420**:
519 139–170.
520
- 521 Bagnall MW, McLean DL. 2014. Modular organization of axial microcircuits in zebrafish.
522 *Science* **343**: 197–200.
523
- 524 Brett JR. 1964. The respiratory metabolism and swimming performance of young sockeye
525 salmon. *J. Fish Res. Board Can.* **21**: 1183–1226.
526
- 527 Brown DA. 2010. Muscarinic acetylcholine receptors (mAChRs) in the nervous system: some
528 functions and mechanisms. *J. Mol. Neurosci.* **41**: 340–346.
529
- 530 Chevallier S, Nagy F, Cabelguen J-M. 2006. Cholinergic control of excitability of spinal
531 motoneurons in the salamander. *J. Physiol. (Lond.)* **570**: 525–540.
532
- 533 Clemente D, Porteros Á, Weruaga E, Alonso JR, Arenzana FJ, Aijón J, Arévalo R. 2004.
534 Cholinergic elements in the zebrafish central nervous system: Histochemical and
535 immunohistochemical analysis. *J. Comp. Neurol.* **474**: 75–107.
536

- 537 Cullheim S, Kellerth JO, Conradi S. 1977. Evidence for direct synaptic interconnections
538 between cat spinal alpha-motoneurons via the recurrent axon collaterals: a morphological study
539 using intracellular injection of horseradish peroxidase. *Brain Res.* **132**: 1–10.
540
- 541 Deardorff AS, Romer SH, Sonner PM, Fyffe REW. 2014. Swimming against the tide:
542 investigations of the C-bouton synapse. *Front Neural Circuits* **8**: 106.
543
- 544 Eccles JC, Fatt P, Koketsu K. 1954. Cholinergic and inhibitory synapses in a pathway from
545 motor-axon collaterals to motoneurons. *J. Physiol. (Lond.)* **126**: 524–562.
546
- 547 Felder CC. 1995. Muscarinic acetylcholine receptors: signal transduction through multiple
548 effectors. *Faseb J.* **9**: 619–625.
549
- 550 Finkel E, Etlin A, Cherniak M, Mor Y, Lev-Tov A, Anglister L. 2014. Neuroanatomical basis
551 for cholinergic modulation of locomotor networks by sacral relay neurons with ascending
552 lumbar projections. *Journal of Comparative Neurology* **522**: 3437–3455.
553
- 554 Gabriel JP, Ausborn J, Ampatzis K, Mahmood R, Eklöf-Ljunggren E, Manira El A. 2011.
555 Principles governing recruitment of motoneurons during swimming in zebrafish. *Nature*
556 *Publishing Group* **14**: 93–99.
557
- 558 González, A., López, J.M., Sánchez-Camacho, C., Marín, O., 2002. Localization of choline
559 acetyltransferase (ChAT) immunoreactivity in the brain of a caecilian amphibian, *Dermophis*
560 *mexicanus* (Amphibia: Gymnophiona). *J. Comp. Neurol.* **448**: 249–267.
561 doi:10.1002/cne.10233
562
- 563 Goulding M. 2009. Circuits controlling vertebrate locomotion: moving in a new direction. *Nat.*
564 *Rev. Neurosci.* **10**: 507–518.
565
- 566 Grillner S, Jessell TM. 2009. Measured motion: searching for simplicity in spinal locomotor
567 networks. *Curr. Opin. Neurobiol.* **19**: 572–586.
568
- 569 Grillner SS. 2003. The motor infrastructure: from ion channels to neuronal networks. *Nat. Rev.*
570 *Neurosci.* **4**: 573–586.

571
572 Hassall CJ, Stanford SC, Burnstock G, Buckley NJ. 1993. Co-expression of four muscarinic
573 receptor genes by the intrinsic neurons of the rat and guinea-pig heart. *Neuroscience* **56**: 1041–
574 1048.
575
576 Herron LR, Miles GB. 2012. Gender-specific perturbations in modulatory inputs to
577 motoneurons in a mouse model of amyotrophic lateral sclerosis. *Neuroscience* **226**: 313–323.
578
579 Höglund AU, Baghdoyan HA. 1997. M2, M3 and M4, but not M1, muscarinic receptor
580 subtypes are present in rat spinal cord. *J. Pharmacol. Exp. Ther.* **281**: 470–477.
581
582 Hornby TG, McDonagh JC, Reinking RM, Stuart DG. 2002. Effects of excitatory modulation
583 on intrinsic properties of turtle motoneurons. *J. Neurophysiol.* **88**: 86–97.
584
585 Hosey MM. 1992. Diversity of structure, signaling and regulation within the family of
586 muscarinic cholinergic receptors. *Faseb J.* **6**: 845–852.
587
588 Huang A, Noga BR, Carr PA, Fedirchuk B, Jordan LM. 2000. Spinal cholinergic neurons
589 activated during locomotion: localization and electrophysiological characterization. *J.*
590 *Neurophysiol.* **83**: 3537–3547.
591
592 Hulme EC, Birdsall NJ, Buckley NJ. 1990. Muscarinic receptor subtypes. *Annu. Rev.*
593 *Pharmacol. Toxicol.* **30**: 633–673.
594
595 Ichiyama RM, Broman J, Roy RR, Zhong H, Edgerton VR, Havton LA. 2011. Locomotor
596 training maintains normal inhibitory influence on both alpha- and gamma-motoneurons after
597 neonatal spinal cord transection. *Journal of Neuroscience* **31**: 26–33.
598
599 Ireland MF, Funk GD, Bellingham MC. 2012. Muscarinic acetylcholine receptors enhance
600 neonatal mouse hypoglossal motoneuron excitability in vitro. *J. Appl. Physiol.* **113**:1024–1039.
601
602 Jordan L, McVagh JR, Noga BR, Cabaj AM, Majczyński H, Sławińska U, Provencher J,
603 Leblond H, Rossignol S. 2014. Cholinergic mechanisms in spinal locomotion-potential target
604 for rehabilitation approaches. *Front Neural Circuits* **8**: 132.

605
606 Kiehn O. 2006. Locomotor circuits in the mammalian spinal cord. *Annu. Rev. Neurosci.* **29**:
607 279–306.
608
609 Kurihara T, Suzuki H, Yanagisawa M, Yoshioka K. 1993. Muscarinic excitatory and inhibitory
610 mechanisms involved in afferent fibre-evoked depolarization of motoneurons in the neonatal
611 rat spinal cord. *Br. J. Pharmacol.* **110**: 61–70.
612
613 Miles GB, Sillar KT. 2011. Neuromodulation of vertebrate locomotor control networks.
614 *Physiology (Bethesda)* **26**: 393–411.
615
616 Miles GB, Hartley R, Todd AJ, Brownstone RM. 2007. Spinal cholinergic interneurons regulate
617 the excitability of motoneurons during locomotion. *Proc. Natl. Acad. Sci. U.S.A.* **104**: 2448–
618 2453.
619
620 Mueller T, Vernier P, Wullimann MF. 2004. The adult central nervous cholinergic system of a
621 neurogenetic model animal, the zebrafish *Danio rerio*. *Brain Res.* **1011**: 156–169.
622
623 Murakami Y, Matsumoto K, Ohta H, Watanabe H. 1996. Effects of oxotremorine and
624 pilocarpine on striatal acetylcholine release as studied by brain dialysis in anesthetized rats.
625 *Gen. Pharmacol.* **27**: 833–836.
626
627 Palstra AP, Tudorache C, Rovira M, Brittijn SA, Burgerhout E, van den Thillart GEEJM,
628 Spaink HP, Planas JV. 2010. Establishing zebrafish as a novel exercise model: swimming
629 economy, swimming-enhanced growth and muscle growth marker gene expression. *PLoS ONE*
630 **5**, e14483.
631
632 Pullen AH, Athanasiou D. 2009. Increase in presynaptic territory of C-terminals on lumbar
633 motoneurons of G93A SOD1 mice during disease progression. *Eur. J. Neurosci.* **29**: 551–561.
634
635 Quinlan KA, Buchanan JT. 2008. Cellular and synaptic actions of acetylcholine in the lamprey
636 spinal cord. *J. Neurophysiol.* **100**: 1020–1031. doi:10.1152/jn.01157.2007
637

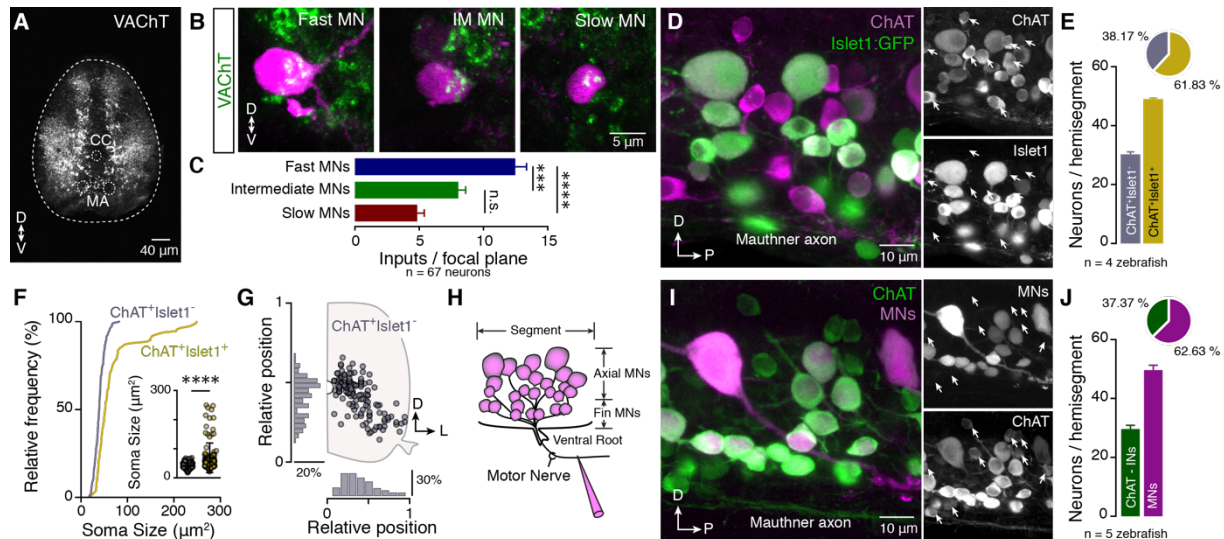
- 638 Satou C, Kimura Y, Higashijima S-I. 2012. Generation of multiple classes of V0 neurons in
639 zebrafish spinal cord: progenitor heterogeneity and temporal control of neuronal diversity.
640 *Journal of Neuroscience* **32**: 1771–1783.
- 641
- 642 Saxena S, Roselli F, Singh K, Leptien K, Julien J-P, Gros-Louis F, Caroni P. 2013.
643 Neuroprotection through excitability and mTOR required in ALS motoneurons to delay disease
644 and extend survival. *Neuron* **80**: 80–96.
- 645
- 646 Skup M, Gajewska-Wozniak O, Grygielewicz P, Mankovskaya T, Czarkowska-Bauch J. 2012.
647 Different effects of spinalization and locomotor training of spinal animals on cholinergic
648 innervation of the soleus and tibialis anterior motoneurons. *Eur. J. Neurosci.* **36**: 2679–2688.
- 649
- 650 Song J, Ampatzis K, Björnfors ER, Manira El A. 2016. Motor neurons control locomotor circuit
651 function retrogradely via gap junctions. *Nature* **529**: 399–402.
- 652
- 653 Stil A, Drapeau P. 2016. Neuronal labeling patterns in the spinal cord of adult transgenic
654 Zebrafish. *Dev Neurobiol* **76**: 642–660.
- 655
- 656 Thiriet G, Kempf J, Ebel A. 1992. Distribution of cholinergic neurons in the chick spinal cord
657 during embryonic development. Comparison of ChAT immunocytochemistry with AChE
658 histochemistry. *Int. J. Dev. Neurosci.* **10**: 459–466.
- 659
- 660 Uemura O, Okada Y, Ando H, Guedj M, Higashijima S-I, Shimazaki T, Chino N, Okano H,
661 Okamoto H. 2005. Comparative functional genomics revealed conservation and diversification
662 of three enhancers of the *isll* gene for motor and sensory neuron-specific expression. *Dev. Biol.*
663 **278**: 587–606.
- 664
- 665 Welton J, Stewart W, Kerr R, Maxwell DJ. 1999. Differential expression of the muscarinic m2
666 acetylcholine receptor by small and large motoneurons of the rat spinal cord. *Brain Res.* **817**:
667 215–219.
- 668
- 669 Wullimann MF, Rupp B, Reichert H. 1996. Neuroanatomy of the zebrafish brain: a topological
670 atlas. Basel: Birkhaeuser Verlag.

- 671 Zagoraiou L, Akay T, Martin JF, Brownstone RM, Jessell TM, Miles GB. 2009. A cluster of
672 cholinergic premotor interneurons modulates mouse locomotor activity. *Neuron* **64**: 645–662.
673
- 674 Zhang H-M, Li D-P, Chen S-R, Pan H-L. 2005. M2, M3, and M4 receptor subtypes contribute
675 to muscarinic potentiation of GABAergic inputs to spinal dorsal horn neurons. *J. Pharmacol.*
676 *Exp. Ther.* **313**: 697–704.

677 **Figures**

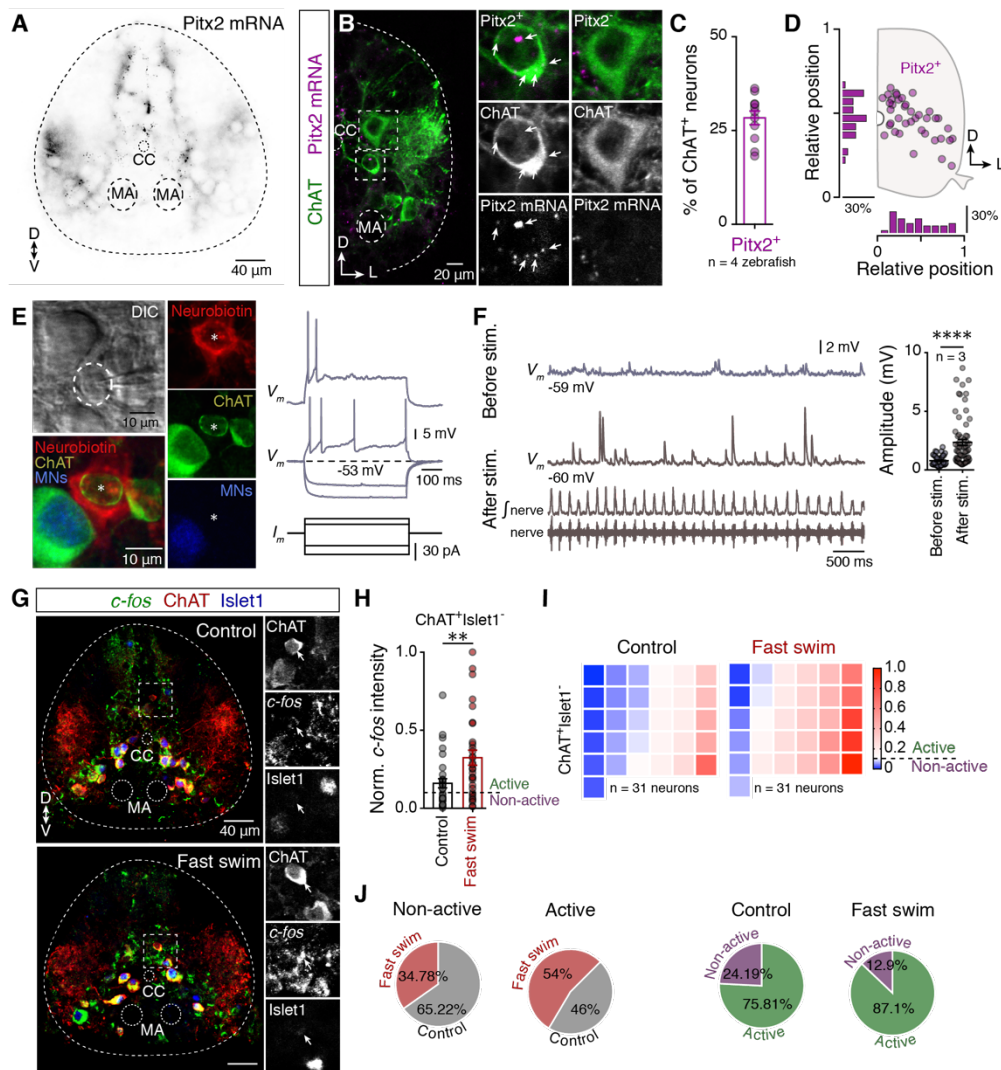
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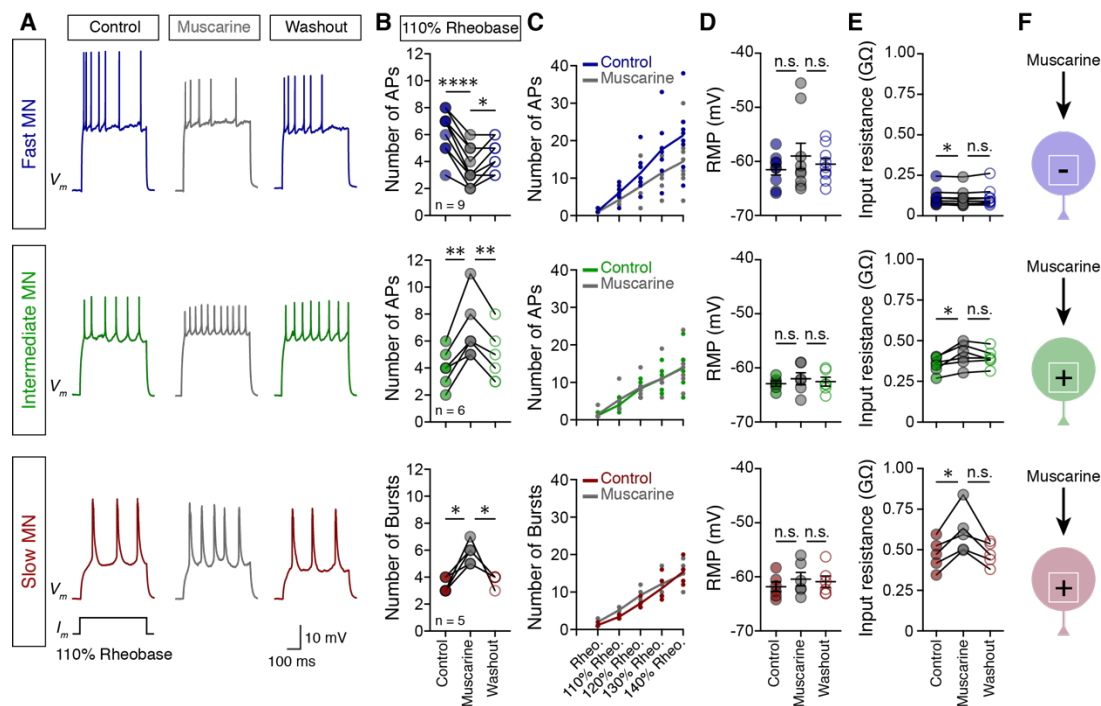
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681 **Figure 1.** Organization of spinal cholinergic system. (A) Distribution of cholinergic terminals within
 682 the adult zebrafish spinal cord. (B) Single optical sections show the cholinergic synapses onto different
 683 types of motoneurons. (C) Cholinergic (ACh) input is different between motoneuron (MN) classes, (n_{slow}
 684 = 10, $n_{\text{interm}} = 27$, $n_{\text{fast}} = 30$ neurons; $n = 5$ zebrafish). (D) Whole mount spinal cord image shows that
 685 cholinergic interneurons $\text{ChAT}^+\text{Islet1}^-$ exist in adult zebrafish. Arrows indicate the $\text{ChAT}^+\text{Islet1}^-$ neurons
 686 (Cholinergic interneurons). (E) Quantification of the adult zebrafish cholinergic neurons, in *Islet1:GFP*
 687 line, per spinal cord hemisegment ($n = 4$ zebrafish). (F) Cholinergic interneurons ($\text{ChAT}^+\text{Islet1}^-$) have
 688 smaller soma sizes compared to motoneurons. (G) Distribution pattern of cholinergic interneurons
 689 ($\text{ChAT}^+\text{Islet1}^-$) within the adult zebrafish spinal cord. (H) Drawing showing the methodological
 690 approach to backfill all the motoneurons (axial and fin) exiting the spinal cord through the ventral root
 691 by injecting a retrograde tracer (Dextran). (I) Whole mount spinal cord immunohistochemistry reveals
 692 that a fraction of ChAT^+ neurons are not backfilled motoneurons. Arrows indicate ChAT^+MN^- neurons
 693 (Cholinergic interneurons; $n = 5$ zebrafish). (J) Quantification of the adult zebrafish cholinergic neurons,
 694 per spinal cord hemisegment ($n = 5$ zebrafish), reveals similar number of cholinergic interneurons as in
 695 the *Islet1:GFP* line. CC, central canal; D, dorsal; L, lateral; MA, Mauthner axon; P, posterior; V, ventral.
 696 Data are presented as mean \pm SEM; *** $p < 0.001$; **** $p < 0.0001$; n.s., non-significant.



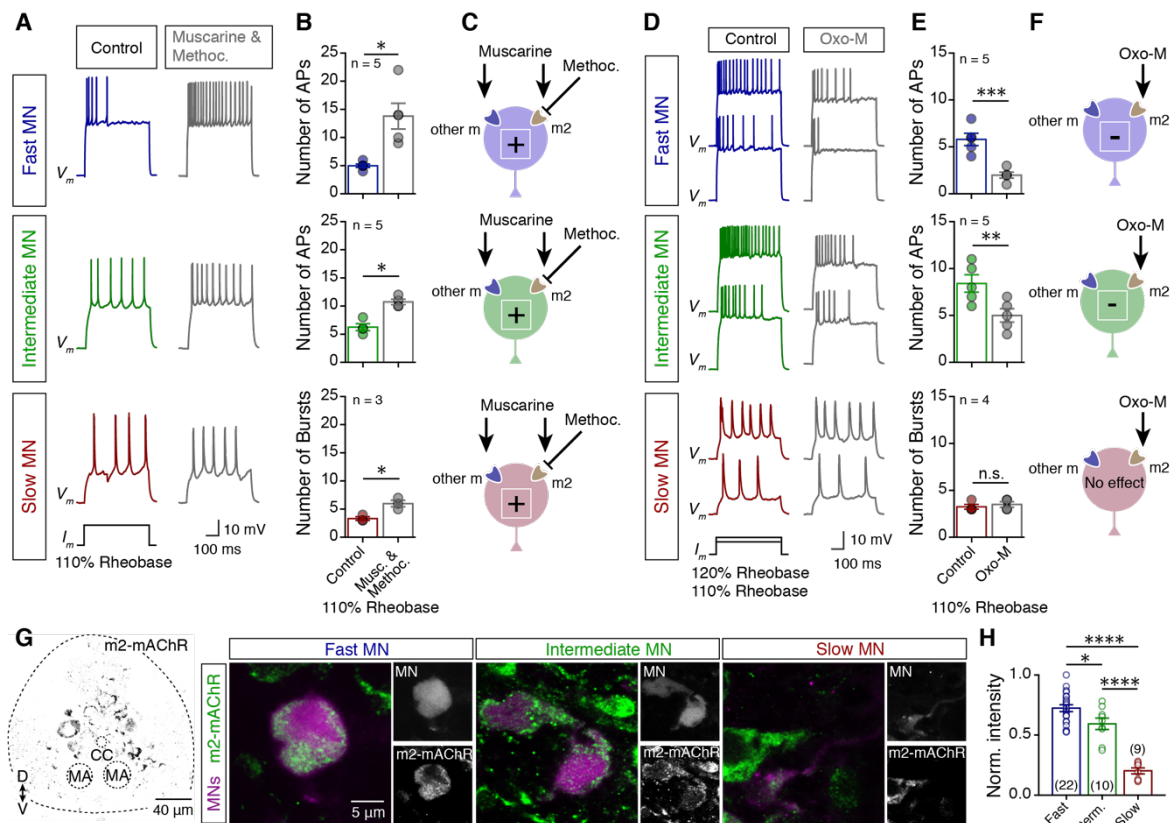
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698 **Figure 2.** Cholinergic interneurons are homologues to mammalian V0c interneurons and they are
 699 recruited at high speed locomotion. (A) Localization of *Pitx2* mRNA in adult zebrafish spinal cord. (B)
 700 Cholinergic *Pitx2*⁺ and *Pitx2* neurons found in adult zebrafish spinal cord. Arrows indicate the location
 701 of mRNA in the cholinergic neuron. (C) Quantification (%) of cholinergic neurons that are *Pitx2*⁺ (n =
 702 4 zebrafish). (D) Distribution pattern of cholinergic *Pitx2*⁺ interneurons within the adult zebrafish spinal
 703 cord. (E) Whole cell patch clamp recording from identified neuron that filled with neurobiotin. Post-
 704 hoc evaluation of the patched neuron that is cholinergic (ChAT⁺) and not motoneuron (MN). Firing
 705 behavior of a representative cholinergic interneuron upon steps of somatic current injections. The
 706 asterisk denotes the neurobiotin filled patched neuron. (F) Increasing number and amplitude of post-
 707 synaptic responses during fictive locomotion, as indicated from the recording of motor nerve activity
 708 (lower trace and rectified trace) after electrical stimulation of descending axons to initiate locomotion
 709 (n = 3 neurons). (G) Microphotographs showing that *c-fos* immunoreactivity increases after prolonged
 710 (2h) forced fast swimming (80% of the Ucrit) in the cholinergic interneurons (ChAT⁺Islet1⁺). (H)
 711 Quantification of normalized *c-fos* intensity in cholinergic interneurons (ChAT⁺Islet1⁺). The threshold
 712 of activity was considered to be 0.11. (I) Heat map displaying the normalized *c-fos* intensity of each
 713 individual cholinergic interneuron (n_{control} = 31 neurons, n_{fast swim} = 31 neurons). (J) Proportions of active
 714 and non-active cholinergic interneurons (ChAT⁺Islet1⁺) between control and after forced fast swimming
 715 based on the normalized *c-fos* intensity. CC, central canal; D, dorsal; L, lateral; MA, Mauthner axon; P,
 716 posterior; V, ventral. Data are presented as mean ± SEM; **p < 0.01; ****p < 0.0001.



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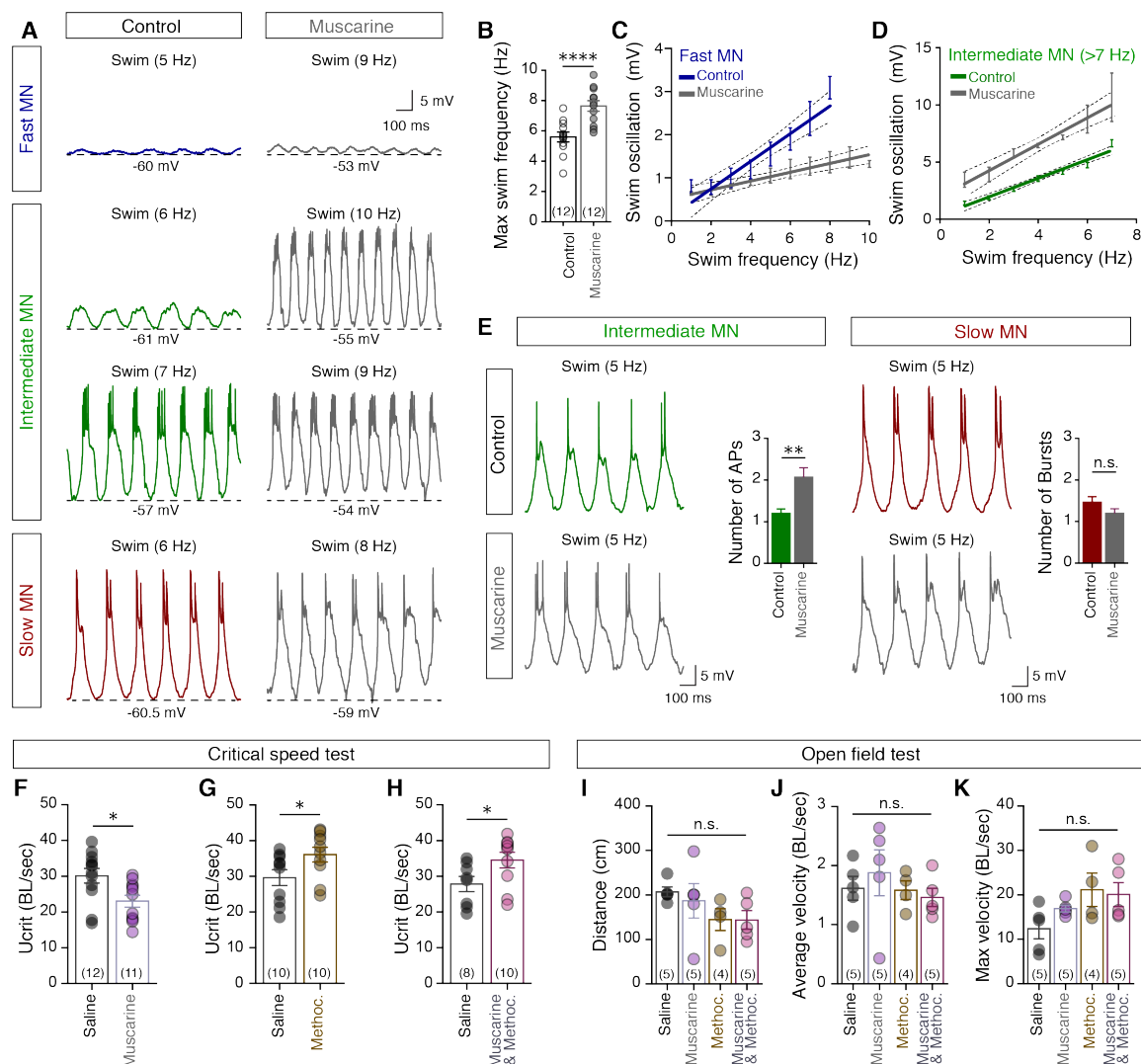
719 **Figure 3.** Muscarinic receptors differentially alter motoneuron excitability. (A) Representative traces of
720 motoneuron (MN) firing in responses to 110% of rheobase somatic current injection, before (colored
721 traces) after bath application of muscarine (15 μ M; gray traces) and followed by washout (colored
722 traces). (B) In the presence of muscarine the fast motoneurons significantly reduce the number of action
723 potentials (APs) in response to 110% of rheobase current injection, while both the intermediate and the
724 slow motoneurons increase their firing rate. During washout, the motoneurons partially recover ($n_{\text{fast}} =$
725 9 neurons, $n_{\text{intermediate}} = 6$ neurons, $n_{\text{slow}} = 5$ neurons). (C) Relation of the number of action potentials
726 (APs) or bursts of APs to current (increments of 10% increase from rheobase) in absence (colored line)
727 and presence of muscarine (gray line). (D) During the application of muscarine (gray circles) and after
728 washout (open colored circles) there is no significant change in the resting membrane potential of the
729 motoneurons. (E) Bath application of muscarine alters the input resistance of all motoneuron types. The
730 input resistance was decreased in the fast motoneurons and increased in the intermediate and slow
731 motoneurons. (F) Overview of the change in excitability of different types of MNs in response to
732 muscarine. The sign represents the alteration of the MN excitability (+ = increase, - = decrease). Data
733 are presented as mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$; n.s., non-significant.



734

735

736 **Figure 4.** Motoneurons possess different muscarinic receptor subtypes. (A) Traces of motoneuron (MN)
 737 firing in response to 110% of rheobase current injection, before (colored traces) and after bath
 738 application of a mixture of muscarine (15 μ M) and methoctramine (10 μ M; gray traces). (B-C) Co-
 739 application of muscarine and methoctramine produce hyperexcitability in all MN pools ($n_{fast} = 5$ neurons,
 740 $n_{intermediate} = 5$ neurons, $n_{slow} = 3$ neurons), derived from the antagonistic effect on m2-mAChRs. (D)
 741 Representative traces of motoneuron (MN) firing in response to 110% and 120% of rheobase current
 742 injections, before (colored traces) and after bath application of oxotremorine-M (Oxo-M; 20 μ M; gray
 743 traces) which preferentially activates the m2-mAChRs. (E-F) In the presence of oxotremorine-M,
 744 excitability decreases in the fast and intermediate motoneurons, whereas the slow motoneurons do not
 745 change their firing rate in response to 110% of rheobase current injection ($n_{fast} = 5$ neurons, $n_{intermediate} =$
 746 $n_{slow} = 4$ neurons). (G) Distribution pattern of m2-mAChRs in relation to different
 747 motoneuron types. A combination of retrograde labeling of different motoneuron pools with
 748 immunostaining for m2-mAChRs reveals that the fast motoneurons exhibit a vast number of m2
 749 receptors compared to other motoneuron types (intermediate and slow). Slow motoneurons were found
 750 to be weakly labeled. The sign represents the excitability alteration of the MN (+ = increase, - =
 751 decrease). Data are presented as mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$;
 752 n.s., non-significant.



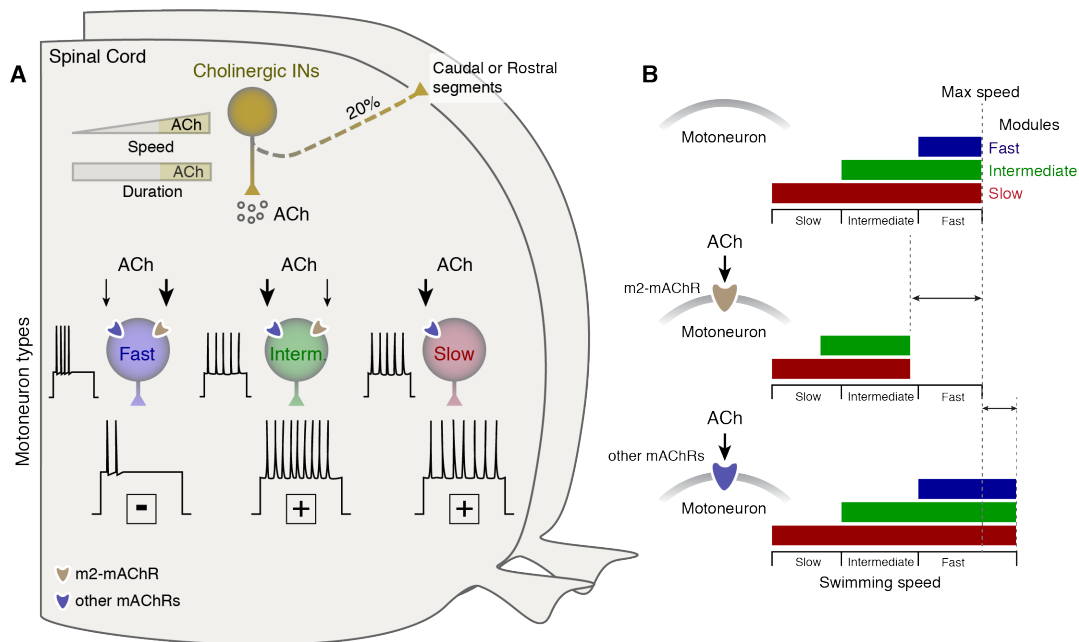
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755 **Figure 5.** Activation of motoneuron muscarinic receptors controls fast locomotion. (A-B) During fictive
756 locomotion, motoneurons display membrane potential oscillations. The highest swimming frequency
757 increases in the presence of muscarine (15 μ M; gray traces) during fictive locomotion (*ex vivo*
758 experiments). Dashed line indicates the membrane potential of the lower part of the oscillations. (C)
759 Averaged slopes of the membrane potential oscillations in relation to different swimming frequencies
760 of fast motoneurons in the absence (blue line), or presence (gray line) of muscarine. The data are
761 presented as interpolation curve (middle line \pm SEM) and 95% confidence band (outer dashed lines). (D)
762 Slopes showing the increasing amplitude of the membrane potential oscillations of the intermediate
763 motoneurons recruited above 7 Hz, in relation to the swimming frequency, before and after the
764 application of muscarine. After muscarine (gray line) the amplitude of the membrane oscillations was
765 significant increased ($F = 856.6$ $p < 0.0001$), in comparison to the control (green line). The data are
766 presented as interpolation curve (middle line \pm SEM) and 95% confidence band (outer dashed lines).
767 (E) During 5 Hz fictive locomotion, in the absence (colored trace) or presence (gray trace) of muscarine,
768 the number of discharges of intermediate motoneurons increased, while slow motoneurons did not
769 display any changes in the number of bursts of action potentials. (F-H) Intraperitoneal administration
770 of muscarine reduces the U_{crit} (BL = 1.84 ± 0.11 cm; $n = 23$ zebrafish), while methoctramine increased
771 the maximum swimming speed (BL = 1.68 ± 0.1 cm; $n = 20$ zebrafish). Co-administration of muscarine
772 and methoctramine increased the maximum obtained swimming speed (BL = 1.71 ± 0.12 cm; $n = 18$
773 zebrafish). (I-K) *In vivo* monitoring of adult zebrafish locomotor behavior. Muscarine and/or
774 methoctramine administration was not found to affect the distance traveled, the average velocity and the
775 maximum velocity of the studied animals ($n = 19$ zebrafish). The data were normalized to body lengths

776 (BL) / sec. Data are presented as mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$; n.s., non-
777 significant.

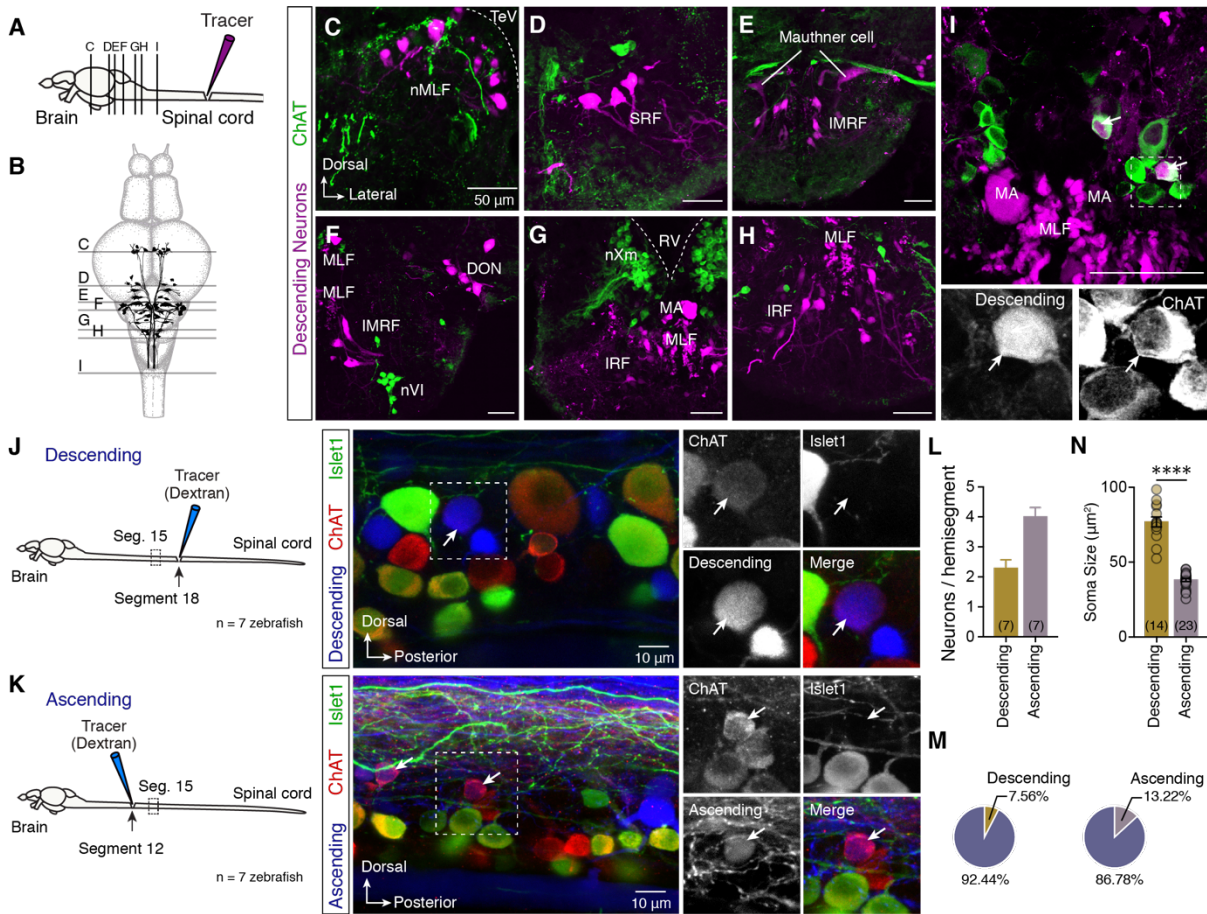
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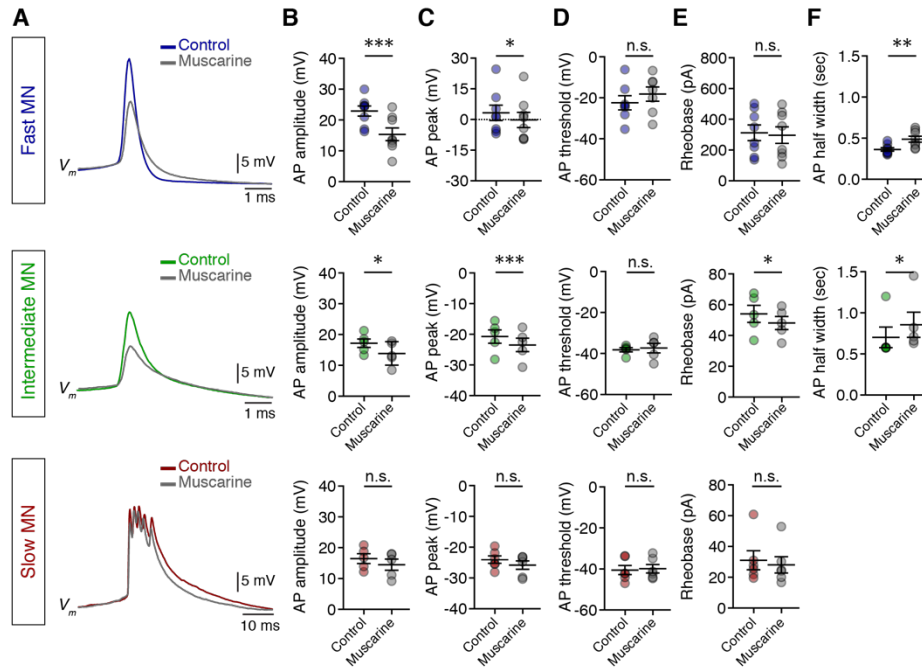
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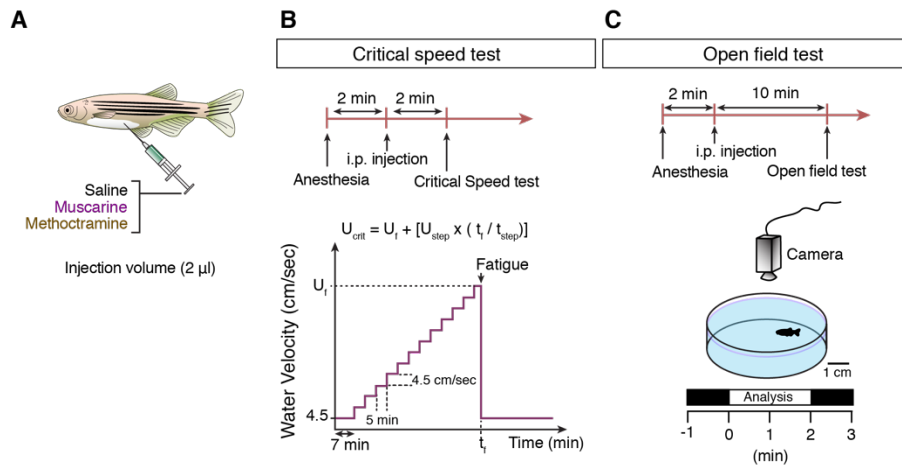
781 **Figure 6.** Differential organization of cholinergic input to MNs permits changes of the operational range
782 of locomotor circuit. (A) Acetylcholine release (after prolonged fast swimming) from spinal cholinergic
783 interneurons affects the excitability of different types of motoneurons in a mAChR dependent manner.
784 Our results clearly demonstrate that acetylcholine can only increase slow motoneuron excitability,
785 whereas the intermediate and fast motoneurons can alter their excitability through the activation of the
786 different mAChRs that they possess. (B) This configuration allows the animals to adjust the recruitment
787 of the locomotor microcircuit modules and alter the operational range of these networks.



Supplementary file 1. All the brain neurons descending to spinal cord are not cholinergic. **(A-B)** Injection of methylrhodamine dextran in the spinal cord retrogradely labels all the descending supra-spinal neurons (N = 8 zebrafish brains). Schematic representation of the distribution of adult zebrafish brain descending neurons with the level of sections that correspond to the following images in C-I. **(C-H)** Confocal images show that none of the descending labeled neuron is ChAT⁺ in all studied brain areas. **(I)** Few descending neurons (n = 4 neurons out of 8 brains) were found to be ChAT⁺ in the initial part of the spinal cord. Arrows indicate the double labeled neurons. **(J)** Injection of a retrograde dextran tracer in spinal segment 18 reveals the descending interneurons located in spinal cord segment 15. Few descending neurons that are cholinergic (ChAT⁺) and not motoneurons (Islet1⁻) were found in the spinal hemisegment of adult zebrafish. Arrow indicates cholinergic descending interneuron. **(K)** Injection of dextran retrograde tracer in spinal segment 12 reveals the ascending interneurons in spinal cord segment 15. Arrows indicate a small number of retrograde traced neurons that are cholinergic interneurons (ChAT⁺ Islet1⁻). **(L)** Analysis of the number of the cholinergic interneurons that possess a descending (2.28 \pm 0.28 neurons / hemisegment; n = 7 zebrafish) or an ascending (4 \pm 0.3 neurons / hemisegment; n = 7 zebrafish) axon. **(M)** Percentage of the descending and ascending cholinergic interneurons per hemisegment of spinal cord. **(N)** The descending and ascending cholinergic interneurons have non-overlapping soma sizes (t = 13.4, p < 0.0001, n = 37 neurons), suggesting that different populations of cholinergic interneurons are ascending or descending in adult zebrafish spinal cord. DON, descending octaval nucleus; IMRF, intermediate reticular formation; IRF, inferior reticular formation; MA, Mauthner axon; MLF, medial longitudinal fascicle; nMLF, nucleus of the medial longitudinal fascicle; nVI, abducens nucleus; nXm, vagal motor nucleus; RV, rhombencephalic ventricle; SRF, superior reticular formation; TeV, tectal ventricle. Data are presented as mean \pm SEM; ****p < 0.0001.



Supplementary file 2. Changes in MN intrinsic biophysical properties after the bath application of muscarine. **(A)** Superimposed representative examples of the first action potential (AP) in fast and intermediate motoneurons and first burst of action potentials (APs) of slow motoneurons before (colored traces) and after (gray traces) muscarine. **(B)** The AP amplitude was significantly reduced by muscarine in intermediate ($t = 4.33$, $p = 0.0123$, $n = 5$) and fast motoneurons ($t = 5.66$, $p = 0.0008$, $n = 8$). **(C)** Following the reduction of AP amplitude also the AP peak was found to be hyperpolarized (Fast MNs: $t = 2.44$, $p = 0.044$, $n = 8$; intermediate MNs: $t = 14.64$, $p = 0.0001$). **(D)** Muscarine application was found not to affect the AP threshold. **(E)** Rheobase, the minimum current injection that generates an AP, was hyperpolarized in intermediate motoneurons ($t = 4.24$, $p = 0.013$, $n = 5$). **(F)** The half-width duration of the AP was significantly increased by muscarine in motoneurons firing single APs (intermediate MNs: $t = 3.79$, $p = 0.019$, $n = 5$; fast-MNs: $t = 4.9$, $p = 0.0017$, $n = 8$). Student's paired t test. Data are presented as mean \pm SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s., non-significant.



Supplementary file 3. Experimental design to investigate the effect of mAChRs during *in vivo* locomotion. **(A)** Intraperitoneal administration of saline, muscarine and/or methoctramine in anesthetized adult zebrafish before the *in vivo* tests. **(B)** The critical speed test protocol. **(C)** Protocol for *in vivo* monitoring of adult zebrafish locomotor behavior.