

1 **Manuscript Title:** Motor neurons involved in fine motor control are labeled by tracing *Atoh1*-lineage  
2 neurons in the spinal cord.

3 **Abbreviated Title:** *Atoh1*<sup>Cre/+</sup>-labeled motor neurons

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## 27 **Abstract**

28 Motor neurons (MNs) innervating the digit muscles of the intrinsic hand and foot (IH and IF)  
29 control fine motor movements. Previous studies suggest that the IH and IF MN pools have a unique  
30 developmental history in comparison to limb MN pools. Consistent with having this unique  
31 development, we find that the IH and IF MN pools are labeled postnatally using a CRE knock-in mouse  
32 line of *Atoh1*, a developmentally expressed basic helix-loop-helix (bHLH) transcription factor, while  
33 limb-innervating MN pools are not. Approximately 60% of the IH and IF MN pools are labeled and are a  
34 mixture of alpha and gamma-MNs. In addition, because *Atoh1* is known developmentally to specify  
35 many cerebellar-projecting neurons, we tested the hypothesis that IH and IF MNs can send axon  
36 collaterals to the cerebellum as a mechanism of corollary discharge. Using intersectional genetic, viral  
37 labeling, and retrograde labeling strategies, we were unable to provide evidence in support of this idea.  
38 As a secondary finding of our viral labeling experiments, we report here that injection of both AAV and  
39 Lentiviruses in the periphery can cross the blood-brain barrier to infect Purkinje cells within the central  
40 nervous system. Altogether, though, we find that labeling of the IH and IF motor neurons using the  
41 *Atoh1* CRE knock-in mouse suggests that IH and IF MNs have a unique developmental history and that  
42 this mouse strain might be a useful tool to target these specific sets of neurons allowing for functional  
43 studies of fine motor control.

## 44 **Significance Statement**

45 Motor neurons (MNs) of the intrinsic hand and foot (IH and IF) are labeled postnatally using a  
46 CRE knock-in mouse line of the basic helix-loop-helix (bHLH) transcription factor *Atoh1* indicating a  
47 unique developmental history. We tested whether IH and IF MNs send axon collaterals rostrally to the  
48 cerebellum as a mechanism of direct corollary discharge from MNs, but the question remains  
49 unresolved. As a resource for the community, we report that injection of both AAV and Lentiviruses in  
50 the periphery can cross the blood-brain barrier and infect Purkinje cells within the central nervous  
51 system.

## 52 **Introduction**

53 Fine motor skills, such as writing or sewing, require exquisite control of the motor neurons  
54 (MNs) innervating the digits of the hand. Over vertebrate evolution, unique molecular pathways are  
55 involved in the elaboration of digits (Shubin et al., 1997). Correspondingly, the MNs innervating the  
56 distal digits have unique developmental programs compared to the neighboring MNs of the lateral  
57 motor column (LMC) that innervate limb muscles (Mendelsohn et al., 2017). Precisely how digit-  
58 innervating MNs adopt their unique identities is unclear. Insight into the development and connectivity  
59 of digit-innervating MNs could reveal distinct functions of these MNs in fine motor behavior.

60 Developmentally, all MNs derive from a progenitor domain expressing the basic helix-loop-helix  
61 (bHLH) oligodendrocyte transcription factor 2 (*Olig2*) in the ventral neural tube (Lu et al., 2002).  
62 Unexpectedly, we found that the digit-innervating MNs were labeled using CRE-loxP lineage tracing of  
63 the bHLH transcription factor atonal homolog 1, *Atoh1*, a transiently expressed gene in the dorsal-most  
64 part of the developing neural tube that specifies spinal cord neurons that project rostrally to the  
65 hindbrain (Bermingham et al., 2001; Gowan et al., 2001; Sakai et al., 2012; Yuengert et al., 2015).  
66 Here, we explored the features of the digit-innervating MNs labeled by *Atoh1* CRE-LoxP lineage  
67 tracing.

68 In the motor field, corollary discharge is a mechanism by which copies of descending motor  
69 signals are sent back to the proprioceptive sensory pathways to distinguish self-generated movements  
70 from externally generated ones (Sperry, 1950; von Holst and Mittelstaedt, 1950; Crapse and Sommer,  
71 2008). In mammals, to the best of our knowledge, there has been no evidence of corollary discharge  
72 occurring at the level of MNs themselves, although retrograde labeling studies of spinocerebellar  
73 neurons have reported cerebellar-projecting neurons whose cell bodies reside in the MN lamina IX of  
74 the spinal cord (Matsushita and Hosoya, 1979; Matsushita et al., 1979; Terman et al., 1998). In  
75 addition, Cooper and Sherrington in their initial description of ascending projections from the spinal cord  
76 observed “large cells” in the ventral horn that degenerated upon cutting their ascending axons (Cooper  
77 and Sherrington, 1940).

78 The fact that *Atoh1*-lineage neurons include many cerebellar-projecting neurons such as those  
79 of the spinocerebellar system, pedunclopontine tegmentum, pontine nuclei, lateral reticular nucleus,  
80 and external cuneate nucleus (Rose et al., 2009), raised the intriguing possibility that *Atoh1* CRE-LoxP  
81 lineage traced MNs might indicate a function of ATOH1 in these MNs to specify axon collaterals to the  
82 cerebellum. Evidence of axon collaterals to the cerebellum from MNs, specifically the MNs involved in  
83 fine motor control, would support a model of corollary discharge directly from MNs themselves. We  
84 attempted to test this model using intersectional genetic, viral tracing, and retrograde tracing strategies,  
85 but each approach had its caveats. Here, we characterize the *Atoh1* CRE-LoxP lineage traced MN  
86 populations and report our efforts testing a model of corollary discharge from fine motor control MNs.

## 87 **Materials & Methods**

### 88 *Mouse strains*

89 The following mouse strains were used: *Atoh1*<sup>Cre/+</sup> knock-in (Yang et al., 2010), *R26*<sup>LSL-tdTom/+</sup>  
90 (Ai14)(Madisen et al., 2010), *R26*<sup>LSL-FSF-tdTom/+</sup> (Ai65)(Madisen et al., 2015), *Hoxa4::Cre* (Huang et al.,  
91 2012), *Chat*<sup>IRES-Cre</sup> (Rossi et al., 2011), *Cdx2::FLPo* (Bourane et al., 2015). The *Chat*<sup>IRES-FLPo</sup> mouse was  
92 generated by Gord Fishell and Rob Machold (unpublished). Briefly, the IRES-FLPo-pA cassette was  
93 knocked into the 3'UTR immediately following the *ChAT* stop codon in B4 ES cells (C57Bl/6). Following  
94 germline transmission of the correctly targeted ChAT-IRES-FLPo allele, the Neo cassette (LoxP  
95 flanked) was removed by crossing the mice with the CMV-Cre deleter line (JAX # 006054) prior to use.  
96 All mice were outbred and thus, are mixed strains (at least C57Bl/6J and ICR). *Atoh1*<sup>Cre/+</sup> knock-in mice  
97 crossed to reporter mice were screened for “dysregulated” expression as previously reported (Yuengert  
98 et al., 2015). All animal experiments were approved by the Institutional Animal Care and Use  
99 Committee at UT Southwestern.

### 100 *Tissue processing*

101 Embryos were timed as E0.5 on the day the vaginal plug was detected and P0 on the day of  
102 birth. Pregnant females were euthanized with CO<sub>2</sub> and cervical dislocation, embryos dissected out of  
103 the uterus, and spinal cords dissected out. Embryonic spinal cords (E14.5) were fixed in 4%

104 paraformaldehyde (PFA)/PBS for 2-3 hrs at 4°C. Early postnatal animals (younger than P7) were  
105 cooled on ice, decapitated, their spinal cords dissected out, and fixed in 4% PFA/PBS for 2 hours at  
106 4°C. Mice older than P14 were anesthetized with Avertin (2,2,2-Tribromoethanol) (0.025-0.030 mL of  
107 0.04 M Avertin in 2-methyl-2-butanol and distilled water/g mouse) and transcardially perfused, first with  
108 0.012% w/v Heparin/PBS and then 4% PFA/PBS. A dorsal or ventral laminectomy exposed the spinal  
109 cord to the fixative. The spinal cords were fixed for 2 hrs and the brains overnight at 4°C. Tissue was  
110 washed in PBS for at least one day and cryoprotected in 30% sucrose dissolved in deionized water.  
111 Tissue was marked with 1% Alcian Blue in 3% acetic acid on one side to keep orientation and were  
112 then embedded in OCT (Tissue-Tek Optimal Cutting Temperature compound). Tissue was sectioned  
113 using a Leica CM1950 Cryostat.

#### 114 *Immunohistochemistry and confocal imaging*

115 Cryosections (30-40  $\mu\text{m}$ ) were blocked with PBS/1-3% normal goat or donkey serum/0.3%  
116 Triton X-100 (Sigma) for up to 1 hour at room temperature (RT) and incubated overnight with primary  
117 antibody at 4°C. After washing 3 times with PBS, the appropriate secondary antibody (Alexa 488, 567,  
118 and/or 647, Invitrogen) was incubated for an hour at RT. Sections were rinsed 3 times in PBS, mounted  
119 with Aquapolymount (Polysciences Inc.), and coverslipped (Fisher). The following primary antibodies  
120 and dilutions were used: 1:500 rabbit anti-dsRed (Clontech), 1:100 goat anti-CHAT (Millipore Sigma),  
121 1:1000 rabbit anti-MMP9 (Abcam), 1:8000 rabbit anti-HB9 (gift of Dr. Sam Pfaff, Salk Institute), 1:3000  
122 guinea pig anti-CPNE4 and 1:8000 guinea pig anti-FIGN (gifts of Dr. Tom Jessell, Columbia Univ.),  
123 1:500 mouse anti-NEUN (Millipore Sigma), 1:100 mouse anti-ERR3 (R&D Systems), 1:3000 alpha-  
124 bungarotoxin 488 (Invitrogen), 1:1000 rabbit anti-Syntaxin1 (gift of Thomas Südhof, Stanford  
125 University), 1:1000 guinea pig anti-VGLUT1 (Millipore Sigma), 1:1000 guinea pig anti-VGLUT2  
126 (Millipore Sigma), 1:1000 goat anti-VACHT (Millipore Sigma). Sections were referenced to the Mouse  
127 Brain Atlas (Paxinos and Franklin, 2007) and Christopher Reeves Spinal Cord Atlas (Watson et al.,  
128 2009).

129           Fluorescent images were taken on a Zeiss LSM710 or LSM880 confocal microscope with an  
130 appropriate optical slice (0.5-10  $\mu\text{m}$ ) depending on the image. Images were pseudocolored using a  
131 magenta/green/blue or magenta/yellow/cyan color scheme using Adobe Photoshop (Adobe) or Fiji  
132 (Schindelin et al., 2012).

### 133 *CTB muscle injections*

134 Mice aged P14 were anesthetized using isoflurane and prepared for injections into muscle. An  
135 approximate total of 500-750 nL of Cholera toxin subunit B (CTB) AlexaFluor 488 or 647 Conjugate  
136 (Invitrogen) (Nanoject II, Drummond Scientific) was injected into 2-3 different locations in the left  
137 forepaw (Intrinsic Hand (IH) MN pool) or hindpaw (Intrinsic Foot (IF) MN pool), or 3-4 different locations  
138 for the gastrocnemius (GS) or tibialis anterior (TA) in 50.6 nL increments. Spinal cords were harvested  
139 5 days after injection.

### 140 *Viral Injections*

141           Mice aged P4-5 or P14-15 were anesthetized using isoflurane (Henry Schein) and prepared for  
142 injections into the hindpaw. The hindpaw was shaved if needed and 70% ethanol and betadine (Avrio  
143 Health L.P.) applied. For P4-5 pups, virus was injected in a single location through the skin. For P14-  
144 15, a midline incision was made on the dorsal surface of the hindpaw. A total of 200-250 nL of AAV8-  
145 hSyn-GFP-Cre (UNC Vector Core,  $6.5 \times 10^{12}$  Vg/mL) or Lenti<sup>FugE</sup>-Cre, a pseudotyped lentivirus  
146 mediating the expression of CRE, was injected in 50.6 nL increments (Nanoject II, Drummond  
147 Scientific) with 1-2 min between injections at 2-3 different locations in the left hindpaw for P14-P15  
148 animal and at a single location through the skin in P4-5 pups. Lenti<sup>FugE</sup>-Cre was pseudotyped with a  
149 fusion glycoprotein enabling efficient retrograde axonal transport (Kato et al., 2014). To generate  
150 Lenti<sup>FugE</sup>-Cre, *Cre* was sub-cloned into the third generation HIV-based lentivirus vector under the  
151 control of a synapsin promoter (FSW-*Cre*). FSW-*Cre* was co-transfected into HEK293 cells with three  
152 packing plasmids, pMDLg/pRRE, pRSV-Rev and pCAGGS-FuG-E to generate Lenti<sup>FugE</sup>-Cre, which was  
153 concentrated with ultracentrifugation to  $2.0 \times 10^{12}$  Vg/mL. The incision was closed with surgical glue

154 (Henry Schein). Carprofen (5 mg/kg) was administered daily 3 days after surgery. Spinal cords were  
155 harvested 21-27 days after injection.

### 156 *Fluorogold Injections*

157 Two P32 *Atoh1*<sup>Cre/+</sup> TOM<sup>+</sup> female mice were injected with 4% (w/v) FG solution in saline  
158 (Fluorochrome). Mice were anesthetized with isoflurane and the area above and around the cerebellar  
159 region was prepared for surgery. A midline incision of 0.75 cm and a craniectomy of approximately 1  
160 mm wide by 1.5 mm long was performed. Bilateral injections at six sites were done at (from Bregma):  
161 rostrocaudal -5.5, -5.9, and -6.3 mm and at mediolateral  $\pm$  0.2-0.4 mm. At each site, several injections  
162 in 50.6 nL increments were performed every 300  $\mu$ m along the dorsoventral axis starting at -1.7 mm  
163 deep for a total of 720 nL of FG on each side in mouse #1, and 270 nL on each side in mouse #2.  
164 Animals were harvested 7 days after injection.

### 165 *Whole tissue imaging*

166 Mouse brainstem and spinal cords were processed following the SHIELD protocol (Park et al.,  
167 2018). Tissues were cleared with SmartClear II Pro (LifeCanvas Technologies, Cambridge, MA) for  
168 several days, mounted in a gel of 0.9% agarose in EasyIndex (LifeCanvas Technologies), and then  
169 incubated in EasyIndex for refractive index matching. Tissues were imaged at 3.6X using a SmartSPIM  
170 light sheet microscope (LifeCanvas Technologies). The *Chat*<sup>Hoxa4</sup> hindbrain sample (Movie 1) was  
171 imaged at 1.8  $\mu$ m x 1.8  $\mu$ m x 4  $\mu$ m resolution. The *Chat*<sup>Hoxa4</sup> spinal cord samples (Movies 2 and 3) were  
172 imaged at 1.8  $\mu$ m x 1.8  $\mu$ m x 2  $\mu$ m resolution. The hindbrain and spinal cord samples were cut to less  
173 than 2.2 cm to fit in the imaging chamber. The spinal cord samples were cut into two pieces (cervical-  
174 thoracic and thoracic-lumbar) and cleared separately. Movies were made in arivis Vision4D 2.12.6.

### 175 *Experimental Design and Statistical Tests*

176 For the percentage of CHAT<sup>+</sup> neurons in the IH and IF MN pools that were *Atoh1*-lineage TOM<sup>+</sup>  
177 neurons (Fig. 1D) and the estimated total number of CHAT<sup>+</sup> neurons in the IH and IF MN pools, IH data  
178 were counted from 3-4 sections per animal from n=2 female (F) mice and IF data were counted from 3-  
179 4 sections per animal from n=3 F mice from two different litters. For estimating the total number of

180 CHAT<sup>+</sup> neurons, counts from 4 MN pools left and right side were counted from sections that  
181 represented a tenth of the MN pool. Therefore, final estimates of the total number of CHAT<sup>+</sup> neurons  
182 were the final counts multiplied by ten. For the percentage of *Atoh1*<sup>Cre/+</sup> TOM<sup>+</sup> MNs that were MMP9<sup>+</sup>  
183 fast twitch MNs (Fig. 1F), IH and IF data were counted from 3 sections per animal from n=2 (1 F, 1  
184 male (M)) mice of the same litter. For the percentage of HB9<sup>+</sup> neurons that were *Atoh1*-lineage TOM<sup>+</sup>  
185 neurons over developmental time (Fig. 3B), IH and IF data were counted from 2-4 sections per animal  
186 from n=2 mice (from the same litter for P3 (gender not noted), P7 (2 F), and P15 (1 F, 1 M); two  
187 different litters for P23 (IH MN pool was from 1 unknown gender and 1 M, IF MN pool was from 1  
188 unknown gender and 1 F). For the percentage of *Atoh1*-lineage TOM<sup>+</sup> neurons that are ERR3<sup>+</sup> or  
189 NEUN<sup>+</sup> (Fig. 4C), IH and IF data were counted from 3 sections per animal from n=2 mice (1 F, 1 M)  
190 from the same litter. For *Chat*<sup>Hoxa4</sup> MNs, 4 sections per animal were counted from n=2 mice (2 M) from  
191 two different litters. For the *Chat*<sup>Cdx2</sup> MNs, 4 sections were counted from one female mouse. The  
192 *Chat*<sup>Hoxa4</sup> mouse processed with SHIELD by LifeCanvas Technologies was male.

193 No statistical tests were required as quantitation of the percentage of particular markers in any  
194 given MN pool were not directly compared to each other. For samples with n=2, the mean is shown  
195 with no standard error of the mean (SEM) since the range between the two data points equals the  
196 mean ± SEM.

## 197 **Results**

198 *Atoh1*<sup>Cre/+</sup> knock-in mice label MN pools involved in fine motor control.

199 We observed using CRE-lineage tracing strategies (*Atoh1*<sup>Cre/+</sup> knock-in mice (Yang et al., 2010)  
200 crossed to tdTomato (TOM) reporter mice (*R26*<sup>LSL-tdTom</sup>, Ai14)(Madisen et al., 2010)) that subsets of  
201 motor neurons (MNs) expressing choline acetyl transferase (CHAT) were labeled in the spinal cord  
202 (Fig. 1A, B; both arrows and arrowheads, CHAT<sup>+</sup>TOM<sup>+</sup>). Based on the anatomical location of the MN  
203 pools along the rostral-caudal axis labeled in *Atoh1*<sup>Cre/+</sup> mice, we predicted that the *Atoh1*<sup>Cre/+</sup> line  
204 labeled MNs of the intrinsic hand (IH) and foot (IF) in thoracic 1 (T1) and lumbar 6 (L6) areas of the  
205 spinal cord (Fig. 1C)(Watson et al., 2009). We tested our prediction by injecting the forepaw and



206 hindpaw with the retrograde tracer cholera toxin B conjugated to Alexa 488 (CTB-488), which labeled  
207 the IH and IF MN pools. We found that the *Atoh1*<sup>Cre/+</sup> TOM<sup>+</sup> MNs indeed labeled the IH and IF MN pools  
208 (Fig. 1A, B, arrows) and made up 60.0% (mean, range 53-67%, n=2) and 61.7% ± 0.9% SEM (mean,  
209 n=3) of the IH and IF MN pools, respectively (Fig. 1D)(See Experimental Design and Statistical Tests  
210 section of the Materials and Methods for details of quantitation throughout the article). We estimate that  
211 the total number of CHAT<sup>+</sup> MNs at P19 in the IH and IF MN pools on one side is IH 370 ± 63 SEM  
212 neurons (counts from 4 MN pools left and right side from n=2 mice) and IF 335 ± 5 SEM neurons  
213 (counts from 4 MN pools left and/or right side from n=3 mice). In addition, we found that a subset of the  
214 TOM<sup>+</sup> MNs were fast twitch MNs (Fig. 1E-F, arrows, MMP9<sup>+</sup>TOM<sup>+</sup>; IH 47.0% (mean, range 38-56%,  
215 n=2) and IF 76.5% (mean, range 74-79%, n=2))(Kaplan et al., 2014). Note that the other TOM<sup>+</sup> cell  
216 bodies in the intermediate spinal cord are from other *Atoh1*-lineage interneurons involved in the  
217 proprioceptive system (Yuengert et al., 2015).

218 To see whether the labeling of *Atoh1*<sup>Cre/+</sup> TOM<sup>+</sup> MNs was specific to the IH and IF MN pools, we  
219 injected CTB-488 into the tibialis anterior (TA) and gastrocnemius (GS) muscles and found that those  
220 MN pools did not have any TOM<sup>+</sup> MNs (Fig. 2A, B, arrowheads). In addition, we sampled sections  
221 throughout the rostral-caudal axis of the spinal cord in *Atoh1*<sup>Cre/+</sup> mice. We found that other MN pools  
222 had TOM<sup>+</sup> expression (Fig. 2C, arrows, CHAT<sup>+</sup>TOM<sup>+</sup>). However, the TOM<sup>+</sup> MN labeling was enriched in  
223 the IH and IF MN pools (Fig. 2C, C8-T1 and L5-6, yellow dashed lines).

224 *Atoh1*<sup>Cre/+</sup> knock-in mice label IH and IF MN pools postnatally.

225 Given that MNs are derived from an *Olig2*-expressing progenitor domain in the ventral neural  
226 tube (Lu et al., 2002; Lai et al., 2016), we tested whether the *Atoh1*<sup>Cre/+</sup> line was labeling IH and IF MNs  
227 early in embryonic development, which would suggest co-expression with the *Olig2*-expressing domain,  
228 or if there was a previously unreported late expression of *Atoh1* in the IH and IF MNs. We found that at  
229 embryonic day 14.5 (E14.5) when the IH and IF MNs first start expressing the unique markers Copine-4  
230 (CPNE4) and Fidgetin (FIGN) (Mendelsohn et al., 2017), the IH and IF MN pools were not yet TOM<sup>+</sup>  
231 (Fig. 3C, C'). In contrast, at postnatal time points, we found that colocalization of TOM<sup>+</sup> MNs in the IH

232 and IF MN pools with the homeobox transcription factor, HB9, marking MN pools, started around P3 (IH  
233 28% (range 25-30%), IF 20% (range 18-21%), mean, n=2) and gradually increased to about 70-80% by  
234 P15 (P7: IH not quantitated (NQ), IF 44% (range 40-48%); P15: IH 83% (range 82-84%), IF 72% (range  
235 71-73%); P23: IH 71% (range 64-77%), IF 65% (range 64-66%); mean, n=2)(Fig. 3A-A''', arrows, and  
236 Fig. 3B). In addition, we confirmed that the IF TOM<sup>+</sup> MNs colocalized with the specific markers CPNE4  
237 and FIGN postnatally (Fig. 3D, D'). To detect postnatal *Atoh1* expression in the IH and IF MNs, we  
238 performed *in situ* hybridization (Gowan et al., 2001 for ISH probe) and RNAscope of *Atoh1* at P14-P15  
239 and P22, but were unable to detect any signal at the mRNA level (unpublished observations). Taken  
240 together, it is likely that the IH and IF MN pools derive from an *Olig2*-expressing progenitor domain and  
241 are labeled postnatally in the *Atoh1*<sup>Cre/+</sup> line.

242 *IH and IF MN pools labeled with Atoh1*<sup>Cre/+</sup> *knock-in mice are both  $\alpha$ - and  $\gamma$ -MNs.*

243 Because *Atoh1*<sup>Cre/+</sup> TOM<sup>+</sup> MNs are enriched in only a subset (~60%) of IH and IF MNs, we  
244 tested whether they were demarcating a specific type of MN ( $\alpha$  or  $\gamma$ ).  $\alpha$ -MNs innervate the striated  
245 extrafusal muscle, are marked by the neuronal marker, NEUN, and receive vesicular glutamate  
246 transporter 1 (VGLUT1<sup>+</sup>) proprioceptive inputs (Friese et al., 2009; Manuel and Zytnicki, 2011; Ashrafi  
247 et al., 2012).  $\gamma$ -MNs innervate the intrafusal muscle spindles and express Estrogen Related Receptor  
248 gamma (ERR3<sup>+</sup>)(Friese et al., 2009). Immunostaining for  $\alpha$ - and  $\gamma$ -MN markers, we found that *Atoh1*<sup>Cre/+</sup>  
249 TOM<sup>+</sup> MNs are a mixture of both  $\alpha$ - and  $\gamma$ -MNs (Fig. 4A, B, NEUN<sup>+</sup>TOM<sup>+</sup> and ERR3<sup>+</sup>TOM<sup>+</sup>, arrows).  
250 The NEUN<sup>+</sup> *Atoh1*<sup>Cre/+</sup> TOM<sup>+</sup> MNs also received VGLUT1<sup>+</sup> proprioceptive inputs (Fig. 4A). Previous  
251 reports found approximately 30% of MNs are  $\gamma$ -MNs and 70% are  $\alpha$ -MNs (Friese et al., 2009). We  
252 found that the *Atoh1*<sup>Cre/+</sup> TOM<sup>+</sup> MNs were IH 23.0% (range 21-25%) and IF 9.5% (range 9-10%)  $\gamma$ -MNs  
253 (ERR3<sup>+</sup>TOM<sup>+</sup>/TOM<sup>+</sup>) and IH 86.5% (range 85-88%) and IF 89.5% (range 79-100%)  $\alpha$ -MNs  
254 (NEUN<sup>+</sup>TOM<sup>+</sup>/TOM<sup>+</sup>)(Fig. 4C, mean, n=2, note that ERR3 and NEUN counts were performed on  
255 different sections). Our results suggest a slight enrichment of  $\alpha$ -MNs in the IH and IF MN pools that  
256 may reflect endogenous differences in  $\alpha$ - and  $\gamma$ -MN distribution in IH and IF MN pools or a preference  
257 for labeling  $\alpha$ -MNs in the *Atoh1*<sup>Cre/+</sup> mouse line. Furthermore, imaging of the hindpaw lumbrical muscle

258 found TOM<sup>+</sup> axons innervating both the extrafusal (Fig. 4D', arrows) and intrafusal muscle (Fig. 4D'',  
259 arrows). Bungarotoxin (BTX<sup>+</sup>) identifies the neuromuscular junctions (NMJs) and syntaxin (STX1<sup>+</sup>)  
260 identifies the muscle spindle (Fig. 4D, open arrowhead) and NMJs. Note that not all NMJs are TOM<sup>+</sup>  
261 (Fig. 4D'-D'', arrowheads) consistent with the fact that only ~60% of the IH and IF MN pools are TOM<sup>+</sup>.  
262 *Ascending projections from caudal cholinergic neurons.*

263 The *Atoh1* transcription factor is known to specify many cerebellar-projecting neurons (Rose et  
264 al., 2009). Given the labeling of the IH and IF MNs in the *Atoh1*<sup>Cre/+</sup> mouse line, we wanted to test  
265 whether IH and IF MNs could send ascending axon collaterals, potentially to the cerebellum. To this  
266 end, we pursued two intersectional genetic strategies to label caudal cholinergic neurons (Fig. 5A, G).

267 The first cross used the *Hoxa4::Cre* and *Chat*<sup>RES-FLPo</sup> alleles crossed to the intersectional  
268 tdTomato reporter (*R26*<sup>LSL-FSF-tdTomato</sup>, Ai65)(hereafter called *Chat*<sup>Hoxa4</sup> neurons)(Madisen et al., 2015).  
269 *Hoxa4::Cre* is expressed in regions caudal to the developing rhombomeres 6/7, which corresponds to  
270 the caudal medulla (Huang et al., 2012; Yuengert et al., 2015), and the *Chat*<sup>RES-FLPo</sup>, generated by Gord  
271 Fishell and Rob Machold (unpublished), is expressed in cholinergic neurons. Therefore, this cross  
272 should label all cholinergic neurons caudal to the lower medulla including spinal cord. We found that  
273 approximately 12.5% (mean, range 10-15%, n=2) of CHAT<sup>+</sup> MN pools were labeled in this cross (Fig.  
274 5B, representative images). When we analyzed the hindbrain sections of two samples, we found  
275 consistent axonal labeling in the intermediate reticular nucleus (IRt), facial nucleus (7N), spinal  
276 trigeminal nucleus (Sp5), and ambiguous nucleus (Amb)(Fig. 5C, orange and green triangles). The  
277 processes in the IRt were cholinergic as the TOM<sup>+</sup> processes colocalized with the vesicular  
278 acetylcholine transporter (VACHT) antibody (Fig. 5F-F', arrows). For Sample 1, we found TOM<sup>+</sup>  
279 processes in the vermis of folia III and paraflocculus (PFI). Curiously, these processes did not express  
280 excitatory marker vesicular glutamate transporter 2, VGLUT2 (VG2), and resided between the NEUN<sup>+</sup>  
281 granule cells (Fig. 5D, E). We were unable to assess whether these processes were cholinergic  
282 because we did not get reliable VACHT antibody staining in the granule cell layer of the cerebellum.  
283 Other areas of the hindbrain that had TOM<sup>+</sup> processes are annotated for both samples in Fig. 5C. We

284 note that axon terminations of sensory neurons labeled in the superficial dorsal horn of *Chat*<sup>Hoxa4</sup> mice  
285 can be seen (Fig. 5B, arrowheads) and are likely due to transient expression of *Chat*<sup>JRES-FLPo</sup> because  
286 *Chat* is not highly expressed in sensory neurons (Sharma et al., 2020).

287 To obtain a three-dimensional view of the axonal trajectories, we cleared the spinal cord and  
288 brain of a *Chat*<sup>Hoxa4</sup> mouse using SHIELD (Park et al., 2018) and imaged with light sheet microscopy. In  
289 this cleared sample, MNs and other cholinergic neurons in the spinal cord such as the sympathetic pre-  
290 ganglionic nucleus (SPN) and V<sub>0c</sub> neurons are labeled (Zagoraiou et al., 2009; Deuchars and Lall,  
291 2015)(Movies 2 and 3). When examining the hindbrain for axonal projections, we found almost no  
292 labeling except for some asymmetric TOM<sup>+</sup> labeling in Crus I and Crus II on only one side of the  
293 cerebellum of unclear origin (Fig. 5C, purple triangles, Movie 1). The cleared *Chat*<sup>Hoxa4</sup> sample had cell  
294 bodies labeled in what appears to be the accessory facial nerve (acs7) and facial nerve (7N)(Movie 1)  
295 and had some processes in Sp5. In the spinal cord of the *Chat*<sup>Hoxa4</sup> cleared sample, we found most MNs  
296 had axonal projections heading to the ventral root (Movies 2 and 3). However, occasionally, axons  
297 heading rostrally were seen, particularly in the cervical-thoracic cleared spinal cord (Movie 2) where  
298 one prominently labeled axon was seen on the left side of the spinal cord (right side of the image).  
299 When following the fluorescence of this axon, it appeared to have originated from the contralateral side  
300 of the spinal cord, but the fluorescence disappeared making it impossible to attribute this axon to any  
301 given neuron.

302 Due to the sparse labeling of MNs in the *Chat*<sup>Hoxa4</sup> mice, it was difficult to conclude whether a  
303 lack of consistent axonal projections in the cerebellum was due to a lack of ascending projections or  
304 lack of sufficient labeling. Therefore, we pursued a second intersectional cross using *Chat*<sup>JRES-Cre</sup> and  
305 *Cdx2::FLPo* crossed to the intersectional Ai65 tdTomato reporter (hereafter called *Chat*<sup>Cdx2</sup> neurons).  
306 *Chat*<sup>JRES-Cre/+</sup> labels all cholinergic cells and cells with transient *Chat* expression (Rossi et al., 2011;  
307 Nasirova et al., 2020) while the *Cdx2::FLPo* labels all cells caudal to the mid-cervical area of the spinal  
308 cord (Bourane et al., 2015). We found that in the spinal cord, labeling of MNs was much more robust  
309 with 92% of the MNs being labeled (n=1) (Fig. 5H). Similar to the *Chat*<sup>Hoxa4</sup> mice, we saw axonal

310 projections from sensory neurons in the dorsal horn indicating some transient expression of *Chat*<sup>JRES-Cre</sup>  
311 in sensory neurons (Fig. 5H, arrowhead). In this mouse, we found many mossy fiber-like terminations in  
312 vermis II and III (Fig. 5I-I', J-J'). In addition, we found prominent TOM<sup>+</sup> processes in the medial part of  
313 the facial nerve (7N)(Fig. 5J'' and insets, arrowhead) that were not cholinergic (VACHT<sup>-</sup>).

314 We note here that we pursued a third intersectional cross of *Atoh1*<sup>Cre/+</sup> and *Chat*<sup>JRES-FLPo</sup> alleles  
315 (unpublished). This cross labeled known *Atoh1*-lineage cholinergic neurons in the pedunculopontine  
316 tegmentum (PPTg) and the lateral dorsal tegmentum (LDTg)(unpublished and Rose et al., 2009).  
317 However, PPTg neurons have been shown to project to deep cerebellar nuclei (Woolf and Butcher,  
318 1989; Jaarsma et al., 1997), thus, confounding any interpretation of potential *Atoh1*-lineage cholinergic  
319 projections from the spinal cord.

#### 320 *Infection of Purkinje cells by injection of viruses in the periphery.*

321 As an alternative strategy to test whether IF MNs could send ascending axon collaterals, we  
322 sought to isolate specifically the IF MN pool and trace their arborizations. We reasoned that injection of  
323 CRE viruses into the hindpaw of tdTomato (TOM) reporter mice (*R26*<sup>LSL-tdTom</sup>, Ai14)(Madisen et al.,  
324 2010) would infect the MN axons allowing for TOM expression in the entire MN. If any ascending axon  
325 collaterals existed, we would see TOM<sup>+</sup> labeling in the cerebellum. We performed multiple experiments  
326 using both AAV and Lentivirus (AAV8-GFP-Cre and Lenti<sup>FugE</sup>-Cre) at early (P4-5) and later (P14-15)  
327 time points with 21-27 days allowed for expression (Fig. 6). We chose to use AAV8, which was reported  
328 to have sparse infection of the MNs at P1 (Foust et al., 2008). Sparse labeling of MNs would allow us to  
329 trace axonal trajectories. In addition, although AAVs have been reported to cross the blood-brain barrier  
330 (BBB) at early postnatal stages, those experiments were performed with intraperitoneal or intravenous  
331 infection, whereas we were targeting specifically the hindpaw area as well as later time points (P14-15)  
332 (Foust et al., 2008; Foust et al., 2009; Gray et al., 2011; Zhang et al., 2011). Furthermore, we tested  
333 infection with Lentivirus, which to the best of our knowledge, is not known to cross the BBB.

334 In all the injections, consistent with infection of MN axons innervating the hindpaw, we found a  
335 small number of TOM<sup>+</sup> neurons in the IF MN pool (Fig. 6A, B, C, D, E, arrows). In the cerebellum, we

336 found that Purkinje cells expressed tdTomato upon unilateral injection of viruses into the hindpaw. In all  
337 cases, axonal arborizations in the dentate nucleus could be seen (Fig. 6A'', B'', C'', D'', E''). In some  
338 cases axons could be seen traveling to the dentate (Fig. 6B', C', E', arrowheads) and a Purkinje cell  
339 (PC) cell body was labeled (Fig. 6E', arrowhead). In general, PC labeling was quite sparse except in  
340 one case where many PCs were labeled on the same side as the injected hindpaw and the axons  
341 projecting to the dentate were on the contralateral side to the injected hindpaw (Fig. 6D'-D'''). In the two  
342 cases where we preserved the orientation of the cerebellum relative to the injection site, we found that  
343 the axons in the dentate were on the side contralateral to the hindpaw injection site (Fig. 6D''', E''').  
344 Altogether, using viral tracing strategies, we could label the IF MNs; however, axons seen in the  
345 cerebellum appeared to come from infected PCs on the periphery of the cerebellum.

#### 346 *Retrograde labeling from the cerebellum.*

347 Lastly, to test whether IF MNs could send axons to the cerebellum, we injected the retrograde  
348 tracer Fluorogold (FG) into the vermis of folia I-V in the cerebellum (Fig. 7A-B). In the IF MN pool of a  
349 FG injected mouse, none of the *Atoh1*-lineage MNs (TOM<sup>+</sup>CHAT<sup>+</sup>) were co-labeled with FG  
350 (arrowheads)(Fig. 7C). Notably, other FG labeled cells in the ventral spinal cord, some of which have a  
351 large cell body morphology like MNs, are not cholinergic (FG<sup>+</sup>CHAT<sup>-</sup>)(Fig. 7D''-D''', arrowheads). The  
352 FG-retrograde labeling worked successfully, though, as other cerebellar-projecting neurons in the  
353 medulla and spinal cord, namely, the external cuneate nucleus (ECu), lateral reticular nucleus (LRt),  
354 inferior olive (IO), and Clarke's column (CC)(Fig. 7B, D) were labeled. Furthermore, consistent with our  
355 previous findings (Yuengert et al., 2015), an occasional CC cell is *Atoh1*-lineage (FG<sup>+</sup>TOM<sup>+</sup>, Fig. 7D',  
356 arrow). Interestingly, only one of the cells in the cluster of retrogradely-labeled cells lateral to CC is  
357 *Atoh1*-lineage (FG<sup>+</sup>TOM<sup>+</sup>, Fig. 7D'', arrow) indicating that few of the *Atoh1*-lineage neurons target Folia  
358 I-V in the cerebellar vermis. Two female mice were injected with FG with similar results, so a  
359 representative animal (mouse #2) is shown in Fig. 7.

#### 360 **Discussion**

361 The impetus for this study stemmed from the serendipitous finding that IH and IF MNs are  
362 labeled in *Atoh1<sup>Cre/+</sup>* mice. This finding led to two lines of inquiry: the development as well as the  
363 potential novel connectivity of IH and IF MNs.

364 *IH and IF MNs have a unique developmental history*

365 We found that *Atoh1<sup>Cre/+</sup>* mice label the IH and IF MNs consistent with these MN pools having a  
366 unique developmental history. Other MN pools such as the gastrocnemius and the tibialis anterior are  
367 not enriched in the *Atoh1<sup>Cre/+</sup>* mouse line indicating that the IH and IF MN pools have distinct molecular  
368 processes that allow for tdTomato expression. We were able to isolate labeling of the IH and IF MNs in  
369 the *Atoh1<sup>Cre/+</sup>* mouse line to the postnatal time period, although the levels of *Atoh1* mRNA at the times  
370 tested were below detectable limits. However, the consistent and robust labeling of IH and IF MNs in  
371 the *Atoh1<sup>Cre/+</sup>* mouse line indicates that these MNs have a unique developmental program compared to  
372 the neighboring LMC MN pools that are not labeled.

373 Although *Atoh1<sup>Cre/+</sup>* is enriched in the IH and IF MN pools, the cell type of the MNs labeled by  
374 the *Atoh1<sup>Cre/+</sup>* mouse line is heterogeneous. We found that *Atoh1<sup>Cre/+</sup>* TOM<sup>+</sup> IH and IF MN pools  
375 represent a mixture of  $\alpha$ - and  $\gamma$ -MNs and with only a subset expressing a marker for fast twitch MNs.  
376 The *Atoh1<sup>Cre/+</sup>* TOM<sup>+</sup> axons innervate both the intrafusal and extrafusal muscle of the hindpaw lumbrical  
377 muscle consistent with both  $\alpha$ - and  $\gamma$ -MNs being labeled. It is also possible, though, that *Atoh1<sup>Cre/+</sup>*  
378 TOM<sup>+</sup> MNs are labeling  $\beta$ -MNs.  $\beta$ -MNs are MNs whose axons bifurcate to simultaneously innervate  
379 both intrafusal and extrafusal muscle (Manuel and Zytnicki, 2011). Currently, to the best of our  
380 knowledge, there are no known molecular markers for  $\beta$ -MNs that would allow us to test whether the  
381 *Atoh1<sup>Cre/+</sup>* TOM<sup>+</sup> MNs are  $\beta$ -MNs. Interestingly, one of the earliest descriptions of  $\beta$ -MNs in mammalian  
382 systems was from the lumbrical muscle of cats (Bessou et al., 1965) and could be a feature of IH and  
383 IF MNs. It is enticing to speculate that the MNs involved in fine motor skill would have  $\beta$ -MNs as a  
384 feature because functionally,  $\beta$ -MNs would allow for simultaneous contraction of intrafusal and  
385 extrafusal muscle, thus resetting the muscle spindle to respond immediately to stretch activation  
386 (Manuel and Zytnicki, 2011).

387 *MNs as a source of corollary discharge*

388       The finding that *Atoh1*, a transcription factor that specifies many cerebellar-projecting neurons,  
389 could be enriched in the IH and IF MNs led to the intriguing hypothesis that *Atoh1* might act in these  
390 MNs to send axon collaterals to the cerebellum. A model by which MNs themselves could send a copy  
391 of their motor information directly back to the cerebellum or other hindbrain areas was enticing. Such a  
392 model would suggest that motor information, in particular fine motor information, would need to be  
393 immediately sent back to the cerebellum as a means of updating the proprioceptive sensory system  
394 about self-generated movements. Furthermore, having a corollary discharge pathway specifically from  
395 digit-innervating MNs might make temporal sense given that fine motor movements would need more  
396 rapid updating of the sensory-motor system compared to whole limb movements. We attempted to test  
397 the hypothesis that IH and IF MNs could send ascending axon collaterals using intersectional genetics,  
398 viral labeling, and retrograde labeling strategies, but were unable to find evidence to support this idea.

399 *Intersectional genetic labeling of caudal cholinergic neurons*

400       To label caudal cholinergic neurons, we pursued two intersectional strategies generating  
401 *Chat<sup>Hoxa4</sup>* and *Chat<sup>Cdx2</sup>* mice, both of which had limitations. The labeling of MNs in the *Chat<sup>Hoxa4</sup>* mice  
402 was too sparse (12.5%) to ensure that the IH and IF MN pools were sufficiently labeled. The lack of  
403 robust MN labeling in the *Chat<sup>Hoxa4</sup>* mice might be due to the dorsal-enriched expression of *Hoxa4::Cre*  
404 that would miss ventrally generated neuronal populations (Yuengert et al., 2015). In contrast, the  
405 *Chat<sup>Cdx2</sup>* mice labeled almost all the MNs (92%), but also labeled many other neurons with potentially  
406 ascending projections in the spinal cord likely due to transient *Chat* expression (Nasirova et al., 2020).  
407 In both mice, other cholinergic neurons in the spinal cord (SPN, *V<sub>oc</sub>*) were labeled, although these  
408 should not have any ascending projections (Zagoraïou et al., 2009; Deuchars and Lall, 2015).

409       Taking both *Chat<sup>Hoxa4</sup>* and *Chat<sup>Cdx2</sup>* mice together, the only places that had common hindbrain  
410 projections were in folia III of the cerebellar vermis and in the facial nerve nucleus (7N). The projections  
411 to the facial nucleus were not cholinergic (Fig. 5J") and are likely evidence of transient cholinergic  
412 expression in these neurons that become glutamatergic in adulthood (Nasirova et al., 2020). The



413 projections to folia III are of unknown neurotransmitter status as antibodies for VACHT do not work well  
414 in the cerebellar granule cell layer and these TOM<sup>+</sup> projections in *Chat*<sup>Hoxa4</sup> mice did not express  
415 VGLUT2. Therefore, it is difficult to identify the origin of the projections to folia III and it is possible that  
416 they also come from cells that transiently express *Chat*. Note the many TOM<sup>+</sup> cells in the spinal cord  
417 that do not colocalize with CHAT antibody (Fig. 5H) representing transiently expressing *Chat* neurons.

418 As for other ascending projections in either the *Chat*<sup>Cdx2</sup> or *Chat*<sup>Hoxa4</sup> mouse, the TOM<sup>+</sup>  
419 processes in IRt are not as prominent in the *Chat*<sup>Cdx2</sup> mouse as in the *Chat*<sup>Hoxa4</sup> mouse indicating that  
420 these cholinergic IRt processes in the *Chat*<sup>Hoxa4</sup> mice might be from cells labeled between the caudal  
421 medulla and mid-cervical spinal cord. This would suggest that the cholinergic innervation of IRt neurons  
422 involved in respiration might come from the cervical spinal cord area (Anderson et al., 2016; Nasirova  
423 et al., 2020). In the *Chat*<sup>Hoxa4</sup> mice, we found areas of the medulla that were previously reported to  
424 contain cholinergic projections in *Chat*<sup>JRES-Cre/+</sup> mice (facial nucleus (7N), spinal trigeminal nucleus  
425 (Sp5), ambiguus nucleus (Amb), and paraflocculus (PFI))(Nasirova et al., 2020) and were able to  
426 isolate these projections as originating from cell bodies caudal to the lower medulla. Furthermore, to get  
427 a three-dimensional view of MN axonal trajectories that could be missed in thin cryosections, we  
428 cleared the spinal cord and hindbrain of a *Chat*<sup>Hoxa4</sup> mouse. In the *Chat*<sup>Hoxa4</sup> mouse, we saw one  
429 ascending projection in the spinal cord and some faint asymmetric TOM<sup>+</sup> labeling in right Crus I and II,  
430 but could not follow the axons of these to identify the originating cell. Altogether, even though we saw  
431 some projections in cerebellar vermis III in both, *Chat*<sup>Hoxa4</sup> and *Chat*<sup>Cdx2</sup> mice, it remains ambiguous as  
432 to whether these projections originated from MNs due to the large number of transiently-expressing  
433 *Chat* neurons that were labeled.

#### 434 *Viral injections in the periphery infect Purkinje cells in the CNS*

435 To analyze the projections specifically of IF MNs, we injected CRE AAV and Lentiviruses into  
436 the hindpaw of CRE-dependent TOM reporter mice. While this resulted in sparse labeling of the IF MN  
437 pool, when we analyzed the cerebellum, we found that Purkinje cells (PCs) were also labeled. The  
438 viruses were injected into the hindpaw where they presumably infected muscle, nerve, and blood

439 vessels. For two animals where the orientation was recorded, the PCs contralateral to the injected  
440 hindpaw were infected.

441         There are at least two potential explanations for how peripheral injection of viruses infect PCs in  
442 the central nervous system (CNS). One possible explanation is that CRE-expressing AAVs are  
443 “hopping” one synapse in an anterograde fashion as has been previously reported (Zingg et al., 2017).  
444 In this scenario, the CRE AAV could potentially reflect a connection between the initially infected MN  
445 and PCs in the cerebellum. We think this possibility is unlikely given that one mouse had a PC labeled  
446 on the edge of Crus I (Fig. 6E') and another on the edge of paraflocculus (Fig. 6B') indicating that the  
447 infection is random. In addition, we saw the same infection of PCs using a CRE-expressing lentivirus,  
448 which has not been reported to travel transynaptically. A second possible explanation is that the viruses  
449 can travel across the blood brain barrier (BBB) to preferentially infect PCs in the CNS. This would  
450 explain why several PCs were labeled in one mouse on both the contralateral and ipsilateral sides and  
451 yet we do not see widespread infection of other cell types (Fig. 6D').

452         Several AAV serotypes including AAV8 have been reported to cross the BBB, particularly at  
453 early postnatal stages (P1) when the BBB is not yet fully developed (Foust et al., 2008; Foust et al.,  
454 2009; Gray et al., 2011; Zhang et al., 2011). AAV8 was reported to sparsely label MNs, which we  
455 thought would work to our advantage in tracing single neuronal axons (Foust et al., 2008). In addition,  
456 we reasoned that local delivery to the hindpaw, rather than the more global intraperitoneal and  
457 intravenous injections described previously, would limit crossing the BBB. However, we found that  
458 infection of the CNS using AAV8 and Lentivirus at postnatal stages slightly later than P1 (namely P4-5  
459 and P14-15) can cross the BBB sparsely infecting PCs. We present the data here as a warning for  
460 future studies looking a peripheral to CNS connectivity.

461 *Reconciling spinocerebellar retrograde tracing studies.*

462         Several retrograde-labeling studies of spinocerebellar neuron in cats, rats, opossum, and  
463 monkeys have identified cerebellar-projecting cells in spinal cord lamina IX where MNs reside (Cooper  
464 and Sherrington, 1940; Matsushita and Hosoya, 1979; Matsushita et al., 1979; Terman et al., 1998).

465 Together with our findings that *Atoh1*-lineage neurons labeled IF MNs, we tested the hypothesis that IF  
466 MNs could send ascending projections to the cerebellum. However, our injections of FG into the  
467 cerebellar vermis did not label the IF MN pool (Fig. 7). Furthermore, cerebellar-projecting cells in the  
468 ventral spinal cord, some of which had a large cell body morphology similar to MNs, were not  
469 cholinergic. Therefore, the developmental origin of these ventral cerebellar-projecting cells remains  
470 unknown. Although evidence for IF MNs sending axon collaterals to the cerebellum is not supported by  
471 our data, it is possible that potential ascending projections terminate in other areas of the cerebellum or  
472 hindbrain that were not targeted in our FG injections.

473 Altogether, we present here that the *Atoh1*<sup>Cre/+</sup> mouse consistently labels MNs of the IH and IF  
474 and that the *Atoh1*<sup>Cre/+</sup> mouse could be used to specifically label these populations. Whether the IH and  
475 IF MNs send axon collaterals to the cerebellum as a mechanism of corollary discharge, as  
476 hypothesized, remains an open question. We tested this hypothesis using three distinct approaches:  
477 intersectional genetics, viral labeling, and retrograde labeling, but were unable to find evidence to  
478 support the idea that IH and IF MNs send axon collaterals to the cerebellum.

## 479 **Legends**

480 **Figure 1. The *Atoh1*<sup>Cre/+</sup> knock-in mouse line labels the intrinsic hand (IH) and foot (IF) motor**  
481 **neuron (MN) pools.** (A, B) Injection of the retrograde tracer CTB-488 into the forepaw and hindpaw  
482 labels the IH and IF MN pools, which are labeled with tdTomato (TOM) when the TOM reporter mouse  
483 is crossed to the *Atoh1*<sup>Cre/+</sup> knock-in mouse. Arrows, CTB<sup>+</sup> CHAT<sup>+</sup> TOM<sup>+</sup>; arrowheads, CTB<sup>-</sup> CHAT<sup>+</sup>  
484 TOM<sup>+</sup>. (C) Diagram of motor neuron pools at T1 and L6. (D) Quantitation of the percentage of the IH or  
485 IF MN pools that are labeled TOM<sup>+</sup> in *Atoh1*<sup>Cre/+</sup> knock-in mice. (E-F) Some of the TOM<sup>+</sup> IH MNs and IF  
486 MNs are fast twitch MNs (MMP9<sup>+</sup>). MMP9<sup>+</sup>TOM<sup>+</sup> arrows; MMP9<sup>+</sup>TOM<sup>+</sup> arrowheads. See text for values  
487 in D, F. Christopher Reeve Atlas referenced for spinal cord MN pools (Watson et al., 2009). Abbrev: P,  
488 postnatal; C, cervical; T, thoracic; L, lumbar; Tr, triceps; Pec, pectoral; Ax, axial; Hm, hamstring; Gl,  
489 gluteus; ExA, external anal sphincter; TA, tibialis anterior; GS, gastrocnemius. Scale bars: 100  $\mu$ m.

490 **Figure 2. The *Atoh1*<sup>Cre/+</sup> knock-in mouse line does not label the TA and GS MN pools and**  
491 **sparsely labels other MN pools.** (A, B) Injection of CTB-488 into the TA and GS shows no labeling  
492 (TOM<sup>+</sup>, arrowheads) of these MN pools in the *Atoh1*<sup>Cre/+</sup> knock-in mouse. (C) Representative images  
493 throughout the rostral-caudal axis of *Atoh1*<sup>Cre/+</sup> knock-in mice crossed to the TOM reporter mouse show  
494 that TOM labels MNs mainly in IH and IF (yellow dashed lines) with sparser labeling of MNs in other  
495 MN pools (arrows). Some MN pools have no TOM<sup>+</sup> expression (arrowheads). Christopher Reeve Atlas  
496 referenced for spinal cord MN pools (Watson et al., 2009). Abbrev: IML, intermediolateral nucleus; ICo,  
497 intercostal; ThAb, thoracic abductor; Q, quadriceps; Ad, adductor; Ps, psoas. Scale bars: 100  $\mu$ m.

498 **Figure 3. The *Atoh1*<sup>Cre/+</sup> knock-in mouse labels IH and IF postnatally.** (A-A'') TOM<sup>+</sup> labeling of the  
499 IF MN pool at several postnatal time points. HB9<sup>+</sup>TOM<sup>+</sup> neurons, arrows. In A, CTB (blue) was injected  
500 into the hindpaw to identify the IF MN pool. (B) Quantitation of percentage of TOM<sup>+</sup> neurons in the IH or  
501 IF MN pools at several time points. (C-C') At E14.5 (embryonic day 14.5), TOM<sup>+</sup> neurons are CPNE4<sup>-</sup>  
502 and FIGN<sup>-</sup>. (D-D') At P6, TOM<sup>+</sup> neurons are CPNE4<sup>+</sup> and FIGN<sup>+</sup> (arrows). Scale bars: 100  $\mu$ m.

503 **Figure 4. Both  $\alpha$ - and  $\gamma$ -MNs are labeled in the *Atoh1*<sup>Cre/+</sup> knock-in mouse.** (A) TOM<sup>+</sup> MNs in the IF  
504 MN pool are NEUN<sup>+</sup> (arrows) and have closely apposed VGLUT1<sup>+</sup> boutons (grey arrows). (B) Some  
505 TOM<sup>+</sup> IF MNs are also ERR3<sup>+</sup> (arrow). (C) Percentage of the TOM<sup>+</sup> MNs in the IH and IF that are  
506 ERR3<sup>+</sup> ( $\gamma$ -MN marker) or NEUN<sup>+</sup> ( $\alpha$ -MN marker). (D-D'') TOM<sup>+</sup> axons in the hindpaw lumbrical muscle  
507 show the neuromuscular junction innervating extrafusal muscle (D', arrows, BTX<sup>+</sup>STX1<sup>+</sup>TOM<sup>+</sup>). TOM<sup>+</sup>  
508 axons also innervate the intrafusal muscle spindle (D, open arrowhead; D'', arrows, BTX<sup>+</sup>STX1<sup>+</sup>TOM<sup>+</sup>).  
509 Arrowheads indicate motor endplates that are TOM<sup>-</sup>. Scale bars: 100  $\mu$ m, inset in A is 10  $\mu$ m.

510 **Figure 5. Intersectional genetic labeling of caudal cholinergic neurons finds ascending**  
511 **projections to the cerebellum.** (A) Diagram of *Chat*<sup>Hoxa4</sup> intersectional cross that labels cholinergic  
512 neurons caudal to the medulla. (B) Sparse TOM<sup>+</sup> (arrows) labeling of MNs (CHAT<sup>+</sup>, green) in *Chat*<sup>Hoxa4</sup>  
513 mice. Axonal terminations from sensory neuronal labeling are seen in the superficial dorsal horn  
514 (arrowheads). (C) Summary of data from three samples of where TOM<sup>+</sup> processes are seen in the  
515 hindbrain. Number of sections (sect) analyzed is stated. (D) Representative image of TOM<sup>+</sup> process

516 (arrow) seen in vermis III that is VGLUT2 (VG2) negative. Maximum Intensity Projection (MIP) of 21  
517  $\mu\text{m}$ . (E) Representative image of TOM<sup>+</sup> process (arrows) in PFI that is VG2<sup>-</sup> and courses between the  
518 NEUN<sup>+</sup> granule cells. Maximum Intensity Projection (MIP) of 30  $\mu\text{m}$ . (F-F') Processes in IRt express  
519 VACHT (arrows). (G) Diagram of *Chat*<sup>Cdx2</sup> intersectional cross that labels cholinergic neurons caudal to  
520 the mid-cervical spinal cord. (H) Caudal cholinergic neurons are well-labeled in *Chat*<sup>Cdx2</sup> mice (arrows,  
521 TOM<sup>+</sup>CHAT<sup>+</sup>). Note that axonal terminations from sensory neuronal labeling are also seen in the  
522 superficial dorsal horn (arrowheads). (I-I', J-J') TOM<sup>+</sup> processes are seen in the folia II and III vermis of  
523 *Chat*<sup>Cdx2</sup> mice. TOM<sup>+</sup> processes are also seen around the facial nerve (7N)(arrowhead). Processes in  
524 the medial facial nerve area do not colocalize with VACHT antibody (J''). Brain pictures taken from  
525 Mouse Brain Atlas (Paxinos and Franklin, 2007). Abbrev: SPN, sympathetic preganglionic nucleus; 7N,  
526 facial nerve; Int, interpositus; IRt, intermediate reticular nucleus; Sp5, spinal trigeminal nucleus; Amb,  
527 ambiguous nucleus; PFI, paraflocculus; PML, paramedian lobule. Scale bars: 100  $\mu\text{m}$  unless otherwise  
528 noted; D, E, F', J'' insets are 10  $\mu\text{m}$ ; F and I are 1 mm.

529 **Figure 6. Peripheral injections of viruses infect Purkinje cells in the cerebellum.** (A-B'') Injection  
530 of AAV8-GFP-Cre at P4 or P5 into the hindpaw of two representative animals (*R26*<sup>LSL-tdTom</sup>) and  
531 harvested 21 days later shows TOM fluorescence within the IF MN pool indicating infection and  
532 recombination (A, B, arrows). Cerebellar sections show axonal processes in the dentate nucleus (A'-  
533 B''). Labeling of a long-projecting axon can be seen (B', arrowheads). (C-D''') Injection of AAV8-GFP-  
534 Cre at P14 into the hindpaw of two representative animals (*R26*<sup>LSL-tdTom</sup>) and harvested 22-24 days later  
535 shows TOM fluorescence within the IF MN pool indicating infection and recombination (C, D, arrows).  
536 Long-projecting axons are seen terminating in the dentate nucleus (C'-C'', D'''). In one example  
537 injection, Purkinje cells were infected on the same side as injection (D', D'') and axons projecting to the  
538 dentate were seen on the contralateral side. (E-E''') Injection of Lenti<sup>FugE</sup>-Cre into the left hindpaw of  
539 *R26*<sup>LSL-tdTom</sup> mice at P4 and harvested 21 days later showed infection of the IF MN Pool (E, arrow). A  
540 Purkinje cell can be seen extending an axon to the dentate nucleus (E', arrowheads) and its terminals  
541 in the dentate nucleus seen in a more caudal section (E'''). (F) Schematic of the proposed hindpaw

542 injections. MN pools identified with CHAT Antibody in B, D, and E. Brain pictures taken from Mouse  
543 Brain Atlas (Paxinos and Franklin, 2007). Distance from Bregma given in mm. Scale bars: 100  $\mu\text{m}$ , E'''  
544 is 10  $\mu\text{m}$ .

545 **Figure 7. Injection of Fluorogold retrograde tracer into the cerebellar vermis does not label the**  
546 **IF MN pool.** (A) Schematic of Fluorogold (FG) injections into the cerebellar vermis of *Atoh1*<sup>Cre/+</sup> knock-in  
547 mice crossed to the TOM reporter mice. (B) FG (green) was injected into the vermis of folia II-V. The  
548 injections were symmetrically spread from the midline, so only one half is shown. Areas in the medulla  
549 that are known to project to the cerebellum (ECu, LRt, and IO) are retrogradely labeled (FG<sup>+</sup>). Many of  
550 the neurons in the ECu, LRt, and a portion of the IO (insets) are also *Atoh1*-lineage (TOM<sup>+</sup>) as  
551 previously reported (Rose et al., 2009). Note that a portion of the IO (arrowhead in bottom IO panel) are  
552 not TOM<sup>+</sup>. (C) There are no FG<sup>+</sup> cells in the IF MN pool (FG<sup>-</sup>TOM<sup>+</sup>CHAT<sup>+</sup>, arrowheads). (D-D''')  
553 Cerebellar-projecting CC cells and cerebellar-projecting cells lateral to CC are retrogradely labeled with  
554 FG (D'-D''). Few of these retrogradely labeled cells are *Atoh1*-lineage (FG<sup>+</sup>TOM<sup>+</sup>, arrows in D-D'').  
555 Cerebellar-projecting cells in the ventral spinal cord are not *Atoh1*-lineage and are not cholinergic (D'''-  
556 D''''', FG<sup>+</sup>TOM<sup>-</sup>CHAT<sup>-</sup>, arrowheads). Brain pictures taken from Mouse Brain Atlas (Paxinos and Franklin,  
557 2007). Abbrev: IC, inferior colliculus; Sim, Simplex; PML, paramedian lobule; ECu, external cuneate  
558 nucleus; LRt, lateral reticular nucleus; IO, inferior olive; Cu, cuneate; CC, Clarke's column. Distance  
559 from Bregma given in mm. Scale bars: 100  $\mu\text{m}$  in all panels except cerebellar sections in B are 1 mm.

## 560 **Multimedia, Figure, and Table**

561 **Movie 1.** Movie of the tdTomato labeled cells in the hindbrain of a SHIELD cleared *Chat*<sup>Hoxa4</sup> mouse.  
562 Some punctate fluorescence is seen in right Crus I.

563 **Movie 2.** Movie of the tdTomato labeled cells in the cervical to thoracic spinal cord of a SHIELD cleared  
564 *Chat*<sup>Hoxa4</sup> mouse. Most MN axons appear to go into the ventral roots. One axon on the left side of the  
565 spinal cord (right side of the image) appears to originate from the contralateral side of the spinal cord,  
566 but the fluorescence cannot be traced to the original neuron.

567 **Movie 3.** Movie of the tdTomato labeled cells in the thoracic to lumbar spinal cord of a SHIELD cleared  
568 *Chat*<sup>*Hoxa4*</sup> mouse. Most MN axons travel to the ventral roots.

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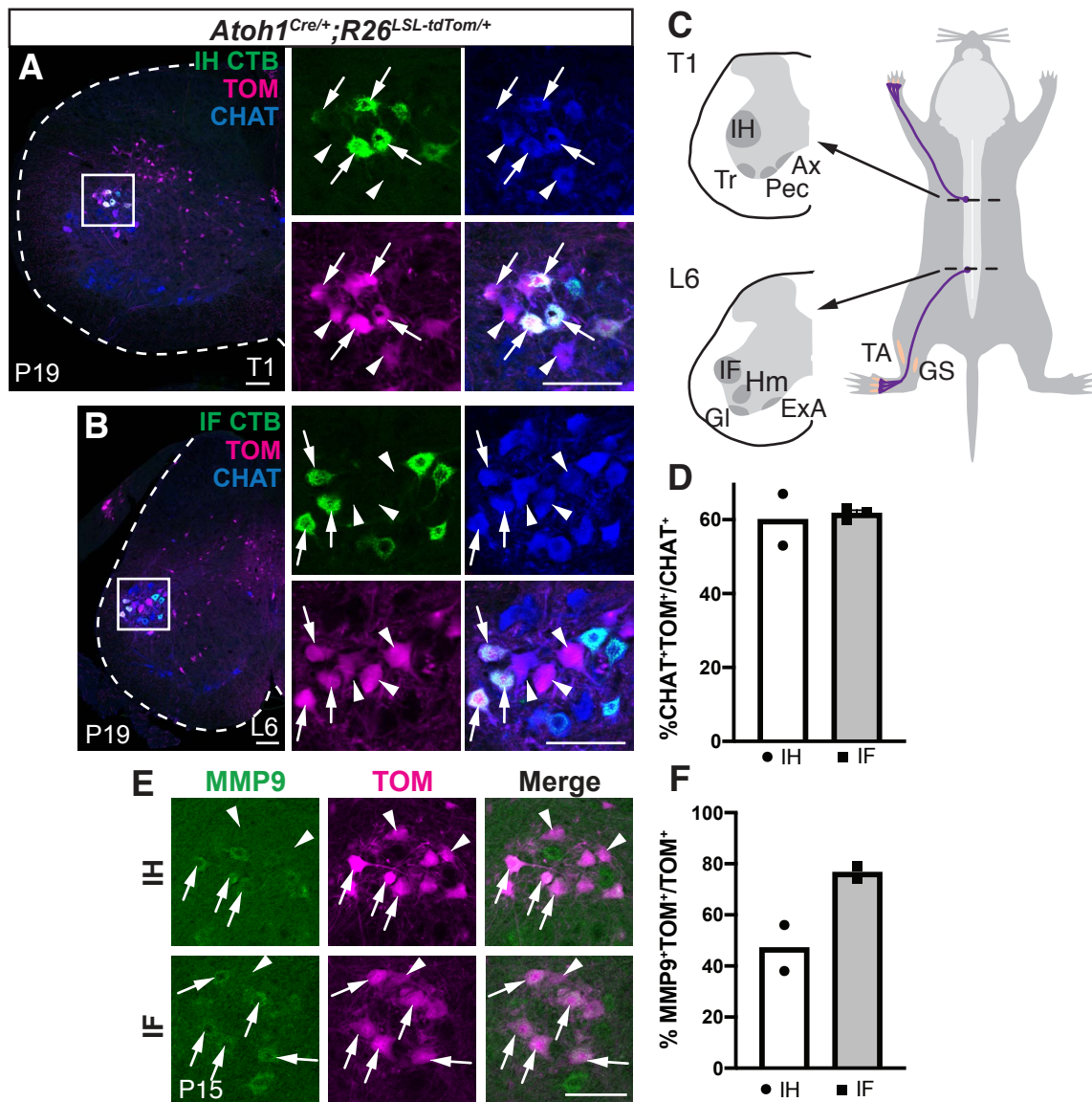
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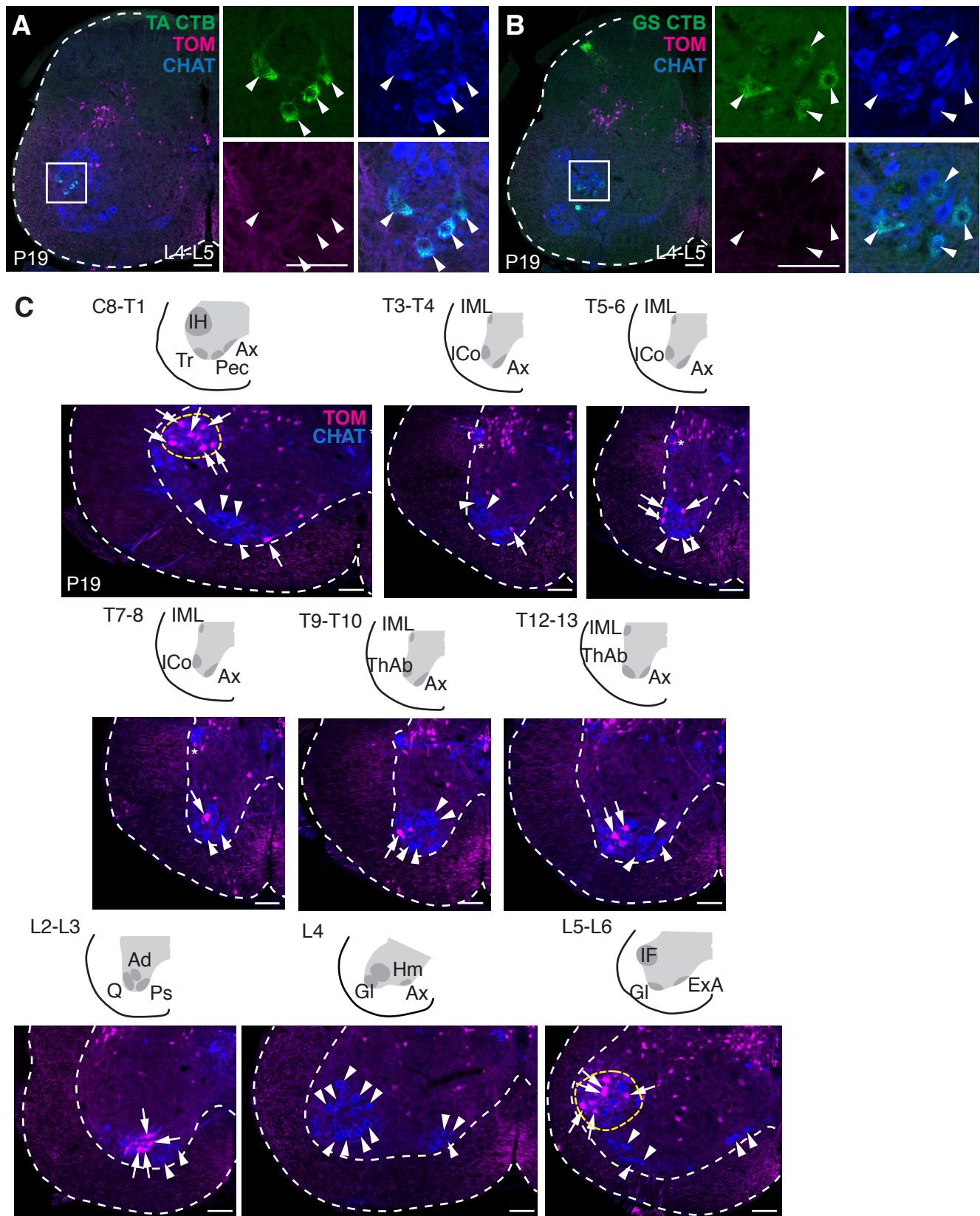
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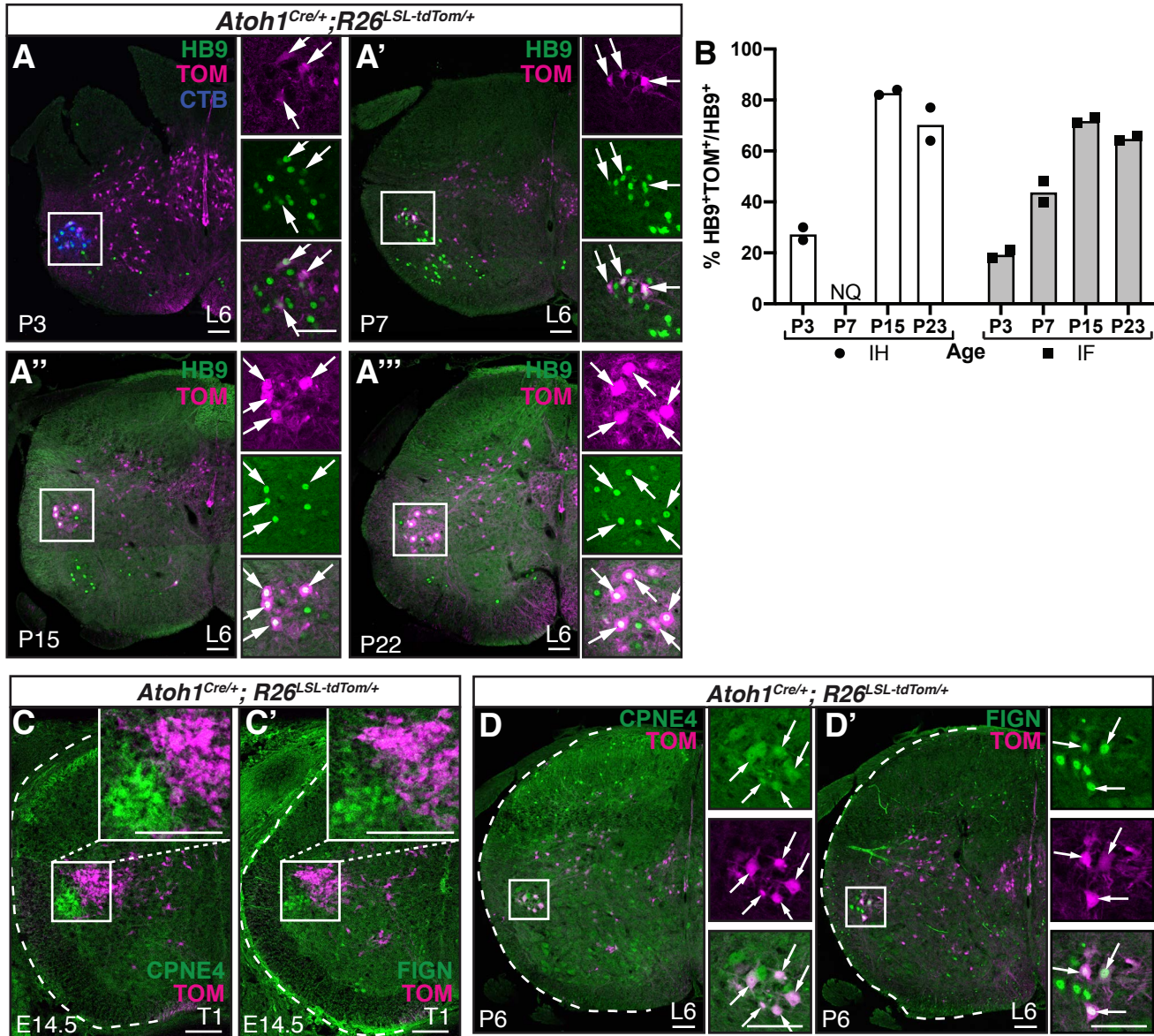
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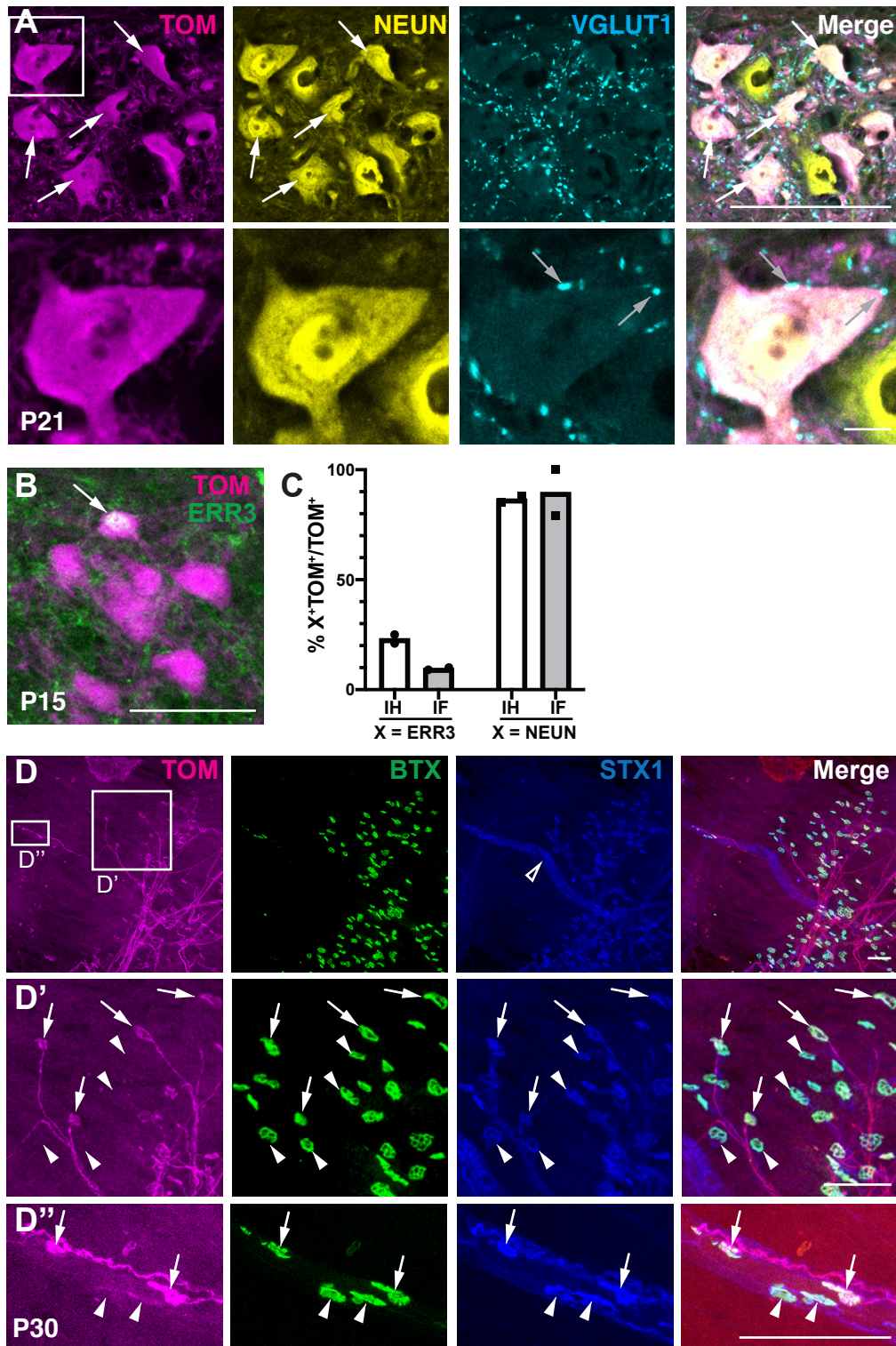
**Figure 1. The *Atoh1<sup>Cre/+</sup>* knock-in mouse line labels the intrinsic hand (IH) and foot (IF) motor neuron (MN) pools. (A, B) Injection of the retrograde tracer CTB-488 into the forepaw and hindpaw labels the IH and IF MN pools, which are labeled with tdTomato (TOM) when the TOM reporter mouse is crossed to the *Atoh1<sup>Cre/+</sup>* knock-in mouse. Arrows, CTB<sup>+</sup> CHAT<sup>+</sup> TOM<sup>+</sup>; arrowheads, CTB<sup>-</sup> CHAT<sup>+</sup> TOM<sup>+</sup>. (C) Diagram of motor neuron pools at T1 and L6. (D) Quantitation of the percentage of the IH or IF MN pools that are labeled TOM<sup>+</sup> in *Atoh1<sup>Cre/+</sup>* knock-in mice. (E-F) Some of the TOM<sup>+</sup> IH MNs IF MNs are fast twitch MNs (MMP9<sup>+</sup>). MMP9<sup>+</sup>TOM<sup>+</sup> arrows; MMP9<sup>+</sup>TOM<sup>+</sup> arrowheads. See text for values in D, F. Christopher Reeve Atlas referenced for spinal cord MN pools (Watson et al., 2009). Abbrev: P, postnatal; C, cervical; T, thoracic; L, lumbar; Tr, triceps; Pec, pectoral; Ax, axial; Hm, hamstring; Gl, gluteus; ExA, external anal sphincter; TA, tibialis anterior; GS, gastrocnemius. Scale bars: 100  $\mu$ m.**



**Figure 2. The *Atoh1*<sup>Cre/+</sup> knock-in mouse line does not label the TA and GS MN pools and sparsely labels other MN pools.** (A, B) Injection of CTB-488 into the TA and GS shows no labeling (TOM<sup>-</sup>; arrowheads) of these MN pools in the *Atoh1*<sup>Cre/+</sup> knock-in mouse. (C) Representative images throughout the rostral-caudal axis of *Atoh1*<sup>Cre/+</sup> knock-in mice crossed to the TOM reporter mouse show that TOM labels MNs mainly in IH and IF (yellow dashed lines) with sparser labeling of MNs in other MN pools (arrows). Some MN pools have no TOM<sup>+</sup> expression (arrowheads). Christopher Reeve Atlas referenced for spinal cord MN pools (Watson et al., 2009). Abbrev: IML, intermediolateral nucleus; ICo, intercostal; ThAb, thoracic abductor; Q, quadriceps; Ad, adductor; Ps, psoas. Scale bars: 100  $\mu$ m.

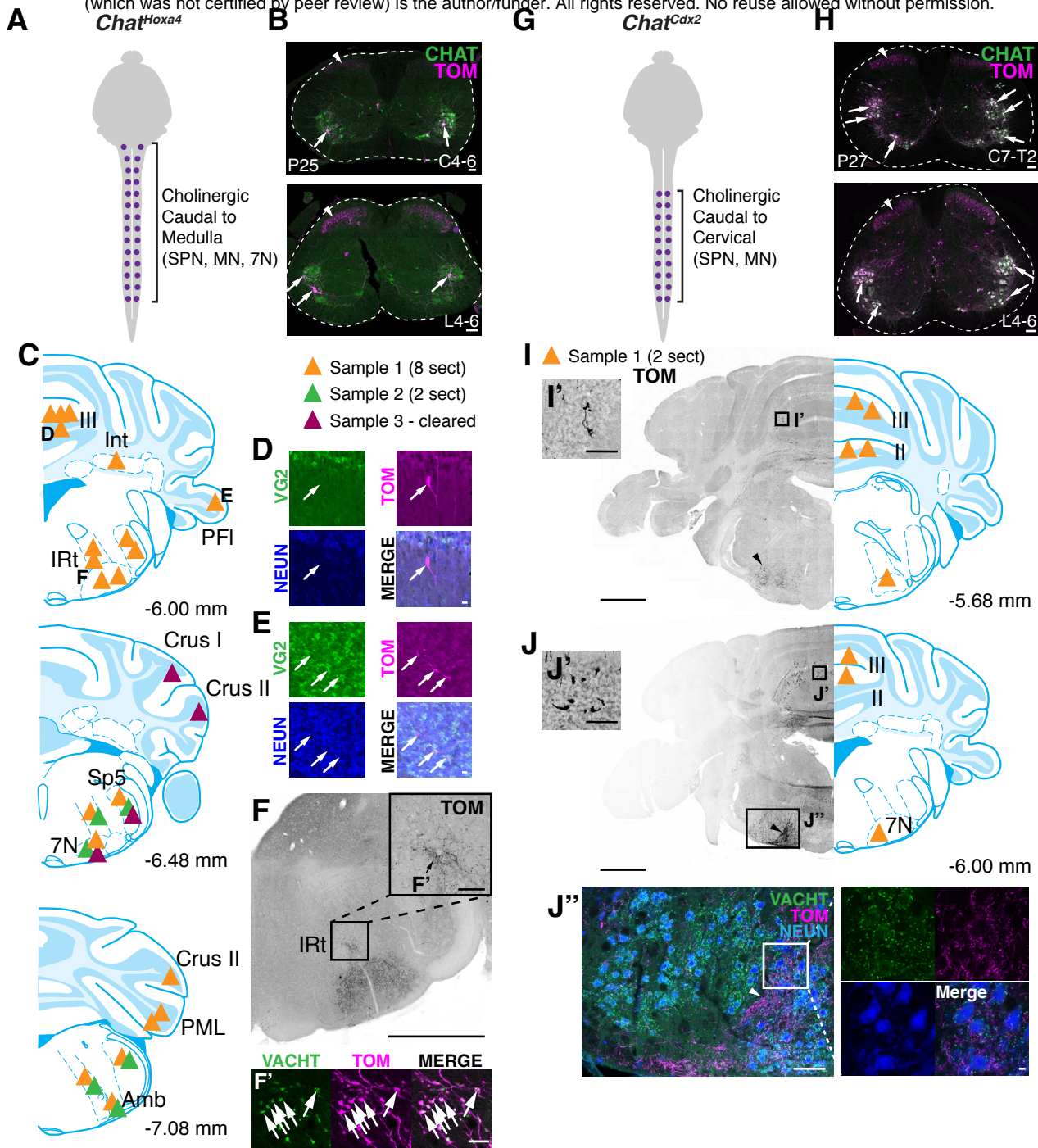


**Figure 3. The *Atoh1<sup>Cre/+</sup>* knock-in mouse labels IH and IF postnatally. (A-A''') TOM<sup>+</sup> labeling of the IF MN pool at several postnatal time points. HB9<sup>+</sup>TOM<sup>+</sup> neurons, arrows. In A, CTB (blue) was injected into the hindpaw to identify the IF MN pool. (B) Quantitation of percentage of TOM<sup>+</sup> neurons in the IH or IF MN pools at several time points. (C-C') At E14.5 (embryonic day 14.5), TOM<sup>+</sup> neurons are CPNE4<sup>-</sup> and FIGN<sup>-</sup>. (D-D') At P6, TOM<sup>+</sup> neurons are CPNE4<sup>+</sup> and FIGN<sup>+</sup> (arrows). Scale bars: 100  $\mu$ m.**



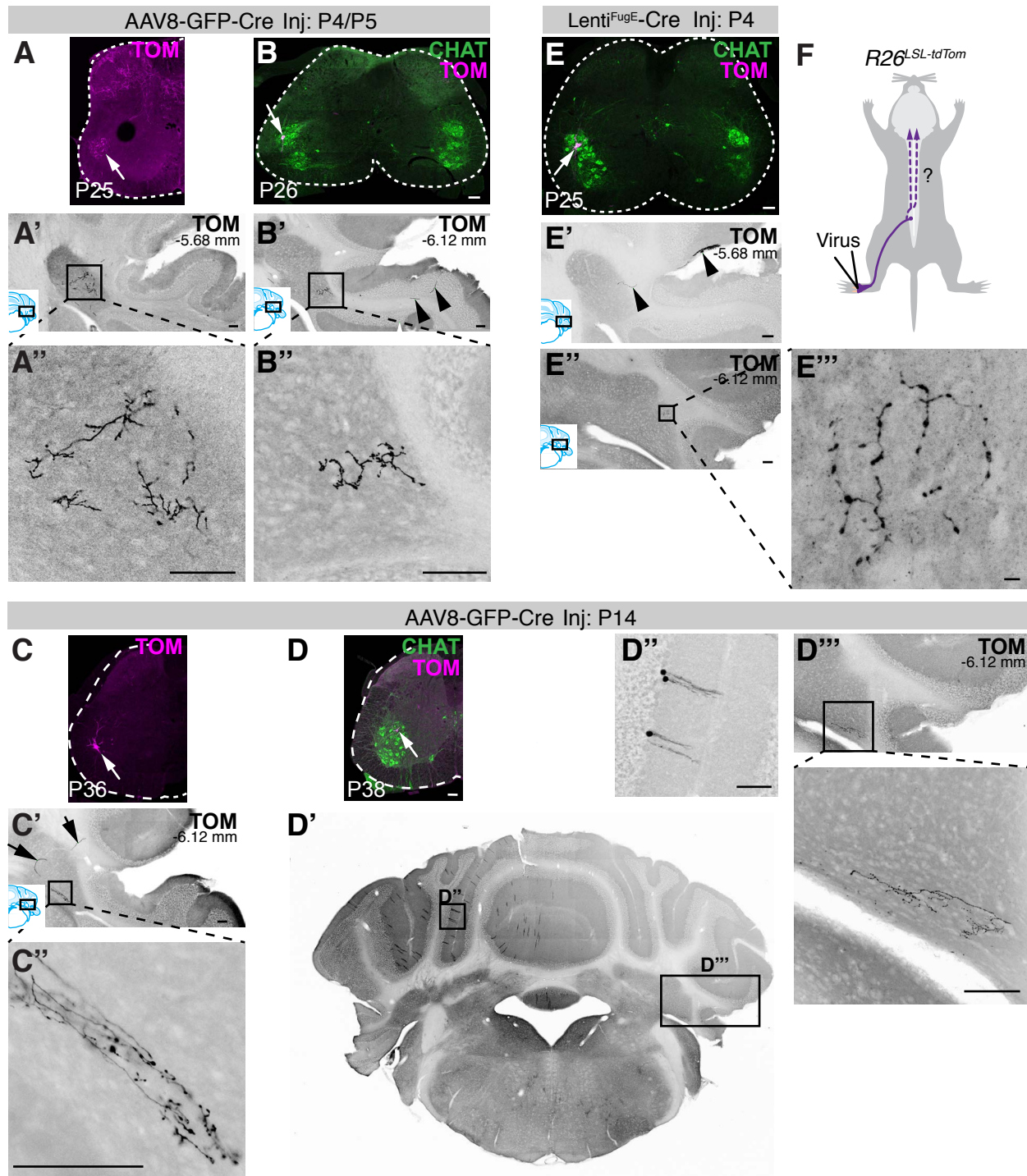
**Figure 4. Both  $\alpha$ - and  $\gamma$ -MNs are labeled in the *Atoh1*<sup>Cre/+</sup> knock-in mouse.** (A) TOM<sup>+</sup> MNs in the IF MN pool are NEUN<sup>+</sup> (arrows) and have closely apposed VGLUT1<sup>+</sup> boutons (grey arrows). (B) Some TOM<sup>+</sup> IF MNs are also ERR3<sup>+</sup> (arrow). (C) Percentage of the TOM<sup>+</sup> MNs in the IH and IF that are ERR3<sup>+</sup> ( $\gamma$ -MN marker) or NEUN<sup>+</sup> ( $\alpha$ -MN marker). (D-D'') TOM<sup>+</sup> axons in the hindpaw lumbrical muscle show the neuromuscular junction innervating extrafusal muscle (D', arrows, BTX<sup>+</sup>STX1<sup>+</sup>TOM<sup>+</sup>). TOM<sup>+</sup> axons also innervate the intrafusal muscle spindle (D, open arrowhead; D'', arrows, BTX<sup>+</sup>STX1<sup>+</sup>TOM<sup>+</sup>). Arrowheads indicate motor endplates that are TOM<sup>-</sup>. Scale bars: 100  $\mu$ m, inset in A is 10  $\mu$ m.



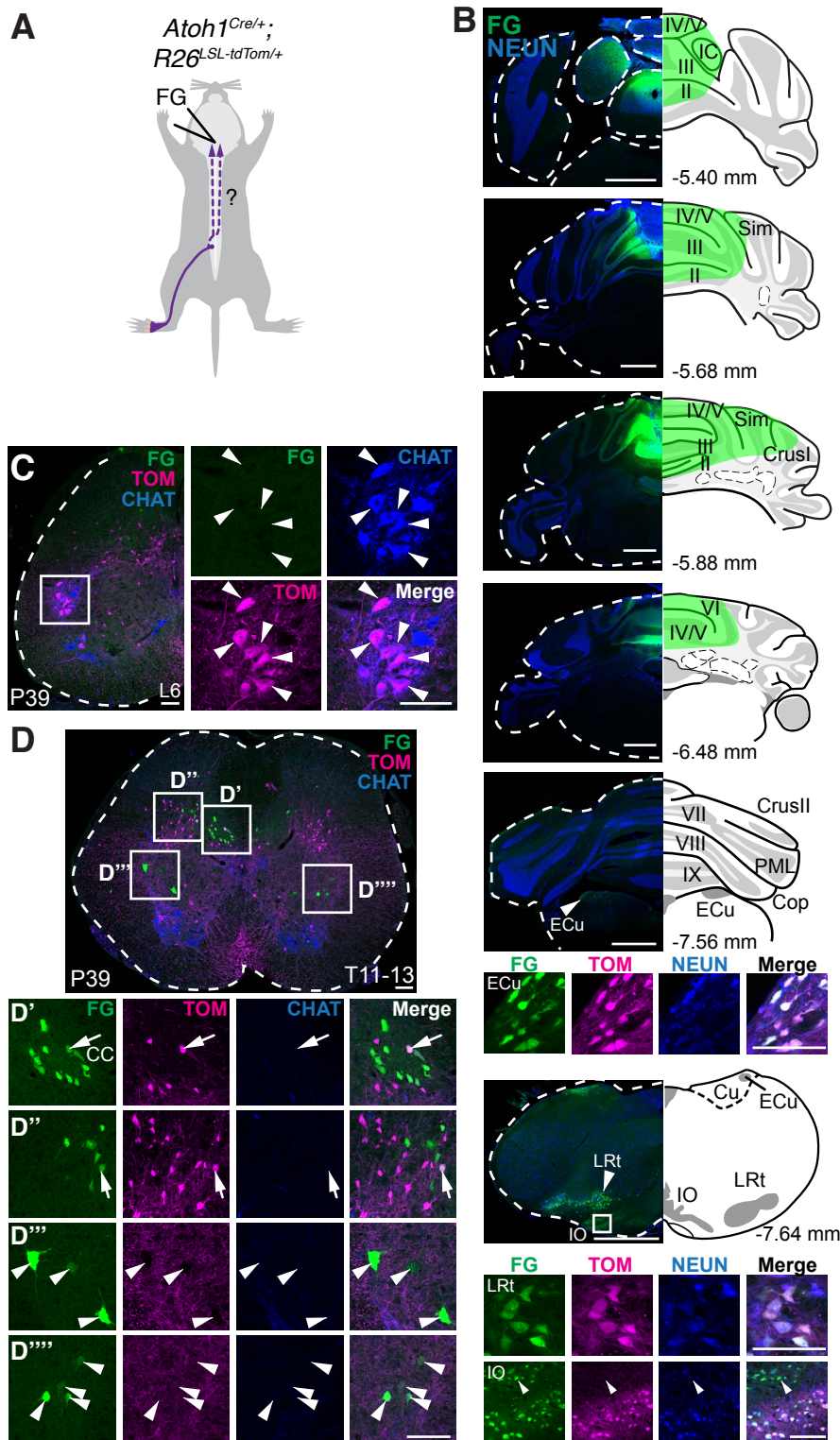


**Figure 5. Intersectional genetic labeling of caudal cholinergic neurons finds ascending projections to the cerebellum.**

(A) Diagram of *Chat<sup>Hoxa4</sup>* intersectional cross that labels cholinergic neurons caudal to the medulla. (B) Sparse TOM<sup>+</sup> (arrows) labeling of MNs (CHAT<sup>+</sup>, green) in *Chat<sup>Hoxa4</sup>* mice. Axonal terminations from sensory neuronal labeling are seen in the superficial dorsal horn (arrowheads). (C) Summary of data from three samples of where TOM<sup>+</sup> processes are seen in the hindbrain. Number of sections (sect) analyzed is stated. (D) Representative image of TOM<sup>+</sup> process (arrow) seen in vermis III that is VGLUT2 (VG2) negative. Maximum Intensity Projection (MIP) of 21  $\mu$ m. (E) Representative image of TOM<sup>+</sup> process (arrows) in PFI that is VG2<sup>-</sup> and courses between the NEUN<sup>+</sup> granule cells. Maximum Intensity Projection (MIP) of 30  $\mu$ m. (F-F') Processes in IRt express VACHT (arrows). (G) Diagram of *Chat<sup>Cdx2</sup>* intersectional cross that labels cholinergic neurons caudal to the mid-cervical spinal cord. (H) Caudal cholinergic neurons are well-labeled in *Chat<sup>Cdx2</sup>* mice (arrows, TOM<sup>+</sup>CHAT<sup>+</sup>). Note that axonal terminations from sensory neuronal labeling are also seen in the superficial dorsal horn (arrowheads). (I-I', J-J') TOM<sup>+</sup> processes are seen in the folia II and III vermis of *Chat<sup>Cdx2</sup>* mice. TOM<sup>+</sup> processes are also seen around the facial nerve (7N)(arrowhead). Processes in the medial facial nerve area do not colocalize with VACHT antibody (J''). Brain pictures taken from Mouse Brain Atlas (Paxinos and Franklin, 2007). Abbrev: SPN, sympathetic preganglionic nucleus; 7N, facial nerve; Int, interpositus; IRt, intermediate reticular nucleus; Sp5, spinal trigeminal nucleus; Amb, ambiguus nucleus; PFI, paraflocculus; PML, paramedian lobule. Scale bars: 100  $\mu$ m unless otherwise noted; D, E, F', and J'' insets are 10  $\mu$ m; F and I are 1 mm.



**Figure 6. Peripheral injections of viruses infect Purkinje cells in the cerebellum.** (A-B'') Injection of AAV8-GFP-Cre at P4 or P5 into the hindpaw of two representative animals (*R26<sup>LSL-tdTom</sup>*) and harvested 21 days later shows TOM fluorescence within the IF MN pool indicating infection and recombination (A, B, arrows). Cerebellar sections show axonal processes in the dentate nucleus (A'-B''). Labeling of a long-projecting axon can be seen (B', arrowheads). (C-D''') Injection of AAV8-GFP-Cre at P14 into the hindpaw of two representative animals (*R26<sup>LSL-tdTom</sup>*) and harvested 22-24 days later shows TOM fluorescence within the IF MN pool indicating infection and recombination (C, D, arrows). Long-projecting axons are seen terminating in the dentate nucleus (C'-C'', D'''). In one example injection, Purkinje cells were infected on the same side as injection (D', D'') and axons projecting to the dentate were seen on the contralateral side. (E-E''') Injection of Lenti<sup>FugE</sup>-Cre into the left hindpaw of *R26<sup>LSL-tdTom</sup>* mice at P4 and harvested 21 days later showed infection of the IF MN Pool (E, arrow). A Purkinje cell can be seen extending an axon to the dentate nucleus (E', arrowheads) and its terminals in the dentate nucleus seen in a more caudal section (E'''). (F) Schematic of the proposed hindpaw injections. MN pools identified with CHAT Antibody in B, D, and E. Brain pictures taken from Mouse Brain Atlas (Paxinos and Franklin, 2007). Distance from Bregma given in mm. Scale bars: 100  $\mu$ m, E''' is 10  $\mu$ m.



**Figure 7. Injection of Fluorogold retrograde tracer into the cerebellar vermis does not label the IF MN pool.** (A) Schematic of Fluorogold (FG) injections into the cerebellar vermis of *Atoh1<sup>Cre/+</sup>* knock-in mice crossed to the TOM reporter mice. (B) FG (green) was injected into the vermis of folia II-V. The injections were symmetrically spread from the midline, so only one half is shown. Areas in the medulla that are known to project to the cerebellum (ECu, LRt, and IO) are retrogradely labeled (FG<sup>+</sup>). Many of the neurons in the ECu, LRt, and a portion of the IO (insets) are also *Atoh1*-lineage (TOM<sup>+</sup>) as previously reported (Rose et al., 2009). Note that a portion of the IO (arrowhead in bottom IO panel) are not TOM<sup>+</sup>. (C) There are no FG<sup>+</sup> cells in the IF MN pool (FG<sup>+</sup>TOM<sup>+</sup>CHAT<sup>+</sup>, arrowheads). (D-D''''') Cerebellar-projecting CC cells and cerebellar-projecting cells lateral to CC are retrogradely labeled with FG (D'-D'''). Few of these retrogradely labeled cells are *Atoh1*-lineage (FG<sup>+</sup>TOM<sup>+</sup>, arrows in D-D'''). Cerebellar-projecting cells in the ventral spinal cord are not *Atoh1*-lineage and are not cholinergic (D''''-D''''', FG<sup>+</sup>TOM<sup>-</sup>CHAT<sup>-</sup>, arrowheads). Brain pictures taken from Mouse Brain Atlas (Paxinos and Franklin, 2007). Abbrev: IC, inferior colliculus; Sim, Simplex; PML, paramedian lobule; ECu, external cuneate nucleus; LRt, lateral reticular nucleus; IO, inferior olive; Cu, cuneate; CC, Clarke's column. Distance from Bregma given in mm. Scale bars: 100  $\mu$ m in all panels except cerebellar sections in B are 1 mm.