Sensory and Motor Systems

An Atoh1 CRE Knock-In Mouse Labels Motor Neurons Involved in Fine Motor Control

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Abstract
Motor neurons (MNs) innervating the digit muscles of the intrinsic hand (IH) and intrinsic foot (IF) control fine motor movements. The ability to reproducibly label specifically IH and IF MNs in mice would be a beneficial tool for studies focused on fine motor control. To this end, we find that a CRE knock-in mouse line of Atoh1, a developmentally expressed basic helix-loop-helix (bHLH) transcription factor, reliably expresses CRE-dependent reporter genes in ~60% of the IH and IF MNs. We determine that CRE-dependent expression in IH and IF MNs is ectopic because an Atoh1 mouse line driving FLPo recombinase does not label these MNs although other Atoh1-lineage neurons in the intermediate spinal cord are reliably identified. Furthermore, the CRE-dependent reporter expression is enriched in the IH and IF MN pools with much sparser labeling of other limb-innervating MN pools such as the tibialis anterior (TA), gastrocnemius (GS), quadricep (Q), and adductor (Ad). Lastly, we find that ectopic reporter expression begins postnatally and labels a mixture of α and γ-MNs. Altogether, the Atoh1 CRE knock-in mouse strain might be a useful tool to explore the function and connectivity of MNs involved in fine motor control when combined with other genetic or viral strategies that can restrict labeling specifically to the IH and IF MNs. Accordingly, we provide an example of sparse labeling of IH and IF MNs using an intersectional genetic approach.

Key words: Atoh1; fine motor control; motor neurons; spinal cord

Significance Statement
Motor neurons (MNs) of the intrinsic hand (IH) and intrinsic foot (IF) are reproducibly labeled in an ectopic manner postnatally using a CRE knock-in mouse line of the basic helix-loop-helix (bHLH) transcription factor Atoh1, serving as a useful genetic tool for future studies of fine motor control.

Introduction
Motor neurons (MNs) innervating the muscles in the digits of the hand allow for exquisite control of fine motor movements required for dexterous skills, such as writing or sewing. Primates are known for their ability to precisely control individual digits of the hand, but skillful movements are not limited to the hand, as people born without arms are able to dexterously manipulate tools with their toes (Shubin et al., 1997; Sustaita et al., 2013; Dempsey-Jones et al., 2019). Precisely how MNs innervating the intrinsic hand (IH) and intrinsic foot (IF) muscles may differ in terms of function and connectivity compared with MNs that innervate limb muscles is unknown. Presumably, IH and IF MNs have unique properties and connectivity that...
contribute to their role in fine motor control, however, examination of the function of specifically IH and IF MNs is underexplored.

In part, lack of exploration of the IH and IF MNs is because of an inability to genetically distinguish these MNs from other limb MNs in mice. Mice also demonstrate remarkable manipulative dexterity when performing fine motor tasks such as eating dried pasta or grasping a pellet (Tennant et al., 2010; Yoshida and Isa, 2018) and, thus, represent a model organism to study fine motor skills. Developing a genetic tool that labels IH and IF MNs would have significant utility in interrogating the function and circuitry of fine motor control. To this end, we found that digit-innervating MNs were labeled using CRE-loxP recombination driven by the bHLH transcription factor atonal homolog 1, Atoh1, a transiently expressed gene in the dorsal-most part of the developing neural tube that is also known to specify excitatory (Vglut2) spinal cord neurons that project rostrally to the hindbrain (Bermingham et al., 2001; Gowan et al., 2001; Sakai et al., 2012; Yuengert et al., 2015; Pop et al., 2020).

Here, we explore the features of the digit-innervating MNs labeled by Atoh1 CRE-LoxP recombination. We find that labeling of IH and IF MNs is ectopic because an Atoh1 mouse line expressing FLPo recombinase that we developed in the lab does not label IH and IF MNs, although it does label the expected Atoh1-lineage neurons in the intermediate spinal cord. The Atoh1 CRE knock-in mouse labels a mixture of α-MNs and γ-MNs in the IH and IF MN pools postnatally, while sparsely labeling other limb-innervating MNs. We further endeavor to show the utility of the Atoh1 CRE knock-in mouse using an intersectional genetic approach and discuss possible future methodologies to interrogate fine motor function and circuitry using this mouse strain.

Materials and Methods

Mouse strains

The following mouse strains were used: Atoh1Cre/– knock-in (Yang et al., 2010), R26SL-FS-fTom/– (Ai65; Madisen et al., 2015), R26SF-fTom/– was generated using CAG-Cre (Sakai and Miyazaki, 1997) crossed to Ai65, R26SF-EGFP/– (named RCE::RFP in Miyoshi et al., 2010), and ChatRES-FLPo (Allaway et al., 2020). All mice were outbred and thus, are mixed strains (at least C57Bl/6J and ICR). Atoh1Cre/– knock-in mice crossed to reporter mice were screened for “dysregulated” expression as previously reported (Yuengert et al., 2015). All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Texas Southwestern.

The Atoh1P2A-FLPo/– mouse was generated using the Easi-CRISPR approach (Quadros et al., 2017). Briefly, a long single stranded DNA cassette consisting of a viral peptide self-cleaving sequence [porcine teschovirus-1 2A (P2A); Kim et al., 2011] and the codon optimized flipase recombinase sequence (FLPo) were inserted after the last amino acid codon and before the stop codon of Atoh1. C57Bl/6N zygotes were microinjected with ribonucleic acid complexes of Cas9 protein, tracrRNA, and crRNA (5’-TGACTCTGATGAGGCCAGTT-3’) along with a ssDNA megamer for homologous recombination (1497 bp containing 60 bp each 5’ and 3’ homology arms and the P2A-FLPo sequence; reagents were procured from IDT, microinjection service was provided by the UT Southwestern Mouse Core Facility). Assembling CRISPR reagents and microinjections were performed as previously described (Jacobi et al., 2017; Miura et al., 2018). The live born mice were first screened for insertion of the P2A-FLPo sequence and of those that were positive, one of the mice contained the full-length cassette. The cassette contained a minor mutation at the end of FLPo (the last isoleucine amino acid was changed to a serine), which could have occurred possibly because of an imprecise DNA repair event. Nevertheless, this amino acid change does not seem to affect the enzymatic function of FLPo. For genotyping, wild-type (WT) 321-bp and mutant 642-bp PCR products were detected using the following primers: WT forward 5’-CCCTAACACGGATGATGCGACAGAGG-3’, WT reverse 5’-GGGGATTGGAAGAGCTGCAGCCGTC-3’, and MUT reverse 5’-CGAACTCGAAGCTGGAGCTGACCGCT-3’. Note that because the P2A sequence self-cleaves near its C terminus, 21 amino acids of the P2A sequence are fused to the C terminus of ATOH1.

Tissue processing

Embryos were timed as embryonic day (E)0.5 on the day the vaginal plug was detected and P0 on the day of birth. Pregnant females were euthanized with CO2 and cervical dislocation, embryos dissected out of the uterus, and spinal cords dissected out. Embryonic spinal cords (E14.5) were fixed in 4% paraformaldehyde (PFA)/PBS for 2–3 h at 4°C. Early postnatal animals (postnatal day 7 (P7) or younger) were cooled on ice, decapitated, their spinal cords dissected out, and fixed in 4% PFA/PBS for 2 h at 4°C. Mice older than P14 were anesthetized with avertin (2,2,2-tribromoethanol; 0.025–0.030 ml of 0.04 m avertin in 2-methyl-2-butanol and distilled water/g mouse) and transcardially perfused, first with 0.012% w/v Heparin/PBS and then 4% PFA/PBS. A dorsal or ventral laminaeotomy exposed the
spinal cord to the fixative. The spinal cords were fixed for 2 h and the brains overnight at 4°C. Tissue was washed in PBS for at least 1 d and cryoprotected in 30% sucrose dissolved in deionized water. Tissue was marked with 1% Alcian Blue in 3% acetic acid on one side to keep orientation and were then embedded in OCT (Tissue-Tek Optimal Cutting Temperature compound). Tissue was sectioned using a Leica CM1950 Cryostat.

Figure 1. The Atoh1\textsuperscript{Cre/+} knock-in mouse line labels the IH and IF 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) in Atoh1\textsuperscript{Cre/+} knock-in mice. MN pools are identified with ChAT antibody. Arrows, CTB \textsuperscript{+} TOM \textsuperscript{+} ChAT \textsuperscript{+}; arrowheads, CTB \textsuperscript{+} TOM \textsuperscript{+} ChAT \textsuperscript{−}. C, Diagram of CTB-488 injections into the IH and IF MN pools, which are located at T1 and L6. D, Injection of CTB-488 into the TA, GS, Q, and Ad muscles retrogradely labels those MN pools, which are sparsely labeled with TOM in Atoh1\textsuperscript{Cre/+} knock-in mice. Arrows, CTB \textsuperscript{+} TOM \textsuperscript{+} ChAT \textsuperscript{+}; arrowheads, CTB \textsuperscript{+} TOM \textsuperscript{−} ChAT \textsuperscript{+}. E, Percentage of the IH, IF, TA, GS, Q, and Ad MN pools that are labeled TOM\textsuperscript{+} in Atoh1\textsuperscript{Cre/+} knock-in mice. F, G, Some of the TOM\textsuperscript{+} IH MNs and IF MNs are fast twitch MNs (MMP9\textsuperscript{+}). Arrows, MMP9\textsuperscript{+} TOM\textsuperscript{+}; arrowheads, MMP9\textsuperscript{+} TOM\textsuperscript{−}. H, Atoh1-lineage neurons in the intermediate spinal cord have extensive overlap in Atoh1\textsuperscript{P2A-FLPo; EGFP/+} mice crossed to Atoh1\textsuperscript{Cre; tdTom} mice (arrowheads, GFP\textsuperscript{+} TOM\textsuperscript{+}, white). However, IH and IF MNs are only labeled in the Atoh1\textsuperscript{Cre/+} mice (TOM\textsuperscript{+}) and not in the Atoh1\textsuperscript{P2A-FLPo; EGFP/+} mice (GFP−; inset). P, postnatal; T, thoracic; L, lumbar. Scale bars: 100 \textmu m.
Immunohistochemistry and confocal imaging

Cryosections (30 μm) were blocked with PBS/1–3% normal goat or donkey serum/0.3% Triton X-100 (Sigma) for up to 1 h at room temperature (RT) and incubated overnight with primary antibody at 4°C. After washing three times with PBS, the appropriate secondary antibody (Alexa Fluor 488, 567, and/or 647, Invitrogen) was incubated for 1 h at RT. Sections were rinsed three times in PBS, mounted with Aquapolymount (Polysciences Inc.), and coverslipped (Fisher). The following primary antibodies and dilutions were used: 1:500 rabbit anti-dsRed (Clontech), 1:100 goat anti-choline acetyltransferase (ChAT; Millipore Sigma), 1:1000 rabbit anti-matrix metalloproteinase-9 (MMP9; Abcam), 1:8000 guinea pig anti-copine-4 (CPNE4) and 1:8000 guinea pig anti-fidgetin (FIGN; gifts of Tom Jessell, Columbia University), 1:500 mouse anti-NEUN (Millipore Sigma), 1:100 mouse anti-ERR3 (R&D Systems), 1:3000 α-bungarotoxin (BTX) 488 (Invitrogen), 1:1000 rabbit anti-Syntaxin1 (gift of Thomas Südhof, Stanford University), and 1:1000 guinea pig anti-vesicular glutamate transporter 1 (VGLUT1; Millipore Sigma). Sections were referenced to the Christopher Reeves Spinal Cord Atlas (Watson et al., 2009).

Fluorescent images were taken on a Zeiss LSM710 or LSM880 confocal microscope with an appropriate optical slice (0.5–10 μm) depending on the image. Images were pseudocolored using a magenta/green/blue or magenta/yellow/cyan color scheme using Adobe Photoshop (Adobe) or Fiji (Schindelin et al., 2012).

CTB muscle injections

Mice aged P14 were anesthetized using isoflurane and prepared for injections into muscle. An approximate total of 500–750 nl of cholera toxin subunit B (CTB) Alexa Fluor 488 or 647 conjugate (Invitrogen; Nanoject II, Drummond Scientific) was injected into two to three different locations in the left forepaw (IH MN pool) or hindpaw (IF MN pool) in 50.6 nl increments. For injections into the tibialis anterior (TA), gastrocnemius (GS), quadriceps (Q), or adductor (Ad), the area of the skin above the muscle was shaved and 70% ethanol and betadine (Avrio Health L.P.) applied. An incision was made above the muscle and 500–750 nl of the CTB-conjugated Alexa Fluor was injected into three to four different locations directly into the muscle. The incision was closed with surgical glue (Henry Schein Medical). Carprofen (5 mg/kg) was administered daily 3 d after surgery. Spinal cords were harvested 5 d after injection. For injections at P0, mice were anesthetized with isoflurane and injected with <250 nl CTB-488 or 647 in one or two different locations of the forepaw and hindpaw and harvested 3 d later.

Experimental design and statistical tests

All details for number of sections counted, biological replicates, and male and female tissue analyzed are given in Results. No statistical tests were required as quantitation of the percentage of particular markers in any given MN pool were not directly compared with each other. Mean ± SEM are reported throughout. For samples with n = 2, the SEM is equal to half of the range between the two data points.

Results

Atoh1Cre/− knock-in mice label MN pools involved in fine motor control

We observed using CRE-lineage tracing strategies (Atoh1Cre/− knock-in mice crossed to tdTomato (TOM) reporter mice (R26SL-Tom Ai14)) (Yang et al., 2010; Madisen et al., 2010) that subsets of MNs expressing ChAT were labeled in the spinal cord (Fig. 1A,B, arrows and arrowheads). Based on the anatomic location of the MN pools along the rostral-caudal axis, we predicted that the Atoh1Cre/− lineage labeled MNs of the IH and IF in thoracic 1 (T1) and lumbar 6 (L6) areas of the spinal cord (Fig. 1C; Watson et al., 2009). We injected the forepaw and hindpaw with the retrograde tracer CTB conjugated to Alexa Fluor 488 (CTB-488) and verified that the IH and IF MN pools were Atoh1Cre/− TOM+ MNs (Fig. 1A,B, arrows). Note that the TOM+ cell bodies in the intermediate spinal cord are from other Atoh1-lineage interneurons.
involved in the proprioceptive system (Yuengert et al., 2015; Pop et al., 2020).

To see whether the labeling of Atoh1Cre/+ TOM+ MNs was specific to the IH and IF MN pools, we injected CTB-488 into the TA, GS, Q, and Ad muscles and found that those MN pools had much fewer TOM+ MNs (Fig. 1D, arrows and arrowheads). In addition, we sampled sections throughout the rostral-caudal axis of the spinal cord in Atoh1Cre/+ mice and found that few other MN pools had TOM+ expression (Fig. 2, arrows). Altogether, the TOM+ MNs labeled 60% of the IH and IF MN pools at P19 [IH: 59 ± 64%, n = 4, 1:3 M:F, four to eight half sections/n; IF: 64 ± 3%, n = 5, 1:4 M:F, two to eight half sections/n; TA: 7 ± 2%, n = 4, 2:2 M:F, two half sections/n; GS: 14 ± 2%, n = 4, 2:2 M:F, two to four half sections/n; Q: 13 ± 4%, n = 4, 2:2 M:F, one to three half sections/n; Ad: 13 ± 3%, n = 4, 2:2 M:F, one to six half sections/n]. MN areas located by CTB-488 injection into appropriate muscle group; Fig. 1E]. We estimate that the total number of ChAT+ MNs in the IH and IF MN pools at P19 on one side is 410 ± 72 neurons for the IH MN pool (n = 3, 1:2 M:F, three to four half sections/n) and 337 ± 7 SEM neurons for the IF MN pool (n = 3, 0:3 M:F, three half sections/n). Counts of sections represented a tenth of the MN pool, so the estimated total number of ChAT+ neurons were the final counts multiplied by ten. Because the TOM+ MNs comprised ~60% of the IH and IF MN pools, we hypothesized that the Atoh1Cre/+ mice might be labeling a particular functional class of MNs such as fast or slow twitch MNs. However, we found that only a subset of the IH and IF TOM+ MNs were labeled by the marker for fast MNs, MMP9, indicating that the IH and IF TOM+ MNs are a mixture of fast and slow MNs (IH: 59 ± 68%, n = 4, 3:1 M:F, three half sections/n; IF: 74 ± 3%, n = 5, 3:2 M:F, three half sections/n; Fig. 1F,G, arrows; Kaplan et al., 2014).

Developmentally, all cholinergic MNs derive from a progenitor domain expressing the basic helix-loop-helix (bHLH) oligodendrocyte transcription factor 2 (Olig2) in the ventral neural tube (Lu et al., 2002), not the transiently-expressed Atoh1 dorsal-most progenitor domain (Lai et al., 2016). Therefore, we tested whether Atoh1 was expressed in MNs by in situ hybridization (Gowan et al., 2001 for ISH probe) and RNAscope of Atoh1 at P14–P15 and P22, ages that we knew had TOM+ expression, but
Figure 4. Both α-MNs and γ-MNs are labeled in the Atoh1Cre+/− knock-in mouse. A, TOM⁺ MNs in the IF MN pool are NEUN⁺ (arrows) and have closely apposed VGLUT1⁺ boutons (gray arrows). B, Some TOM⁺ IF MNs are also ERR3⁺ (arrow). C, Percentage of the TOM⁺ MNs in the IH and IF that are ERR3⁺ (γ-MN marker) or NEUN⁺ (α-MN marker). D, Percentage of the IH and IF MN pools that are ERR3⁺. E-E″, TOM⁺ axons in the hindpaw lumbrical muscle show the NMJ innervating extrafusal muscle (E, arrows, BTX⁺STX1⁺TOM⁺). TOM⁺ axons also innervate the intrafusal muscle spindle (E, open arrowhead; E″, arrows, BTX⁺STX1⁺TOM⁺). Arrowheads indicate motor endplates that are TOM⁻. Scale bars: 100 μm; 10 μm (A, inset).
we were unable to detect any signal at the mRNA level (our unpublished observations). Therefore, to corroborate the labeling of IH and IF TOM+ MNs in the Atoh1Cre/− mouse line, we crossed these mice to an Atoh1P2A-FLPo/+ mouse and FLPo-dependent GFP reporter mouse (R26SF-EGFP/+), such that neurons from the Atoh1Cre/− mouse line were labeled with tdTomato and those from the Atoh1P2A-FLPo/+ mouse were labeled with EGFP. Strikingly, while the IH and IF MN pools were TOM−, they were clearly GFP− (Fig. 1H, insets) indicating that the TOM+ IH and IF MNs are ectopically labeled in the Atoh1Cre/− mouse line, either because of differences in CRE or FLPo recombinase expression themselves or differences in recombination efficiency in the CRE and FLPo lines. Notably, Atoh1-lineage neurons in the intermediate spinal cord had substantial overlap of GFP− and TOM− fluorescence (Fig. 1H, arrowheads) indicating these neurons are reliably from the Atoh1-lineage.

**Atoh1Cre/+ knock-in mice label IH and IF MN pools postnatally**

Because the Atoh1Cre/+ line was labeling IH and IF MNs ectopically, we sought to determine when the ectopic expression occurred during development. We found that at E14.5 when the IH and IF MNs first start expressing the unique markers CPNE4 and FIGN (Mendelsohn et al., 2017), the IH and IF MN pools were not yet TOM− (Fig. 3C,C). In contrast, at postnatal time points, we found that TOM− expression started around P3 and gradually increased until it reached ~60% in the IH and IF MN pools at P22 using the homeobox transcription factor, HB9 as a marker for MN pools (Arber et al., 1999; P3: IH, 26 ± 2%, n = 4, sex was not noted because of young age, four to six half sections/n; IF, 20 ± 1%, n = 4, sex was not noted because of young age, five to six half sections/n; P7: IH, 57 ± 3%, n = 3, 1:2 M:F, two to six half sections/n; IF, 40 ± 2%, n = 6, 2:4 M:F, three to four half sections/n; P15: IH, 74 ± 6%, n = 4, 3:1 M:F, four half sections/n; IF, 71 ± 3%, n = 5, 3:2 M:F, four half sections/n; P22: IH, 67 ± 5%, n = 3, 2:1 M:F, four to eight half sections/n; IF, 62 ± 2%, n = 4, 1:3 M:F, four to eight half sections/n; Fig. 3A–C)). In addition, we confirmed that the IF TOM− MNs colocalized with the specific markers CPNE4 and FIGN postnatally (Fig. 3D,D).

**IH and IF MN pools labeled with Atoh1Cre/+ knock-in mice are both α and γ-MNs**

Because Atoh1Cre/+ TOM− MNs are enriched in only a subset (~60%) of IH and IF MNs, we asked whether they were demarcating a specific type of MN (α or γ). α-MNs innervate the striated extrafusal muscle, are marked by the neuronal marker, NEUN, and receive VGLUT1− proprioceptive inputs (Fries et al., 2009; Manuel and Zytnicki, 2011; Ashrafi et al., 2012). γ-MNs innervate the intrafusal muscle spindles and express estrogen-related receptor γ (ERR3−; Fries et al., 2009). Immunostaining for α-MN and γ-MN markers, we found that Atoh1Cre/+ TOM− MNs were a mixture of both α-MNs and γ-MNs (% ERR3−: IH, 24 ± 2%, n = 4, 3:1 M:F, three to four half sections/n; IF, 15 ± 4%, n = 5, 3:2 M:F, two to four half sections/n; % NEUN−: IH, 88 ± 4%, n = 4, 3:1 M:F, three half sections/n; IF, 93 ± 4%, n = 5, 3:2 M:F, three half sections/n; Fig. 4A–C). Counts for the percentage of TOM− neurons that are ERR3− or NEUN− were performed on different sets of sections because of the fact that they are both mouse antibodies. Therefore, the percentages do not necessarily add up to 100% and the error in counts may be attributed to variablity between sections. The NEUN− TOM− MNs also received VGLUT1− proprioceptive inputs (Fig. 4A). We confirmed that ~30% of MNs are γ-MNs in the IH and IF MN pool as has been reported for other MN pools (IH: 33 ± 3%, n = 2, 2:0 M:F, three half sections/n; IF: 28 ± 4%, n = 3, 2:1 M:F, two to three half sections/n; Fig. 4D; Friese et al., 2009). Therefore, our results suggest that the Atoh1Cre/+ mouse line has a slight preference for labeling α-MNs. Furthermore, imaging of the hindpaw lumbilical muscle found TOM− axons innervating both the extrafusal (Fig. 4E, arrows) and intrafusal muscle (Fig. 4E′, arrows), BTX− identifies the neuromuscular junctions (NMJs) and syntaxin (STX1−) identifies the muscle spindle (Fig. 4E, open arrowhead) and NMJs. Note that not all NMJs are TOM− (Fig. 4E″, arrowheads) consistent with the fact that only ~60% of the IH and IF MN pools are TOM−.

**Intersectional strategy for labeling IH and IF MNs**

Because Atoh1-lineage neurons are glutamatergic and reside in the intermediate spinal cord, we sought a way to isolate specifically the ectopically labeled IH and IF MNs
using an intersectional strategy crossing Atoh1<sup>Cre<sup>+</sup></sup> and Chat<sup>RES-FLPo<sup>+</sup></sup> alleles to the intersectional tdTomato reporter (R26<sup>LSL-FSF-tdT</sup>omato, Ai65). We found that the intersection of the IH and IF MNs was sparse in the Chat<sup>RES-FLPo<sup>+</sup></sup> mouse line alone with only ~30% of the MN pool being labeled (IH, 26 ± 5%, n = 2, 2:0 M:F; six half sections/n; IF, 31 ± 5%, n = 3, 3:0 M:F; 4–10 half sections/n; Fig. 5A,C). Thus, for the intersection of Atoh1<sup>Cre<sup>+</sup></sup> and Chat<sup>RES-FLPo<sup>+</sup></sup>, very few IF and MNs were labeled (IH, 17 ± 4%, n = 2, 1:1 M:F; two to three sections/n; IF, 15 ± 1%, n = 3, 2:1 M:F, three to four half sections/n; Fig. 5B,C). Therefore, this intersectional cross could be useful for sparse labeling of the IH and IF MN pools.

**Discussion**

To understand how dexterous movements of the hand and foot is achieved, we must have some knowledge about the function and circuitry of MNs involved in fine motor control. Thus, obtaining genetic tools in mice that could reproducibly label the IH and IF MNs could help address questions as to how these neurons differ perhaps in their electrophysiological properties and connectivity compared with MNs innervating limb muscles involved in gross motor control. To this end, we found that MNs that innervate the IH with MNs innervating limb muscles involved in fine motor control also serve as a cautionary tale of relying on CRE-recombinase expression.

Atoh1Cre/<sup>+</sup>-lineage neurons in the intermediate rhombomere R2 expression (Mendelsohn et al., 2017) although these would need further characterization of spatial and temporal overlap with the Atoh1<sup>Cre<sup>+</sup></sup> line to determine their suitability for future studies. Furthermore, Osmr and Colba1 could be considered for exploration of specifically IF MNs (Mendelsohn et al., 2017).

An alternate strategy would be to use viruses to restrict labeling to just the IH or IF MN pools. For example, the Atoh1<sup>Cre<sup>+</sup></sup> line could be crossed to an intersectional line expressing a reporter of choice (i.e., fluorescent, optogenetic, or chemogenetic reporter) and an AAV-FLPo injected into either the spinal cord or in the forepaw and hindpaw to be taken up retrogradely to the IH and IF MNs. However, a caveat of this approach is the potential damage to the spinal cord or muscles of the forepaw and hindpaw because of the needle injection. Thus, appropriate injection controls would be needed with this approach.

Altogether, we present here that the Atoh1<sup>Cre<sup>+</sup></sup> mouse consistently labels MNs of the IH and IF and that the Atoh1<sup>Cre<sup>+</sup></sup> mouse could be used to probe the function and connectivity of MNs in fine motor control. Our findings also serve as a cautionary tale of relying on CRE-recombinase mouse lines to faithfully report endogenous gene expression and speak to the need for careful follow-up experiments to appropriately interpret reporter expression.

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