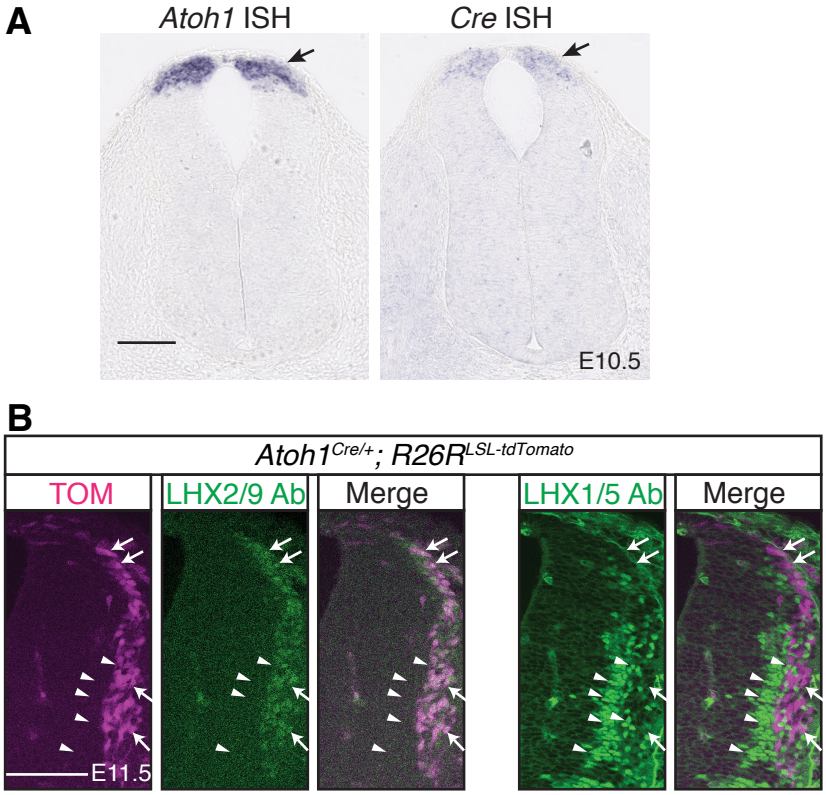

Figure S1, related to Figure 1. *Atoh1*$^{Cre/+}$ knock-in mouse recapitulates *Atoh1* expression. (A) *Cre* recombinase mRNA is expressed in a similar pattern as *Atoh1* endogenous mRNA at E10.5 (arrows) in the *Atoh1*$^{Cre/+}$ knock-in mouse. (B) *Atoh1*-lineage neurons (*Atoh1*$^{Cre/+}$*; R26R*$^{LSL\text{-}tdTomato}$, TOM+, magenta) express the appropriate downstream lineage markers, LHX2/9 (green, arrows), and not the lineage markers of neighboring spinal cord populations, LHX1/5 (green, arrowheads). Scale bars are 100 µm.

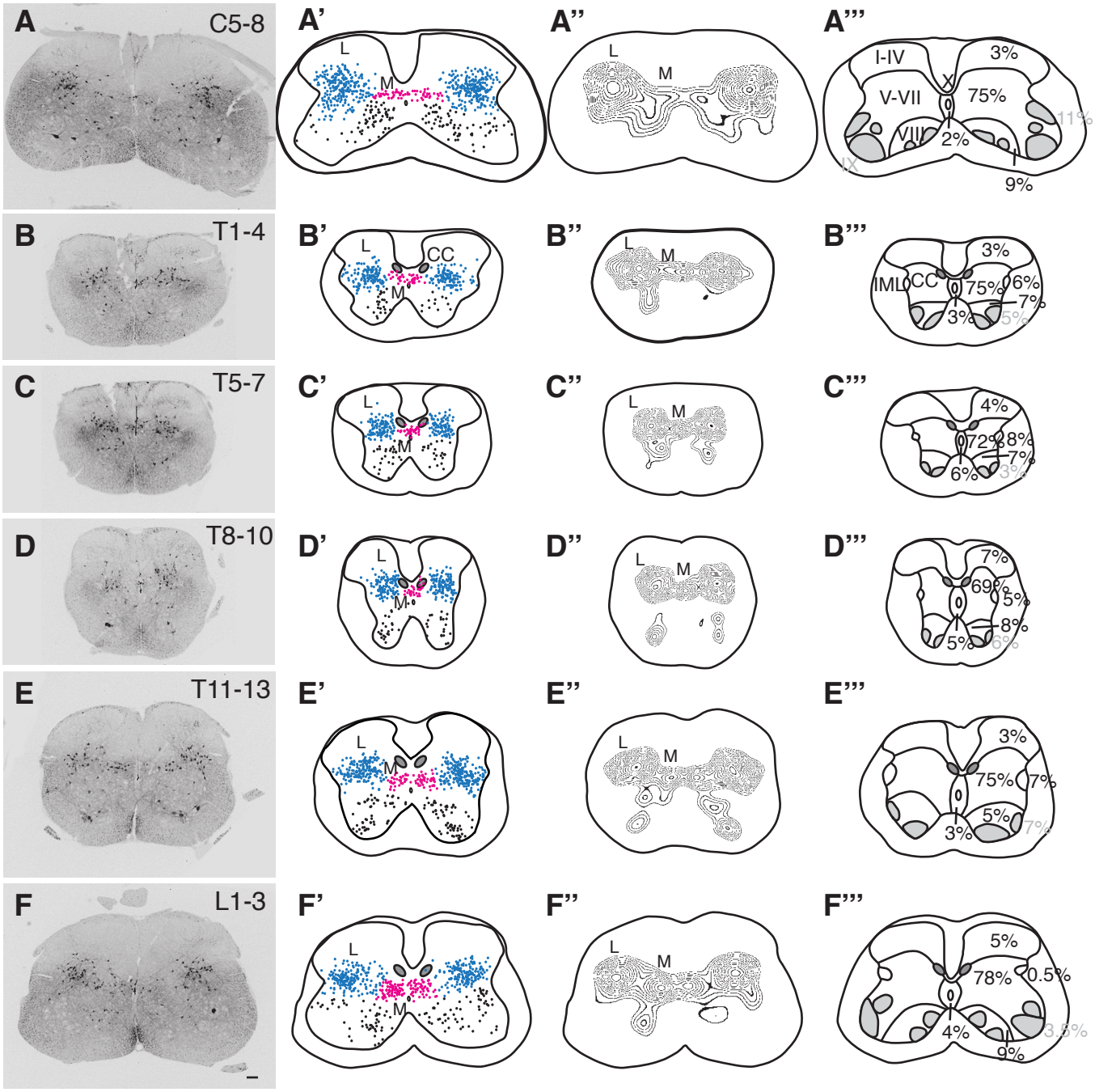

Figure S2, related to Figure 3. *Atoh1*-lineage neurons form a medial and lateral population in laminae V-VII throughout the rostral-caudal axis. (A-F) Representative sections of *Atoh1*-lineage cells (TOM+ fluorescence in grayscale) from lower cervical (C5-8), thoracic (T1-13), and upper lumbar (L1-3). (A'-F') *Atoh1*-lineage cell distribution from six representative sections overlayed on to one spinal cord diagram of a given spinal cord region. Each dot represents one cell: Medial (M) population in pink, Lateral (L) population in blue. (A''-F'') Dot plots from A'-F' represented as contour plots using ImageJ Interactive 3D Surface plot. The contour plot delineates pixel density from A'-F' and were used to determine M and L cell populations. (A'''-F''') Percentage of *Atoh1*-lineage TOM+ cells in laminae I-IV, V-VII, VIII, IX (grey), X, and the intermediolateral (IML) nucleus as determined using a fluorescent Nissl stain from n=3 spinal cords. Clarke's column (CC) in dark grey. Scale bar is 100 μm.

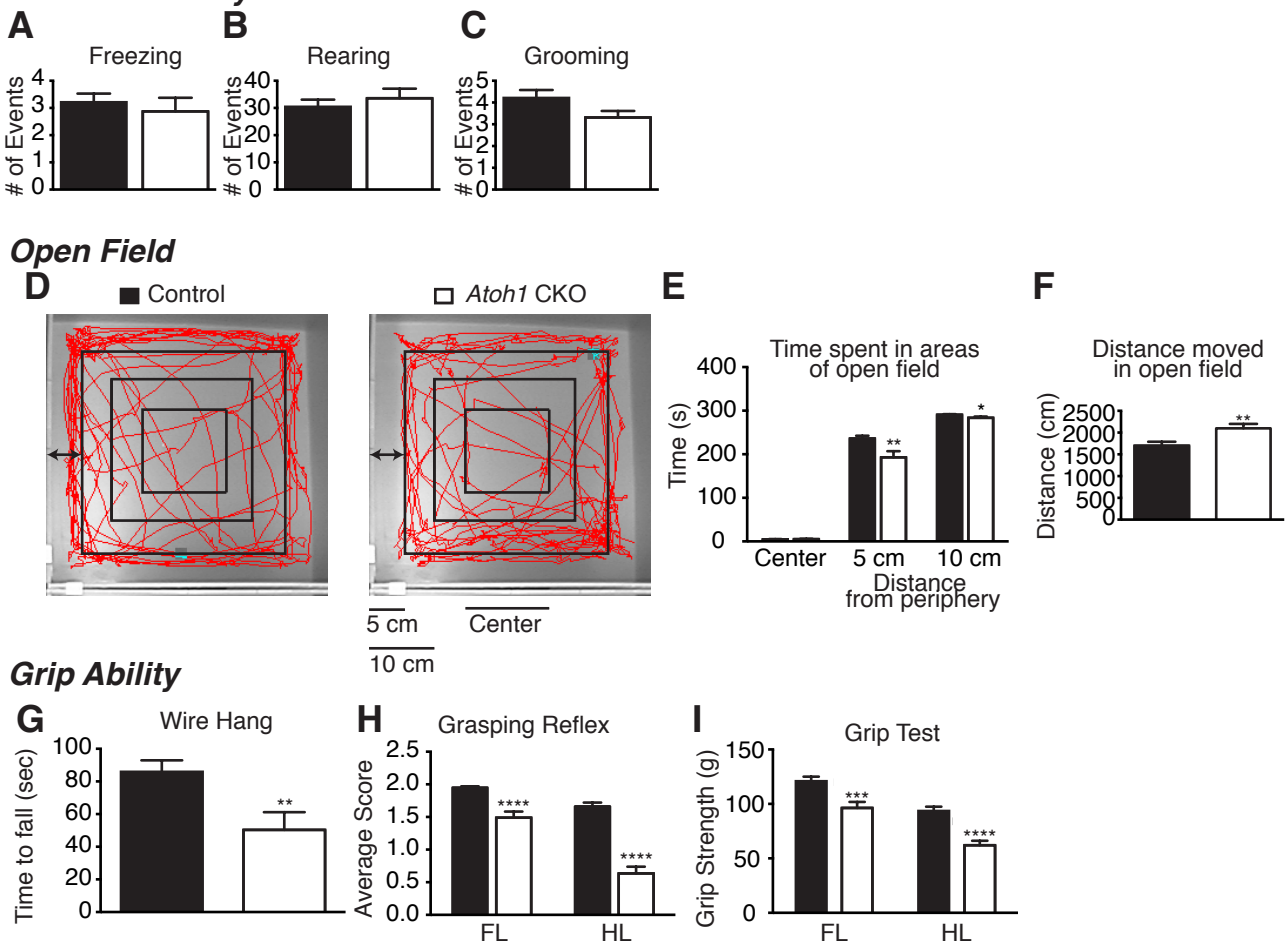

Figure S3, related to Figure 7. Additional behavioral analyses for *Atoh1* CKO mice. (A-C) During a five minute activity test, control (black) and *Atoh1* CKO (white) mice freeze, rear, and groom a similar number of times. (D-F) *Atoh1* CKO mice spend their time slightly further away from the periphery (10 cm) than control mice. In representative open field traces, tracking the mouse's movements, note that the control mouse spends more time 5 cm from the periphery (double arrow) compared to the *Atoh1* CKO mouse (quantitated in E). *Atoh1* CKO mice are just as anxious as control mice (E, center). (F) The total distance moved is greater in the *Atoh1* CKO mice. (G-I) The grip ability of *Atoh1* CKO mice is impaired. They fall more quickly on the wire hang test (G) and have diminished grasping reflex (H) and grip strength (I) in both forelimbs (FL) and hindlimbs (HL). n=23-24 for control, n=14-16 for *Atoh1* CKO. Mean ± SEM shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Supplemental Movies

Movie S1, related to Figure 7. Control mouse activity test. Video clip of a control mouse exploring a novel environment. Note that forepaws are placed on the side of the wall during supported rearing.

Movie S2, related to Figure 7. *Atoh1* CKO mouse activity test. *Atoh1* CKO mice exhibit some odd behaviors when exploring a novel environment. The mouse "bobs" up and down during unsupported rearing with forepaws close to the body. The mouse also "reaches" the wall during supported rearing and keeps one paw retracted while freezing.

Movie S3, related to Figure 7. Control mouse ladder rung test. A control mouse has no trouble traversing a horizontal ladder.

Movie S4, related to Figure 7. *Atoh1* CKO mouse ladder rung test. An *Atoh1* CKO mouse clearly slips and cannot correct paw misplacement when traversing a horizontal ladder.

Supplemental Experimental Procedures

Experimental Animals

We note that approximately 47% of the *Atoh1*$^{Cre/+}$;*R26R-tdTomato,* TOM+ mice have "dysregulated" expression of the reporter gene (77 out of 163 TOM+ mice). Dysregulation was defined as widespread reporter expression in non-neural tube tissues. A noticeable, highly variable expression of TOM fluorescence in the skin and mesenchyme can be seen by whole mount. These animals were pre-screened by fluorescence detection in P0 or P1 pups and discarded. The dysregulation is not germline and appears to be reflective of variability at the *Atoh1* locus.

Tissue Preparation, Immunohistochemistry (IHC), in situ hybridization (ISH), and Microscopy

Postnatal day 10-14 (P10-14), P30, or adult mice were anesthetized with Avertin (2,2,2 Tribromoethanol) (0.02 mL of 0.04 M Avertin in 2-methyl-2-butanol and distilled water/g mouse), and then transcardially perfused, first with PBS and then with 4% paraformaldehyde. A ventral laminectomy exposed the spinal cord to the fixative. Tissue was incubated at 4°C for 2 hrs to overnight, washed, and cryoprotected in 30% sucrose. Spinal cords were cut into lower cervical (C5-8), thoracic (T1-4, T5-7, T8-10, T11-13), and upper lumbar (L1-3) segments before embedding in OCT and sectioned on a cryostat. For birth dating studies, pregnant female mice with embryos at age E9.5, E10.5, and E11.5 were injected with Tamoxifen (Sigma, St. Louis, MO) in sunflower oil (Sigma) at 62.5 mg per kg of mouse.

Cryosections (20-40 μm) were blocked with a PBS/1-3% normal goat or donkey serum/0.1% NP-40 for up to one hour at room temperature (RT) and incubated overnight with primary antibody at 4°C. The appropriate secondary antibody (Alexa 488 and/or 657, Invitrogen, Carlsbad, CA) was incubated for an hour at RT. The following primary antibodies and concentrations were used: 1:100 rabbit anti-ATOH1 (Helms and Johnson, 1998), 1:4000 rabbit

anti-LHX2/9 (gift of Dr. Thomas Jessell)(Liem et al., 1997), 1:100, mouse-anti LHX1/5 (4F2) (Developmental Studies Hybridoma Bank) (Tsuchida et al., 1994),1:200 rabbit anti-ISLET1/2 (gift of Dr. Thomas Jessell), 1:2000 rabbit anti-HB9 (gift of Dr. Sam Pfaff), 1:5000 guinea pig anti-VGLUT1 (Millipore, Billerica, MA), 1:1000 rabbit anti-PV28 (Swant, Marly, Switzerland) , 1:1000 mouse anti-NeuN (Millipore), 1:1000 rabbit anti-OLIG2 (Millipore), 1:1000 rabbit anti-SOX2 (Millipore), 1:500 chick anti-GFP (Millipore and Aves), 1:100 goat anti-FOXP2 (Santa Cruz Biotechnology), 1:500 rabbit anti-PAX2 (Invitrogen). NeuroTrace® 500/525 Green or 640/660 Deep-Red Fluorescent Nissl (Invitrogen) staining (1:300) was incubated for 20 min. after all other primary and secondary antibodies were applied.

For simultaneous IHC and ISH, ISH was performed as per standard protocols with the exception that the proteinase K step was replaced with a one hour incubation in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris pH 8.0)(protocol available upon request) (Gray, 2013). After incubation, regular IHC was performed. Vectashield (Vector labs, Burlingame, CA) was added before coverslipping. ISH for *Atoh1* (Gowan et al., 2001), *Lhx2* (Lee et al., 1998), and *Barhl2* (Saba et al., 2005) was performed as per standard protocols and is available upon request.

Fluorescent or double IHC/ISH images were taken on a Zeiss (Oberkochen, Germany) LSM510 confocal with a 3 μm or 10 μm optical slice as appropriate, on a Nikon ECLIPSE 80i confocal (Tokyo, Japan), or on the MicroBrightField (MBF) System using an Olympus BX51 (Center Valley, PA) epifluorescence microscope. Images were pseudocolored using a magenta/green/blue color scheme using Adobe Photoshop (Adobe, San Jose, CA). ISH sections were imaged with a Nanozoomer C9600-12 (Hamamatsu).

For Figure 3 and Figure S2, three P14 spinal cords were divided into lower cervical (C5-8), thoracic (T1-4, T5-7, T8-10, T11-13), and upper lumbar (L1-3) regions. Cell counts for each

spinal cord region were calculated by sampling six 30 μm sections 300 μm apart and multiplying by ten to cover 1.8 mm of tissue for each given spinal cord region. For Figure 6A'', B'', C'', *Vglut1* ISH and PV and HB9 immunostaining were evaluated for "control" and "Atoh1 CKO" genotypes from three P10 spinal cords each. Percentage of PV+ spinal cord area was evaluated using ImageJ (Abramoff, 2004). Percentages and counts were calculated by sampling six 30 μm sections 240 μm apart and multiplying by eight to cover 1.44 mm of tissue for each given spinal cord region.

Electrophysiology

Atoh1$^{Cre/+;}$ *Pv*$^{IRES-Cre/+}$; *R26R*$^{LSL-ChR2(H134R)-EYFP-WPRE}$; *R26R*$^{LSL-tdTomato}$, or *Atoh1*$^{Cre/+;}$ *R26R*$^{LSL-ChR2(H134R)-EYFP-WPRE}$; *R26R*$^{LSL-tdTomato}$ mice aged P12-P15 were anesthetized with isoflurane (Butler Schein, Dublin, OH) and decapitated. Spinal cords (T9-L3) were dissected out in either oxygenated (95% O_2/ 5% CO_2) artificial ASCF (aACSF in mM): 125 NaCl, 26 $NaHCO_3$, 1.25 $NaH_2PO_4 \cdot 2H_2O$, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 0.4 Ascorbic Acid, 2 Sodium Pyruvate, 26 D-glucose, pH 7.3-7.4, 305-320 mOsm, or ice cold dissecting buffer (in mM): 80 NaCl, 26 $NaHCO_3$, 1.25 $NaH_2PO_4 \cdot 2H_2O$, 2.5 KCl, 0.5 $CaCl_2$, 3.5 $MgCl_2$, 0.4 Ascorbic Acid, 2 Sodium Pyruvate, 75 Sucrose, 11 D-glucose. Meninges were taken off and the spinal cord was embedded in 3.25% Agarose. Acute spinal cord transverse slices (350-400 μm) were sectioned on a Leica (Solms, Germany) Vibratome 1000 Plus in oxygenated ice cold aASCF or dissecting buffer. Sections were kept in oxygenated aACSF for at least an hour at room temperature (RT) before recording or incubated at 34°C for 15 min and then RT until recording.

Whole cell patch voltage clamp recordings were performed with an Axopatch 200A (Molecular Devices, Sunnyvale, CA). The internal pipet solution contained (in mM): 130 Potassium Gluconate, 5 Sodium Gluconate, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES (pH 7.2), 4 mM Na_2ATP, 0.5% Biocytin, pH 7-7.2, 290-305 mOsm (adjusted with Sucrose). Pipet resistances

ranged from 5-7 MOhms. For the channelrhodopsin experiments, 10 ms of blue light (465-495 nm, X-cite 200DC (Lumen Dynamics) illuminated a 100-250 μm diameter circle through a 10x objective (Nikon Eclipse E600FN). Spinal cord slices were 1-1.5 mm wide. For experiments with AMPA blocker, 20 μm DNQX in aACSF was perfused over the slice at 1 mL/min for 10-12 minutes and washed out for 40-50 min. with aACSF. Data were analyzed with ClampFit 9.2 (Molecular Devices).

Biocytin staining

Acute slices were immediately placed in 4% formaldehyde following recordings. Slices were rinsed with PBS and then incubated for 6-8 hours in 1%Triton/PBS at RT. Slices were then incubated overnight at RT in 1:500 guinea pig anti-rat VGLUT1 antibody (Millipore), rinsed with PBS, and incubated in a solution containing goat anti-guinea pig Alexa 647 (Invitrogen) and Alexa 488-avidin (Invitrogen) overnight at RT. The slices were then rinsed with PBS and mounted between two coverslips with Vectashield (Vector labs) and sealed along the edges with vacuum grease. Dendrites and axons were traced in Neurolucida v10 (MBF Bioscience, Williston, VT) on the MicroBrightField (MBF) System to assess the dendritic and axonal processes (polar coordinate plots). Overlaid traces were aligned using the central canal and midline as landmarks. Axons were distinguished from dendrites morphologically as a thin process compared to the dendrites which were thicker and had spines.

Stereotaxic Injections

Adult animals were anesthetized with ketamine (120 mg/kg)/xylazine (16mg/kg) and prepared for stereotaxic injections into the cerebellum. Cholera toxin subunit B (50 nL) was injected into the following coordinates adjusted from Bregma: AP axis -5 to -6.12, ML axis 0, DV axis -1 to -1.5 mm; Brains and spinal cords were harvested 7 days after injection.

Behavioral tests

All tests were performed on 21-24 “control” animals and 13-16 “Atoh1 CKO” animals (Figure 7 and Figure S3). Male and females were evenly distributed among control animals. The Atoh1 CKO cohort had 12 males and 4 females. All behavior tests were performed at 9-13 weeks of age except for the von Frey hairs test (17-21 weeks) and the locomotor and open field tests (20-27 weeks). All testers were blind to genotype. Three control genotypes were pooled together as no significant differences were detected between the three control genotypes. Left and right scores were averaged for forelimbs and hindlimbs as no lateral defects were detected. Mice were acclimated to the testing room for 0.5-1 hour on the day of testing. While most *Atoh1* CKO mice lived well into adulthood (8-9 months old), three of the original 19 *Atoh1* CKO mice died before 9 weeks of age when the first behavior tests were performed. Of the remaining 16 *Atoh1* CKO mice, two died, one at 4 months and one at 5 months. In contrast, none of the control mice died.

Activity Test

Animals were filmed for five minutes in a novel environment. Number of events for each animal were recorded for the following behaviors: freezing, rearing, grooming, retracting forepaws during unsupported rearing, and “reaching” for the wall during supported rearing. Holding a forepaw retracted while exhibiting freezing behavior was scored as 0 or 1 for each event and the scores averaged for a given animal.

Open Field

Mice were placed in a 40 cm x 40 cm box with areas designated as center (14 cm x 14 cm box in the middle) and border (5 and 10 cm from the periphery) for 5 min. Tracking software by Noldus Ethovision (Wageningen, The Netherlands) followed the centroid of the mouse to determine the time spent in each area and total distance moved.

DigiGait

Mice acclimated for one minute to the DigiGait system (Mouse Specifics, Quincy, MA) and started at a speed of 10 cm/s ramping up to a testing speed of 20 cm/s in 10 sec. At least 5

reproducible steps per paw were used for analyses. In addition to several gait parameters measured by the system (refer to the DigiGait manual), animal length and width were also recorded. Ataxia Coefficient = (max stride length - min stride length)/ mean stride length.

Balance Beam

Mice traversed a 75 cm beam of increasing difficulty (18 mm, 9 mm, 5 mm widths) toward a dark housing on the opposite side. Three trials were performed per beam width before proceeding to the narrower beam. Time to cross and number of foot slips were recorded.

Ladder Rung

Mice were filmed (60 frames/sec, interlaced, and rendered to 30 frames/sec for playback) going across an 81 cm horizontal ladder with 1.5 cm rung separation. Steps for all limbs were scored as follows: 0 - Total miss,1 - Deep slip, 2 - Slight slip, 3 - Replacement, 4 - Correction, 5 - Partial placement, 6 - Correct placement (Farr et al., 2006; Tennant and Jones, 2009). Detailed descriptions available upon request. The average score per step for 3 trials run over 3 days is reported. A score of 0-2 was counted as a misstep.

Rotarod

Mice were placed on an accelerating rotarod (IITC Life Science Series 8, Woodland Hills, CA) from 5 to 45 RPM in 300 sec. Four trials were performed on one day with at least a 30 minute wait time between trials.

Wire Hang

Mice were allowed to grip a wire mesh and then hung upside down. Time to fall was recorded and trial was stopped at 120 seconds if mouse did not fall.

Grasping Reflex

Grasping reflex was assessed when a blunt instrument was stroked on the underside of the palm of a suspended animal. Reflexes were scored as 0 for absent, 1 for weak, and 2 for strong. Left and right forelimbs were scored together as a mouse would usually grab the instrument with both limbs simultaneously. Left and right hindlimbs were scored separately and

then averaged together to give a hindlimb score. Six trials over 3 days were averaged together for the final score for each animal.

Grip Test

Grip force was testing using a grip strength system from San Diego Instruments. Five trials were performed per forelimb or hindlimb pair.

Hot Plate

Mice were placed on a 52°C hot plate (IITC Life Science Series 8). Time to first visible reaction (for example, paw licking) was recorded.

Footshock

Mice were acclimated in chambers with parallel metal rods for one minute (Med Associates Inc., St. Albans, VT). Electrical current starting at 0.05 mA for 2 sec. was run through the rods. Every 5 sec. the current was increased by 0.05 mA. The current required to flinch, jump (all four feet off the rods), and vocalize were recorded.

Von Frey Hairs

Sensitivity to mechanical pain was assayed by the von Frey hairs up-down method (Chaplan et al., 1994). Mice were acclimated in acrylic cylinders on a wire mesh for 1-2 hours on the day of testing. Graded filaments from 0.4 to 15 g were applied for approximately 3 seconds to the plantar hindpaw with at least 5 minutes between each application. A response was recorded as toe spreading, flinching, or licking.

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