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## Supplemental Information

# Compromised Survival of Cerebellar Molecular Layer Interneurons Lacking GDNF Receptors GFRα1 or RET Impairs Normal Cerebellar Motor Learning

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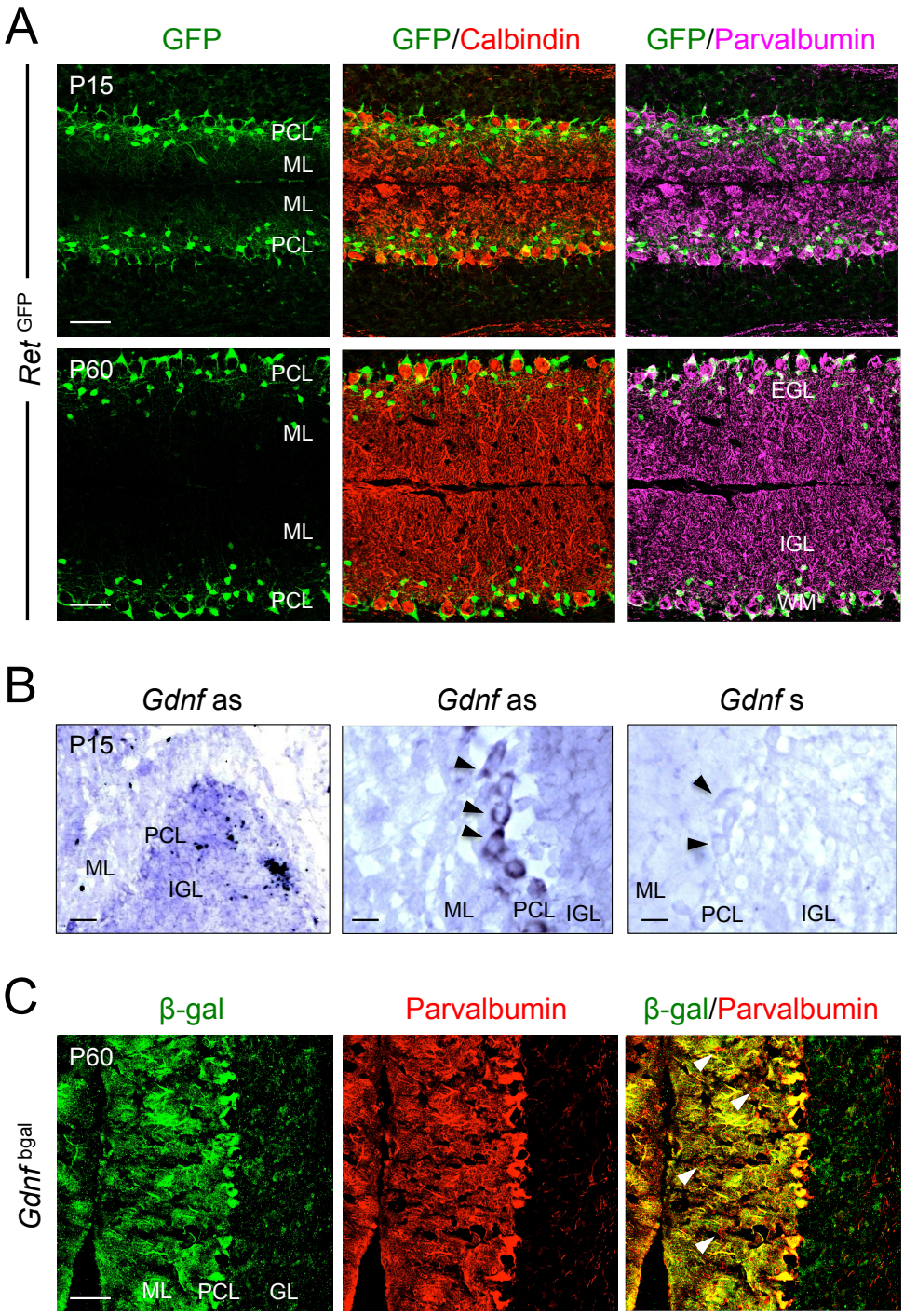
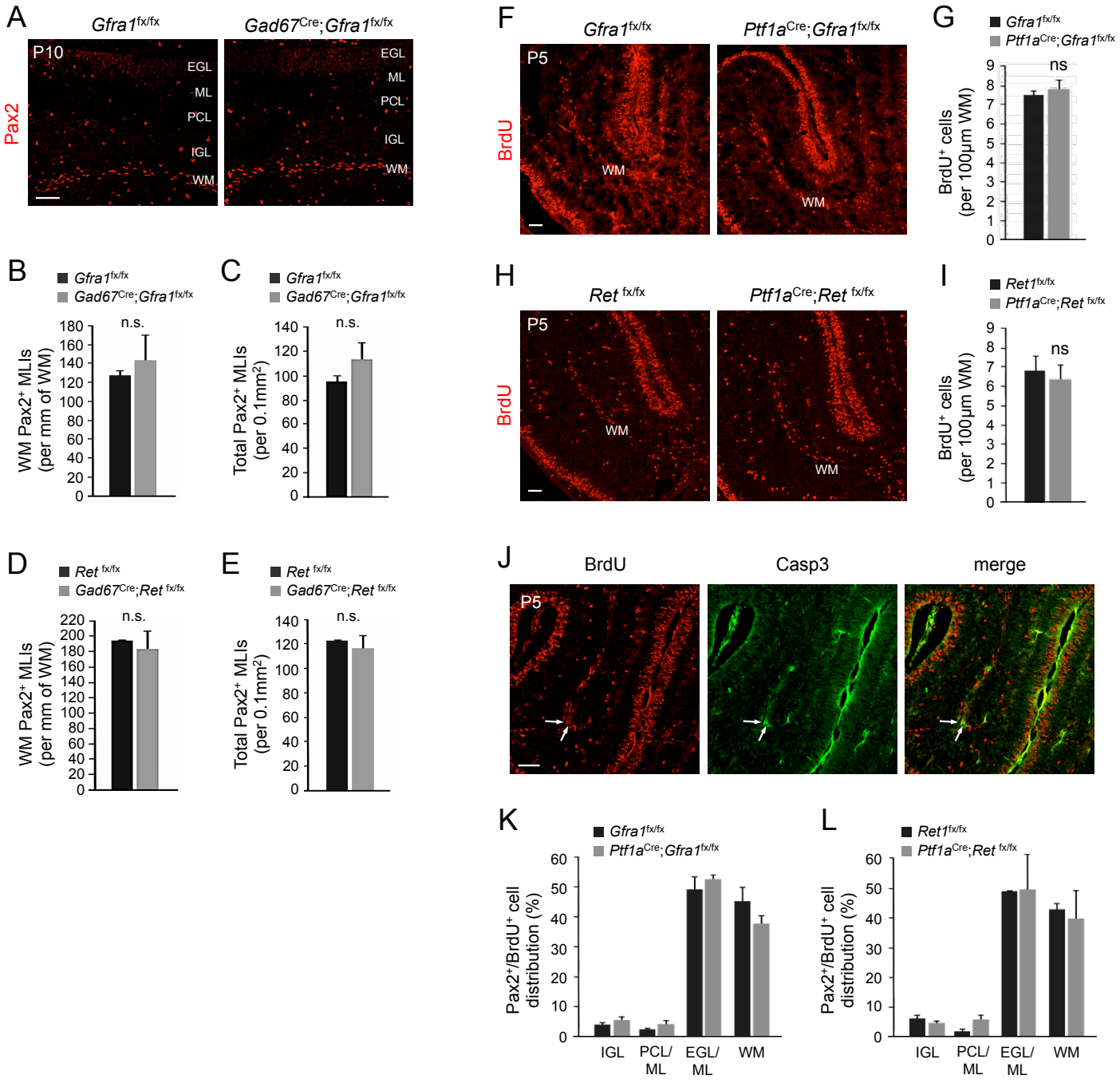



**Supplementary Figure S1. Related to Fig. 1. Expression of RET and GDNF during postnatal cerebellar development**

(A) Sagittal cerebellar sections from P15 and P60 *Ret*$^{GFP}$ mice immunostained for GFP (green), Calbindin (red) and Parvalbumin (purple). GFP was localized to the inner part of the molecular layer. GFP$^+$ axons were seen enveloping Calbindin$^+$ PC bodies, a characteristic of basket cells. GFP$^+$ signal co-localized with Parvalbumin in MLIs but not in PCs, which also express PV. PCL: Purkinje cell layer, ML: molecular layer. Scale bar, 50μm.

(B) Sagittal cerebellar sections from P15 wild type mice hybridized with *Gdnf* antisense (as) and sense (s) riboprobes. Arrowheads point to PCs strongly expressing the *Gdnf* mRNA in sections incubated with the antisense probe. Lower intensity signal was detected in the IGL. No signal was detected in the ML or in sections incubated with the sense probe. ML: molecular layer, PCL: Purkinje cell layer, IGL: internal granule layer. Scale bar, 500μm (far left) and 100 μm (center and far right).

(C) Sagittal cerebellar sections from P60 *Gdnf*$^{bgal}$ mice immunostained for β-galactosidase and Parvalbumin (PV). GDNF expression was mainly found in PCs (labeled here with anti-PV antibodies), with lower levels present in the granule layer (GL). No GDNF expression was observed in PV$^+$ MLIs (arrowheads). ML: molecular layer, PCL: Purkinje cell layer, GL: granule layer. Scale bar, 50μm.




**Supplementary Figure S2. Related to Fig. 2. No effect of deletion of *Gfra1* or *Ret* with *Gad67*$^{CRE}$ in cerebellum and normal proliferation and migration of MLI progenitors in *Gfra1* and *Ret* conditional mutants**

(A) Cerebellar sections from P10 *Gad67*$^{Cre}$;*Gfra1*$^{fx/fx}$ conditional mutant mice and *Gfra1*$^{fx/fx}$ controls stained with Pax2 antibodies. EGL: external granule layer, ML: molecular layer, PCL: Purkinje cell layer, IGL: internal granule layer, WM: white matter. Scale bar, 50µm.

(B) Quantification of Pax2$^+$ progenitor density in the folial WM of P10 *Gad67*$^{Cre}$;*Gfra1*$^{fx/fx}$ conditional mutant mice and *Gfra1*$^{fx/fx}$ controls. Values represent mean ±SEM. N=3 mice per group. n.s; not significantly different.

(C) Quantification of the total Pax2$^+$ MLI progenitor density in the cerebellar cortex of P10 *Gad67*$^{Cre}$;*Gfra1*$^{fx/fx}$ conditional mutant mice and *Gfra1*$^{fx/fx}$ controls. Values represent mean ±SEM. N=3 mice per group. n.s; not significantly different.

(D) Quantification of Pax2$^+$ progenitors in the folial WM of P10 *Gad67*$^{Cre}$;*Ret*$^{fx/fx}$ conditional mutant mice and *Ret*$^{fx/fx}$ controls. Values represent mean ±SEM. N=3 mice per group. n.s; not significantly different.

(E) Quantification of the total Pax2$^+$ MLI progenitor density in the cerebellar cortex of P10 *Gad67*$^{Cre}$;*Ret*$^{fx/fx}$ conditional mutant mice and *Ret*$^{fx/fx}$ controls. Values represent mean ±SEM. N=3 mice per group. n.s; not significantly different.

(F) Cerebellar sections from P5 *Gfra1* conditional mutant mice (*Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$) and controls (*Gfra1*$^{fx/fx}$) stained with BrdU antibodies 2h after a BrdU pulse. WM: white matter. Scale bar, 50µm.

(G) Quantification of BrdU$^+$ cells in WM of conditional *Gfra1* mutant mice and controls. Values represent mean ±SEM. N=6 and 4 mice per group, respectively. n.s; not significantly different.

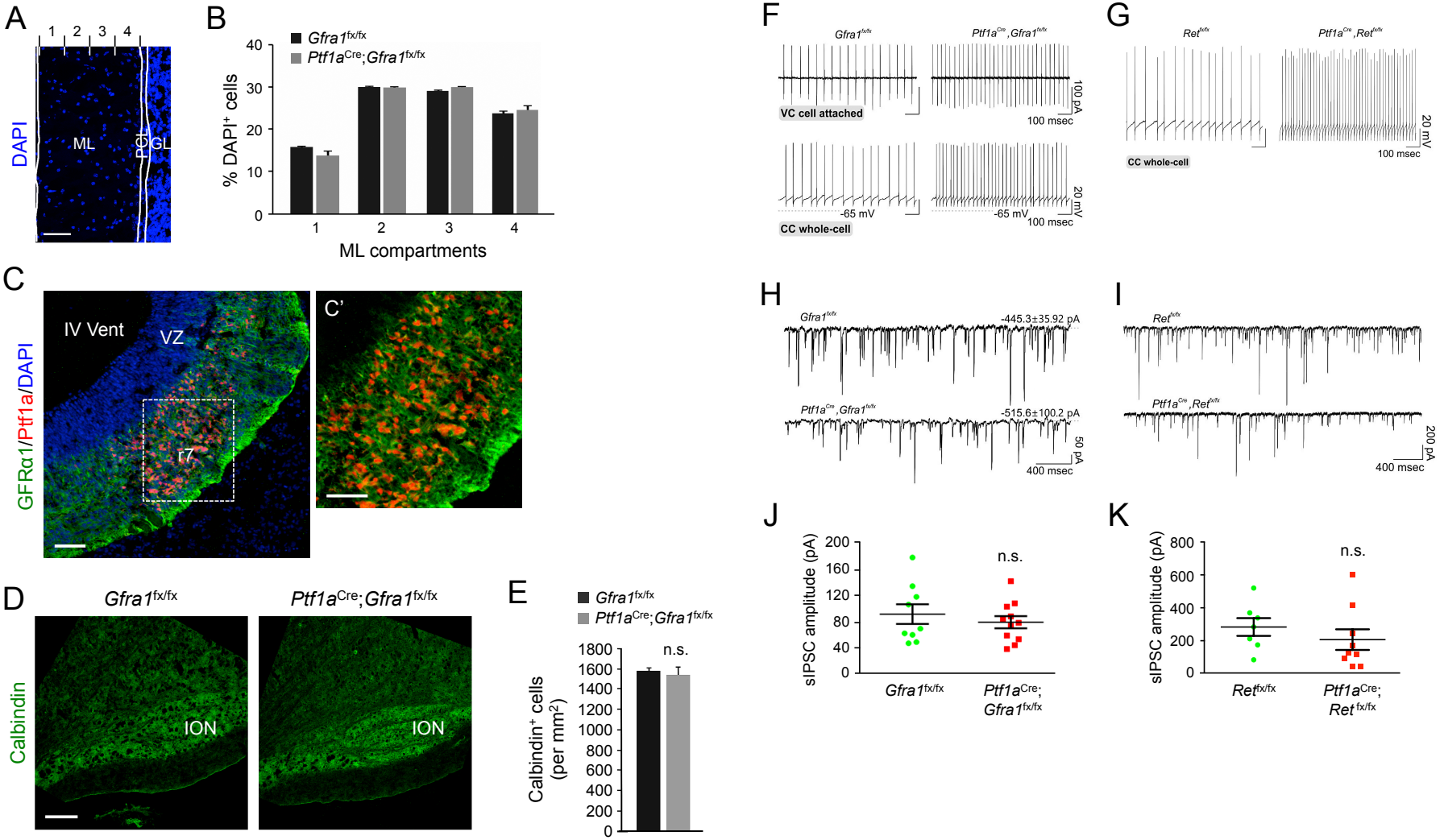
(H) Cerebellar sections from P5 *Ret* conditional mutant mice (*Ptf1a*$^{Cre}$;*Ret*$^{fx/fx}$) and controls (*Ret*$^{fx/fx}$) stained with BrdU antibodies 2h after a BrdU pulse. WM: white matter. Scale bar, 50µm.

(I) Quantification of BrdU$^+$ cells in WM of conditional *Ret* mutant mice and controls. Values represent mean ±SEM. N=5 and 6 mice per group, respectively. n.s; not significantly different.

(J) Activated caspase-3 and BrdU in WM of conditional *Gfra1* mutant mice. Arrows indicate cells that incorporated BrdU and show activation of caspase-3. Scale bar 50µm.

(K) Quantification of the percentage of Pax2$^+$/BrdU$^+$ double-positive cells in each layer of the cerebellar cortex relative to the total number of double-labeled cells in *Gfra1* conditional mutant mice (*Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$) and controls (*Gfra1*$^{fx/fx}$) injected with BrdU at P5 and sacrificed at P10. EGL: external granule layer, ML: molecular layer, PCL: Purkinje cell layer, IGL: internal granule layer, WM: white matter. Values represent mean ±SEM. N=5 and 4 mice per group, respectively. n.s: not significantly different.

(L) Quantification of the percentage of Pax2$^+$/BrdU$^+$ double-positive cells in each layer of the cerebellar cortex relative to the total number of double-labeled cells in *Ret* conditional mutant mice (*Ptf1a*$^{Cre}$;*Ret*$^{fx/fx}$) and controls (*Ret*$^{fx/fx}$) injected with BrdU at P5 and sacrificed at P10. Values represent mean ±SEM. N=3 mice per group. n.s: not significantly different.




**Supplementary Figure S3. Related to Fig. 3. Normal MLI distribution in cerebellum of adult *Gfra1* conditional mutants. Co-expression of GFRα1 and Ptf1a in the embryonic inferior olivary nucleus, but no loss of calbindin positive cells in adult *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ conditional mutant mice. Normal MLI distribution and PC sIPSC amplitudes in the cerebellum of adult *Gfra1* and *Ret* conditional mutants**

(A) Distribution of DAPI$^+$ cells in the ML of 2 month old *Gfra1* conditional mutant mice and controls. The ML was divided in 4 strata as shown. Scale bar, 50µm.

(B) Quantification of the percentage of DAPI$^+$ cells in each ML stratum. ML: molecular layer, PCL: Purkinje cell layer, GL: granule layer. Values represent mean ± SEM. N=3 mice per group.

(C) Expression of GFRα1 (green) and Ptf1a (red, from *Ptf1a*$^{Cre}$;*Rosa26*$^{dTOM}$) in E12.5 mouse brainstem. Counterstaining with DAPI in blue. Rostral is to the right. r7, rhombomere 7; IV Vent, IVth ventricle; VZ, ventricular zone. Scale bar, 100µm; inset (C'), 50µm.

(D) Calbindin expression in inferior olivary nucleus (ION) of adult *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ conditional mutant mice and *Gfra1*$^{fx/fx}$ control. Scale bar, 100µm.

(E) Quantification of the density of calbindin$^+$ cells in the adult inferior olivary nucleus (ION) of adult *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ conditional mutant mice and *Gfra1*$^{fx/fx}$ controls. Values represent mean ± SEM. N=6 mice per group. n.s., not significantly different (p>0.05).

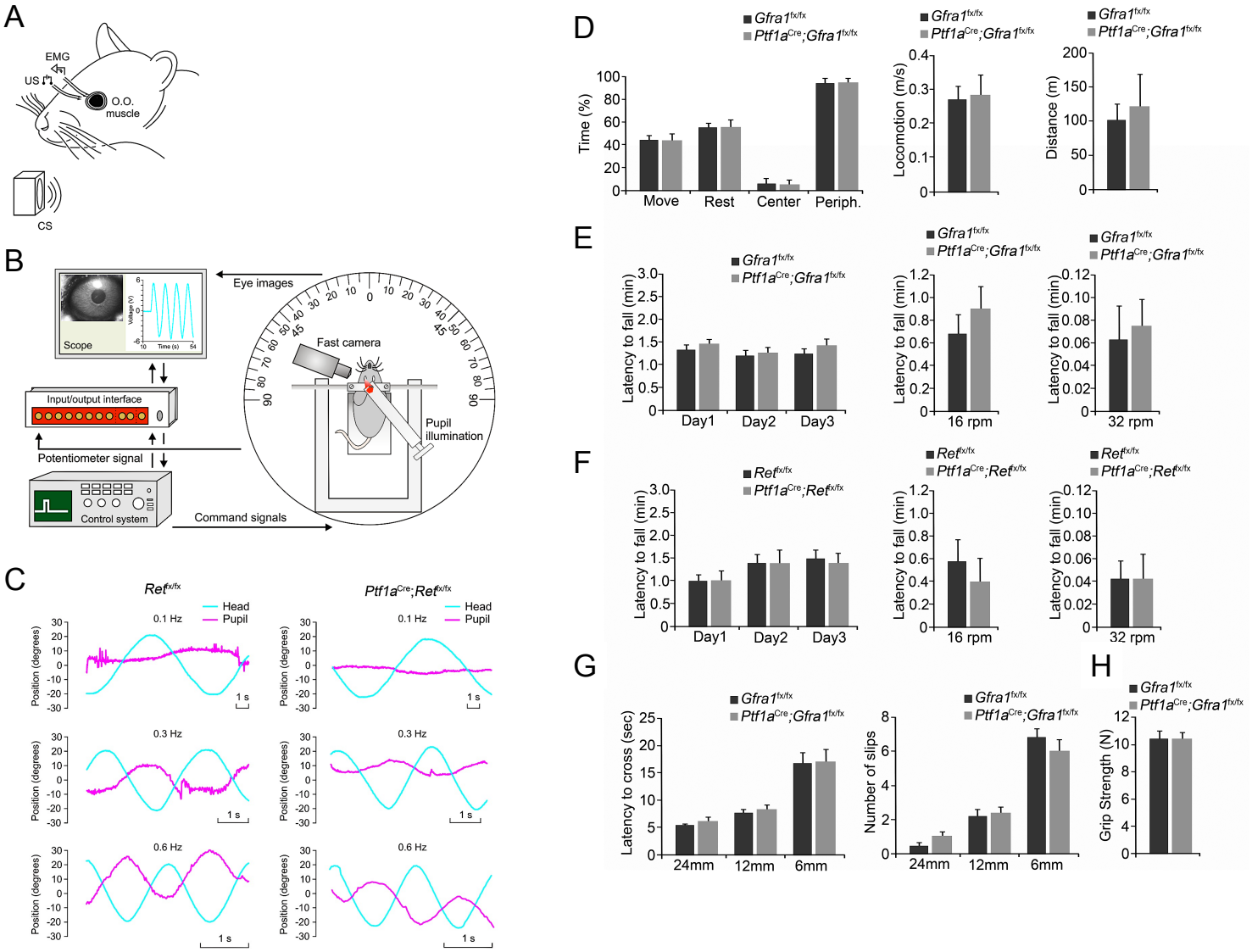
(F) Examples of patch clamp recordings in cell-attached voltage-clamp (top) and whole-cell current-clamp (bottom) modes from PCs in cerebellar slices from *Gfra1*$^{fx/fx}$ (left) and *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ (right) mice. Note increased firing frequency in the conditional mutant.

(G) Examples of patch clamp whole-cell recordings from PCs in cerebellar slices from *Ret*$^{fx/fx}$ (left) and *Ptf1a*$^{Cre}$;*Ret*$^{fx/fx}$ (right) mice. Note increased firing frequency in the conditional mutant.

(H) Whole-cell patch clamp recordings from PCs performed in voltage clamp mode from *Gfra1*$^{fx/fx}$ (top) and *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ (bottom) mice. Spontaneous inhibitory postsynaptic currents (sIPSCs) were isolated by the application of CNQX and AP-5 to block ionotropic glutamatergic transmission (command potential -60 mV). Note decreased frequency of sIPSCs in mutant mice.

(I) Whole-cell patch clamp recordings of sIPSCs from PCs performed in voltage clamp mode from *Ret*$^{fx/fx}$ (top) and *Ptf1a*$^{Cre}$;*Ret*$^{fx/fx}$ (bottom) mice, performed as described for (H). Note lower sIPSCs frequency in mutant mice.

(J-K) sIPSC amplitude in PCs from cerebella of 2 month old *Gfra1* (E) or *Ret* (F) conditional mutant mice and controls as calculated from whole-cell voltage-clamp recordings in voltage-clamp mode. Each dot represents the mean of values obtained from 300-second recording samples from an individual PC. At least three mice per genotype were used for the recordings. The mean and SEM are shown with parallel lines. N=9 , 11 (J) and 7, 9 (K) PCs per group. n.s: not significantly different.




**Supplementary Figure S4. Related to Fig. 4. Experimental designs for analysis of classical eyeblink conditioning and vestibulo-ocular reflex. Gfra1 and Ret conditional mutants have normal motor behavior**

(A) For classical eyeblink conditioning, animals were presented with a tone as a conditioned stimulus (CS), delivered from a loudspeaker located 50 cm from the animal's head. For unconditioned stimulus (US), mice were implanted with stimulating electrodes on the left supraorbital nerve. To quantify conditioned responses (CRs), mice were implanted with bipolar electrodes to record the electromyographic (EMG) activity of the ipsilateral orbicularis oculi (O.O.) muscle.

(B) For vestibulo-ocular reflex, animals were placed on a turn-table and rotated (± 20°) at three selected frequencies (i.e. 0.1, 0.3, and 0.6 Hz, respectively). Compensatory eye movements were recorded using a fast-camera pupil-tracking system.

(C) Representative examples (averaged 3 times) of vestibulo-ocular (VOR) reflexes evoked in 2 month old *Ret* mutants and controls at the indicated frequencies.

(D) Quantitative analysis of general locomotor behavior of 3 month old *Gfra1*$^{fx/fx}$ and *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ mice in the openfield. Histograms show the percent of time spent moving, resting, in center or in periphery (left), the locomotion speed (middle) and the distance covered (right). Histograms show average ± SEM, N=25 mice per group.

(E) Quantitative analysis of rotarod behavior of 3 month old *Gfra1*$^{fx/fx}$ and *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ mice. Histograms show the latency to fall off the rod in an accelerating (4-40 rpm, left) and two constant (16 and 32 rpm, middle and right) speed protocols Histograms show average ± SEM, N=25 mice per group.

(F) Quantitative analysis of rotarod behavior of 3 month old *Ret*$^{fx/fx}$ and *Ptf1a*$^{Cre}$;*Ret*$^{fx/fx}$ mice. Histograms show the latency to fall off the rod in an accelerating (4-40 rpm, left) and two constant (16 and 32 rpm, middle and right) speed protocols Histograms show average ± SEM, N=10 mice per group.

(G) Quantitative analysis of behavior of 3 month old *Gfra1*$^{fx/fx}$ and *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ mice in the balance beam test using beams of three different sizes as indicated. Histograms show average ± SEM for the latency to cross the beam (left) and the number of slips (right), N=20 mice per group.

(H) Quantitative analysis of the grip strength in Newton (N) of 3 month old *Gfra1*$^{fx/fx}$ and *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ mice. Histograms show average ± SEM, N=20 mice per group.

# Supplemental Experimental Procedures

## Key Resource Table

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| **Antibodies** | | |
| goat anti-GFRα1 | R&D | AF560 |
| rabbit anti-Calbindin | Chemicon | AB1778 |
| mouse anti-Calbindin | Swant | #300 |
| rabbit anti-Somatostatin | Peninsula | T-4103 |
| chicken anti-GFP | Abcam | AB13970 |
| rabbit anti-beta galactosidase | Cappel | #55976 |
| rat anti-BrdU | Accurate Chemicals | OBT0030 |
| rabbit anti-Pax2 | Invitrogen | #71-6000 |
| rabbit anti.Parvalbumin | Swant | PV25 |
| rabbit anti-Cleaved Caspase3 | Cell Signaling | #9661 |
| mouse anti-Dystroglycan | Millipore | #05-298 |
| rabbit anti-Kv1.2 | Millipore | AB5924 |
| guinea pig anti-VGAT | Synaptic systems | #131004 |
| donkey anti-goat Alexa Fluor 488 | Invitrogen | A11055 |
| donkey anti-goat Alexa Fluor 568 | Invitrogen | A11057 |
| donkey anti-rabbit Alexa Fluor 488 | Invitrogen | A21206 |
| donkey anti-rabbit Alexa Fluor 555 | Invitrogen | A31572 |
| donkey anti-rabbit Alexa Fluor 647 | Invitrogen | A31573 |
| donkey anti-mouse Alexa Fluor 488 | Invitrogen | A21202 |
| donkey anti-mouse Alexa Fluor 555 | Invitrogen | A31570 |
| donkey anti-mouse Alexa Fluor 647 | Invitrogen | A31571 |
| donkey anti-rat DyLight 549 | Jackson ImmunoResearch | 712-506-153 |
| donkey anti-chicken DyLight 488 | Jackson ImmunoResearch | 703-485-155 |
| donkey anti-guinea pig Alexa Fluor 546 | Invitrogen | A11074 |
| | | |
| **Bacterial and Virus Strains** | | |
| n. a. | | |
| | | |
| **Biological Samples** | | |
| n. a. | | |
| | | |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| 4′-6-diamidino-2-phenylindole (DAPI, D1306, Sigma) | Sigma | D1306 |
| Tamoxifen | Sigma | T5648 |
| BrdU | Sigma | B5002 |
| Corn oil | Sigma | C8267 |
| | | |
| **Critical Commercial Assays** | | |
| n. a. | | |
| | | |
| **Deposited Data** | | |
| n. a. | | |

| | | |
|---|---|---|
| **Experimental Models: Cell Lines** | | |
| n. a. | | |
| | | |
| **Experimental Models: Organisms/Strains** | | |
| Mouse: GDNF+/- | Pichel et al., 1996 | |
| Mouse:GDNFbgal/+ | Moore et al.,1996 | |
| Mouse:RET GFP/+ | Jain et al.,2006 | |
| Mouse:RET fx | Kramer et al., 2006 | |
| Mouse:GFRa1 fx | by Mart Saarma and Jaan-Olle Andressoo | |
| Mouse:GAD67Cre/+ | Tolu et al.,2010 | |
| Mouse:Rosa26YFP/+ | Madisen et al.,2010 | |
| Mouse:GFRa1CreERT2/+ | Sergaki et al.,2017 | |
| Mouse:Ptf1aCre/+ | Kawaguchi et al.,2002 | |
| | | |
| **Oligonucleotides** | | |
| n. a. | | |
| | | |
| **Recombinant DNA** | | |
| n. a. | | |
| | | |
| **Software and Algorithms** | | |
| ImageJ software | http://imagej.nih.gov/ij/ | |
| Matlab scripts | available upon request | |
| Actimot Software | www.TSE-Systems.com | |
| GraphPad Prism 6 | Graphpad.com | |
| | | |
| **Other** | | |
| n. a. | | |
| | | |

## Animals

Mice were housed in a 12- hour (h) light/dark cycle and fed a standard diet. The following mouse lines were used: *Gdnf*$^{+/-}$ {Pichel:1996en}, *Gdnf*$^{bgal}$ {Moore:1996io}, *Ret*$^{GFP}$ {Jain:2006ie}, *Ret*$^{fx}$ (Kramer et al., 2006), *Gfra1*$^{fx}$ (kindly provided by Mart Saarma and Jaan-Olle Andressoo, University of Helsinki), *Ptf1a*$^{Cre}$ {Kawaguchi:2002hk}, *Gad67*$^{Cre}$ {Tolu:2010bu}, *Rosa26*$^{YFP}$ {Madisen:2010fi} and *Gfra1*$^{CreERT2}$ {Sergaki:2017uy}. All lines used were in the C57BL/6J background, except *Gdnf*$^{+/-}$ and *Gfra1*$^{fx}$ which were in CD1 background. All studies were performed on mice of both sexes. The day of vaginal plug was considered as embryonic day 0.5 (E0.5) and the day of birth as postnatal day 0 (P0). Control and mutant pups were derived from the same litter. All animal experiments were approved by Stockholm North Ethical Committee for Animal Research (protocols no. N27/15, N173/15 and N26/15).

## Histological studies

Postnatal and adult mice were anesthetized with isofluorane and transcardially perfused with PBS followed by 4% PFA. Brains were removed and postfixed in 4% PFA o/n. Tissue samples were washed in PBS, cryoprotected in 20% sucrose at 4°C, embedded

in OCT compound and frozen in dry ice. 14µm sections were obtained across the sagittal plane, collected onto Superfrost Plus slides (Thermo Fischer Scientific), air dried and stored at −20°C until use. For immunostaining, cerebellar sections were blocked for 1h in PBS containing 5% normal donkey serum and 0.1% Triton X-100. Incubation with primary antibodies, diluted in blocking solution, was done o/n at 4°C. The primary antibodies used were as follows: goat anti-GFRα1 (1:200; AF560, R&D), rabbit anti-Calbindin (1:500; AB1778, Chemicon), mouse anti-Calbindin (1:5000; #300, Swant), rabbit anti-Somatostatin (1:500; T-4103, Peninsula), chicken anti-GFP (1:500, AB13970, Abcam), rabbit anti-beta galactosidase (1:500; #55976, Cappel), rat anti-BrdU (1:500, OBT0030, Accurate Chemicals), rabbit anti-Pax2 (1:500, #71-6000, Invitrogen), rabbit anti.Parvalbumin (1:5000; PV25, Swant), mouse anti-Parvalbumin (a:1000; PV235, Swant), rabbit anti-Cleaved Caspase3 (1:500, #9661, Cell Signaling), mouse anti-Dystroglycan (1:500, #05-298, Millipore), rabbit anti-Kv1.2 (1:250; AB5924, Millipore) and guinea pig anti-VGAT (1:250, #131004, Synaptic systems). Sections were washed 3x10minutes (min) in PBS and then incubated with fluorescently labeled secondary antibodies (diluted in blocking solution) and 1µg/ml 4′-6-diamidino-2-phenylindole (DAPI, D1306, Sigma) for counterstaining for 2h at room temperature (R.T). The secondary antibodies used in 1:1000 dilution were as follows: donkey anti-goat Alexa Fluor 488 (A11055) or 568 (A11057, Invitrogen) ; donkey anti-rabbit Alexa Fluor 488 (A21206), 555 (A31572) or 647 (A31573, Invitrogen); donkey anti-mouse Alexa Fluor 488 (A21202), 555 (A31570) or 647 (A31571, Invitrogen); donkey anti-rat DyLight 549 (712-506-153); donkey anti-chicken DyLight 488 (703-485-155, Jackson ImmunoResearch) and donkey anti-guinea pig Alexa Fluor 546 (A11074, Invitrogen). The slides were finally washed 3x10min in PBS and mounted with DAKO fluorescent medium.

**Genetic fate mapping and BrdU labeling**

For genetic fate mapping, *Gfra1*$^{CreERT2}$;*Rosa26*$^{YFP}$ mice received a single subcutaneous injection of 2mg/30g Tamoxifen (Tmx, Sigma) dissolved in corn oil (Sigma) containing 10% ethanol at P0, P15 and P90.

For BrdU labeling, pups were injected subcutaneously with 25mg/kg BrdU (Sigma) in PBS at P5. Embryos were collected 2h after injection for proliferation analysis or 5 days later for migration studies. For BrdU detection, sections were incubated in 2N HCl, 0.1% Triton X-100 at 37°C for 20min, washed with 0.1M Sodium Borate for 15min, washed 2x5min with PBS and incubated with rat anti-BrdU antibody.

**Image analysis**

All fluorescent images were captured with a Carl Zeiss LSM710 confocal microscope using ZEN 2009 software (Carl Zeiss) and cell counts were made with ImageJ software (http://imagej.nih.gov/ij/). For caspase-3 analysis, counts were made in the entire length of folial WM from 6 sagittal sections (14 µm thick, one section every 140 µm) per animal from medial to lateral planes. For Pax2 MLI and Golgi cell counts, two images, containing all layers of the cerebellar cortex, were obtained from each folium, approximately at the same location, and 2 midsagittal sections (14 µm) were analyzed per mouse. For counts in adult tissue, DAPI$^{+}$, PV$^{+}$ cells or Dystroglycan$^{+}$ puncta were analyzed in the molecular layer; Kv1.2$^{+}$ synapses on PC cell bodies.

**Cerebellar slice recordings**

Cerebellar slices were prepared from 40-50 day-old male and female mice. Following anesthesia with pentobarbital and decapitation, the brain was rapidly removed and

placed in an ice-cold and oxygenated (95%O2/5%CO2) "slicing" solution containing the following: 250mM Sucrose, 26mM NaHCO3, 10mM D(+)□Glucose, 4mM MgCl2, 3mM myo□inositol, 2.5 mM KCl, 2mM Sodium Pyruvate, 1.25mM NaH2PO4, 0.5mM Ascorbic acid, 0.1mM CaCl2 and 1mM Kynurenic acid, pH 7.4. The meninges were gently removed, and the brain was mounted and cut on a vibratome (Leica VT1000). Parasagittal slices (300 µM) were transferred to extracellular artificial cerebrospinal fluid (aCSF): 126mM NaCl, 24mM NaHCO3, 1mM NaH2PO4, 2.5mM KCl, 2.5mM CaCl2, 2mM MgCl2 and 10mM D(+)Glucose, pH 7.4 (osmolarity = 295305 mOsmol). Slices were incubated at room temperature for one hour prior to recording and then transferred to a recording chamber of an upright Axio Examiner D1 Zeiss microscope with infrared differential interference contrast optics that was continuously perfused with aCSF (flow rate = 4ml/min). Whole cell recordings were made from Purkinje cell somata primarily located in lobules 4-7 {Larsell:1952um} at near-physiological temperature (34°C). Whole-cell current- and voltage-clamp recordings were performed with pipettes (2-4 MΩ) made from borosilicate glass capillaries (World Precision Instruments) pulled on a P-97 Flaming/Brown micropipette puller (Sutter Instruments). The holding potential in voltage-clamp recordings was 60 mV. The intracellular recording solution used in experiments contained 140mM Kgluconate, 10mM KCl, 1mM MgCl2, 10mM HEPES, 0.02mM EGTA, 4mM Mg□ATP, 0.4mM Na2□GTP, pH was adjusted to 7.3 with KOH (osmolarity = 278□285 mOsmol).

Recordings of spontaneous inhibitory postsynaptic currents (sIPSCs) were performed in voltage-clamp mode using micropipettes filled with intracellular solution containing (in mM), 150mM KCl, 1mM MgCl2, 10mM HEPES, 0.02mM EGTA, 4mM Mg□ATP, 0.4mM Na2□GTP, pH adjusted to 7.3 with KOH in the presence of 10µM CNQX and 25µM AP-5 to block ionotropic glutamatergic neurotransmission. All sIPSCs recordings were concluded by application of 10µM Gabazine to confirm the GABAergic nature of events by the complete loss of all synaptic currents. Recordings were performed using a Multiclamp 700B amplifier, a DigiData 1440 and pClamp10.2 software (Molecular Devices). Slow and fast capacitative components were automatically compensated for. Access resistance was monitored throughout the experiments, and recordings in which the series resistance exceeded 12 MΩ or changed ≥20% were excluded from further analysis. Liquid junction potential was 9.4 mV and not compensated. The recorded current was sampled at 10 kHz and filtered at 2 kHz. Data analysis was performed with GraphPad Prism6 and custom written Matlab scripts (available upon request). Postsynaptic currents were analyzed using Mini Analysis 6.0.9 (Synaptosoft, Decatur, GA). Detection threshold was set at three-fold the root-meansquare (RMS) noise level, which typically was 3–6 pA. Cumulative frequency was calculated from recordings in voltage clamp mode. Frequency, inter-event-interval, and amplitudes were calculated as a mean of the values obtained from 300-second recording samples. All measurements were taken from at least three mice per genotype.

**Behavioral studies**

Experiments were carried out on mice of both sexes. *Ret*$^{fx/fx}$ (n = 10) and *Ptf1a*$^{Cre}$;*Ret*$^{fx/fx}$ (n = 8) mice were transferred from the Karolinska Institute in Stockholm to the Pablo de Olavide University in Seville. Animals were housed in individual cages until the end of the experiment on a 12-h light/dark cycle with constant ambient temperature (21 ± 1°C) and humidity (55 ± 5%), with food and water available ad libitum. Experiments were carried out in accordance with the guidelines of the European Union Council (2010/276:33-79/EU) and Spanish (BOE 34:11370-421, 2013) regulations for the use of laboratory

animals in chronic studies, and approved by the local Ethics Committee of the Pablo de Olavide University.

For chronic behavioral studies, mice were anesthetized with 0.8-3% halothane delivered from a calibrated Fluotec 5 (Fluotec-Ohmeda, Tewksbury, MA, USA) vaporizer at a flow rate of 1-2 L/min oxygen. Animals were implanted with bipolar recording electrodes in the left orbicularis oculi muscle and with bipolar stimulating electrodes on the ipsilateral supraorbital nerve. Electrodes were made of 50 µm, Teflon-coated, annealed stainless steel wire (A-M Systems, Everett, WA, USA). Electrode tips were bare of the isolating cover for 0.5 mm and bent into a hook to facilitate a stable insertion in the upper eyelid. Two 0.1-mm bare silver wires were affixed to the skull as a ground. The 6 wires were connected to two 4-pin sockets (RS-Amidata, Madrid, Spain). The sockets were fixed to the skull with the help of 2 small screws and dental cement. A holding system was also fixed to the skull for a proper stabilization during head rotation and eye movement recordings. Further details of this chronic preparation have been explained elsewhere {Gruart:2006fh}.

Classical conditioning was achieved using a delay paradigm (Figure S4A). Animals (6 at a time) were placed in separate small (5x5x10 cm) plastic chambers located inside a larger (35x35x25 cm) Faraday box. As conditioned stimulus (CS) animals were presented with a tone (2.4 kHz, 85 dB) lasting for 350 ms. The unconditioned stimulus (US) consisted of a cathodal, square pulse applied to the supraorbital nerve (500 µsec, 3 x threshold) at the end of the conditioned stimulus. A total of 2 habituation, 10 conditioning, and 5 extinction sessions were carried out for each animal. A conditioning session consisted of 60 CS-US presentations and lasted 30 min. For a proper analysis of the evoked conditioned responses, the conditioned stimulus was presented alone in 10% of the cases. Paired conditioned stimulus-unconditioned stimulus presentations were separated at random by 30 ± 5 sec. For habituation and extinction sessions, only the conditioned stimulus was presented, also for 60 times per session, at intervals of 30 ± 5 sec. Training sessions lasted for about 30 min {Gruart:2006fh}. Unrectified EMG activity of the orbicularis oculi muscle, and 1-V rectangular pulses corresponding to conditioned and unconditioned stimuli were stored digitally in a computer through an analog/digital converter (1401-plus; CED; Cambridge, UK) for quantitative off-line analysis. Collected data were sampled at 10 kHz for EMG recordings, with an amplitude resolution of 12 bits. A computer program (Spike2 from CED) was used to display the EMG activity of the orbicularis oculi muscle.

For vestibular stimulation, a single animal was placed on a home-made turning-table system (Figure S4B). Its head was immobilized with the help of the implanted holding system, while the animal was capable to walk over a running wheel. Table rotation was carried out by hand following a sinusoidal display in scope. Actual rotation of the table was recorded with a potentiometer attached to the rotating axis. The animal was rotated by ± 20º at three selected frequencies (0.1, 0.3, and 0.6 Hz) for about ten cycles with intervals of 2 min between frequencies. Recording sessions were repeated three times per animal. The eye of the mouse was illuminated with an infrared emitter (wavelength: 880 nm) attached to the head holding system. Eye positions during head rotation were recorded with a fast infrared CCD camera (Pike F-032, Allied Technologies, Stadtroda, Germany) at a rate of 60 pictures/sec. Eye positions for each frequency and animal were averaged (10 complete rotations x 3 recording sessions) for offline analysis of gain and phase {deJeu:2012hc}. The number of compensatory eye saccades were also quantified as it is known improper performance of the VOR are partially compensated by eye

saccades {Macdougall:2012fi}. Head and eye positions were stored digitally in the same analog/digital converter and processed off-line with the help of a MATLAB based (MathWorks, Natick, MA) home-made tracking program. Gain was computed as the ratio between the changes in pupil and head angles during a head turn. Phase was computed as the angle difference between head and pupil.

Open field behavior was investigated with the TSE Actimot system (www.TSE-Systems.com) composed of 480 x 480mm transparent acrylic boxes equipped with light-beam strips to record locomotor behavior. Mice of each genotype 2-3 months old were tested for 15 min without habituation (25 mice per genotype for *Ptf1a*$^{Cre}$;*Gfra1*$^{fx/fx}$ mutants and 10 mice per genotype for *Ptf1a*$^{Cre}$;*Ret*$^{fx/fx}$ mutants). Actimot Software was used to calculate general locomotion parameters, including distance travelled, speed, rearing events and wall-hugging behavior.

For the rotarod test, mice 2–3 month old were first habituated for 1 min to a rotarod apparatus equipped with automatic fall detector rotating at 4 rpm. Mice were then trained in accelerating speed (4 to 40 rpm) during 3 trials 15 min apart per day on 3 consecutive days. The latency to fall from the rod was registered and best daily performance of each animal was recorded for analysis. A trial was otherwise terminated if the animal rotated passively with the rod or after 5 min. One day after the training period, mice were subjected to a steady speed trial (16 or 32 rpm) and the latency to fall was recorded as above for 3 trials per animal. Again the best score for each mouse was used.

For the balance test, animals were required to sequentially traverse three horizontal elevated narrow beams of different thicknesses starting from the thickest (24mm) to medium (12mm) and finally to the thinest (6mm). The beams were 1m long and were supported on vertical poles (50cm high). A soft pad was put under the beam in case the animal fell off. A cage with bedding material covered with a black blanket was placed on the one edge of the beam. Each mouse was placed on the free edge of the beam and the latency to reach the cage as well as the number of slips was recorded. Two trials per mouse were performed for each beam. If the mouse failed to cross the beam within 5 min the trial was aborted.

**Statistical analysis**

All experiments were performed blind to the genotype until the end of the analysis. Experimental groups were subsequently generated according to mice genotype without any randomization. The variation between groups was automatically calculated by GraphPad Prism 6 and was similar between groups.

For image analysis, Student's t-test was used for evaluation of the statistical significance of the results. All data are presented as means ± standard error of the mean (SEM). Significance levels are indicated as: * = $p<0.05$; ** = $p<0.01$ and *** = $p<0.001$.

For slice recordings, two-sample Kolmogorov-Smirnov (K-S2) test was used to compare pooled cumulative frequency distributions. Otherwise, statistical significance was set at $p<0.05$ and was determined using the appropriate two-tailed Student's t-test. All data are presented as means ± SEM. Significance levels are indicated as: * = $p<0.05$; ** = $p<0.01$ and *** = $p<0.001$.

For behavioral studies, statistical analyses were carried out using the SPSS package (SPSS Inc., ILL, USA) for a statistical significance level of $p<0.05$. Unless otherwise indicated, mean values are followed by SEM. Collected data were analyzed using a two-

way ANOVA test, with time or session as repeated measure, coupled with contrast analysis when appropriate. When necessary, the Student's t-test was used for the comparison between two independent means and the paired t-test when related to the same measurements.