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Altered lipid homeostasis is associated with cerebellar neurodegeneration in SNX14 deficiency

Authors

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Abstract

Dysregulated lipid homeostasis is emerging as a potential cause of neurodegenerative disorders. However, evidence of errors in lipid homeostasis as a pathogenic mechanism of neurodegeneration remains limited. Here, we show that cerebellar neurodegeneration caused by Sorting Nexin 14 (SNX14) deficiency is associated with lipid homeostasis defects. Recent studies indicate that SNX14 is an inter-organelle lipid transfer protein that regulates lipid transport, lipid droplet (LD) biogenesis, and fatty acid desaturation, suggesting that human SNX14 deficiency belongs to an expanding class of cerebellar neurodegenerative disorders caused by altered cellular lipid homeostasis. To test this hypothesis, we generated a mouse model that recapitulates human SNX14 deficiency at a genetic and phenotypic level. We demonstrate that cerebellar Purkinje cells (PCs) are selectively vulnerable to SNX14 deficiency while forebrain regions preserve their neuronal content. Ultrastructure and lipidomic studies reveal widespread lipid storage and metabolism defects in SNX14 deficient mice. However, pre-degenerating SNX14 deficient cerebella show a unique accumulation of acylcarnitines and depletion of triglycerides. Furthermore, defects in LD content and telolysosome enlargement in pre-degenerating PCs, suggest lipotoxicity as a pathogenic mechanism of SNX14 deficiency. Our work shows a selective cerebellar vulnerability to altered lipid homeostasis and provides a mouse model for future therapeutic studies.

47 **MAIN TEXT**

48 **Introduction**

49 Neurodegenerative disorders are characterized by a progressive loss of specific neuronal types often
50 associated with the accumulation of toxic protein aggregates (1). To better understand disease mechanisms
51 and find therapeutic alternatives, the field has principally focused on the study of protein quality control
52 pathways, including autophagy (2, 3). In contrast, little attention has been paid to lipid homeostasis
53 pathways despite their well-established association with neurodegeneration and relevance for the function
54 and integrity of cellular organelles (4-7).

55 Genetic disorders affecting regulators of lipid homeostasis often show neurodegeneration, particularly
56 affecting the cerebellum and spinal cord (8, 9). The cerebellum integrates motor function with cognition,
57 emotion, and language, and its dysfunction is documented in a wide spectrum of neurological disorders
58 (10-12). Among cerebellar disorders, childhood onset spinocerebellar ataxias are the most severe. In
59 addition to impaired motor coordination and balance, spinocerebellar ataxia in children is often
60 accompanied by additional neurologic and systemic symptoms, including neurodevelopmental delay and
61 intellectual disability (13, 14). Recent efforts that combine patient registry assemblies with advances in
62 sequencing technologies are revealing a new class of childhood cerebellar neurodegenerative disorders
63 caused by dysfunction of lipid homeostasis pathways (8, 15).

64 Mutations in *Sorting Nexin 14* (*SNX14*) are the cause of a childhood-onset ataxia known as
65 Spinocerebellar Ataxia Recessive 20 (SCAR20), characterized by progressive cerebellar degeneration and
66 severe intellectual disability (16-18). We previously discovered that SCAR20 is associated with enlarged
67 lysosomes and altered autophagy in neural cells derived from patients (16). These findings were also
68 reproduced in patient skin fibroblasts and SNX14 deficient U2OS cell lines but deemed secondary to
69 defects in cholesterol distribution and neutral lipid metabolism (19). Subsequent studies identified SNX14

70 as a regulator of cholesterol homeostasis in two independent genome wide perturbation screens (20, 21).
71 Although the mechanisms by which SNX14 regulates cholesterol trafficking is still unknown, recent
72 reports demonstrate that SNX14 is recruited to the endoplasmic reticulum (ER)-lipid droplet (LD) contact
73 sites to facilitate the incorporation of fatty acids (FA) into triglycerides (TGs) of growing LDs (22). In
74 this process, SNX14 interacts with SCD1, an ER anchored FA desaturase, to cooperate in FA
75 incorporation into LDs (23). Consequently, SNX14 deficient cells show enhanced toxicity to saturated
76 FAs and defective FA-stimulated LD biogenesis (22, 23). Furthermore, recent structural predictions
77 suggest that SNX14 and its SNX-RGS family members may be involved in intracellular lipid transfer
78 (24). However, it is currently unknown if the role of SNX14 in lipid homeostasis regulation is implicated
79 in the pathogenesis of SCAR20.

80 To shed light on the cellular and molecular mechanisms that lead to cerebellar degeneration and
81 intellectual disability in SNX14 deficiency, we generated the first *Snx14* full body knock out mouse (*Snx14*
82 KO) that survives to adulthood. Our work shows that *Snx14* KO mice recapitulate cerebellar atrophy, and
83 motor and cognitive defects of SCAR20 patients. Whereas cerebellar atrophy is associated with Purkinje
84 cell (PC) degeneration, forebrain regions responsible for cognitive behavior remain protected from
85 neurodegeneration. Guided by transcriptomic analyses that pointed to lipid dysregulation as a potential
86 cause of selective cerebellar degeneration, we identify tissue specific alterations of lipid profiles in *Snx14*
87 KO mice. Particularly, non-degenerating *Snx14* KO cerebral cortices exhibit reduced
88 phosphatidylethanolamine (PE) levels that may be associated with synaptic dysfunction, while
89 accumulation of Acylcarnitines (AcCa-s) is unique to pre-degenerating cerebella and likely associated
90 with selective cerebellar neurodegeneration. Finally, we show that SNX14 deficiency reduces LD content
91 and causes lipid storage defects in cerebellar PCs. Together, our work provides evidence for the

involvement of lipid homeostasis defects in selective neurodegeneration and uncovers lipid targets for therapeutic interventions.

Results

SNX14 deficiency causes partial embryonic lethality and developmental delay in mice

SCAR20 patients share clinical features of developmental delay and perinatal onset neurodegeneration of the cerebellum. Previous work suggested that the severity of developmental phenotypes is species-specific, with SNX14 deficient mice showing fully penetrant embryonic lethality, while dogs and zebrafish display neurological and metabolic defects reminiscent of SCAR20 patients (25). However, by randomly introducing a frameshift 1 bp deletion in the exon 14 of *Snx14* (c.1432delG; p.Glu478Argfs*18), we successfully generated SNX14 deficient mice (*Snx14* KO) that are viable and thrive despite a complete loss of SNX14 protein and 90% reduction of the transcript when the mutation is in homozygosity (Figure 1A and Supplemental Figure 1A-D).

Although *Snx14* KO mice survive to adulthood, we noticed that they were born in lower than the expected Mendelian ratio (observed 9.9% vs expected 25%) (Figure 1B). To test if the reduced birth ratio was due to embryonic lethality, we genotyped embryos produced by heterozygous breeding pairs and uncovered that about half of *Snx14* KO embryos die between embryonic day (E)10 and E15. The other half were distinguishable by their small size, a feature that persisted throughout neonate and adulthood (Figure 1C-E). Notably, similar to SCAR20 patients (16-18), adult *Snx14* KO mice showed dysmorphic facial features characterized by an upturned nose, bulging forehead, and eye defects (Figure 1F- G). These data indicate that SNX14 deficiency in mice causes developmental delay phenotypes reminiscent of SCAR20.

Snx14 KO mice display motor and cognitive behavioral defects

115 Unlike SCAR20 patients who show severe gait abnormalities typical of cerebellar degeneration,
116 *Snx14* KO mice were undistinguishable from their wild type (WT) littermates based on their home cage
117 walking activity. However, Catwalk gait analysis revealed a mild gait disruption characterized by longer
118 paw stand time and faster swing speed of the limbs (Figure 2A-B). Functional gait disruption was seen on
119 the horizontal Metz ladder where mice cross a series of rungs separated by varying distances. Here, *Snx14*
120 KO mice had significantly more foot slips than control mice (Figure 2C). Moreover, *Snx14* KO mice
121 underperformed when challenged with complex motor tasks that require coordination and balance. On the
122 accelerating Rotarod, *Snx14* KO mice showed difficulty maintaining balance (Figure 2D) similar to other
123 cerebellar ataxia mouse models (26). In addition, the accelerating Rotarod procedure was performed in
124 three consecutive days to assess motor learning. While WT mice improved their performance over trial,
125 *Snx14* KO learning rate was low, especially for females (Figure 2E).

126 Given that intellectual disability is also a hallmark of SCAR20, we wondered whether *Snx14* KO
127 mice had broader behavioral deficits. To answer this question, we performed a test for social preference
128 and recall (27). During a choice phase of the procedure, the *Snx14* KO mice showed typical preference
129 for a social cue relative to an inanimate object. However, in the recall phase, *Snx14* KO mice failed to
130 discriminate between a familiar and a stranger mouse (Figure 2G). Thus, *Snx14* KO mice showed similar
131 preference to the social cue, but their lack of preference toward exploration of the novel mouse suggests
132 a social memory deficit likely caused by dysfunction of brain regions, including the cerebellum (28, 29).

133

134 **Behavioral defects are associated with cerebellar atrophy**

135 Having established that SNX14 deficient mice recapitulate developmental, motor, and behavioral
136 delays of SCAR20, we looked for the underlying neuropathologic causes. Similar to humans, in mice,
137 SNX14 is widely expressed in the developing and adult brain with a slight enrichment in older brains

138 (Supplemental Figure 2A). In line with the expression pattern, the gross brain morphology of *Snx14* KO
139 mice appeared normal during the first month of life but showed defects as mice became older. Specifically,
140 we found that *Snx14* KO mice had smaller cerebella than WT littermates starting at 2.5 months of age
141 while forebrain areas were mostly intact (Figure 3A), suggesting that the cerebellum is particularly
142 vulnerable to SNX14 deficiency.

143

144 **SNX14 deficiency causes selective PCs degeneration**

145 To further determine vulnerabilities of SNX14 deficiency at a cellular level, we histologically
146 analyzed cerebellar and forebrain tissue. Recent single cell transcriptomic data show that within the
147 cerebellum, *Snx14* expression is enriched in Golgi cells and PCs (30) (Supplemental Figure 2B).
148 Accordingly, RNAscope *in situ* hybridization showed an enrichment of *Snx14* in PCs (Supplemental
149 Figure 2C-D). PCs are some of the largest neurons in the nervous system and their loss is a hallmark of
150 cerebellar ataxias (31). Thus, we first analyzed PCs in 1-, 2.5-, and 4-month-old cerebellar sections by
151 immunofluorescence (IF) staining with Calbindin 1 (CALB1) antibody. At 1 month of age, both WT and
152 *Snx14* KO cerebellar stainings showed perfectly aligned somas in the PC layer and PC dendrites extended
153 into the molecular layer (ML). However, by 2.5 months of age, patches of missing PCs were evident in
154 *Snx14* KO cerebella (Figure 3B). Quantification of PC number per mm of PC layer confirmed significantly
155 lower PC density in lobule III of 2.5- and 4-month-old *Snx14* KO cerebella compared to WT (Figure 3B
156 bottom left graph). The loss of PCs in *Snx14* KO cerebella was followed by a reduced thickness of the
157 molecular layer that was first detectable at 4 months of age (Figure 3B bottom right graph). Upon closer
158 examination of CALB1 staining, we identified vacuole-like structures within *Snx14* KO PC dendrites and
159 soma (Figure 3C, Supplemental Figure 3A). Although, these vacuoles were more abundant and larger in
160 older cerebella, they were sparsely detected in 1-month-old PCs, suggesting that these vacuoles may be a

161 pathological sign that precedes PC neurodegeneration. Remarkably, IF staining with anti-LAMP1
162 antibody revealed that enlarged vacuoles overlap with lysosomal structures and *Snx14* KO PCs display
163 larger lysosomes in comparison to WT (Figure 3D).

164 Given that PC degeneration is often followed by disorganization of Bergmann Glia (BG) processes
165 and gliosis, we also immunostained sagittal cerebellar sections with anti-GFAP and anti-IBA1 antibodies.
166 Concurrent with PC loss, anterior lobes of 2.5-month-old *Snx14* KO cerebella showed abnormal branching
167 of GFAP⁺ BG processes (Figure 3E) and an accumulation of IBA1⁺ microglia within the ML (Figure 3F).
168 Moreover, we found that reactive astrocytes progressively accumulate nearby the PC layer from 2.5 to 4.5
169 months of age (Figure 3E). Interestingly, these findings were specific of the anterior lobes of *Snx14* KO
170 cerebella while posterior lobes (VIII and IX) did not show signs of neurodegeneration until 11 months of
171 age (Supplemental Figure 3B-D). Notably, we did not detect neuronal loss or signs of gliosis in cortical
172 and hippocampal regions of the forebrain (Figure 3G and Supplemental Figure 4A-C).

173 Taken together, our results indicate that despite the wide expression of SNX14 in the whole brain,
174 the forebrain and posterior cerebellum are protected from neurodegeneration, while anterior PCs
175 selectively neurodegenerate in SNX14 deficient mice after 2 months of age.

176

177 **Lipid response genes are dysregulated in pre-degenerating *Snx14* KO mice cerebella**

178 To gain insights into the molecular mechanisms of selective cerebellar PC degeneration, we next
179 analyzed the transcriptome of *Snx14* KO mice cerebella at pre- and post-degenerating stages (1-month-
180 old and 1-year-old, respectively) and compared them with cerebral cortices, which do not show signs of
181 neurodegeneration. After RNA sequencing, we defined differentially expressed genes (DEG) as those
182 showing absolute $\log_2(\text{FC}) > 0.50$ with $p\text{-adj} < 0.05$ between *Snx14* KO and WT tissue. As expected, *Snx14*
183 was downregulated in all *Snx14* KO samples (Figure 4A-B and Supplemental Figure 1C-D). Few

184 differences between *Snx14* KO and WT cerebral cortex transcriptomes were detected at <2 month of age
185 (7 DEGs including *Snx14*) and only 37 downregulated and 3 upregulated DEGs at 1 year of age (Figure
186 4A). None of these DEGs suggested changes in specific cell type composition, which is consistent with
187 the lack of neurodegeneration or neuroinflammation in our histological analyses (Supplemental Data 1).
188 We then tested if cortical DEGs were enriched in specific cellular and molecular functional annotations.
189 Given the short list of DEGs at 1-month-old cortices, we only performed functional annotation analysis
190 on the 37 downregulated genes at 1 year of age. Results revealed a significant enrichment for genes
191 involved in synaptic vesicle membrane (i.e. *Doc2b*, *Sv2c*) (Figure 4A, C). Accordingly, SNX14 has been
192 shown to promote synaptic transmission in mouse cortical neuronal cultures (32). To further validate these
193 data, we analyzed cortical sections by IF staining of pre-and post-synaptic puncta markers and by WB
194 analysis of synaptic vesicle protein, SV2A, levels. Remarkably, IF and WB results were consistent with a
195 reduction of excitatory and inhibitory synaptic puncta (Supplemental Figure 4D) and SV2A protein levels
196 (Supplemental Figure 4E), suggesting that cognitive behavioral deficits in SNX14 deficiency are likely
197 caused by defects in synaptic signaling of forebrain cortical neurons.

198 Unlike cerebral cortices, cerebellar transcriptomes were markedly different between *Snx14* KO
199 and WT mice, with 160 upregulated and 6 downregulated DEGs at 1 month of age and 142 up- and 222
200 downregulated DEGs at 1-year *Snx14* KO (Figure 4B, D-G and Supplemental Figure 5A). We reasoned
201 that the increase in the amount of downregulated DEGs from 1 month to 1 year of age could reflect the
202 progressive PC loss in *Snx14* KO cerebella. Accordingly, most downregulated DEGs in 1-year *Snx14* KO
203 cerebella correspond to PC markers, such as *Calb1*, *Pcp2*, *Car8*, and *Rgs8* (Figure 4B). To unbiasedly test
204 this observation, we analyzed 1-year-old DEGs for functional annotation enrichments. Furthermore, we
205 compared DEGs with a list of a recently reported mouse cerebellar single nuclear RNAseq dataset (30).
206 Results confirmed that downregulated DEGs are enriched in genes predominantly expressed in PCs

207 (Supplemental Figure 5B-C). In contrast, most of the upregulated DEGs genes are sparsely expressed
208 across various cerebellar cell types, with a group of them typically expressed in astrocytes and
209 macrophage/microglia (*Lyz2*, *C4b*, *Cd68*, *Trem2*, *ApoE*, *Gfap*) or associated with cell death (Casp3)
210 (Figure 4B and Supplemental Figure 5B- C). Notably, 1-month-old DEGs were not enriched for PC or
211 astroglia specific functional annotations indicating a later onset of neurodegeneration, consistent with our
212 histological analyses (Figure 4B and Supplemental Figure 5B). Additionally, functional annotation
213 analysis revealed enrichments of genes localized in synaptic, dendritic, and ER compartments in pre- and
214 post-degenerating cerebella (Figure 4D and Supplemental Data 2).

215 Given the lack of neurodegenerative signs in histology or transcriptomic data (Figure 3 and 4), we
216 anticipated that DEGs at 1-month-old cerebella could point us to the molecular causes that precede PC
217 neurodegeneration. However, considering that PCs only constitute ~1% of the total cerebellum,
218 transcriptomic changes in PCs may only contribute to small fold changes in bulk transcriptomic data. To
219 account for these small changes, we analyzed our RNAseq data with Gene Set Enrichment Analysis
220 (GSEA). Interestingly, GSEA revealed cerebellar specific enrichments in biological processes involved
221 in oxidative stress (e.g. ‘response to oxygen containing compounds’ in 1-month-old cerebella and
222 ‘response to reactive oxygen species’ in 1-year-old cerebella), fatty acid or lipid homeostasis regulation
223 (e.g. ‘response to positive regulation of unsaturated fatty acid biosynthetic process’ in 1-month-old
224 cerebella and ‘response to lipid’ in 1-year-old cerebella) and iron accumulation (i.e. ‘regulation of iron
225 ion transmembrane transport’ and ‘iron ion binding’ in 1-year-old cerebella) (Figure 4E-G). Remarkably,
226 genes contributing to GSEA enrichments in pre-degenerating cerebella include upregulated *Fabp5*, which
227 encodes a protein involved in interorganelle lipid transport (33), and *Dcn* encoding a protein released by
228 cells dying by ferroptosis (34) (Figure 4B, E-G). These data suggest that lipid homeostatic defects may
229 precede selective cerebellar degeneration in SNX14 deficiency.

230

231 ***Snx14* deletion alters lipid metabolite levels in a tissue specific manner**

232 We next set out to analyze lipid metabolite composition of pre-degenerating cerebella in 2-month-
233 old WT and *Snx14* KO mice by unbiased lipidomic analysis. As a control of a non-degenerating tissue,
234 we included their cerebral cortices in the analysis. Since the liver is a lipid rich organ with high content
235 of TGs stored in LDs, we included liver lipid extracts as a control for lipid metabolite detection. Finally,
236 to distinguish tissue specific lipids from those circulated by their blood supply, we also extracted plasma
237 lipids from circulating blood.

238 The lipid extracts were analyzed by ultraperformance liquid chromatography-high resolution mass
239 spectrometry (UPLC-HRMS) as previously described (35) and after normalization with lipid internal
240 standards, we quantitatively identified >200 lipid species per sample (Supplemental Data 3). Overall,
241 *Snx14* KO and WT tissues had similar total lipid concentrations (Supplemental Figure 6A) and each tissue
242 analyzed was distinguishable by their relative lipid class abundance. For instance, liver displayed the
243 highest abundance of TGs while the cerebral cortex and cerebellum had phosphatidylcholines (PCh) as
244 the most abundant lipid class (Supplemental Figure 6B). This data is consistent with the literature (36,
245 37), thus validating our methodology.

246 Next, we aimed to determine how SNX14 deficiency affects tissue specific lipid composition. To
247 this end, we compared the concentration of each lipid specie in *Snx14* KO and corresponding WT tissue.
248 Given SNX14's role in facilitating the incorporation of FAs into TG during LD biogenesis (22), we
249 hypothesized that SNX14 deficiency would result in a depletion of TG levels. Although TGs were
250 undetectable in all cerebellar and cortical samples, *Snx14* KO livers displayed a significant reduction of
251 TGs (Figure 5A and Supplemental Figure 6B), further confirming our hypothesis and the reliability of our
252 lipidomic analysis.

253 Additionally, results showed that the cerebral cortex and cerebellum are the tissues with the largest
254 amount of altered lipid species upon SNX14 depletion (Figure 5A). Using p-value < 0.05 as a cutoff, we
255 identified 58 and 36 altered lipid species in cerebral cortices and cerebella, respectively. Furthermore,
256 only cerebellar samples clustered by genotype in a principal component analysis (Supplemental Figure
257 6C), suggesting SNX14 has a larger impact on lipid homeostasis in cerebella than in the other tissues we
258 analyzed.

259 Among the 58 altered lipids in cerebral cortices, 54 had lower concentrations in KOs, and 40
260 belong to the phosphatidylethanolamine (PE) class (Figure 5A- C). PEs provide fluidity and curvatures to
261 membranes which may facilitate vesicular budding and membrane fusion essential for synaptic vesicle
262 formation (38). Thus, changes in PE species may alter cerebral cortex-dependent behaviors and executive
263 functions in SNX14 deficiency. The remaining 4 lipid species had higher concentrations in *Snx14* KO
264 than in WT and all were sphingomyelins (SMs) (Figure 5A and Supplemental Figure 6E). Similarly, *Snx14*
265 KO cerebella exhibited increased levels of total SM concentrations (Figure 5A and Supplemental Figure
266 6E). While some PEs were lower in *Snx14* KO cerebella, these did not influence total PE concentration
267 (Figure 5A-B). In addition, *Snx14* KO cerebella were distinguishable from the cortex, liver, and plasma
268 given the increased levels of several acylcarnitine (AcCa) species (Figure 5A, E-F). Specifically, 6 out of
269 16 increased lipids in *Snx14* KO cerebella were AcCa-s. This accounted for the majority of AcCa-s
270 detected in cerebella (6 out of 8) and resulted in an overall increase of total AcCa concentration in *Snx14*
271 KO cerebella. *Snx14* KO cerebellar samples were also the ones with the largest amount of accumulated
272 lipid species among all the analyzed tissues.

273 To further determine region specific differences in lipid metabolite abundance *in situ*, we next
274 analyzed brain sections with matrix-assisted laser desorption ionization and mass spectrometry imaging
275 (MALDI-MSI). Results uncovered differences between WT and *Snx14* KO brain lipid patterns consistent

276 with UPLC-HRMS results, including reduced PE C38:2 levels (Figure 5F). Furthermore, two TGs were
277 reduced in *Snx14* KO cerebella, one of which (TG 53:2) is specific to the outermost layer of the cerebellar
278 cortex comprised by PC soma and dendrites (Figure 5F). L-carnitine signal also overlapped this area and
279 was more intense in *Snx14* KO than WT (Figure 5F). Given L-carnitine's involvement in AcCa
280 metabolism, this increase in signal may be associated with the accumulation of AcCa in this cerebellar
281 region.

282 Taken together, the bulk and *in situ* lipidomic analyses show tissue specific lipid metabolite
283 defects including a cerebellar specific AcCa accumulation that may be associated with the selective
284 cerebellar neurodegeneration characteristic of SNX14 deficiency.

285

286 ***Snx14* deletion impairs lipid storage *in vivo***

287 Under conditions of high energy demand or nutrient deprivation, AcCa-s carry FAs into the
288 mitochondria for beta oxidation. Elevated concentrations of AcCa, however, can become cytotoxic and
289 disrupt mitochondrial function. Here, LDs are vital by storing excessive FAs and preventing AcCa induced
290 toxicity (39). Accordingly, increased AcCa and decreased TG levels in *Snx14* KO cerebella could be a
291 consequence of defects in LD biogenesis. In line with this idea, SNX14 interacts with LDs and its
292 deficiency leads to impaired LD content and morphology in cell cultures (22). Thus, we investigated
293 whether *Snx14* deletion alters LD biogenesis in the cerebellum by staining *Snx14* KO and WT mice
294 cerebella with Bodipy 493/503 (BD493), a fluorescent dye that stains neutral lipids typically stored in
295 LDs. As a control, we stained the liver, a LD rich tissue, and detected abundant BD493 positive LDs in
296 WT sections. Results also revealed a prominent reduction of LD amounts in *Snx14* KO liver (Figure 6A),
297 which is consistent with the reduction of TG levels in *Snx14* KO liver lipidomics (Figure 5A and

298 Supplemental Figure 6B). These data suggest that SNX14 is necessary for LD biogenesis *in vivo*, at least
299 in the liver.

300 Next, we focused our attention on the cerebellum. Here, BD493 staining showed few, if any,
301 structures resembling LDs, even in WT PCs (Supplemental Figure 7A). To further explore the possibility
302 that *Snx14* deletion affects LD biogenesis in PCs, we stimulated LD biogenesis in cerebellar cultures by
303 supplementation with oleic acid (OA). As expected, OA induced LD biogenesis in WT PCs (Figure 6B).
304 In contrast, the number of LDs detected in *Snx14* KO PCs was half the number in WT PCs (Figure 6B),
305 indicating that SNX14 is necessary for LD biogenesis also in PCs.

306 To assess for LDs or alternative lipid storage defects in *Snx14* KO PCs in tissue, we analyzed
307 cerebellar sections by transmission electron microscopy (TEM) after imidazole-buffered osmium
308 tetroxide staining to highlight LDs (40) (Figure 6C-I). An overview of PC integrity in TEM images
309 confirmed that most *Snx14* KO PCs are still intact at pre-degenerating ages (2 months) (Figure 6C) while
310 a gradient of degenerating PCs is observed at 6 months of age (Figure 6D and Supplemental Figure 7B).
311 Again, TEM studies failed to identify LDs in the cerebellum at pre-degenerating (2 months) or post-
312 degenerating stages (6 months). Nonetheless, TEM results revealed that at pre-degenerating ages, *Snx14*
313 KO PCs have less and larger telolysosomes, which are lipid rich lysosomal storage organelles (Figure 6E-
314 F and Supplemental Figure 7B). Interestingly, this is consistent with larger lysosome compartments we
315 observed in *Snx14* KO PCs (Figure 3D) and in SCAR20 patient neural cell lines (16). These results
316 suggests that SNX14 may have a specialized function regulating lipid clearance or storage through the
317 lysosomal compartment in PCs.

318 Less, yet enlarged telolysosomes in PCs, and elevated AcCa-s at pre-degenerating *Snx14* KO
319 cerebella, suggest that lipid homeostasis defects underlay PC degeneration in SNX14 deficiency (39).
320 Nevertheless, increased AcCa can also be a consequence of mitochondrial damage. Therefore, to

determine if AcCa accumulation is a consequence of lipid storage defects or caused by mitochondrial damage, we assessed mitochondrial ultrastructure of PCs by TEM. Results showed mostly intact mitochondria in pre-degenerating *Snx14* KO PCs (Figure 6G and Supplemental Figure 7C), suggesting that AcCa accumulation is the result of lipid storage and clearance defects, not mitochondrial damage. Furthermore, at 6 months, ultrastructure analysis also revealed a progressive enlargement of ER as PCs degenerate. Damaged mitochondria with enlarged and disorganized cristae were only observed in most degenerated PCs (Figure 6I).

Together, our work indicates that lipid storage and clearance defects are associated with PC neurodegeneration in SNX14 deficiency contributing to the expanding list of neurodegenerative disorders associated with lipid homeostasis defects.

Discussion

SNX14 deficiency causes a childhood onset cerebellar degeneration syndrome clinically defined as SCAR20 and characterized by cerebellar ataxia and intellectual disability. Previous work identified lysosome and autophagy specific defects in cultured patient neural progenitor like cells (16, 17) and recent evidence implicates SNX14 in LD biogenesis, FA desaturation, and non-vesicular interorganelle lipid transport (19, 22-24). However, most of these studies were performed in cultured cells with unclear relevance for SCAR20 pathology. To overcome this limitation and study pathogenic mechanisms that selectively affect the cerebellum, we generated a *Snx14* KO mouse that closely recapitulates SCAR20 at genetic and phenotypic level. Consistent with a widespread expression of SNX14, we find that SNX14 deficiency *in vivo* leads to tissue specific lipid metabolite and storage defects that likely result from cell type specific lipid homeostatic requirements. Remarkably, pre-degenerating *Snx14* KO cerebella is distinguishable from non-degenerating cerebral cortex by a unique accumulation of AcCa and L-Carnitine,

344 including depletion of TG levels. These data, combined with reduced LD numbers and enlarged
345 telolysosomes in pre-degenerating PCs, suggest that lipid homeostasis defects cause cerebellar
346 degeneration in SNX14 deficiency. However, due to lack of cellular resolution, our data does not rule out
347 the possibility that other cell types in the cerebellum may also contribute to the lipidomic changes and
348 neurodegeneration in *Snx14* KO cerebella.

349 Lipid homeostasis disruption is associated with many cerebellar neurodegenerative disorders (8).
350 Little is known, however, about the mechanisms that preserve lipid homeostasis in the cerebellum. PCs
351 are fast and high frequency spiking neurons with a large membrane area, which makes them particularly
352 susceptible to oxidative stress induced by membrane lipid peroxidation (41). Portions of membranes that
353 contain peroxidated lipids are often cleared by autophagy, which leads to an overproduction of FAs and
354 their storage in LDs as a protective mechanism from excess FA induced damage (39, 42, 43). Although
355 neurons produce few LDs, recent evidence implicates autolysosome derived structures in the clearance of
356 toxic lipids through exocytosis in neurons (44). Therefore, our data showing abnormal lipidomic profiles
357 and lipid storage and clearance organelles in pre-degenerating cerebella, fit with a model implicating
358 SNX14 in the storage (lipid droplet) and clearance (lysosome) of toxic lipids generated in PCs. Notably,
359 SNX14 has been associated with lysosome function regulation (16) and recent structural predictions
360 suggest a role in inter-organelle lipid transport (24) that may be important for PC specific lipid
361 homeostasis. Furthermore, lipid clearance and storage defects have recently been associated with neuronal
362 ferroptosis (44) and our transcriptomic data show upregulation of ferroptosis associated genes (i.e. *Dcn*
363 and *Fabp5*) in pre-degenerating *Snx14* KO cerebella and genes associated with iron at older ages. These
364 data suggest an exciting hypothesis implicating lipotoxicity induced ferroptosis as a pathogenic
365 mechanism of cerebellar degeneration in SCAR20 that warrants future investigation.

366 Given the widespread expression of *Snx14*, it is possible that other cell types contribute to PC
367 degeneration. Indeed, glia cells have a central role in the clearance and metabolism of neuronal lipids (44-
368 46). Remarkably, loss of PCs in *Snx14* KO cerebella overlaps with a robust gliosis. Given the enrichment
369 of *Snx14* expression in cerebellar PCs reported in the literature and our RNAscope analyses, we predict
370 that PC degeneration is primary to SNX14 deficiency which then triggers gliosis in *Snx14* KO cerebella.
371 In agreement, the pre-degenerating *Snx14* KO cerebellar transcriptomic data shows an upregulation of
372 *Dcn*, which encodes a protein that stimulates the immune response after being released by cells dying
373 from ferroptosis (34). Recent reports also suggest that gliosis is induced by PC degeneration in cerebellar
374 ataxias. For example, PC specific expression of mutant *ataxin1* in *Scal154Q/2Q* mice is enough to induce
375 astrogliosis and microgliosis (47) and deletion of mutant *ataxin-7* from PCs prevents gliosis in *SCA7-92Q*
376 *BAC* mice (48). Future studies will investigate if the loss of SNX14 affects lipid homeostasis in glia and
377 whether this contributes to the selective cerebellar degeneration in SNX14 deficiency.

378 Similar to recently reported SNX14 deficient mice (25, 49), the homozygous 1bp deletion in our
379 *Snx14* KO mice causes loss of full length SNX14 protein and low RNA counts across all coding exons.
380 Unlike previous models that showed fully penetrant embryonic lethality (25, 49), ~a third of our *Snx14*
381 KO mice develop and survive to adulthood with a phenotype that resembles SCAR20. This finding
382 suggests that SNX14 deficiency in humans may also interrupt embryonic development, and cause
383 SCAR20 only when embryonic lethality is circumvented. Although we still do not know what factors
384 determine the developmental success or failure in SNX14 deficiency, there is a striking difference in the
385 genetic architecture of *Snx14* mutations between organisms that show full and partial embryonic lethality.
386 For instance, SNX14 deficient mice that completely fail to develop carry deletions of at least one full exon
387 while SCAR20 patients and animal models, including our *Snx14* KO mice, dogs (50) and zebrafish (25)
388 carry truncating point mutations or small indels. This observation has interesting implications for the

389 generation of animal models of human disorders and for the pathogenic prediction of truncating genetic
390 mutations that warrant further investigation.

391 Another factor that can influence the outcome of SNX14 deficient embryos is the environment and
392 diet lipid composition. In line with this idea, SNX14 deficient cells are more vulnerable than control cells
393 to saturated FAs (25) and treatment with valproic acid, a branched short-chain FA, partially rescued PC
394 degeneration in a conditional mouse model (49). Furthermore, previous studies have shown that maternal
395 diet lipid composition can modulate brain lipidome either embryonically by maternal feeding or in adult
396 mice (37). Altogether, these data open a window to alter the course of SCAR20 through therapeutic diets.
397 In this regard, further elucidating mechanisms that preserve lipid homeostasis in neurons, and particularly
398 in the cerebellum is of crucial relevance.

399 Overall, our work highlights the relevance of lipid homeostasis for neurodegenerative disorders
400 and suggest a mechanism for increased susceptibility of the cerebellum to the expanding class of disorders
401 caused by disrupted lipid metabolism pathways. Furthermore, our study provides a mouse model and
402 molecular targets for future therapeutic studies.

403

404 **Materials and Methods**

405 Detailed materials and methods are available in Supplemental Methods.

406

407 **Sex as a biological variable**

408 Our study examined male and female animals, and similar findings are reported for both sexes.

409

410 **Animals**

411 ***Generation of mouse model***

412 *Snx14* KO mice were generated by pronuclear injection of 5ng/ul Cas9 mRNA and 2.5ng/ul sgRNA (5'-
413 GTAAACACGTTCTCCAAC-3') in 1 cell stage fertilized embryos obtained from superovulated
414 C57BL/6J females mated with C57BL/6J males. Pups carrying *Snx14* indel alleles were selected for
415 backcross with WT C57BL/6J mice for 3-6 generations (to filter out potential off targets) and further
416 expanded as an experimental model. Only the *Snx14* c.1432delG carriers generated homozygous pups.

417

418 **Behavior analysis**

419 ***Experimental design***

420 Behavior analysis was performed with three cohorts of WT and *Snx14* KO littermates starting at 8 months
421 of age. Each cohort contained mixed genotype and sex of animals. Behavior tests were performed in the
422 following order: accelerating Rotarod, Catwalk, Metz Ladder and Social choice/recall. Investigators were
423 blinded during scoring of behavioral assessments. Whenever possible, offline analysis by computer
424 software was utilized to enhance rigor.

425

426 ***Accelerating Rotarod***

427 On day 1, mice were habituated to the stationary Rotarod for 2 minutes. This was immediately followed
428 by a trial where rotation was programmed to rise from 4-40rpm in 300 seconds. After a 30-minute intertrial
429 interval (ITI), a second trial was performed, followed by another ITI and third trials. Three additional
430 trials were performed on the next 2 consecutive days, for a total of 9 trials. A trial was terminated when a
431 mouse fell, made one complete revolution while hanging onto the rod, or after 300s. Latency to fall (time
432 stayed until falling or riding the rod for a single revolution) was determined. Learning rate was calculated
433 as followed: learning rate = (Trial 9 latency to fall – Trial 1 latency to fall)/8, 8 is the number of inter-trial
434 intervals in this study.

435

436 ***Catwalk gait analysis***

437 In the Catwalk gait analysis assay, mice were placed on a meter-long illuminated glass plate walkway in
438 a dark room. A high-speed video camera below the plate recorded the paw prints, as the mice traversed a
439 20cm section of the alley. The paw print footage was analyzed by CatWalk XT program (Noldus,
440 Leesburg, VA).

441

442 ***Metz ladder rung waking test***

443 The Metz procedure used a 1-meter-long horizontal ladder, which was about 1cm wider than the mice.
444 The Plexiglas walls were drilled with 3mm holes to accept the metal rungs. The gaps between the rungs
445 were randomly spaced 1-5 cm apart so that the mice had to adjust the projection of the landing of each
446 paw. Mice were trained to run the ladder with all rungs in place, 1cm apart before the test trials began. In
447 the test, each mouse was placed at the beginning of the ladder. Five trials were performed on consecutive
448 days and videotaped. The pattern of the rungs was changed after each trial to prevent animals from
449 adapting. Trials were recorded by a high-definition digital camera. Foot slip(s) of each trial was quantified
450 later by an investigator blinded to group designation with video.

451

452 ***Social choice and recall test***

453 Mice were tested for social preference and recall as described previously (51). The testing apparatus was
454 a rectangular Plexiglas three chamber arena (60 cm (L) × 40 cm (W) × 20 cm (H)). The chamber was
455 continuous with areas at the ends designated for the placement of vented cylinders to hold the cues. The
456 social cues were juvenile, sex-matched C57BL/6J mice. The inanimate cues were smooth rocks that
457 approximate the size of the social cues. The procedure consisted of a habituation phase whereby the

458 experimental mouse was placed into the center chamber with empty cylinders in the side chambers for 10
459 minutes. After habituation, the choice phase immediately began. The cylinders were loaded with either a
460 social cue (young mouse, M1) or inanimate cue. The experimental mouse was allowed to explore the cues
461 for 10 minutes. Immediately after the choice phase, the recall phase was performed. The now familiar
462 social cue, M1 remained in a cylinder while a novel mouse, M2 was loaded into the cylinder that
463 previously held the inanimate cue. The experimental mouse was allowed to freely explore the 2 social
464 cues for 10 min. The bouts and duration of explorations (nose \leq 1 cm proximity) with the cylinders was
465 determined with ANYmaze software (Stoelting Co. Wood Dale IL.).

466

467 **Histology**

468 ***Immunofluorescence staining***

469 Mice were anesthetized with isoflurane (Terrell) and perfused trans-cardially with 20ml 1X PBS and 20ml
470 4% paraformaldehyde (PFA) (Electron Microscopy Sciences). Brains dissected out from scalp were post-
471 fixed in 4% paraformaldehyde for 18h in RT and washed 3 x 10 mins in 1X PBS. Brains were sliced into
472 50um sections using a vibratome (Leica).

473

474 On the day of staining, slides were washed with 1X PBS, permeabilized and blocked with PBS+0.3%
475 Triton X-100 (PBST) and 4% goat serum (G9023, Sigma-Aldrich) for 45 min at room temperature. Slides
476 were then incubated with primary antibodies (see Supplemental Methods) in 2% goat serum in PBST at
477 4°C on the shaker overnight. Next day, slides were washed with PBST 3 x 10 mins and incubated with
478 Alexa Fluor-conjugated secondary antibodies at 1:500 in 2% normal goat serum in PBST for 2h at room
479 temperature (RT). Slides were washed in PBST 3 x 10 mins, incubated with 300 nM DAPI (D3571,
480 Invitrogen) for 10 min at RT and mounted on microscope slides with ProLong Gold antifade (P36930,

481 Invitrogen) or Mowiol (#81381, Sigma) covered with a coverslip. Immunostainings were imaged with a
482 Leica TCS SP8 X confocal microscope and images processed and quantified with ImageJ (NIH).

483

484 **RNAscope *In situ* hybridization**

485 The RNAscope *in situ* hybridization was performed as recommended by the manufacturer with reagents
486 from Advanced Cell Diagnostic (USA) (see Supplemental Methods). Once RNAscope was completed,
487 immunofluorescent staining was immediately performed as described above. Sections were imaged with
488 a Leica TCS SP8 X confocal microscope and images processed and quantified with ImageJ.

489

490 **BODIPY staining**

491 Fixed brain and liver tissue was sliced into 50 μ m sections using vibratome, rinsed in PBS and incubated
492 with 2 μ M BODIPY 493/503 (D3922, Invitrogen) for 30 min at RT with gentle rocking. Then, the sections
493 were rinsed in PBS 3 x 10 mins and mounted on microscope slides with Mowiol and covered with
494 coverslips.

495

496 **Transmission Electron Microscopy (TEM)**

497 Mice were perfused with 20 mL of PBS, followed by 20 mL 2% PFA and 2% glutaraldehyde in sodium
498 cacodylate buffer. Cerebella were dissected, trimmed to 1 mm thickness, and processed for TEM at the
499 University of Delaware's Bio-Imaging Center. Briefly, tissues were washed 3 x 15 min in 0.1M sodium
500 cacodylate buffer pH 7.4 and post-fixed for 2 h with freshly prepared 1% osmium tetroxide and 1.5%
501 potassium ferrocyanide in 0.1M sodium cacodylate buffer pH 7.4 or alternatively, to improve lipid droplet
502 detection, with 1% osmium tetroxide in 0.1M imidazole pH 7.5. The tissue was washed with water,
503 dehydrated through an ascending acetone series, and then infiltrated with Embed-812 resin. The next day,

504 samples were embedded in flat-bottom capsules and polymerized at 60°C overnight. Ultrathin sections
505 were cut using a Leica UC7 ultramicrotome and placed onto single hole 1500-micron copper aperture
506 grids with a formvar/carbon film. Sections were post-stained with 2% uranyl acetate in 50% methanol
507 and Reynolds' lead citrate and examined on a ThermoFisher Scientific Talos L120C transmission electron
508 microscope operating at 120kV. Images were acquired with a ThermoFisher Scientific Ceta 16M camera.
509 Quantification of area and numbers was done by ImageJ.

510

511 ***Matrix assisted laser desorption ionization coupled to time-of-flight mass spectrometry (MALDI-TOF***
512 ***MS) Imaging***

513 MALDI-TOF MS imaging was carried out in MALDI MS Imaging Joint Facility at Advanced Science
514 Research Center of City University of New York Graduate Center.

515 8-weeks-old mouse brains were cryosectioned (10 µm thickness) sagittally and gently transferred onto
516 the pre-cooled conductive side of indium tin oxide (ITO)-coated glass slides (Bruker Daltonics) for
517 MALDI imaging. Mounted cryosections were desiccated in vacuum for 45 min at RT, followed by matrix
518 deposition using HTX M5 sprayer (HTX Technologies, LLC) on DHB matrix (40 mg/mL in
519 methanol/water (70/30, v/v), flow rate of 0.05 mL/min and a nozzle temperature of 85 °C for 8 cycles) to
520 detect metabolites and lipids. MALDI mass spectra were acquired in positive ion mode (DHB) acquired
521 by MALDI-TOF MS Autoflex (Bruker Daltonics). MALDI imaging data were recorded and processed
522 using FlexImaging v3.0, and further analyzed using SCiLS (2015b). Ion images were generated with root-
523 mean square (RMS) normalization and a bin width of ± 0.15 Da. The spectra were interpreted manually,
524 and analyte assignment was achieved by comparing with LC-MS/MS experiment results (52). The signal
525 intensity of the cortex and cerebellum regions of three animals of each genotype were quantified using

526 SCiLS and further analyzed using GraphPad. P-value between control and mutant animals were analyzed
527 by Student's t-test using three animals of each group.

528

529 **Cell Culture**

530 ***Purkinje Cell Culture***

531 Primary mixed cerebellar cultures were generated and maintained as described (53). Briefly, cerebellums
532 were isolated from E16.5 of WT or *Snx14* KO mice, dissociated and plated at 50,000 cells on coverslips
533 coated with 0.1 mg/mL poly-D-lysine in recovery media (DMEM/F-12, (#11330032, Gibco)
534 supplemented with 1% Penicillin-Streptomycin (#15140122, Gibco), 1X B-27 (# 17504044, Gibco), 10%
535 FBS (#101, Tissue Culture Biologicals), 20 ug/mL Insulin (#I9278, Millipore Sigma), and 100 ug/mL
536 IGF-1 (#100-11, PeproTech). Two hours later, recovery media was removed and replaced with complete
537 media (DMEM/F-12, supplemented with 1% Penicillin-Streptomycin, 1X B-27, 1% FBS, 20 ug/mL
538 Insulin, and 100 ug/mL IGF-1). Purkinje cells were cultured for 7 days *in vitro* before processing for
539 experiments.

540

541 ***Purkinje Cell Lipid droplet Analysis***

542 To promote LD biogenesis, cerebellar cultures were incubated with 600 uM Oleic Acid (#O1008, Sigma)
543 conjugated to 100 uM fatty acid-free BSA (A1595, Sigma) overnight. Cells were fixed 10 min with 4%
544 PFA at RT, washed with 1X PBS and blocked in blocking buffer (1.5% Glycine, 3% BSA, 0.01% Saponin
545 in 1X PBS) for 1h at RT. Cells were incubated overnight at 4°C with primary antibodies in antibody
546 solution (1% BSA, 0.01% Saponin in 1X PBS). The following day cells were washed, incubated in
547 secondary antibodies with 300nM DAPI, and 2µM BODIPY 493/503 for 2h at RT and mounted with
548 Fluoromount-G (#00-4958-02, Invitrogen). Images for quantification were captured with Leica TCS SP8

549 X confocal microscope and BODIPY 493/503 positive puncta quantified with ‘analyze particles’ plug-in
550 in Fiji-ImageJ after processing with “Intermodes” algorithm.

551

552 **Biochemical studies**

553 ***Western blot***

554 Mouse tissue was dissected, fast-froze, and stored in -80°C until use. On the experiment day, tissue was
555 homogenized in RIPA buffer (#9806, Cell Signaling) supplemented with a protease inhibitor cocktail
556 (P8340, Sigma-Aldrich) and incubated for 15 minutes at 4°C. After centrifugation at 13,200 rpm,
557 supernatant containing protein extract was collected, mixed with 1X LDS loading buffer (B0007,
558 Invitrogen) supplemented with 200 mM DTT (BP172-5, Fisher Scientific) and loaded on a 4-15% Mini-
559 Protean TGX Precast Protein Gel. Proteins were transferred onto PVDF membranes in Mini Gel Tank at
560 80V for 180 min. Membranes were blocked with 5% milk-TBST or EveryBlot Blocking Buffer
561 (#12010020, Bio-Rad) for 1h at RT then probed with primary antibodies diluted in 5% milk-TBST or
562 EveryBlot Blocking Buffer solution overnight at 4°C. The next day, membranes were washed and probed
563 with horseradish-peroxidase-conjugated secondary antibodies for 1h at RT, incubated in either Pierce™
564 ECL Western Blotting Substrate kit (#32106, Thermo Scientific) or SuperSignal™ west dura extended
565 duration substrate (34076, Invitrogen) and exposed on autoradiography film following development in
566 AFP Mini-Med 90 X-Ray Fil Processor. Exposed films were scanned, and protein bands were quantified
567 using ImageJ.

568

569 ***RNA-seq***

570 1-month-old or 1-year-old mice were euthanized, and tissue was dissected on ice, fast frozen, and stored
571 in -80°C until RNA extraction. Total RNA from the cerebellum or cerebral cortex were isolated using

572 TRIzol (15596026, Invitrogen) reagent according to the manufacturer's instructions. Strand-specific
573 mRNA-seq libraries for the Illumina platform were generated and sequenced at GENEWIZ or Novogene
574 following the manufacturer's protocol with sample specific barcodes for pooled sequencing. After
575 sequencing in Illumina HiSeq or Novoseq platform with 2x150 PE configuration at an average of 15
576 million reads per sample, sequenced reads were trimmed to remove possible adapter sequences and poor-
577 quality nucleotides and trimmed reads mapped to the *Mus musculus* GRCm38 reference genome using
578 Spliced Transcripts Alignment to a Reference (STAR v2.7.3a) software. Reads were counted using
579 FeatureCounts from the subread package (v2.0.1) (54). Transcripts Per Million (TPM) values were
580 calculated from featureCounts-derived counts. Heatmap of gene expression were generated using the
581 tidyverse R package with z-score of the $\log_2(\text{tpm}+1)$. Differential gene expression analysis was performed
582 with DEseq2 (v1.38.3). Raw p-values were adjusted using the Benjamini-Hochberg method. Differentially
583 expressed genes (DEGs) were defined as having an adjusted p value (P_{adj}) < 0.05. Volcano plots were
584 generated with the EnhancedVolcano R package. Functional enrichment analysis was conducted utilizing
585 the enrichR R package. Gene Set Enrichment Analysis (GSEA) was performed on the *Mus musculus*
586 msigdb database in the C5 ontology category. Relevant lipid, oxygen, or iron -related terms were
587 manually selected and displayed in the waterfall plots, generated through the tidyverse R package.

588

589 ***UPLC-HRMS whole lipidome analysis***

590 *Sample preparation*

591 2-months-old mice were euthanized and, after heart blood collection, cortex, cerebellum, and liver were
592 dissected, snap-frozen in liquid nitrogen and stored at -80 until lipid extraction. For lipid extraction,
593 plasma samples were prepared as previously reported (55). ~10 mg of frozen tissue fragments were
594 weighted, chopped and mixed with 0.6 mL 80% methanol (MeOH) and 10 μ L of internal standard mix

595 (SPLASH® LIPIDOMIX #330707 from Avanti Polar Lipids, Alabaster, AL). Samples were pulse
596 sonicated in ice for 30x 0.5 second, incubated for additional 20 min in ice, vortexed 3x 30 seconds and
597 tissue homogenates transferred to a 10 mL glass Pyrex tube with screw cap. Then, 5 mL methyl tert-butyl
598 ether (MTBE) was added to each tube and vigorously shaken for 30 minutes, followed by the addition
599 of 1.2 mL water and 30 second vortex. Samples were centrifuged for 10 min at 1000 g and the top clear
600 phase was collected to a clean glass Pyrex tube and dried down under nitrogen. For the analysis, dried
601 samples were resuspended in 100 µL MTBE/MeOH=1/3 (v/v), spun down at 10,000g for 10 min at 4°C.
602 The top 50 µL were transferred to a HPLC vial and 2µL were injected for LC-MS analysis.

603 *Liquid chromatography high resolution -mass spectrometry (LC-HRMS) for lipids*

604 Separations were conducted on an Ultimate 3000 (Thermo Fisher Scientific) using an Ascentis Express
605 C18, 2.1 × 150 mm 2.7µm column (Sigma-Aldrich, St. Louis, MO). For the HRMS analysis, a recently
606 calibrated QE Exactive-HF mass spectrometer (Thermo Fisher Scientific) was used in positive ion mode
607 with an HESI source. Control extraction blanks were made in the same way using just the solvents instead
608 of the tissue homogenate. Untargeted analysis and targeted peak integration was conducted using
609 LipidsSearch 4.2 (Thermo Fisher Scientific) as described by Wang et al (56). Lipids quantification was
610 done from the full scan data. The areas were normalized based on the amount of the internal standard
611 added for each class. All amounts were then normalized to the original tissue weight.

612

613 **Statistics**

614 Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, Inc.). When possible,
615 data was analyzed blind to the genotype. Sample size for each experiment was determined based on similar
616 studies. To compare the means of groups where normal distribution and similar variance between groups
617 was confirmed, two-tailed Student's *t*-test (for two samples), one-way ANOVA (for more than two

618 samples) or two-way ANOVA followed by Sidak's or Tukey's post hoc test (for multiple variables) was
619 used. A *P* value less than 0.05 was considered statistically significant. Outliers were removed in two
620 behavioral studies using the ROUT method with $Q=1\%$, $p < 0.0002$.

621

622 **Study approval**

623 All animal procedures were performed according to NIH guidelines and approved by the Institutional
624 Animal Care and Use Committee (IACUC) at Children's Hospital of Philadelphia.

625

626 **Data availability**

627 RNAseq data was deposited in GEO under the GSE215834 reference. Whole data from lipidomic
628 analysis is available in Lipidomic data file. All other data are available in the Supporting Data Values
629 file.

630

631 **Author contributions**

632 Study conceptualization and design: Y.Z., V.S., and N.A. Validation and maintenance of mouse colony:
633 Y.Z., T.J., and N.A. Behavioral study design, execution, and data collection: B.C. and T.O. Behavioral
634 data analysis: B.C., T.O., Y.Z., and H.T. Histology, cell culture, and TEM studies: Y.Z., V.S., M.F., and
635 D.Y. RNA extraction and RNAseq analysis: Y.Z. Lipidomic analysis: Y.Z., P.X., and C.M. MALDI MS
636 Imaging analysis: S.L. and Y.H. Data interpretation: Y.Z., V.S., M.H., and N.A. Supervision and project
637 administration: N.A. Manuscript preparation: Y.Z., V.S., and N.A. Manuscript edit and review: All
638 authors.

639

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651

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777 **Figures**
778 **Figure 1**

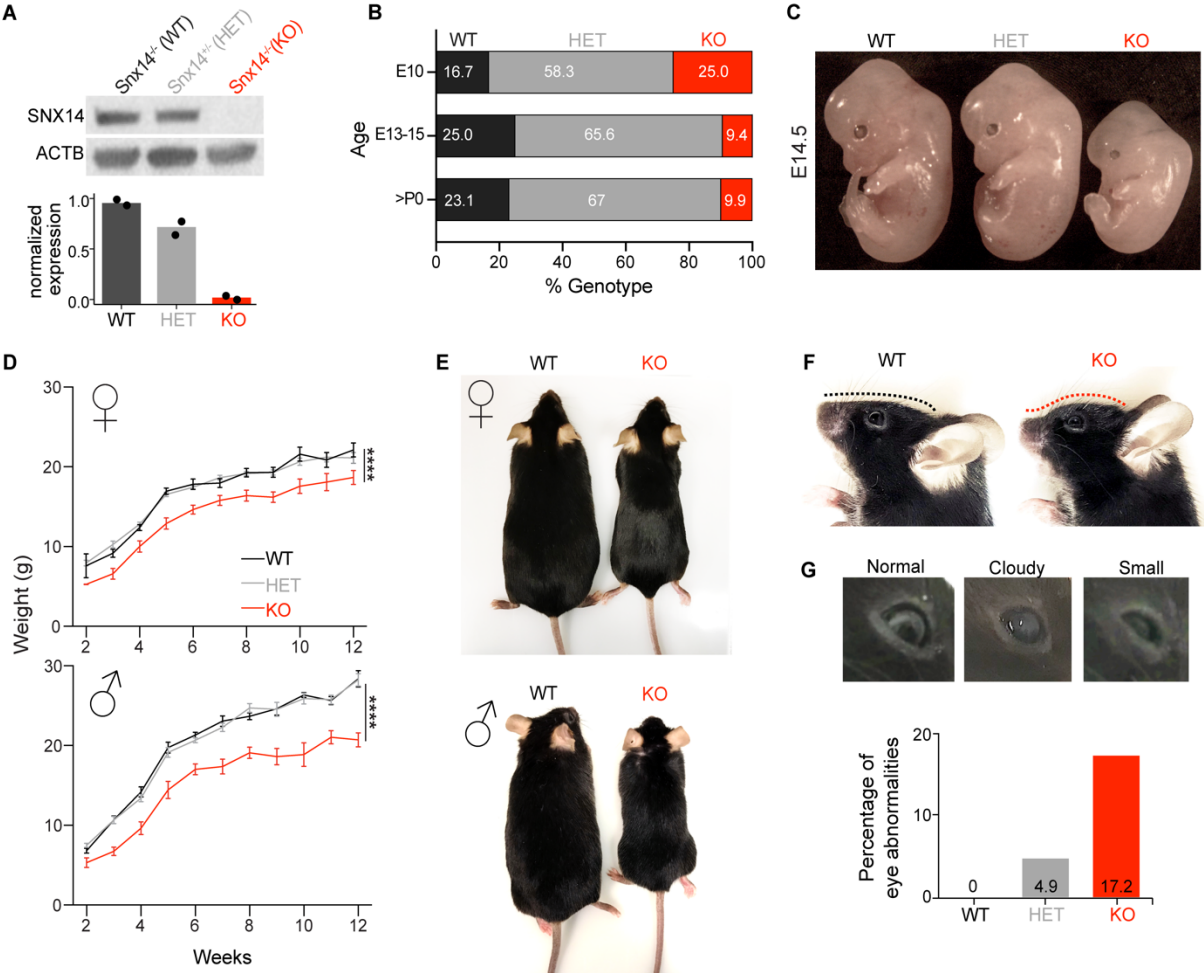
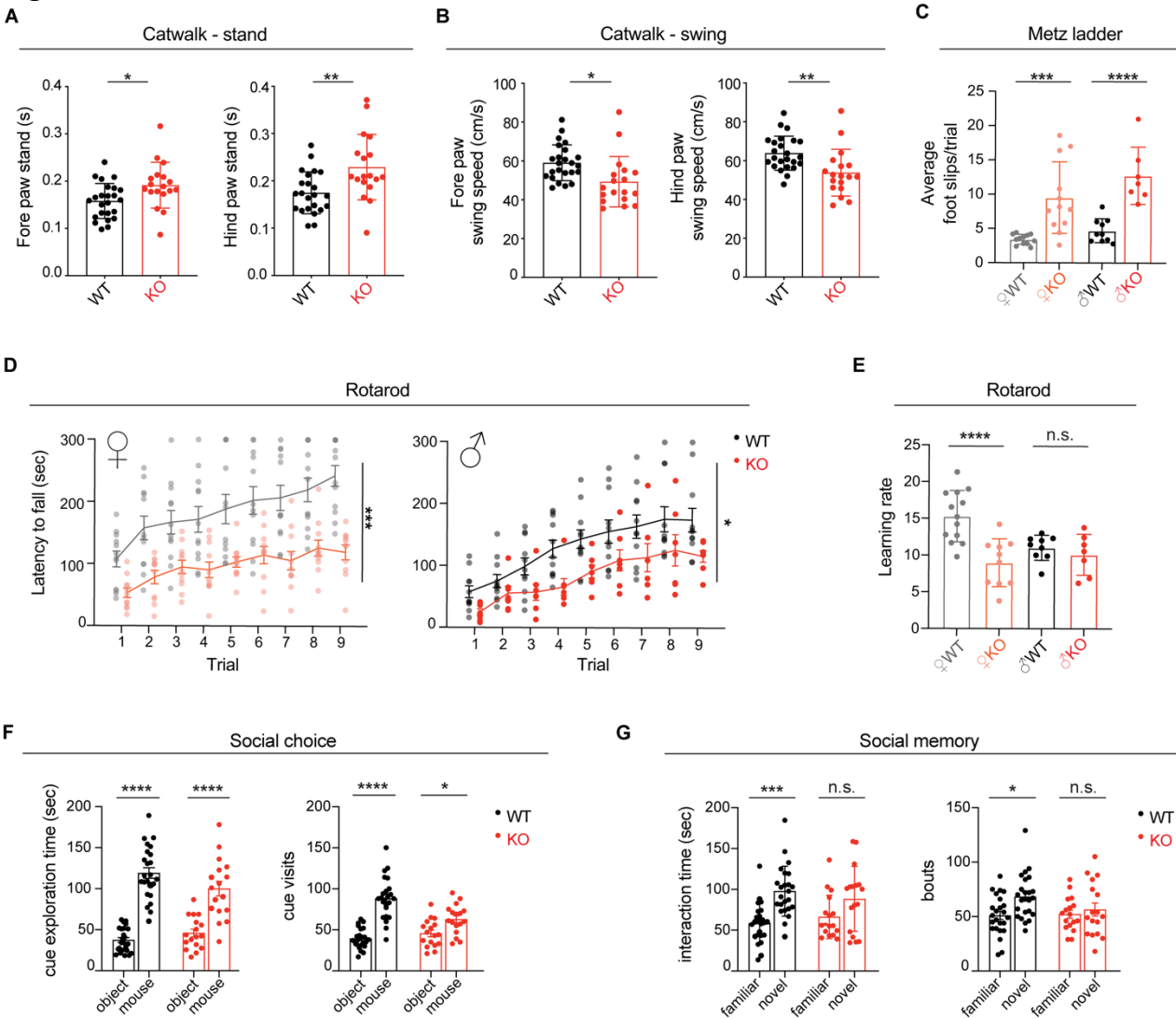


Figure 1. SNX14 deficient mice show developmental delay and atypical facial features. (A) Representative western blot (WB) images show loss of SNX14 expression in *Snx14* KO mice tissue. Beta-actin (ACTB) was used as loading control. Bar graph shows WB band densitometry quantification of SNX14 relative to ACTB. n=2 for each genotype. (B) Percentage of embryos/mice with the indicated genotypes obtained from heterozygous parent matings. Chi-square test shows significant discrepancy between >P0 observed and expected values ($P = 0.001$) indicating embryonic lethality of KOs. E10, n=13; E13-15, n=36; >P0, n=91. (C) Representative image of WT, HET, and KO E14.5 embryos showing smaller size of KOs. (D) Growth curves show consistently lower body weight of 2–12-week-old *Snx14* KO males and females. Data represent mean \pm S.E.M of n \geq 3. Two-way ANOVA test shows significant effect of genotype (**** $P < 0.0001$). (E) Representative images of 9-month-old WT and KO littermates of each gender. (F) Representative images showing the atypical face with forehead protrusion of 6-month-old KO mice (red line) compared to WT littermate. (G) Representative images of 8-month-old KO mice showing eye abnormalities, including cataracts (cloudy), and microphthalmia (small). Bar graph shows percentages of mice with eye abnormality for each genotype.

801 **Figure 2**



802 **Figure 2. SNX14 deficiency in mice recapitulates motor and behavioral deficits of SCAR20.** (A-B) Catwalk analysis shows
803 altered gait of KO mice with a longer stand (A) and shorter swing (B) than WT mice. Bar graphs represent mean \pm S.E.M of
804 $n=24$ WT and $n=18$ KO mice. Two-tailed Welch's t -test. (C) Metz ladder rung test shows altered limb placing and coordination
805 of KO males and females. Bar graphs represent mean foot slip of 5 trials performed in consecutive days \pm S.E.M of $n=10$ WT
806 males, $n=7$ KO males, $n=12$ WT females, $n=12$ KO females. Two-way ANOVA followed by Sidak's test. (D) Accelerating
807 rotarod reveals defects in motor performance of KO mice in the 9 trials performed over 3 consecutive days. Graphs show mean
808 latency to fall \pm S.E.M of $n=11$ WT males, $n=7$ KO males, $n=13$ WT females, $n=11$ KO. Two-way ANOVA test shows
809 significant effect of genotype. (E) KO females show impaired learning rate on accelerating rotarod performance over time
810 (between trial 1 and 9). Bar graph shows mean learning rate \pm S.E.M of $n=9$ WT males, $n=7$ KO males, $n=13$ WT females,
811 $n=10$ KO females. Two-way ANOVA followed by Sidak's test. (F-G) Three-chamber social interaction test showing similar
812 preference for a mouse over an object between WT and KO mice (F) but impaired social novelty preference in KO mice (G).
813 Bar graphs represent mean and S.E.M of WT $n=24$, KO $n=17$. Two-way ANOVA followed by Tukey's test. n.s. = non-
814 significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.
815

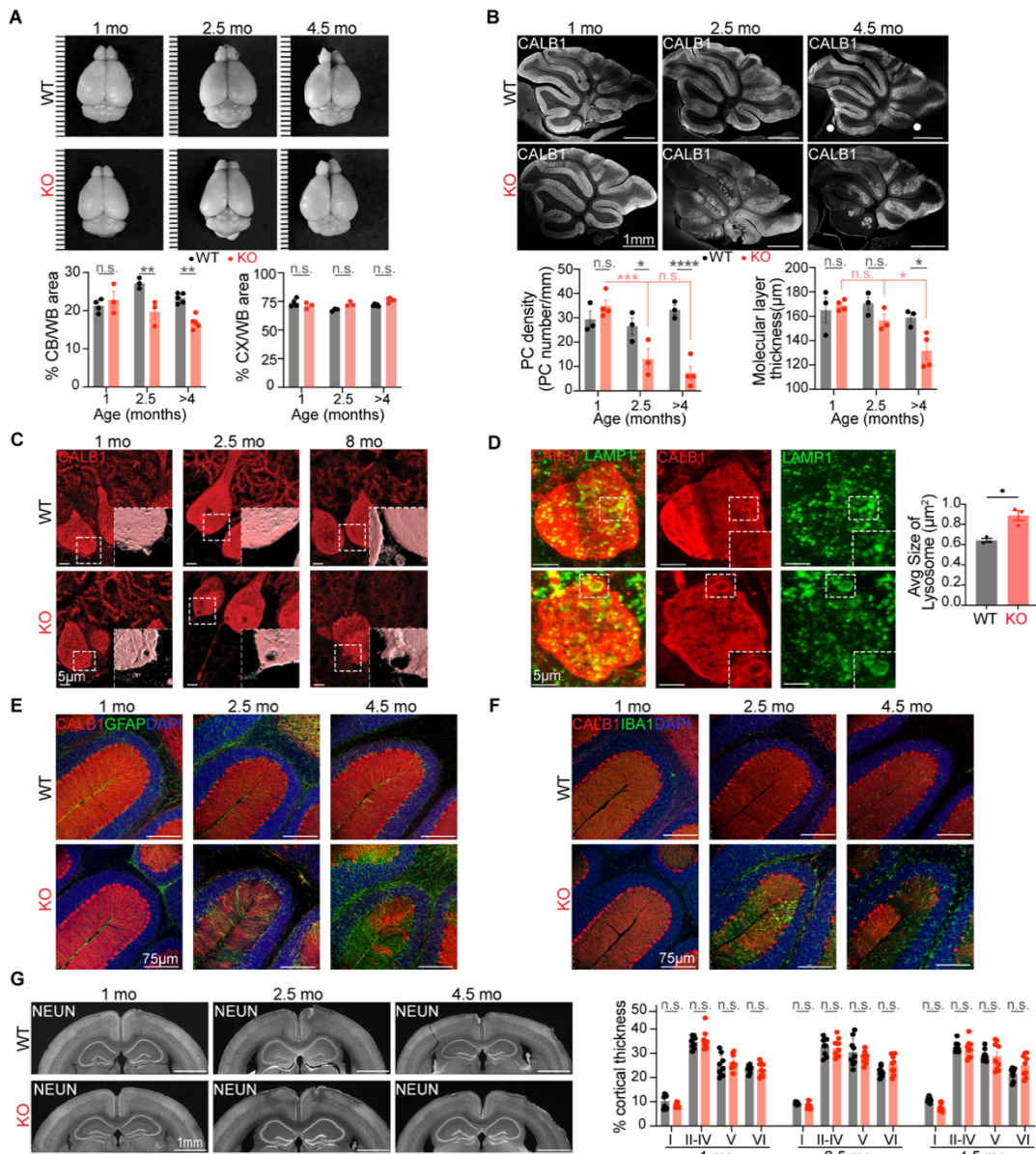


Figure 3. SNX14 deficiency causes selective cerebellar degeneration. (A) Representative brain images from WT and KO mice at indicated age shows shrinkage of KO cerebellum (CB) over time. Ruler marks separated by 1mm. Bar graphs show percentage area of CB or cerebral cortex (CX) relative to the whole brain (WB) in n=3-5 mice. Two-way ANOVA followed by Sidak's test. (B) Representative cerebellar sagittal sections immunostained with PC specific anti-CALB1 antibody reveal progressive loss of PCs in KO mice. Bar graphs show PC linear density (right) and thickness of the molecular layer (left) in the Cerebellar Lobule III of n=3-4 mice. Two-way ANOVA followed by Sidak's test. (C) Representative immunostaining of PCs with anti-CALB1 antibody reveals progressive accumulation of vacuoles in KO mice. (D) Immunostaining of PCs with anti-CALB1 and lysosomes with anti-LAMP1 show enlarged lysosomes in KO mice. Bar graph shows average lysosome size per mouse. n=3 mice (in WT = 29 PCs and 4,033 lysosomes were counted and in KO = 30 PCs and 3,247 lysosomes). Two-tailed *t*-test. (E-F) Representative immunostaining showing progressive accumulation of astrocytes labeled with anti-GFAP (E) and microglia with anti-IBA1 (F) in degenerating KO cerebella (base of Lobule III & IV). (G) Coronal sections of cerebral cortices immunostained with anti-NeuN do not show differences between WT and KO mice. Bar graphs show percentage thickness occupied by each cortical layers (I-VI) in 4-5 cortical regions of 2 mice per genotype and age. Two-way ANOVA followed by Sidak's test. In all graphs, data represent mean \pm S.E.M. n.s. = non-significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, *****P* < 0.0001.

839 **Figure 4**

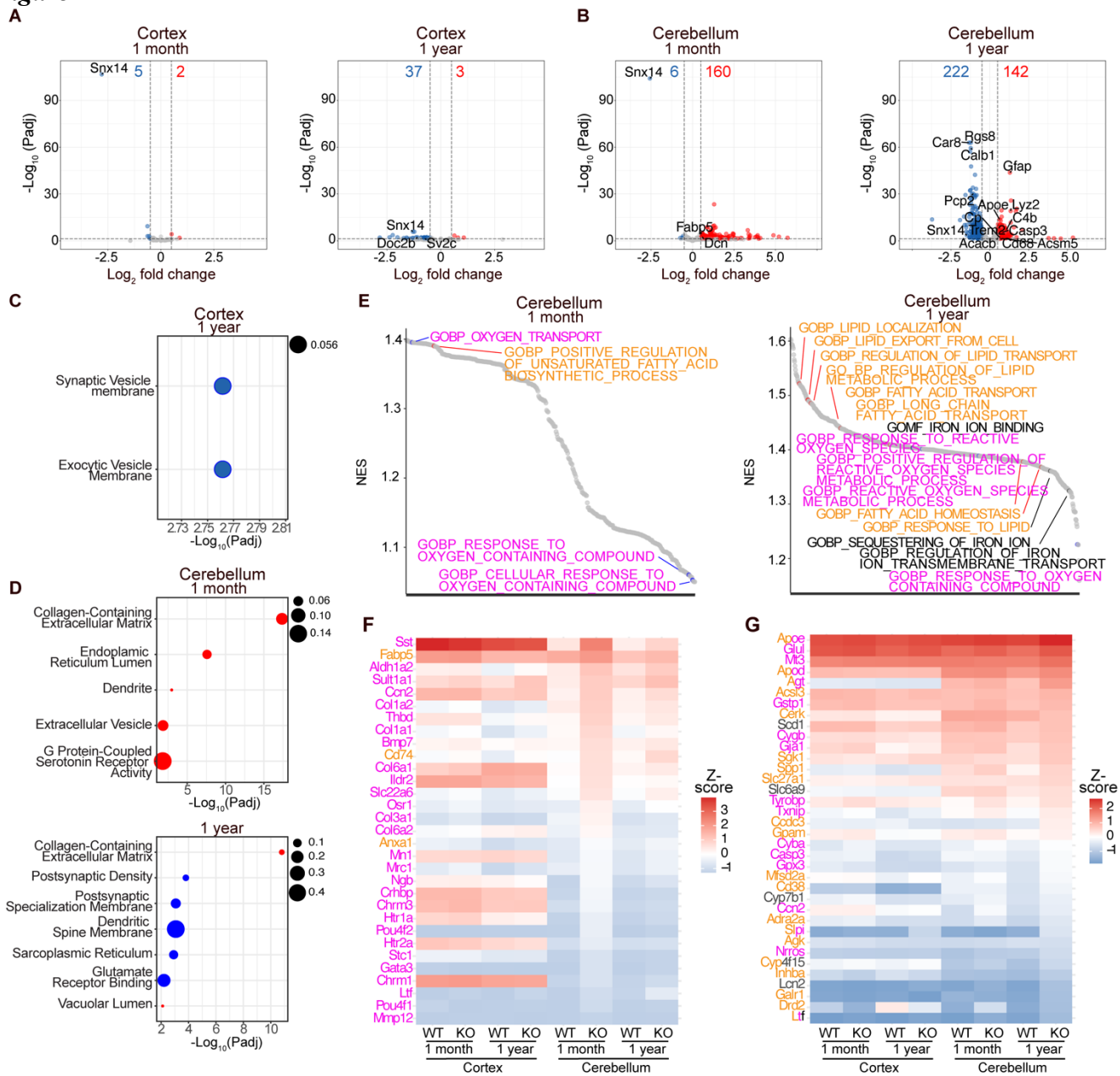
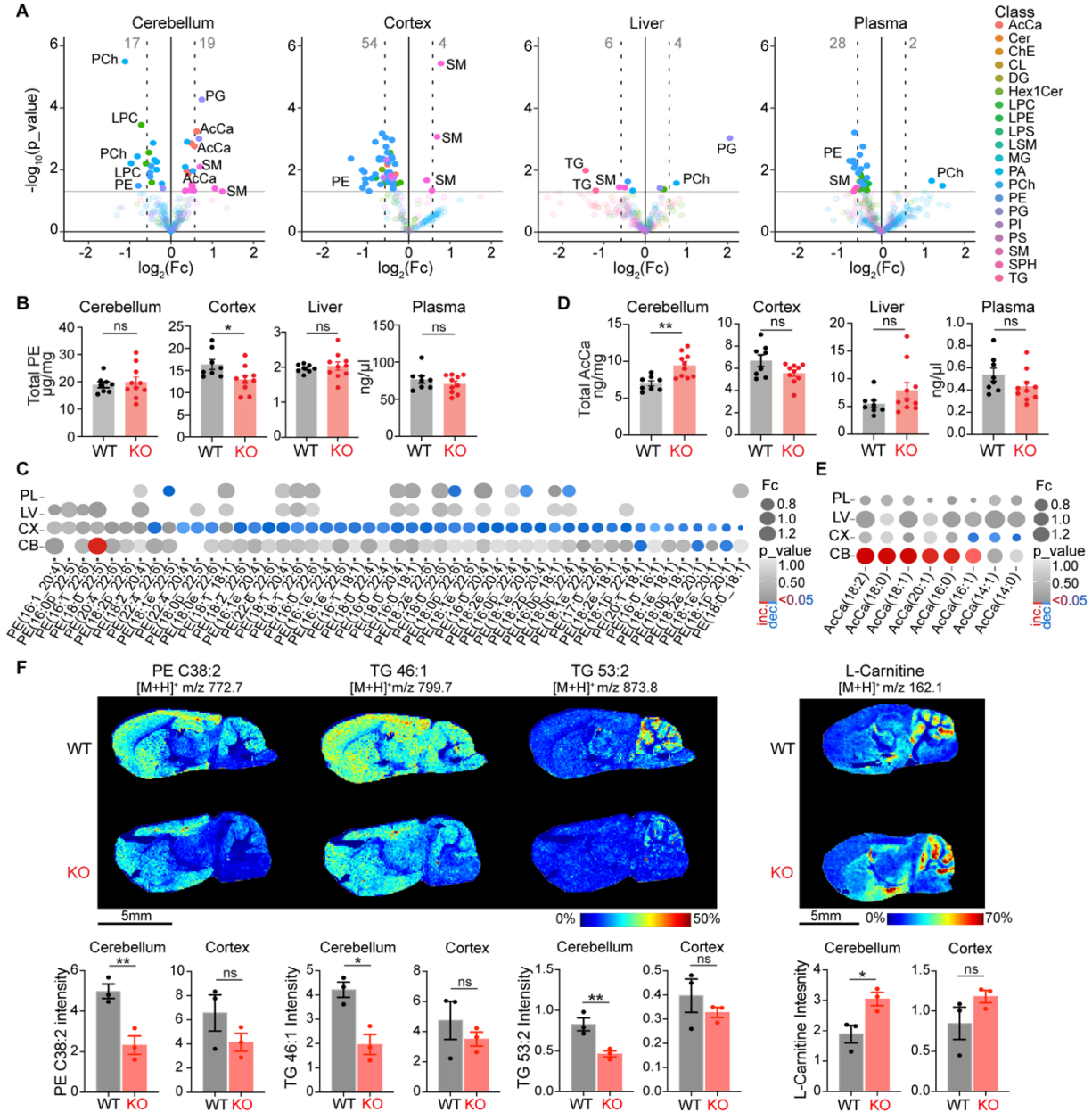


Figure 4. Genes involved in lipid response are differentially expressed in SNX14 deficient cerebella. (A-B) Volcano plots of differentially expressed genes (DEGs) in the Cortex and Cerebellum of WT vs. KO mice at 1 month and 1 year. Dashed lines indicate statistical significance cut off ($-\log_{10}(\text{Padj}) > 1.301$ and $\log_2(\text{FC}) = \pm 0.5$). Number of significantly down and up-regulated genes are displayed on the top of each plot in blue and red, respectively. **(C-D)** Dot plots of gene ontology (GO) analysis of the DEGs, with down- and upregulated genes marked in blue and red, respectively. Dot size indicates proportion of DEGs relative to the total number of genes in each category. **(E)** Waterfall plots of Gene Set Enrichment Analysis (GSEA) of cerebellar specific significant gene ontology terms. Terms in orange, magenta, and black, are related to lipid, oxygen, and iron, respectively. **(F)** Heatmap of the top 20 leading edge genes of each term displayed in the 1-month Cerebellum GSEA (E). **(G)** Heatmap of the top 10 leading edge genes of each term displayed in the 1-year Cerebellum GSEA (E).



855 **Figure 5. Unique deregulation of lipid metabolites in pre-degenerating KO cerebella.** (A) Volcano plots show deregulated
856 lipids in 2-month-old *Snx14* KO cerebellum (CB), cerebral cortex (CX), liver, and plasma. Horizontal gray lines indicate $P <$
858 0.05 cut-off. Data shows increased concentrations of Acylcarnitine (AcCa) species specifically in KO CB. (B) Bar graphs show
859 total PE concentrations per tissue in $n=8$ WT and $n=10$ KO mice. Two-tailed t -test. PEs are significantly reduced in *Snx14* KO
860 CX. (C) Dotplot depicting fold change (Fc) (proportional to dot size) and p -value (in grey intensity scale) of PE species
861 detected in cerebral cortices for all analyzed tissues. Red dots represent significantly increased lipids while blue dots represent
862 significantly decreased lipids. (D) Bar graphs show total AcCa concentrations in $n=8$ WT and $n=10$ KO mice. Two-tailed t -
863 test. AcCa-s are significantly increased only in KO CB. (E) Dotplot depicting Fc and p -value of AcCa species detected in
864 cerebellar samples for all analyzed tissues. Red dots represent significantly increased lipids while blue dots represent
865 significantly decreased lipids. (F) MALDI-MS imaging of brain cryosections show reduction of PE C38:2, TG 46:1 and TG
866 53:2, and cerebellar accumulation of L-carnitine in KO. The molecules were revealed in positive ion mode using DHB matrix
867 and the m/z (mass-to-charge ratio) of $[M+H]^+$ are indicated. Heatmap colors depict the relative abundance of each metabolic
868 species. Bar graphs show cerebellar or cortical intensity of each lipid species in $n=3$ per genotype. In all panels, graphs show
869 mean \pm S.E.M. n.s. = non-significant, * $P < 0.05$, ** $P < 0.01$. Key of lipid class is found in supplemental data.

A