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1 **Defective tubulin detyrosination causes structural brain abnormalities with**
2 **cognitive deficiency in humans and mice**

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26 **Abstract**

27 Reversible detyrosination of tubulin, the building block of microtubules, is crucial for neuronal
28 physiology. Enzymes responsible for detyrosination were recently identified as complexes of
29 vasohibins 1 or 2 with small vasohibin-binding protein (SVBP). Here we report three
30 consanguineous families, each containing multiple individuals with biallelic inactivation of
31 SVBP caused by truncating variants (p.Q28* and p.K13Nfs*18). Affected individuals show
32 brain abnormalities with microcephaly, intellectual disability and delayed gross motor and speech
33 development. Immunoblot testing in cells with pathogenic *SVBP* variants demonstrated that the
34 encoded proteins were unstable and non-functional, resulting in a complete loss of vasohibin
35 detyrosination activity. SVBP knock-out mice exhibit drastic accumulation of tyrosinated
36 tubulin and a reduction of detyrosinated tubulin in brain tissue. Similar alterations in tubulin
37 tyrosination levels were observed in cultured neurons and associated with defects in axonal
38 differentiation and architecture. Morphological analysis of the SVBP knockout mouse brains
39 by anatomical MRI showed a broad impact of SVBP loss, with a 7% brain volume decrease,
40 numerous structural defects and a 30% reduction of some white matter tracts. SVBP knockout
41 mice display behavioral defects, including mild hyperactivity, lower anxiety and impaired
42 social behavior. They do not, however, show prominent memory defects. Thus, SVBP deficient
43 mice recapitulate several features observed in human patients. Altogether, our data demonstrate
44 that deleterious variants in *SVBP* cause this neurodevelopmental pathology, by leading to a
45 major change in brain tubulin tyrosination and alteration of microtubule dynamics and neuron
46 physiology.

47

48 **Introduction**

49

50 Microtubules are dynamic, polarized polymers composed of α/β tubulin dimers which have a
51 wide range of cellular functions. They are crucial to cell division, cell shape and motility, and
52 intracellular organization and transport. Thus, microtubules are key to normal development of
53 the central nervous system. In recent years an increasing number of human brain malformations
54 and neurological disorders have been associated with mutations in α and β tubulin genes (1, 2).
55 Examples of genes associated with these “tubulinopathies” include *TUB1A* and *TUBB2B* where
56 mutations in these genes result in a range of cortical malformations such as lissencephaly and
57 polymicrogyria (3-6). Moreover, deficiency of some microtubule binding proteins such as
58 DCX, LIS1 or MAP1B are also well known to cause severe neurodevelopmental disorders (7,
59 8).

60 Microtubules display functional specialization by not only having several tubulin
61 isoforms but also through diverse post-translational modifications occurring generally at their
62 C-termini which are located on the microtubule surface. These modifications control
63 interactions with the many microtubule-associated proteins including molecular motors and
64 stabilizing/destabilizing proteins. Among them is the reversible detyrosination of α -tubulin.
65 Detyrosinated tubulin is generally thought to be associated with longer-lived microtubules,
66 whereas more dynamic microtubules are mostly tyrosinated (9, 10). In the cycle of
67 detyrosination/tyrosination, the C-terminal tyrosine of α -tubulin is removed by a
68 carboxypeptidase and re-added by a ligase. The enzyme catalyzing the tyrosination, the tubulin
69 tyrosine ligase (TTL), was identified over 25 years ago (11) and mice lacking this enzyme have
70 been shown to die perinatally, with poorly developed neuronal networks (12). In contrast,
71 enzymes catalyzing the detyrosination (tubulin carboxypeptidases, TCPs) were discovered only
72 very recently (13, 14). Vasohibins (VASHs) were shown to be the major tubulin detyrosinating

73 enzymes, and their partner SVBP (small vasohibin binding protein) to be a prominent regulator
74 of their stability and activity (15-17). SVBP binds to vasohibins with high affinity (17, 18) and
75 the tubulin carboxypeptidase function is achieved by the VASH-SVBP complexes.

76 Tyrosination is an important regulatory signal for neuronal physiology. It is involved in
77 brain development and neuron functioning, including axonal guidance, neurite extension and
78 retrograde transport (12, 19-24). Moreover, knock-down experiments for each of the proteins
79 in the detyrosinating VASH-SVBP complex were recently shown to cause severe neuronal
80 differentiation defects and to alter neuronal migration in the developing mouse brain (13).
81 Interaction of VASH with SVBP and association of the complex to microtubules was shown to
82 regulate axon specification of neurons (17).

83 Using a combination of whole-genome sequencing (WGS) and whole-exome
84 sequencing (WES) we now show that biallelic inactivating *SVBP* variants in humans cause a
85 syndrome involving brain anomalies, intellectual disability and delayed gross motor and speech
86 development. We demonstrate that the *Svbp* knockout mouse model recapitulates several aspects
87 of the neuropathology observed in the human subjects.

88

89 **Results**

90 **Clinical descriptions of patients with *SVBP* mutations**

91 **Family 1**

92 The proband (individual V-6) is the 6th child in a sibship of 7 surviving children (Figure 1a)
93 born to first-cousin parents of South Asian ancestry. She showed early signs of gross motor
94 developmental delay, sitting at 9 months, standing at 19 months and was over 2 years of age
95 before she walked independently. She has gained a few single words of expressive language.
96 She is relatively microcephalic (Table S1). She developed lower limb spasticity with brisk
97 tendon reflexes. She received a number of botulinum toxin injections to relieve Achilles

98 contractures. She has marked mirror movements of her fingers. Cranial nerve examination is
99 normal. She has very short 3rd and 4th toes on both feet. An MRI scan of the brain showed
100 irregular ventricular margins and a thin corpus callosum.

101 Individual V-7 is the younger sister of the proband. She presented in a similar fashion
102 to her sister with severe global developmental delay and an evolving spastic paraparesis. She
103 also sat at 9 months, stood at 13 months and walked at 24 months. She has developed only a
104 few single words of speech. She had a single episode of status epilepticus as a two-year-old and
105 had an ongoing seizure disorder which resolved in adolescence. She also displays mirror
106 movements of the fingers. Her head circumference was noted to be below the 0.4th centile for
107 her age. Her height is between the 9th and 25th centiles. An MRI brain scan performed at age
108 4 years showed a small corpus callosum with dysmorphic ventricles. There was slight
109 prominence of CSF spaces in keeping with a degree of volume loss, including the lateral
110 cerebellar hemispheres. The cisterna magna was normal. There was completed myelination
111 with no white matter abnormality and normal deep grey structures.

112 Individual IV-3 is the maternal aunt of the proband. Prior to her niece being referred to
113 our service, she had been diagnosed with learning disability and short stature. She was noted to
114 have short 3rd, 4th and 5th metacarpals which resulted in her being investigated for
115 pseudohypoparathyroidism. However, all biochemical and metabolic investigations, including
116 sequence analysis of *GNAS1* identified no specific underlying cause. At 43 years of age she has
117 very little speech. She had marked wasting of the intrinsic muscles of her hands with clawing
118 of the fingers. She has delayed gross motor development and walks with the use of a frame. No
119 cranial MRI was available.

120 Individual IV-4 is the maternal uncle of the proband. He was 37 years of age when first
121 seen in our clinic. He also has learning disability with a spastic paraparesis requiring several
122 operations to release contractures at his hips and ankles. He had seizures as an infant and child,

123 resolving in adolescence. He walked at 5 years and has attained only a few single words of
124 expressive language. He has hypothyroidism. He has coarse facial features similar to those seen
125 in his affected sister and nieces. His thumbs are short and he has marked muscle wasting of the
126 small muscles of his hands with clawing of his fingers. He has a spastic paraparesis with brisk
127 lower limb reflexes and extensor plantar responses. No cranial MRI was available.

128 **Family 2**

129 The proband (II-3) is a 6-year-old female referred to the Kuwait medical centre aged 2 years
130 due to developmental delay. She is the third of four offspring (Figure 1b), born weighing 2.9
131 kg to phenotypically normal 1st-cousin-once-removed parents of Kuwaiti ancestry. Increased
132 muscle tone was observed, especially in both lower limbs. She has ankle clonus, easily elicited
133 reflexes and an unsteady spastic gait with toe walking. Occipital frontal circumference (OFC)
134 was <0.4th centile. At age 6 she could speak only a few words. MRI of her brain showed a thin
135 corpus callosum with dilated ventricles and poor white matter volume. Cranial nerves
136 examinations, electromyography, plasma amino acids and urinary organic acids were all
137 normal.

138 Her elder brother (II-2) is similarly affected with an OFC of 49.7 cm aged 7 years (0.4th
139 centile). He has generalized hypertonia predominantly in the lower limbs, brisk reflexes, an
140 unsteady spastic gait, mirror hand movement and spoke using only a few words.

141 **Family 3**

142 The proband (II-3, Figure 1c) is the third child of first-cousin parents from a multiply
143 consanguineous South Asian family. She was referred to the genetics clinic at 3 years of age
144 with progressive microcephaly, progressive difficulty in walking and global developmental
145 delay. The antenatal scans were reportedly normal and her birth weight was 2.7 kg at term (10th
146 centile). Head circumference at birth was 31.5 cm (<0.4th centile). There were no concerns in
147 early infancy and she was walking independently at 14 months of age but remained unsteady

148 on her feet and would trip easily. Upon examination she was seen to be of proportionate short
149 stature and a failure to thrive had been noted. She also demonstrated spasticity with delayed
150 gross and fine motor development. She has profound intellectual disability with delayed speech
151 and language development and demonstrated autistic behavior. Neurometabolic investigations
152 were normal. Hyperpigmentation of the skin in the form of small café au lait macules was noted.
153 In view of the microcephaly and the café au lait macules, a DNA repair disorder was considered
154 as a possible differential diagnosis.

155 Her older sibling (II-1) was initially assessed at 3 years of age because of microcephaly,
156 progressive difficulty in walking and developmental delay. His birth weight and head
157 circumference were reported on the 3rd centile although no measurements were available. At 3
158 years of age his head circumference was 46 cm (<0.4th centile). He was subsequently lost to
159 follow-up and was seen with his younger sister when he was 21 years old. At that time, he had
160 only 10 words. He had spastic diplegia and was non-ambulant. Additional features included 2-
161 4 toe cutaneous syndactyly and coarse facial features.

162

163 **Genetic findings**

164 **Family 1**

165 We first identified a consanguineous family of Pakistani ancestry in which four individuals,
166 two sisters and their uncle and aunt, had a syndrome characterized by learning disability, spastic
167 paraparesis, a thin corpus callosum and decreased cerebral white matter volume (Table S1).
168 Individual V-6 underwent WGS as part of the WGS500 project. We carried out linkage analysis
169 under a recessive model, combining the WGS data with SNP array data from other relatives
170 (Figure 1a). This revealed a single significant linkage peak (Figure S1): a 12.7 Mb region on
171 chr1:40,921,145-53,713,336 (GRCh37/hg19) that had a LOD score of 3.01 and was

172 homozygous in all affected individuals. This region contained 145 protein-coding genes, and
173 was interrogated for rare simple recessive variants in the WGS data.

174 There was only one coding variant that was sufficiently rare and that disrupted a well
175 conserved position. This was a homozygous transition, chr1:43,282,134G>A which predicts a
176 LoF variant c.82C>T; p.Q28* in *SVBP* (NM_199342.4). The variant co-segregated with the
177 phenotype in all family members for whom DNA was available. It was seen in a single
178 heterozygous individual from a population cohort of 7,446 exome-sequenced British Pakistani
179 and Bangladeshi adults (East London Genes and Health;
180 www.genesandhealth.org/research/scientific-data-downloads), but absent from the gnomAD
181 resource of 123,136 exomes and 15,496 genomes from individuals without severe paediatric
182 disease (<https://gnomad.broadinstitute.org>). There were also no high-confidence homozygous
183 LoF variants in either of these cohorts. We also searched for potentially causal variants under
184 other inheritance models and outside the coding region in this family (see Supplemental
185 Methods), but found none more likely to be pathogenic than the LoF variant in *SVBP*.

186

187 **Family 2**

188 In the hope of replicating the results from Family 1, we searched the CentoMD mutation
189 database (25) for candidate variants in *SVBP* and identified a 5 year old female (II-3, CentoGene
190 ID 1099053) with a homozygous c.39_42del; p.K13Nfs*18. This variant had been identified by
191 trio WES analysis, with the unaffected parents both shown to be heterozygous. The only other
192 potential candidate variant identified in this family highlighted was a c.3049G>A; p.E1017K
193 in *SZT2* (NM_015284.3), a gene associated with Early Infantile Epileptic Encephalopathy
194 (OMIM *615463). Both *SVBP* and *SZT2* variants were homozygous in the elder affected
195 brother (II-2). DNA from unaffected siblings has not yet been tested, however the proximity
196 of *SZT2* to *SVBP* means the variants will likely co-segregate and thus testing unaffected siblings

197 is unlikely to rule *SZT2* out conclusively. However, *SZT2* is a large gene and c.3049G>A
198 predicts a missense change with poor SIFT and polyphen scores. Most variants in this gene
199 which have been reported to be pathogenic in ClinVar (www.ncbi.nlm.nih.gov/clinvar/) are
200 LoF alleles. Epilepsy is a common feature whereas II-3 had no history of seizures. The *SZT2*
201 variant was therefore assessed as being one of unknown significance. The proband's similarly
202 affected brother (II-2) had previously undergone exome analysis in another laboratory and this
203 had identified variants in *PLP1* (c.-102C>T) and *WDR81* (c.2051A>C; p.Q684P). Segregation
204 analysis of these two variants in the parents and in II-3 excluded both variants from being
205 causative for the phenotype.

206

207 **Family 3**

208 By searching through genetic data from 49,434 individuals from the 100,000 Genomes Project,
209 we identified one additional family with biallelic LoF variants in *SVBP*. Both affected children
210 in this family had been diagnosed with intellectual disability and were homozygous for the same
211 c.82C>T; p.Q28* variant as seen in Family 1. Both unaffected siblings were heterozygous for
212 this variant (Figure 1c). Using SNP array data, Families 1 and 3 were shown to share a 3.9 Mb
213 region of identity by descent on chromosome 1, between rs9659165 and rs7515271. Using
214 WGS, this shared region was refined to 39,550,261 – 43,442,698 (rs76104968 and rs1770796).
215 The chr1:43,282,134G>A (p.Q28*) variant is thus likely to be an ancestral variant rather than
216 a recurrent mutation.

217

218 **Clinical comparison across families**

219 Clinical details for affected individuals are reported in Table S1. Most individuals exhibited
220 microcephaly (on or below the 0.4th centile, where data were available), moderate to severe
221 learning disability and speech delay. A detailed comparison of the MRI data available in these

222 families demonstrated a subtle but consistent pattern comprising reduced brain volume, a thin
223 corpus callosum and abnormal ventricles in all three families (Figure 1 d,e and data not shown).
224 Affected individuals also exhibited spasticity and in most cases this involved predominantly
225 the lower limbs. More variable features included mirror movements observed in 4/8, coarse
226 facial features in 5/8, seizures in 3/8, short stature in 3/8 and digital abnormalities in 4/8.

227

228 **Consequences of *SVBP* pathogenic variants on VASH enzymatic activity**

229 We obtained whole-blood RNA from individuals from Family 1, and found no evidence that
230 the p.Q28* variant resulted in nonsense mediated decay (Figures S2 and S3). Hypothesizing
231 that these protein-truncating variants would nonetheless impair the function of SVBP, we
232 examined the ability of mouse VASH1 enzyme to detyrosinate α -tubulin in the presence of
233 SVBP proteins either in their native form, or with the p.Q28* and p.K13Nfs*18 variants
234 observed in human patients (Figure 2a). Expression of VASH in HEK293T cells alone resulted
235 in a slight increase of detyrosinated tubulin (due to the presence of endogenous SVBP in cells),
236 whereas expression with native SVBP resulted in a substantial increase in detyrosinated tubulin
237 and a large loss of tyrosinated tubulin, thus revealing the detyrosination activity due to
238 exogenous VASH-SVBP complex. When VASH was expressed with the SVBP mutants, the
239 levels of modified tubulin did not differ from the conditions observed in the absence of SVBP
240 (Figure 2a). This is mostly due to instability of the SVBP mutants: whereas native SVBP was
241 easily detected, in similar experimental conditions p.Q28* was hardly detected and
242 p.K13Nfs*18 could not be detected at all (see Flag signals in Figure 2a). Moreover, even the
243 expression of the VASH was affected by the expression of the SVBP mutants (GFP signals), as
244 expected from the known instability of the VASH in the absence of its chaperone SVBP (13,
245 14). Thus, the p.Q28* and p.K13Nfs*18 variants effectively result in a functional knockout of
246 *SVBP*.

247

248 To investigate the function of *Svbp* *in vivo*, KO mice were produced by CRISPR/Cas9
249 technique. Homozygous mice, having a deletion after the third residue of SVBP generating a
250 frameshift adding only four chimeric residues (Figure S4), were obtained by selective breeding.
251 PCR and Sanger sequencing results confirmed the deletion of targeted region. These *Svbp* KO
252 mice are viable, fertile, exhibited normal general development and do not exhibit weight loss
253 compared to wild-type mice.

254

255 **Defects in tubulin tyrosination in brain of SVBP knockout mice**

256 We analyzed the levels of tyrosinated, detyrosinated and $\Delta 2$ α -tubulin (a modification that
257 follows, and thus depends on, detyrosination) in the brain of *Svbp* KO mice (Figure 2 b-e).
258 Detyrosinated and $\Delta 2$ tubulin drastically decreased (-39.9 ± 2.3 % and -33.0 ± 2.3 %, respectively),
259 and a 3-fold increase in tyrosinated tubulin was observed ($+233.4 \pm 7.9\%$). Thus
260 the absence of the VASH-stabilizing SVBP chaperone leads to drastic changes of α -tubulin
261 modifications, with a large reduction of tubulin detyrosination. The remaining pool of
262 detyrosinated α -tubulin could be due to $\alpha 4$ -tubulin (which genetically lacks the C-terminal
263 tyrosine) or to other detyrosinases (13, 14). The accumulation of tyrosinated tubulin could be
264 the result of neosynthesis of tyrosinated tubulin that cannot be detyrosinated at specific stage
265 of brain development.

266

267 **SVBP-null neurons show anomalies of their differentiation and morphology.**

268 We then examined the three pools of α -tubulin (tyrosinated, detyrosinated and $\Delta 2$) in
269 hippocampal neurons prepared from wild-type and *Svbp* KO mice brains embryos (Figure S5)
270 cultured 2, 7 and 17 days *in vitro* (2, 7 and 17 DIV). In young neurons, the absence of SVBP
271 leads to a drastic decrease of detyrosinated tubulin (-63.6 ± 1.8 % and -73.8 ± 1.5 % at day 2

272 and day 7 of DIV, respectively) and $\Delta 2$ tubulin almost disappeared. In mature *Svbp* KO neurons
273 (17 DIV), the decrease of detyrosinated tubulin is maintained but less pronounced (-37.7 ± 11.7
274 %) and $\Delta 2$ tubulin present ($36 \% \pm 9.1 \%$ of the controls). On the other hand, tyrosinated tubulin
275 accrued extensively in the KO neurons, with a 1.5-fold increase observed at 2 DIV and a 3-fold
276 increase observed after 7 DIV.

277 As in brain, the remaining pools of detyrosinated α -tubulin in neurons missing SVBP
278 (and thus vasohibin-SVBP detyrosinating activity) could be due to $\alpha 4$ -tubulin or to other
279 detyrosinases^{13,14}. Yet, the notable accumulation of tyrosinated tubulin in neurons observed
280 before 7 DIV strongly suggest that, at these early stages of neuronal differentiation, the
281 detyrosination of large pools of α -tubulin cannot be performed by other enzymes than
282 vasohibin-SVBP complexes.

283 Confocal images of *Svbp* KO neurons cultured 2 DIV showed a large decrease in the
284 amount of detyrosinated tubulin (Figure 3a) compared to wild type neurons. Remaining
285 detyrosinated pools are concentrated in axons, whereas the other neurites exhibit tyrosinated
286 tubulin. Moreover, SVBP deficiency led to a large decrease of neurons bearing an axon at 2
287 DIV (from $55.8 \pm 4.0 \%$ to $29.7 \pm 3.3 \%$), revealing a clear delay in axonal differentiation
288 (Figure 3b). *Svbp* KO neurons at 2 DIV developed an increased number of primary neurites and
289 extra branches whereas the axon length was significantly reduced ($-26.5 \% \pm 4.4 \%$) (Figure
290 3c-e). At 17 DIV, after complete neuronal maturation, *Svbp* KO neurons still show a significant
291 increase in their dendritic branching compared to wild-type neurons within the first 150 μm
292 close to the soma, as measured by Sholl analysis (Figure 3f).

293 Altogether, in neurons, SVBP deficiency resulted in marked changes in the pools of modified
294 α -tubulin and in a delay of axon differentiation associated with severe morphological defects.

295

296 **Brain morphological defects associated with *Svbp* deletion in mice**

297 We performed brain imaging and morphometric assessments of wild-type and *Svbp* KO mice
298 using anatomical MRI (Figure 4). *Svbp* KO mice show a significant decrease of the whole brain
299 volume (7.3 %) affecting a large number of the brain regions including the cortex (7.8 %), the
300 striatum (10.5 %), the hypothalamus (10.0 %), the thalamus (10.4 %) and the brainstem (10.0
301 %). We also performed measurements of white matter structures and found a strong significant
302 reduction of several structures, for example the corpus callosum (33.7 %), the fimbria (31.7 %)
303 and the anterior commissure (38.9 %). Thus, *Svbp* deletion in mice results in a microcephaly
304 affecting more severely the white matter, revealing deficiencies of axonal tracts.

305

306 **Behavioral defects associated with *Svbp* deletion in mice**

307 A comprehensive behavioral analysis of *Svbp* KO mice as compared to wild-type mice was
308 performed. Firstly, we assayed locomotor activity in an open field. The total distance traveled
309 by a mouse cumulated over 30 min was recorded. The responses exhibited by *Svbp* KO mice
310 were enhanced compared with the wild-type (Figure 5a; WT: 7306 ± 464 cm, *Svbp* KO: $9397 \pm$
311 595 cm), indicating that the KO mice were more active than their wild-type counterparts.

312 We next evaluated cognitive abilities by investigating memory and social behavior. We
313 monitored spontaneous alternation in a Y-maze, a test of spatial learning and memory. We
314 found no difference between wild-type and *Svbp* KO mice in the alternation frequency (Figure
315 S6a) indicating that the memory and willingness of KO mice to enter new environments was
316 not affected. Social behavior was then assayed using the Resident-Intruder Test. Male mice are
317 territorial and will react to an unfamiliar male placed in their home cage. We analyzed the
318 behavior of a resident male, isolated either 1 or 3 weeks, when exposed to an intruder by
319 measuring the time spent in active investigation. As shown in Figure 5b, *Svbp* KO mice
320 exhibited a significant reduction of social investigation, compared to wild-type mice (WT:
321 150.1 ± 9.2 s and 142.6 ± 12.7 s, *Svbp* KO: 105.3 ± 12.6 s and 84.3 ± 3.1 s, respectively for 1

322 and 3 weeks of isolation). In addition, we investigated the exploratory behavior of mice in the
323 Hamlet test (26). Animals were repeatedly trained in the apparatus and analyses of the time
324 spent in each houses during training days showed a significant increase in the presence of *Svbp*
325 KO mice in the Hide house, a trend to decreased presence in the Interact house and no change
326 in the 3 other houses, Run, Eat and Drink (Figure 5d), as compared to the group of wild-type
327 mice. Data analyzed in terms of number of entries failed to show any difference whatever the
328 house.

329 In parallel, we monitored possible depressive/anxiety-like status of *Svbp* KO mice. For
330 that we performed the Novelty Suppressed Feeding (NSF) test where the animal's motivation
331 to eat competes with the aversive signal of bright illumination. We measured the latency to eat
332 and found that *Svbp* KO mice behave as wild-type mice (Figure S6b). We next used the Elevated
333 Plus Maze test, using a maze composed of two open and two enclosed arms. Mice exhibit
334 aversion to open spaces and will remain in enclosed spaces. In this test, *Svbp* KO mice behave
335 as wild-type mice (Figure S6c). Finally, we used the Marble Burying Test, a test based on the
336 fact that mice will bury harmful objects in their bedding. Each mouse was introduced in a cage
337 containing 24 marbles for 30 min, and the number of buried marbles was then counted. As
338 shown in Figure 5c, *Svbp* KO mice buried significantly less marbles than wild-type mice (WT:
339 12.77 ± 1.2 , *Svbp* KO: 8.00 ± 0.81) indicating a possible lessening of anxiety and of interaction
340 with the environment.

341 In summary, *Svbp* KO mice were more active than wild-type mice, do not exhibit severe
342 memory defects, and seem to be less anxious than wild-type mice. Interestingly, they exhibit
343 an impaired social behavior, showed by reduced interaction with the stranger mouse in the
344 resident intruder test and a trend in the Hamlet, together with a higher time spent in the safe
345 place provided by the Hide house in this device.

346

347 **Discussion**

348 In this study, we initially assessed a large consanguineous Pakistani kindred (family 1) using a
349 combination of linkage analysis and WGS, identifying a c.82C>T; p.Q28* variant in *SVBP* as
350 the most likely candidate. Although genetic co-segregation (Figure 1a) was confirmed in June
351 2012, the gene was then known as *CCDC23* and little was known about the function of the
352 encoded 66 amino-acid protein. The variant could therefore not conclusively be confirmed to
353 be causative for the intellectual disability, microcephaly and spasticity observed in affected
354 family members. Although initially identified as a secretory chaperone for VASH1 and
355 proposed to play a regulatory role in angiogenesis (18), it was the recent studies confirming
356 *SVBP*'s importance in tubulin detyrosination and neuron differentiation (13, 14) that prompted
357 the functional testing required to confirm this variant as the causative allele. The protein-
358 truncating changes were associated with an absence of a catalytically active enzymatic complex
359 VASH-SVBP, as shown by in cell-activity tests of the variants. Results from HEK293T cells
360 were then recapitulated in the brains of KO mice, where reduced levels of detyrosinated tubulin
361 were observed, together with concomitant increases in tyrosinated tubulin. This is the first
362 demonstration of the link between the clinical characteristics observed in patients with *SVBP*
363 mutations and the underlying molecular defect of tubulin detyrosination. This study also
364 highlights the importance of reassessing clinical genomic data (27, 28).

365 Additional evidence supporting pathogenicity comes from our recent identification of *SVBP*
366 mutations in two additional families, with affected individuals sharing significant phenotypic
367 overlaps. Although we registered the phenotype and genes in the chromosome 1 linkage region
368 from Family 1 on GeneMatcher (<https://genematcher.org/>), this genetic replication came
369 through a more unconventional route; Family 2 was identified during a two-week trial of the
370 CentoMD mutation database (25). Family 3 was ascertained through access to the 100K

371 Genomes Project; this study represents one of the first novel genes to be reported from that
372 program.

373 Whilst this manuscript was in preparation, Iqbal *et al* described four individuals with
374 intellectual disability, microcephaly, hypotonia and ataxia who were homozygous for the same
375 p.Q28* variant (29). Although their two kindreds were of Syrian and Pakistani ethnicity, a
376 single ancestral disease haplotype of 1.8 Mb was shared across both families. Where a
377 genotype-phenotype relationship is based on a single disease haplotype, caution should be
378 exercised as it is possible that an unobserved pathogenic mutation may lie on the same
379 haplotype as the candidate variant (30, 31). Without WGS data, it is difficult to exclude the
380 possibility that a rare non-coding variant or a cryptic structural variant could have been missed.
381 In contrast, we have WGS data for both families harboring the p.Q28* disease haplotype and
382 the uniformity of coverage means we are better able to exclude such scenarios. The genotype-
383 phenotype correlation is further corroborated by the results for Family 2 where a different
384 truncating *SVBP* variant was detected in patients exhibiting the same phenotype.

385 Detailed phenotypic descriptions of the 8 affected individuals described here allows us
386 to start to delineate the range of phenotypic features associated with defective detyrosination in
387 humans. In combination with the 4 patients reported by Iqbal *et al* (29), it is clear that the
388 disorder comprises several common features as well as a number of variable characteristics.
389 All patients presented with intellectual disability (moderate to profound), delayed speech and
390 delayed gross motor development. Microcephaly was another common feature in human
391 patients and this mirrors what was observed in *Svbp* KO mice where there was a 7% loss in
392 global brain volume. Neuroradiological assessment of human MRI data (Figure 1 d,e)
393 highlighted consistent albeit subtle features including enlarged ventricles and a thin corpus
394 callosum. Notably in KO mice this last brain region was also reduced by 33.7%. The most
395 conspicuous difference between the families described here and those reported by Iqbal *et al*

396 (29) is that the previous group of patients were all reported to have hypotonia, whereas the
397 majority of patients we identified had increased muscle tone, predominantly in the lower limbs.
398 Other variable features observed multiple times in our case series include digital abnormalities,
399 mirror movements, coarse facial features and infantile seizures. Iqbal and colleagues reported
400 chest abnormalities in 2 of their 4 patients but this feature was not noted in our families.
401 Behavioral characteristics may include aggression, described in two patients (29) and autism,
402 described in one patient reported here. Although behavioral traits are difficult to model in
403 rodents, we note that the *Svbp* KO mice in this study also exhibited a significant reduction of
404 social investigation and possibly lower levels of anxiety associated with a low interest for their
405 physical environment.

406 The tubulin tyrosination/detyrosination cycle is one of several post-translational
407 modifications of neuronal tubulin. Other modifications include acetylation, polyglycylation
408 and polyglutamylation, and collectively these are known as the “tubulin code”. Over the last
409 decade, studies in mice have linked tubulin acetylation and polyglutamylation to
410 neurodegeneration (32-35). More recently, a causal relationship between the CCP1
411 deglutamylase and neurodegeneration was found in humans (36). CCP1 deglutamylase is
412 responsible for the successive removal of the two glutamate amino acids immediately preceding
413 the C-terminal tyrosine, yielding $\Delta 2$ - and $\Delta 3$ - α -tubulin (37). Detyrosinated α -tubulin is a
414 substrate for the CCP1 enzyme and so it is not surprising that decreased levels $\Delta 2$ - α -tubulin
415 were observed in brains of the *Svbp* KO mice (Fig 2 b,e). Whilst the patients described in the
416 present study did not show signs of cerebellar atrophy, shared features seen in both SVBP and
417 CCP1 patient cohorts include microcephaly, hypotonia, spasticity, developmental delay and
418 corpus callosum dysplasia.

419 The most frequent form of hereditary spastic paraplegia is caused by mutations in
420 *SPAST* (38). *SPAST* encodes an enzyme that shows microtubule-severing activity, a process

421 which in turn is regulated by tubulin glutamylation (39), another type of post-translational
422 modification that is common in mammalian brain tissue. It is also worth noting that
423 detyrosinated microtubules are known to play a role in modulation of mechanotransduction in
424 muscle (40). It is therefore unsurprising that the abnormal phenotype seen in patients included
425 muscular component and in most cases this was in the form of lower limb spastic paraparesis.
426 Interestingly, this feature was not recapitulated in the *Svbp* KO mouse model, where the open
427 field locomotor activity was demonstrated to be increased.

428 The present study is the first detailed description of *Svbp* KO mice. Anatomical MRI
429 indicates microcephaly and this whole brain volume reduction affects all brain structures
430 analyzed. The brain size reduction is not associated with a general loss of body weight,
431 indicating a specific alteration. *Svbp* deletion results in a severe reduction of the white matter
432 with both intra-hemispheric (corpus callosum) and cortical tracts (fimbria, anterior
433 commissure) defects.

434 The observed reduction of brain volume most possibly originates from an abnormal
435 differentiation and maturation of deficient neurons. In agreement, *Svbp* KO cultured neurons
436 showed a clear delay of their axon differentiation and severe morphological defects. Moreover,
437 downregulation of SVBP was shown to disrupt neuronal migration in developing mouse
438 neocortex (13). Anomalies of progenitor proliferation might also occur since VASH-SVBP
439 complexes were recently shown to be critical during mitosis (41). Finally, the reduction of brain
440 volume might also involve a reduction of synapse density, as suggested by shRNA knockdown
441 experiments in rat hippocampal neurons (29). A whole *in vivo* analysis of neurons morphology
442 and spines integrity will be needed to obtain a full picture of synaptic functioning in the absence
443 of SVBP. Altogether our work clearly indicates that *Svbp* deletion results in brain defects more
444 probably originating from neurodevelopmental anomalies. VASH-SVBP complexes are major
445 regulators of detyrosination and thus of microtubule dynamics in neuronal cells (13, 17). The

446 defects linked to *Svbp* deletion reported in this work highlight the importance of microtubules
447 integrity for brain homeostasis and support the view that microtubule dysfunction can lead to
448 neurodevelopmental disorders and neurodegeneration.

449 The comprehensive behavioral analysis of *Svbp* KO mice indicates several abnormal
450 parameters. *Svbp* KO mice exhibit an enhanced locomotor sensitivity reaction when exposed to
451 novelty. The possible anxious state of the mutant mice was investigated using the Marble
452 Burying Test and the Elevated Plus Maze, however these results were difficult to interpret. *Svbp*
453 KO mice showed no anxious behavior in the Elevated + Maze, whereas they buried a
454 substantially lower number of marbles than wild-type mice in the Marble Test, possibly
455 indicative of a low anxious state. This latter defect might rather be attributed to a lower interest
456 for their environment. This result is in agreement with (a) the reduced interest of SVBP mice
457 for their congener observed in the resident intruder test, and (b) the results of the Hamlet test.
458 Indeed, training of the animals in this complex environment providing distinct functionalized
459 spots for eating, drinking, running, hiding and interacting identified only two differences in the
460 *Svbp* KO mice behavior as compared with wild-type littermates, namely a trend to decrease
461 interaction with the stranger mouse and a significant increase of time spent in the tunnel of the
462 Hide house. Assessment of intellectual disability related defects in mice often relies on analysis
463 of the working memory. *Svbp* KO mice do not exhibit deficit in Y-maze test which relies on
464 measuring memory related to spatial exploration while factors such as motivational or
465 emotional states are minimized and in which learning a rule is not involved. This result suggests
466 that the working memory *per se* is not affected by *Svbp* deletion.

467 In summary, we have shown that rare biallelic variants in *SVBP* result in loss of tubulin
468 detyrosination activity, causing anatomical brain deficits associated with intellectual disability.
469 The three families we describe have several features which are recapitulated in the KO mouse
470 model, which is described here for the first time. This study thus adds to the growing list of

471 diseases linked to aberrant microtubule cytoskeleton and point to the importance of microtubule
472 dynamics.
473

474 **Materials and methods**

475 **Whole-genome or whole-exome sequencing and variant filtering**

476 Appropriate informed consent was obtained for all human subjects.

477 **Family 1**

478 Individual V-6 from family 1 underwent WGS in December 2011 using a HiSeq2000 machine
479 (Illumina) and v3 chemistry, as part of the WGS500 project. Further details of the sequencing,
480 read alignment and variant calling have been described previously (42). The proband's
481 unaffected mother (IV-2), affected sister (V-7) and affected uncle and aunt (IV-3 and IV-4)
482 were genotyped on either the Illumina CytoSNP12 or the OmniExpress microarray (Figure 1a).
483 Since the parents of both pairs of affected siblings were first cousins, we considered the simple
484 recessive model most likely, so focused on regions that were identical-by-descent for both
485 alleles (IBD2) and homozygous in all the affected individuals. However, a compound recessive
486 model would also fit the pedigree, and so we also considered regions that were IBD2 and
487 heterozygous. To identify these regions, we selected SNV markers for linkage with linkdatagen
488 (43) and then ran MERLIN (44) using the Kong and Cox exponential model (45), specifying
489 a recessive model.

490 We then searched for rare coding variants within the linked regions. We excluded
491 variants with a frequency greater than 0.5% in the 1000 Genomes project (46), the NHLBI
492 Exome Sequencing project (<http://evs.gs.washington.edu/EVS/>), gnomAD, or in our own
493 WGS500 project (42), and those that were seen as homozygotes in any of these projects (other
494 than in individual V-6). We also prioritized variants in regions that were highly conserved
495 across the 46 vertebrate species in the UCSC conservation track. Candidate variants were
496 genotyped in the other unaffected siblings by Sanger sequencing to check segregation.
497 Since there was insufficient evidence based on the WGS500 project to implicate a pathogenic
498 variant in these patients' condition the family were later recruited to the 100,000 Genomes

499 Project (<https://doi.org/10.6084/m9.figshare.4530893.v4>), a national project that aims to
500 establish the use of WGS in the National Health Service and which has been described in
501 detail elsewhere (47). Reads were mapped to GRCh37 using the Isaac aligner and variants
502 were called using the Isaac variant caller (Illumina). Structural variants were called with
503 Manta and Canvas (Illumina). As part of this study, samples were also genotyped using the
504 InfiniumCoreExome-24v1 array (Illumina).

505

506 **Families 2 and 3**

507 The affected proband (II-3) from family 2 and her parents underwent WES analysis at
508 CentoGene. WES workflows and results from the 1000 families were described previously
509 (48). Results for family 2 were incorporated into the searchable CentoMD mutation database
510 (CentoGene, www.centogene.com/pharma/mutation-database-centomd.html) (25).

511 Family 3 were whole-genome sequenced as a quad, as part of the 100,000 Genomes
512 Project (as described above). Samples were also genotyped using the InfiniumCoreExome-
513 24v1 array (Illumina). Family 3 was identified from the rare disease subgroup of the 100,000
514 Genomes Project using a customized bcftools script designed to search through vcf.gz files
515 specifically looking for loss of function (LoF) variants in *SVBP*. Genotypes from WGS and
516 from the InfiniumCoreExome-24v1 arrays were used to assess identity by descent sharing
517 across chromosome 1 by looking for extended regions sharing identical genotype calls.

518

519 ***In cellulo* tubulin detyrosination assay**

520 A plasmid encoding mouse SVBP (NM_024462) with C-terminal myc and Flag tags (SVBP-
521 myc-Flag) was obtained from OriGene (MR200054). Using this plasmid we deleted residues
522 28-66 (mimicking p.Q28*) or replaced residues 13-66 by the 17 residues resulting from the
523 frameshift p.K13Nfs*18. Mouse Vash1 cDNA (NM_177354) was PCR-amplified from

524 plasmid Vash1-myc-DDK (Origin, MR222250) and inserted into pEGFP-N1 vector (Clontech),
525 to generate vasohibin-1 with EGFP fused to its C-terminus (V1-GFP). All plasmids were
526 verified for sequence integrity.

527 HEK293T cells were maintained under standard conditions and were transfected using JetPEI
528 transfection reagent (Polyplus-Transfection). A ratio of 1:1 was used for cDNA co-transfections
529 (for VASH1 with SVBP, and with variants). Protein extracts from HEK293T cells were
530 prepared directly by scraping the cell layer in Laemmli buffer.

531

532 **Generation of *Svbp* KO mice**

533 The *Svbp* mutation was generated by CRISPR/Cas9 editing. Guide selection and off-target
534 predictions were made with CRISPOR software (49). Exon 2 target sequence
535 (GATTTTCTTTCCGGGCAGG) was unique in the genome. Predicted off-target sequences
536 on the same chromosome were all non exonic, and with at least 4 mismatches (partly localized
537 close to the PAM) with on-target sequences. A dual guide RNA was prepared by combining
538 synthetic tracrRNA (TriLink BioTechnologies) and crRNA (Eurogentec, Angers, France).
539 Sequences are given in Table S2. A cloning free procedure (50) was used to modify the *Svbp*
540 locus, by microinjection of Cas9/RNA complexes into *in vitro* fertilized B6D2F1xFVB
541 embryos. F0 mosaic animals born after reimplantation of microinjected embryos were
542 genotyped by PCR and Sanger sequencing, and then mated to C57BL/6 mice to provide F1
543 founder mice. Among these, a mouse was selected for the frameshift introducing as few extra
544 amino acids as possible after the third amino-acid of SVBP. The allele was designated
545 *SvbpP4Rfs*6* and the corresponding protein sequence is *MDPRKIQS* (extra amino-acids in
546 italics). At least 8 additional back-crosses were performed with C57BL/6 mice before
547 phenotyping in order to avoid possible confounding effects due to off-target mutations. The
548 study protocol was approved by the local animal welfare committee (Comité Local Grenoble

549 Institute Neurosciences, C2EA-04) and complied with EU guidelines (directive 2010/63/EU).
550 Every precaution was taken to minimize stress and the number of animals used in each
551 experiment. All experiments were conducted on wild-type and *Svbp* KO adult (3-6 months)
552 littermates with a C57BL/6 genetic background.

553

554 **Antibodies**

555 Tyrosinated, detyrosinated and $\Delta 2$ α -tubulin were detected in cells and tissues by immunoblots
556 using specific antibodies, as described previously (37). The other primary antibodies used were
557 as follows: rabbit anti-Flag from Molecular Probes (1:10,000), rabbit anti-GFP from Chromotek
558 (1:5,000), rabbit anti-GAPDH from Sigma (1:10,000).

559

560 **Immunoblot analysis of α -tubulin variants in neuron and brain protein extracts**

561 For neurons protein extracts analysis, cells were collected after 2, 7 or 17 DIV. After washing
562 with phosphate-buffered saline (PBS) medium at 37°C, neurons were directly lysed in Laemmli
563 buffer.

564 For analysis of mice tissues, crude protein extracts were prepared using a FastPrep
565 Instrument (MP Biomedicals, Illkirch, France). Brain proteins were extracted in 100 mM 1,4-
566 piperazinediethanesulfonic acid at pH 6.7, 1 mM EGTA, 1 mM MgCl₂, and protease inhibitors
567 (Complete Mini EDTA-free; Roche Diagnostics, Meylan, France). Cell remnants were
568 eliminated by a 10 min centrifugation at 10,000 × *g*, and Laemmli buffer was added to the
569 supernatant.

570 Protein extracts were then loaded on 10% acrylamide gels (Mini-PROTEAN® TGX
571 Stain-Free™, Biorad) and transferred with Trans-Blot® Turbo (BioRad). Membranes were
572 incubated with primary antibodies, with secondary antibodies conjugated with HRP (1:10 000,
573 from Jackson ImmunoResearch), and finally revealed with Chemidoc camera (Biorad). For

574 analysis and graphical representations of immunoblots (Fig. 3b,c and S6c), protein bands were
575 quantified from triplicate blots of 3 different experiments using Image Lab software (Bio-Rad).
576 Tyrosinated, detyrosinated or $\Delta 2$ α -tubulin signals were normalized to the protein content of
577 the sample estimated from its GAPDH signal.

578

579 **Neuronal cultures and morphometric analysis**

580 Hippocampal neurons were cultured from E17.5 WT or *Svbp* KO embryos as described (12).

581 For axonal and neurite morphometric analysis, neurons were fixed after 2 DIV,
582 immunolabeled and analyzed as previously described (13). 20 neurons per embryo, from 6
583 different WT or *Svbp* KO embryos were analyzed.

584 For Sholl analysis, 1% of cultured hippocampal neurons were infected with 30 MOI of a
585 lentivirus containing GFP (pWPT-GFP addgene.org/12255) and plated onto a monolayer of
586 non-infected neurons. After 17 DIV, neurons were fixed and images were taken with an 20x/0.5
587 objective with an inverted Nikon microscope equipped with a camera EMCCD (Coolsnap,
588 photometrics) piloted by Metaview software. Images were processed by the Tubeness filter,
589 thresholded and converted to mask. Final masks were analyzed using the Sholl Analysis plugin
590 of ImageJ. 15 to 20 neurons per embryo, from 4 WT and 3 *Svbp* KO embryos were analyzed.

591

592 **Brain preparation for *ex vivo* MRI acquisitions**

593 Brains were prepared as previously described (51). Briefly, brains were fixed before harvesting
594 from animals by a transcardiac perfusion of a 4% paraformaldehyde solution in phosphate
595 buffered saline added with a MRI contrast agent (6.25 mM of Gd-DOTA; Guerbet Laboratories,
596 Roissy, France). The contrast agent was used here to reduce MRI acquisition time. After
597 removing surrounding skin and muscles, the skulls containing intact brains were immersed in
598 the fixing solution for four days, and then transferred to a Fomblin (FenS chemicals, Goes,

599 Netherlands) bath for at least seven days after brain fixation. This schedule provided
600 homogeneous distribution of the Gd-DOTA throughout the whole brain (52).

601

602 **MRI acquisitions**

603 *Ex vivo* 3D MRI acquisitions with a high spatial resolution were performed at 9.4 T (Bruker
604 Biospec Avance III; IRMaGe facility) using a volume coil for transmission and a head surface
605 cryocoil for reception. A 3D T_{1w} gradient-echo MRI sequence was used for brain segmentation
606 and volumetric analysis (repetition time: 35.2 ms, echo time: 8.5 ms, flip angle: 20°, field of
607 view: 12 · 9 · 16 mm³, isotropic spatial resolution: 50 μm, 4 signal accumulations, total
608 acquisition time per brain: 2 h 32 min).

609

610 **Quantitative analysis of brain volumes**

611 Brain regions were defined by MRI segmentation on the 3D T_{1w} MR images. Each cerebral
612 structure was manually delimited using regions of interest (ROI) drawn every five slices on the
613 coronal orientation of the 3D T_{1w} MRI, using Fiji software (53). Using the Segmentation editor
614 plug-in (http://fiji.sc/Segmentation_Editor) the whole cerebral structure was reconstructed by
615 interpolation between ROIs. Then, all ROIs were manually corrected based on the Allen mouse
616 brain atlas (<http://atlas.brain-map.org/atlas>). Measurements were obtained for the following
617 structures: the anterior commissure (ac), the brainstem (BS), the caudate putamen (CPU), the
618 cerebellum (CB), the colliculi (CO), the corpus callosum (cc) the cortex (CX), the fimbria (fi),
619 the globus pallidus (GP), the hippocampal formation (HF), the hypothalamus (HP), the
620 olfactory bulb (OB) and the thalamus (TH). Each structure was color-coded, and its 3D
621 representation and volume were determined using the 3D viewer plug-in in the Fiji software
622 (https://imagej.net/3D_Viewer). For each region, the volume was calculated as the number of

623 voxels \times the voxel volume. All segmentations were done blind to the genotype. All data passed
624 the normality test (except the BS for WT), we thus used a student t-test for group comparison.

625

626 **Behavioral tests**

627 Open Field: Locomotor activity of mice was video-recorded via a camera mounted above an
628 open field and connected to a computer. Horizontal activity was analyzed using Ethovision
629 XT14 software (Noldus, Wageningen, Netherlands). In the open field condition, the activity of
630 four mice was simultaneously monitored in a white arena (L50 x 150 x h45 cm) for 30 min.
631 Within each genotype, mice were randomly placed in the different arenas.

632 The Marble Burying Test was performed as described previously (54). More precisely,
633 the mouse was placed in a clear plastic box (L42 \times 126 \times h15 cm), containing 24 glass marbles
634 (1.6 cm in diameter) evenly spaced (six rows of four marbles) on 4 cm of litter. Thirty minutes
635 later, the animals were removed from the cages, and the number of marbles buried more than
636 two thirds in the litter was scored.

637 Resident-Intruder Test: home-cage social interaction was assessed, as described (55).
638 The duration of sniffing investigation displayed by a resident male mouse in response to
639 presentation of an intruder male mouse was measured. In this test, wild-type and *Svbp* KO mice
640 were housed individually during 1 or 3 weeks prior to testing to allow establishment of a home-
641 cage territory. The intruder mice, housed in groups, were unfamiliar to the resident mouse. In
642 each trial, an intruder was placed in a corner of the resident home-cage (L36 \times 120 \times h14 cm),
643 and the exploration activity of the resident was video-recorded for 5 minutes. The time spent
644 sniffing in close contact with the intruder was measured manually from video recordings.

645 The Hamlet test is a novel complex environment device that allows mice to be trained
646 in groups in a more ecological space than the housing cage (26). The maze (160 cm diameter)
647 is composed of a central place, streets expanding from it in a star shape and 5 houses that have

648 been functionalized. The houses contained a food dispenser (physiological function encoded:
649 Eat), two water dispensers (Drink), a novomaze[®] (Viewpoint, Lissieu, France) (Hide), a running
650 wheel (Run) or a compartment for a stranger mouse (Interact). Animals were placed in the
651 Hamlet in groups of 7 from the same housing cage, for 4 h per day during a 2-weeks training
652 period. The videotracking system (Viewpoint) recorded activity in each house in terms of
653 number of entries and duration of presence.

654

655

656 **Supplemental data**

657 Supplemental data includes additional methods, six figures and two tables.

658

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665

666 **Conflict of Interest statement**

667 P.B. is Chief Scientific Officer of Centogene AG. Other authors declare no competing interests.

668

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684

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843

844 **Figures titles and legends**

845

846 **Figure 1. Pedigrees and brain imaging results for the three families described in this study.**

847 (a) Pedigree for family 1 with key indicating the genetic data generated in this study. *SVBP* status is
848 indicated under analysed individuals. (b) Pedigree for family 2. (c) Pedigree for family 3. (d) MRI
849 images for individual II-3 from family 2 taken at age 6 years 3 months – sagittal T1-weighted (left) and
850 axial T2-weighted images through the level of the basal ganglia (middle) and corona radiata (right) show
851 a generally thin but fully formed corpus callosum and reduced bulk of the periventricular white matter
852 with enlargement and scalloping of the ventricular outlines. There is no gliotic damage to the white

853 matter, myelination is appropriate and the basal ganglia/cortex appear normal. (e) MRI images as in (d)
854 for individual II-3 from family 3 at age 3 years 5 months. Similar findings are observed, with a thin
855 corpus callosum and reduced periventricular white matter bulk with mildly enlarged/scalloped
856 ventricles. For family 1, MRI analysis identified similar features (images not available).

857

858 **Figure 2. Impact of SVBP deficiency on detyrosination parameters**

859 (a) Effect of pathogenic SVBPs on detyrosination activity of VASH1. Immunoblots of tubulin
860 detyrosination assays performed in HEK293T cells. Plasmids encoding VASH1-eGFP and wild-type
861 (WT) or mutants SVBP-myc-Flag were cotransfected into cells. Q28 and K13 respectively correspond
862 to p.Q28* and p.K13Nfs*18, with myc-Flag epitopes added at their C-terminus. Antibodies specific to
863 detyrosinated tubulin are used to assess detyrosinase activity. Antibodies against GAPDH, eGFP
864 (VASH1) or Flag (SVBP) reveal the amounts of protein in the extract. Non-transfected cells reveal the
865 endogenous levels of detyrosinated tubulin. (+) refers to wild-type VASH1 or SVBP. (b-e) Comparison
866 of α -tubulin modifications in wild-type and *Svbp* KO brain extracts. (b) Representative immunoblots
867 obtained with brain protein extracts from 3 wild-type and 3 *Svbp* KO mice. Specific antibodies were
868 used to detect tyrosinated, detyrosinated and $\Delta 2$ α -tubulin pools. GAPDH staining is representative of
869 the amount of proteins in the extract. (c-e) Quantification of results obtained as in (b). Triplicate
870 immunoblots of the 6 protein extracts were analysed. Quantity in *Svbp* KO is expressed as percentage
871 of quantity in wild-type (after correction using GAPDH signal) for (a) tyrosinated tubulin level, (b)
872 detyrosinated tubulin level, and (c) $\Delta 2$ tubulin levels. Student *t* test two tails, *** $p < 0.001$, **** $p <$
873 0.0001.

874

875 **Figure 3. Effect of SVBP deficiency on neurite outgrowth and axonal differentiation.** Hippocampal
876 neurons were cultured from E17.5 wild type or *Svbp* KO mice brain embryos. (a-e) After 2 days of
877 differentiation in vitro (2 DIV), neurons were fixed and immunolabeled. (a) Tyrosinated and
878 detyrosinated α -tubulin levels were imaged using the same antibodies as in immunoblots. (b-e) 20
879 neurons per embryo, from 6 different wild-type or *Svbp* KO embryos were analyzed using NeuronJ
880 plugging from ImageJ software on immunofluorescence images (generated as in a). (b) Proportion of

881 stage III neurons (bearing an axon, an index of neuronal differentiation) is represented as mean \pm SEM
882 (n = number of analyzed embryos). (c-e) Morphometric analyses of 2 DIV neurons including axonal
883 length (c) and branching parameters (d,e) are represented as means \pm SEM (n = number of analyzed
884 neurons). Mann-Whitney test, ** p<0.01 and * p<0.05. (f) Sholl analysis of dendritic arborization of
885 WT and *Svbp* KO neurons cultured 17 DIV. The graph shows the mean number of dendritic branches
886 as a function of radial distance (n=60 neurons per condition, 10 to 20 neurons per embryo, 4 WT and 3
887 *Svbp* KO embryos). Statistics calculated by Two-Way ANOVA followed by Sidak's Multiple
888 Comparisons test. Error bars indicate SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.

889

890 **Figure 4. *Svbp* deletion induce brain morphological defects**

891 (a) Representative 3D reconstructions of wild-type brain areas built from high spatial resolution MRI-
892 T_{1w} data. Each brain structure is represented with a specific color: cerebellum (CB, pink); cortex (CX,
893 yellow); olfactory bulbs (OB, green); brainstem (BS, light yellow); hippocampal formation (HF, dark
894 green); corpus callosum (cc, dark purple); caudate putamen (CPU, dark blue); colliculi (CO, red);
895 thalamus (TH, magenta); fimbria (fi, light green); hypothalamus (HP, khaki); globus pallidus (GP, light
896 blue) and anterior commissure (ac, light pink). (b) Quantification of the volumes of the different cerebral
897 regions represented in panel A. The dot plots show volumes for these regions (in mm³) of wild-type and
898 KO mice. Percent volume reduction in SVBP-KO mice is indicated in red. Black bars represent the
899 mean (n = 8 for wild-type and *Svbp* KO mice, Student *t* test two tails, * p < 0.05; ** p < 0.01; *** p <
900 0.001).

901

902 **Figure 5. *Svbp* KO mice exhibit behavioural defects**

903 Wild-type (WT) and *Svbp* KO mice were compared for (a) the travelled distance in the open field, (b)
904 the time spent in social exploration time by the resident after either 1 or 3 weeks of isolation. n = 9 and
905 11, respectively for WT and KO, and (c) the number of buried marbles at the end of the marbles burying
906 test. (d) Average time spent in each house by each group of mice (n = 7 for WT and KO) during the 10
907 days of training in the Hamlet. Horizontal bars represent means, Mann-Whitney test, two tails: * p <
908 0.05; ** p < 0.01.

909

910 **Abbreviations**

911 Days of differentiation in vitro (DIV)

912 Knockout (KO)

913 Loss of Function (LoF)

914 Magnetic resonance imaging (MRI)

915 Multiplicity of infection (MOI)

916 Whole genome sequencing (WGS)

917 Wildtype (WT)

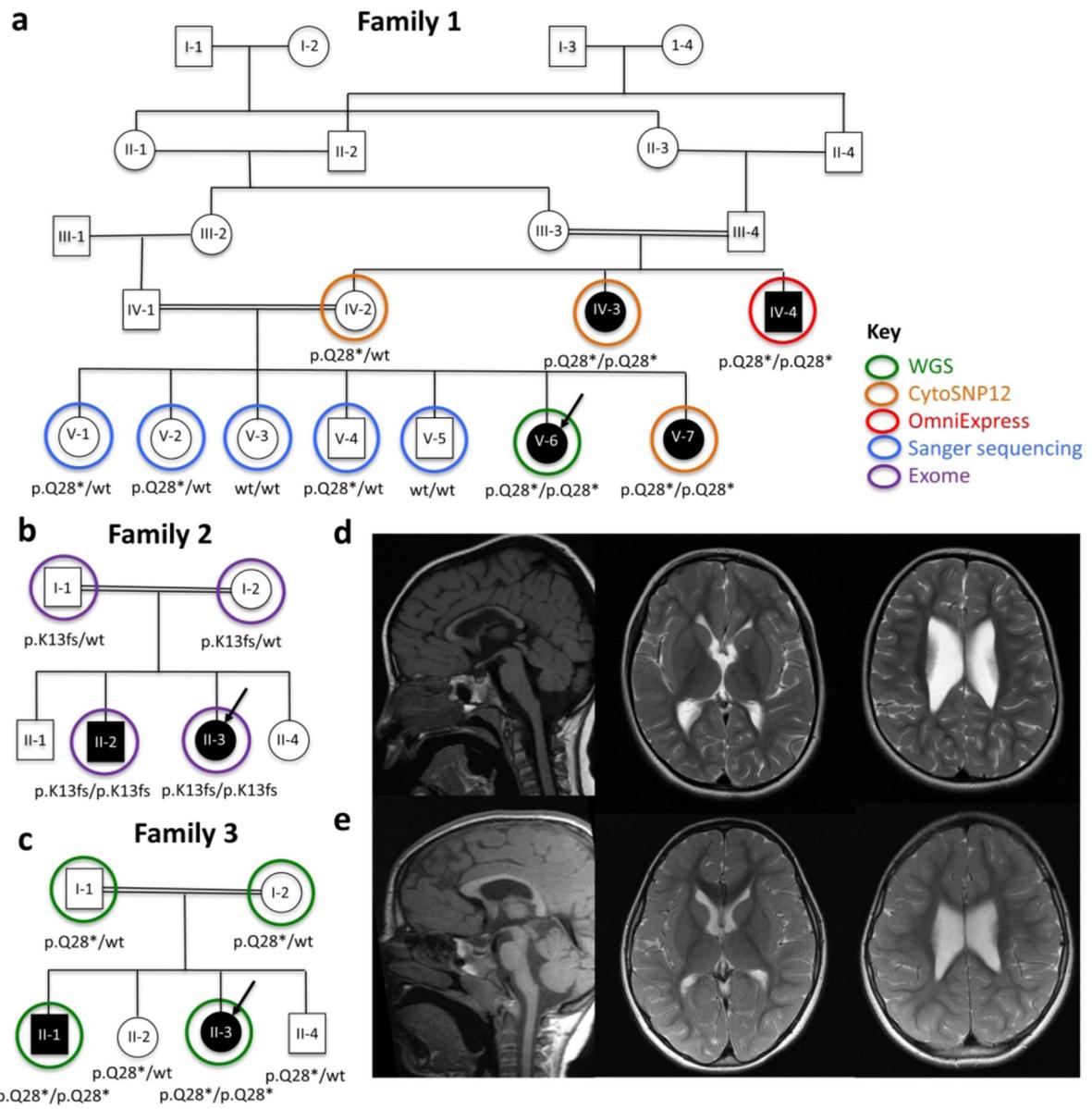
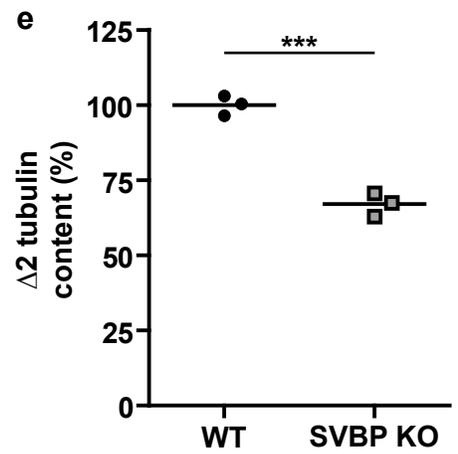
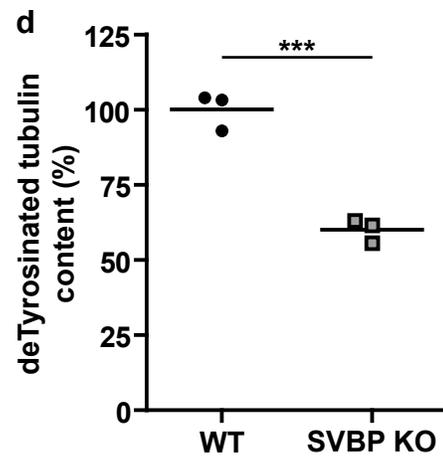
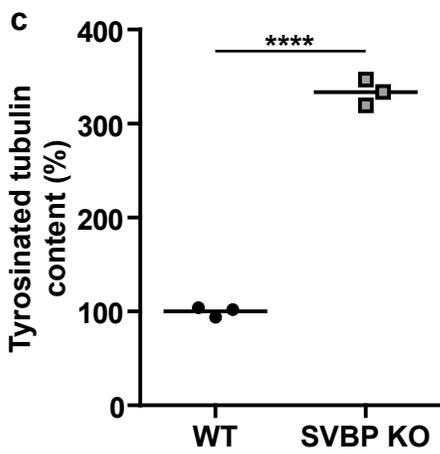
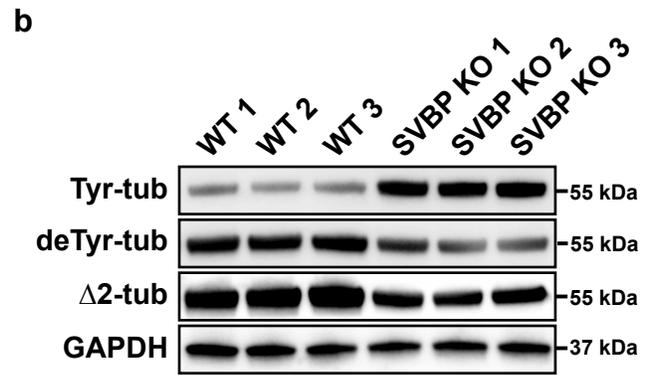
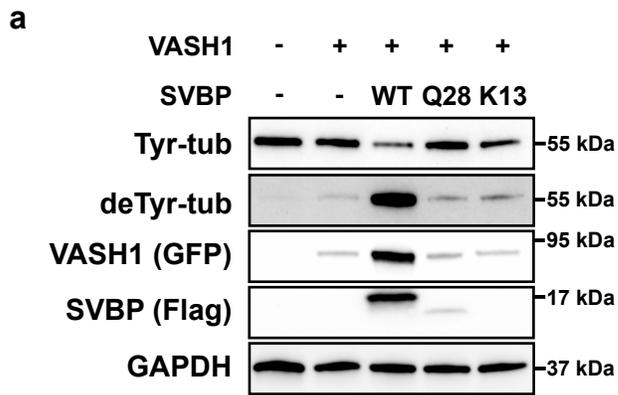
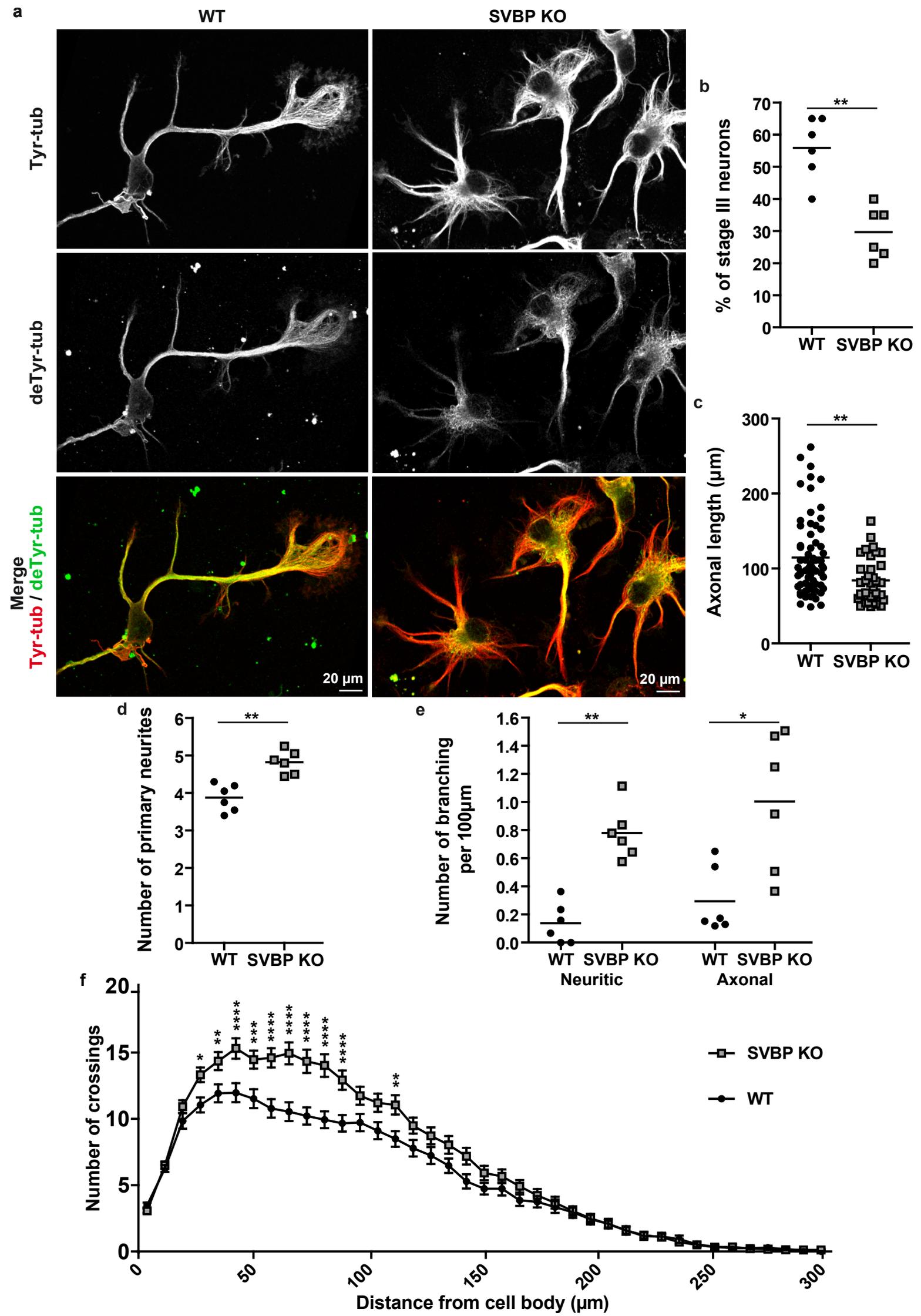
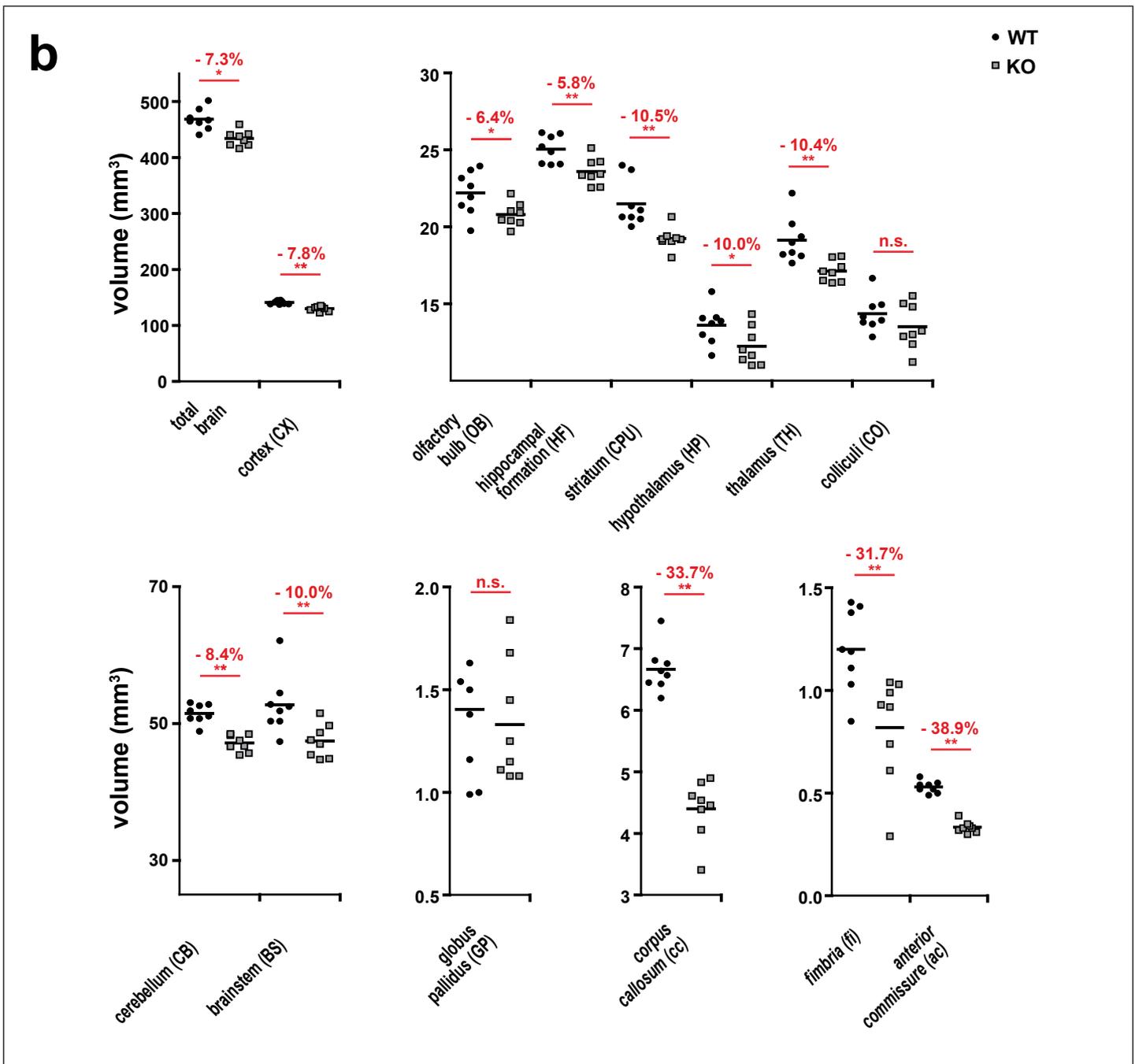
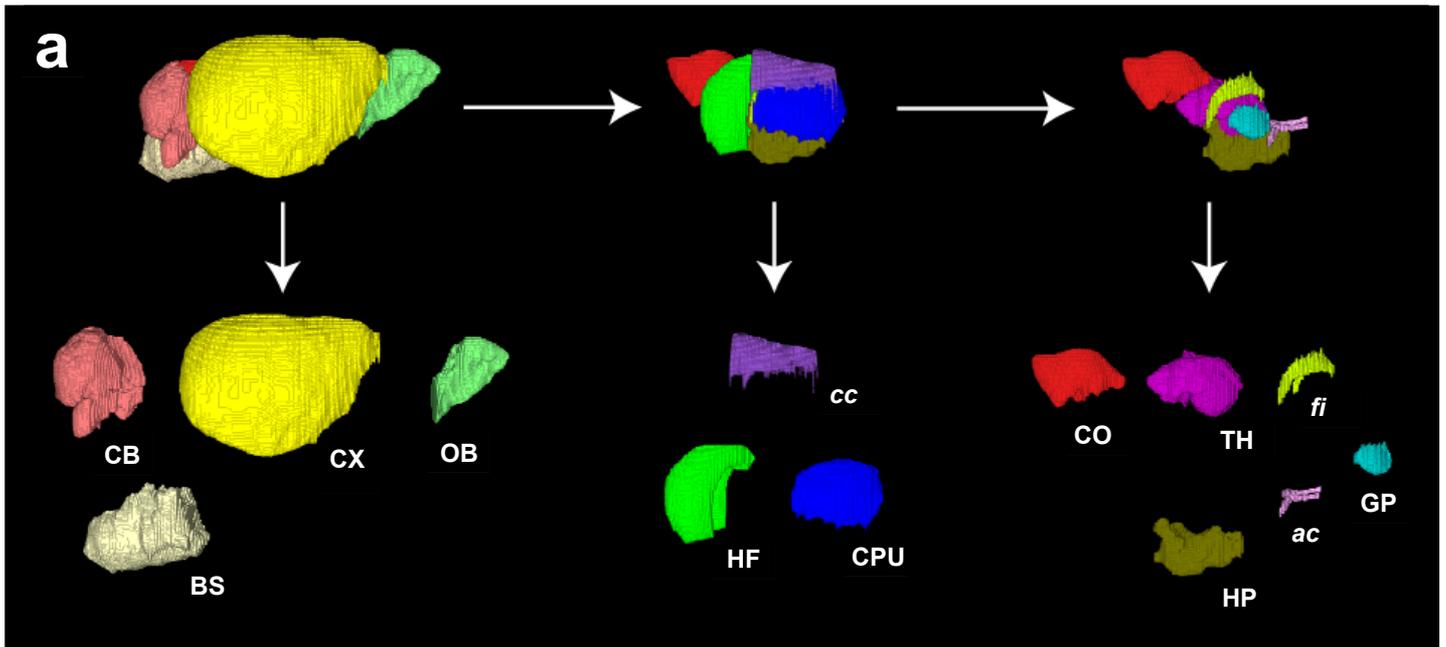
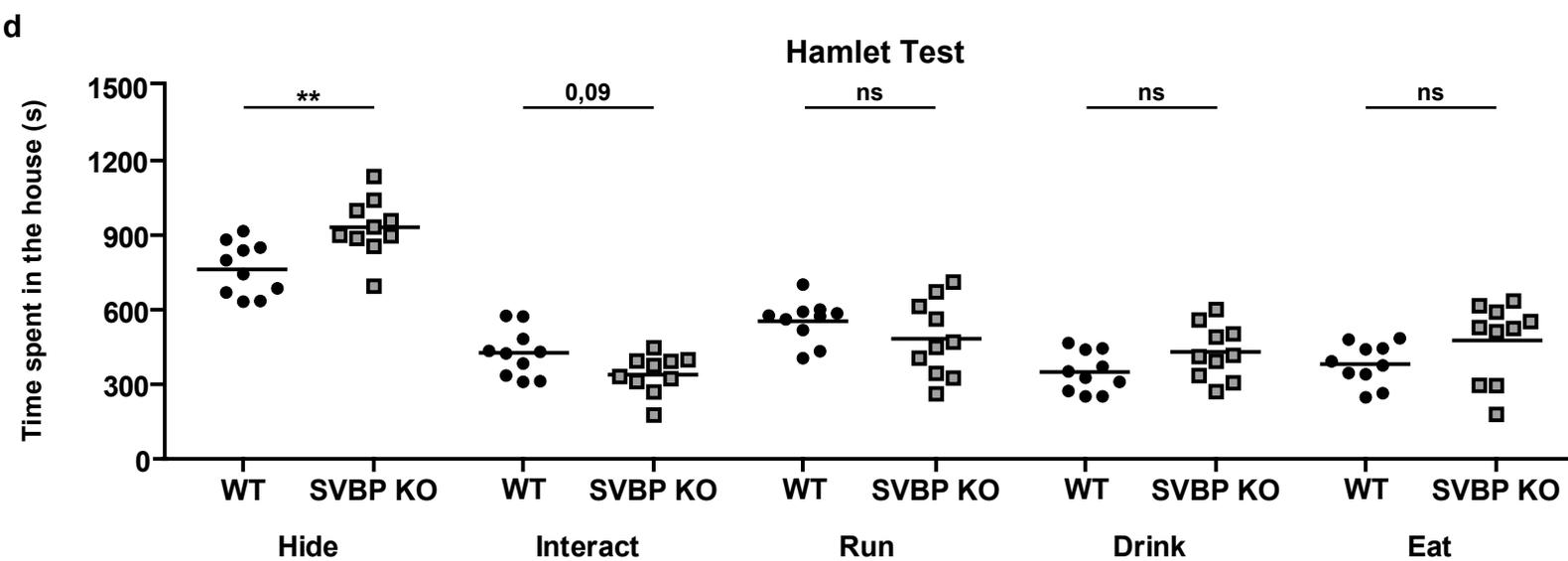
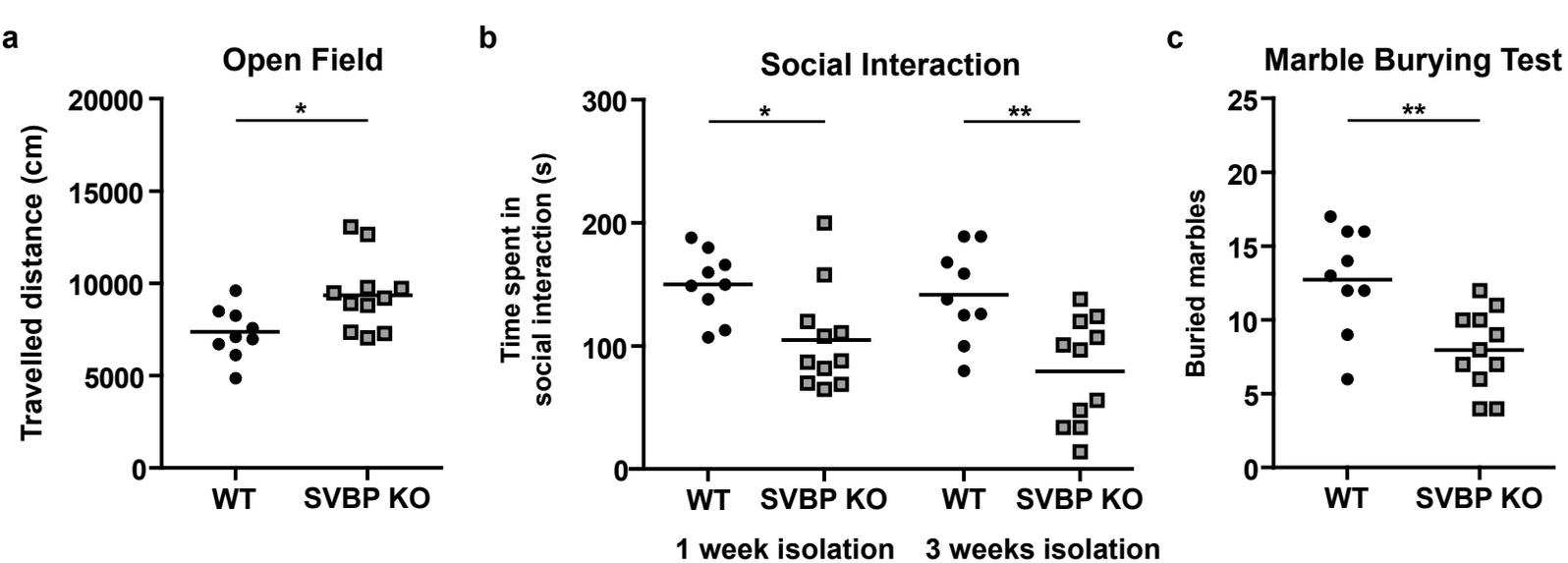


Fig 1: Pedigrees and brain imaging results for the three families described in this study









Supplemental Methods

Search for other candidate pathogenic variants in WGS data from Family 1

In addition to the simple recessive model, we also considered a compound heterozygous model in the three regions that were IBD2 between all affected individuals but not homozygous (totalling 10 Mb), but no good candidates were found.

Additionally, several methods were used to search for copy number variants (CNVs), as described.¹ These included a) generating count profiles in 10 kb bins along the genome and correcting for noise due to biases in sequence composition, b) OncoSNP-SEQ² and c) ExomeDepth.³ In addition to these, GenomeSTRiP⁴ was used to search for intermediate-sized deletions (50-50,000 bp) that would have been missed by arrayCGH and Platypus.⁵ We filtered the calls based on their frequency in other WGS500 samples (MAF < 0.5%). We considered the closest gene to each deletion as the affected gene, and, for the compound recessive model, treated large deletions and SNVs/small indels together. We found no rare structural variants within the linkage region.

We also considered noncoding variants with MAF < 0.5%. To narrow the search, we focused on variants that were within conserved blocks of DNA identified using phastCons⁶, and that fell in regulatory regions annotated by the Ensembl Variant Effect Predictor⁷, which were identified using information about various chromatin marks and transcription factor binding sites. This did not reveal any variants that seemed more likely to be causal than the p.(Q28*) variant in *SVBP*.

To allow for the possibility of a pedigree error, we analyzed the WGS data from individual V-6 alone to check that there were no more likely causal variants. We retained only high confidence variants with at least 5x coverage, a call quality of at least 20 and which passed upstream pipeline filtering. We also required for variants to be seen in both +ve and -ve strand reads. In a previous study, analysis of data from the WGS500 project identified regions of the genome where variant genotype calls are typically unreliable and varied based on which analytical pipeline had been used.⁸ We therefore excluded variants situated in these regions as well.

We then applied a number of population allele frequency filters, removing variants that were seen at > 0.1% in at least one of the following datasets: the 1000 genomes project (phase3v5b), NHLBI ESP exomes (ESP6500SI-V2), ExAC (v0.3.1; using subpopulation with highest allele frequency), gnomAD (v2.0.1; using subpopulation with highest allele frequency).

We then restricted to coding variants (missense, in-frame indels, frameshifting indels, or stop gain, +/- 2 bp splice site variants and variants predicted to disrupt splicing by MaxEntScan). Lastly, we filtered for variants that were called homozygous and were seen in at least 80% of reads.

Sanger sequencing for variant validation and confirmation of segregation

For Sanger validation of the c.82C>T; p.(Q28*) variant we PCR amplified a 269 bp DNA fragment with primers 5'-GCTCGCTGTGTCAGTTCTCCTT-3' and 5'-AAGATCTGCCTCTTTCTGTACCA-3' in 25 µl reaction volumes using the FastStart Taq DNA Polymerase PCR kit (Roche). Amplicons were run on a 1.8% agarose gel for size confirmation and then purified using exonuclease I and shrimp alkaline phosphatase. Sanger sequencing was performed using BigDye chemistry (v3.1).

RNA analysis of clinical samples

2.5 ml of whole blood was collected from all available family members (IV-2, IV-3, IV-4, V-6 and V-7) into PAXgene blood RNA tubes (PreAnalytiX). RNA was extracted using PaxGene blood RNA extraction kit (Qiagen) according to the manufacturer's guidelines. RNA was quantified using the nanodrop ND-1000 and RIN values were obtained using the 2100 Bioanalyser using RNA 6000 Pico chips (Agilent Technologies). RIN values were all 7.7-8.8, indicating good quality RNA. Reverse transcriptase (RT) reactions were performed using 500 ng of heat-denatured RNA as template with the QuantiTect kit (Qiagen) which uses a mix of oligo-dT and random primers. RT-negative reactions were set up in parallel to ensure there was no amplification from any residual genomic DNA.

A 265 bp segment spanning exons 2 and 3 of *SVBP* cDNA was amplified with primers SVBP-2F 5'-AGAAATATCCAGAAGTCAAGCCA-3' and SVBP-3R 5'-TCTCAAAGCTCTCAGGATCCC-3'.

Amplicons were size-checked and then purified as described above. Sanger sequencing was performed using BigDye chemistry (v3.1) and run on an ABI 3730XL machine.

Quantitative PCR was also performed on the same cDNA samples using the SVBP-2F/3R primers and the iQ5 SYBR Green Supermix, using the iQ5 realtime-PCR machine (BIO-RAD). Ct values derived from a 234 bp *ACTB* amplicon were used to normalise expression. Expression was then compared to two control cDNA samples (prepared using the same method) using the $2^{-\Delta\Delta Ct}$ formula.⁹ qPCRs were performed in triplicate and the experiment was performed three times.

Genotyping and backcrosses

PCR amplifications were performed directly on alkaline lysates of toe clips. Briefly, each toe was incubated 30 min at 95°C in 100 µL of alkaline solution (NaOH 25 mM, EDTA 0.2 mM, pH 12.0). Neutralization was performed by adding 100 µl Tris 40 mM, pH 5.0. Two microliters of lysates were analyzed by PCR (25 µL) with appropriate primers (Table S1) and GreenTaq Mix (Euromedex), according the following protocol: 95°C for 10 min, 33 cycles of [95°C for 30 sec / 58°C for 30 sec / 72°C for 30 sec], 72°C for 10 min. Indels in F0 mice were identified by Sanger sequencing of PCR products (Eurofins). The sequencing Sanger electropherograms obtained from mosaic F0 mice were analyzed using the TIDE software which decomposes sequencing traces and estimates the respective contribution of each PCR fragment.¹⁰ We thus selected a mosaic F0 mouse carrying a 11 nt deletion introducing a frameshift and only four additional chimeric residues. This F0 mouse was further backcrossed with C57BL/6 mice to select the $\Delta 11$ deletion among the F0 mosaic indels. Tail fragments of the F1 mice and their successive backcrosses were lysed as above. Two microliters of lysates were then analyzed by PCR (25 µL), using forward primers targeting either the wild-type allele or the $\Delta 11$ deletion junction, a common reverse primer (Table S1) and GreenTaq Mix, according the following protocol: 95°C for 5 min, 33 cycles of [95°C for 30 sec / 50°C for 30 sec / 72°C for 30 sec], 72°C for 2 min. Amplicons were run on a 1.8% agarose gel for presence and size confirmation. PCR with the 5' WT-for primer generates a fragment only with the DNAs of wild-type and heterozygous mice. PCR with the 5' $\Delta 11$ -for primer generates a fragment only with the DNAs of *Svbp* KO and heterozygous mice.

Y-Maze test

The Y-Maze test was performed as described previously.¹¹ Spontaneous alternation in a Y-maze is based on the innate tendency of rodents to explore new environments, and the short-term storage and retrieval of relevant information are related to working memory. The apparatus consists of three walled arms (L20 x l8 x h16 cm) radiating at an angle of 120° from each other. Without prior habituation to the apparatus, each mouse was placed into the starting arm and allowed to move freely through the maze during 5 min. A mouse was considered to have entered an arm when all four paws were positioned in the arm runway. An alternation was defined as entries into all 3 arms on consecutive choices. The alternation score (%) for each mouse was calculated as the ratio of the actual number of alternations to the possible number (defined as the total number of arm entries minus 2), as shown by the following equation: % alternation = [(Number of alternations) / (Total arm entries-2)] × 100.

Novelty Suppressed Feeding (NSF) test

The NSF test was performed as described previously.¹² It is an anxiety-based conflict test where the motivation to eat competes with fear of a brightly lit area. The NSF test was performed for 15 min. Briefly, animals had no access to food in their home cage for 20 h prior to performing the test. For the test, a single food pellet was placed on a brightly-lit white paper platform placed in the center of a plastic box (L37 x l57 x h20 cm) where the floor was covered with approximately 2 cm of bedding. The animal was placed in a corner of the box and the latency to eat was recorded.

Elevated Plus Maze

This labyrinth consists of two intersecting tracks, placed at 60 cm from the ground. One track had no walls (two open arms), while the other had dark walls 15 cm high (two arms closed). Each arms measure L30 cm x l7 cm. The mice were placed in the center of the labyrinth, facing an open arm, and allowed to explore for 5 min. A camera placed above the labyrinth recorded the movements of the animal and the tests were analyzed using the Ethovision XT14 software (Noldus, Wageningen, Netherlands). Total distance travelled, number of entries and time spent in each arm were monitored.

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Author contributions statement

HCM analyzed the genetic data for family 1 and together with JCT, EB and MJM jointly directed the project. ATP performed co-segregation testing, RNA analysis and reviewed genomic data for families 2 and 3. EMB, AAE, CD, SJ, SM, IW, PB and LL helped provide and review clinical data. AS performed neuroradiological assessment of human subjects. CB, AA and MJM oversaw the mice generation project. CB assisted by ED developed the CRISPR/Cas9 and genotyping strategies, and engineered plasmids constructs. FV assisted for plasmids amplifications and mice genotyping. PH and FV analyzed the effect of pathogenic SVBPs on deetyrosination and tubulin modifications levels in cells and tissues supervised by MJM. JCD provided expertise on mice brain imaging and morphometry, and prepared brains for MRI. ELB supervised preclinical anatomical MRI performed by IU. PH performed morphometric analysis, and ED images processing and statistical analysis. PH and SC performed mice behavior experiments supervised by SG, TM and AA. LP performed the neuron experiments and analyzed the data with the help of PH. HCM, MJM, AA and ATP wrote the manuscript with the contribution of all co-authors.

Supplementary Figures and Tables

Supplemental data include six figures and two tables

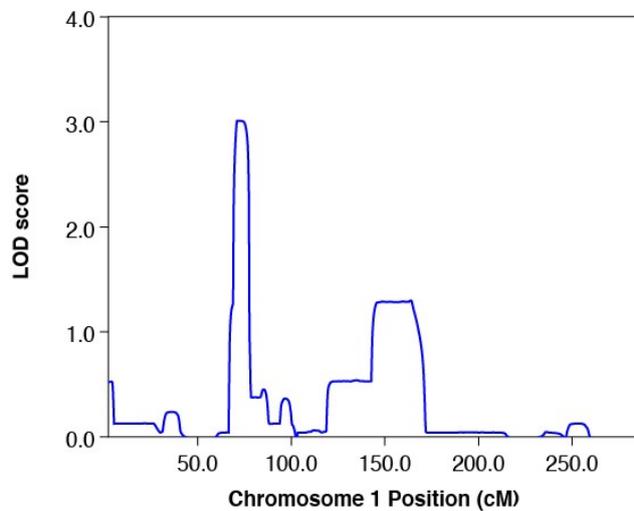


Figure S1. Results from linkage analysis in Family 1. We show the LOD scores on chr1 only since this was the only chromosome containing a significant linkage peak.

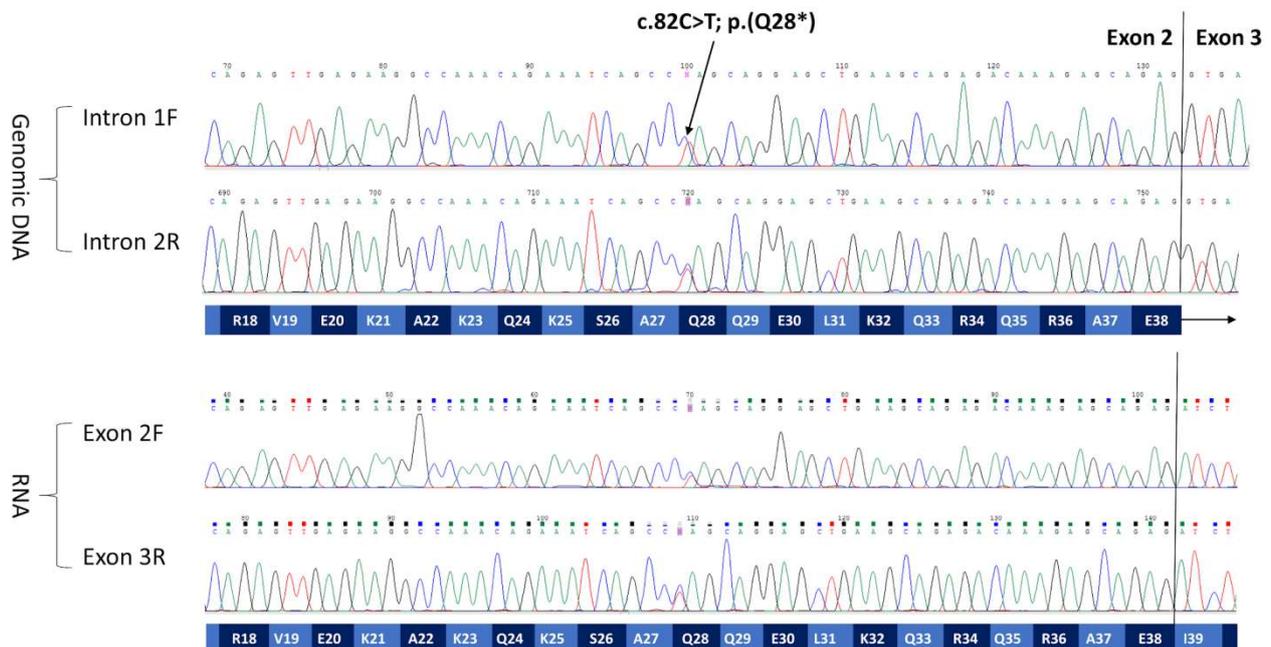


Figure S2. Bidirectional Sanger sequencing of genomic DNA and RNA for heterozygous individual IV-2. For ease of comparison, electropherograms from RNA are shown directly below the trace obtained using the genomic DNA. The positions of the primers used for sequencing are labelled on the left of the figure. Peaks corresponding to the wild-type and c.82C>T alleles are of similar height, matching what is seen for genomic DNA. This suggests that the mutant RNA is not degraded significantly. Sequencing of RNA from all 4 affected family members (IV-3, IV-4, V-6, V-7; data not shown) further confirmed homozygosity of the variant.

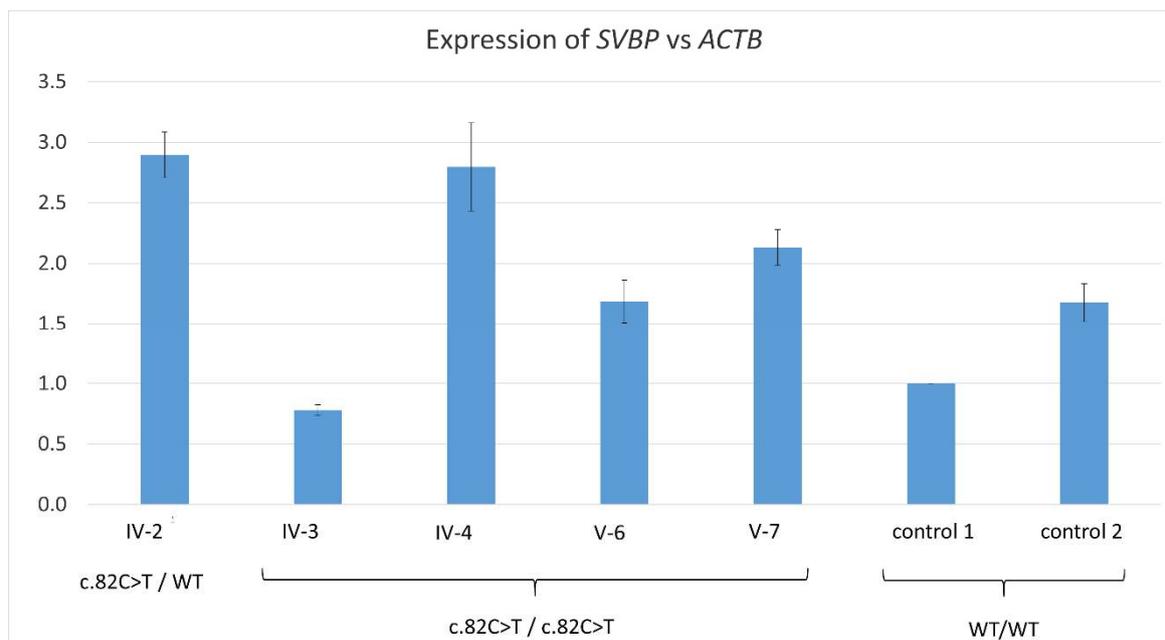


Figure S3. Quantitative RT-PCR of RNA from individuals IV-2, IV-3, IV-4, V-6 and V-7. Expression of *SVBP* was normalised with respect to *ACTB*. Relative RNA expression in family member was then compared to the first of two control samples. qPCRs were performed in triplicate and the experiment was then repeated twice. The error bars represent the standard errors observed across the three experiments. These results are consistent with the mutant *SVBP* transcript not being degraded significantly.

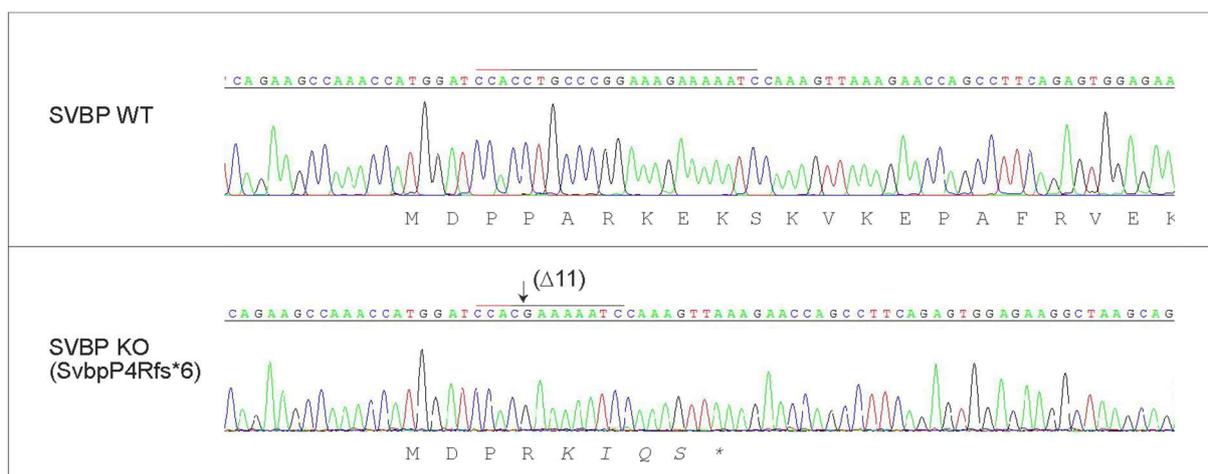


Figure S4. *Svbp* gene DNA Sanger sequencing of control C57BL/6 mouse (WT) and of knock-out mouse SVBP. Black and red lines respectively indicate the positions of the crRNA and protospacer adjacent motif (NGG), both located on the complementary strand. Indel site (arrow) and nature of the indel are indicated above the sequence. Translations of SVBP products are shown below the electropherograms, with frameshifted amino-acid in italic.

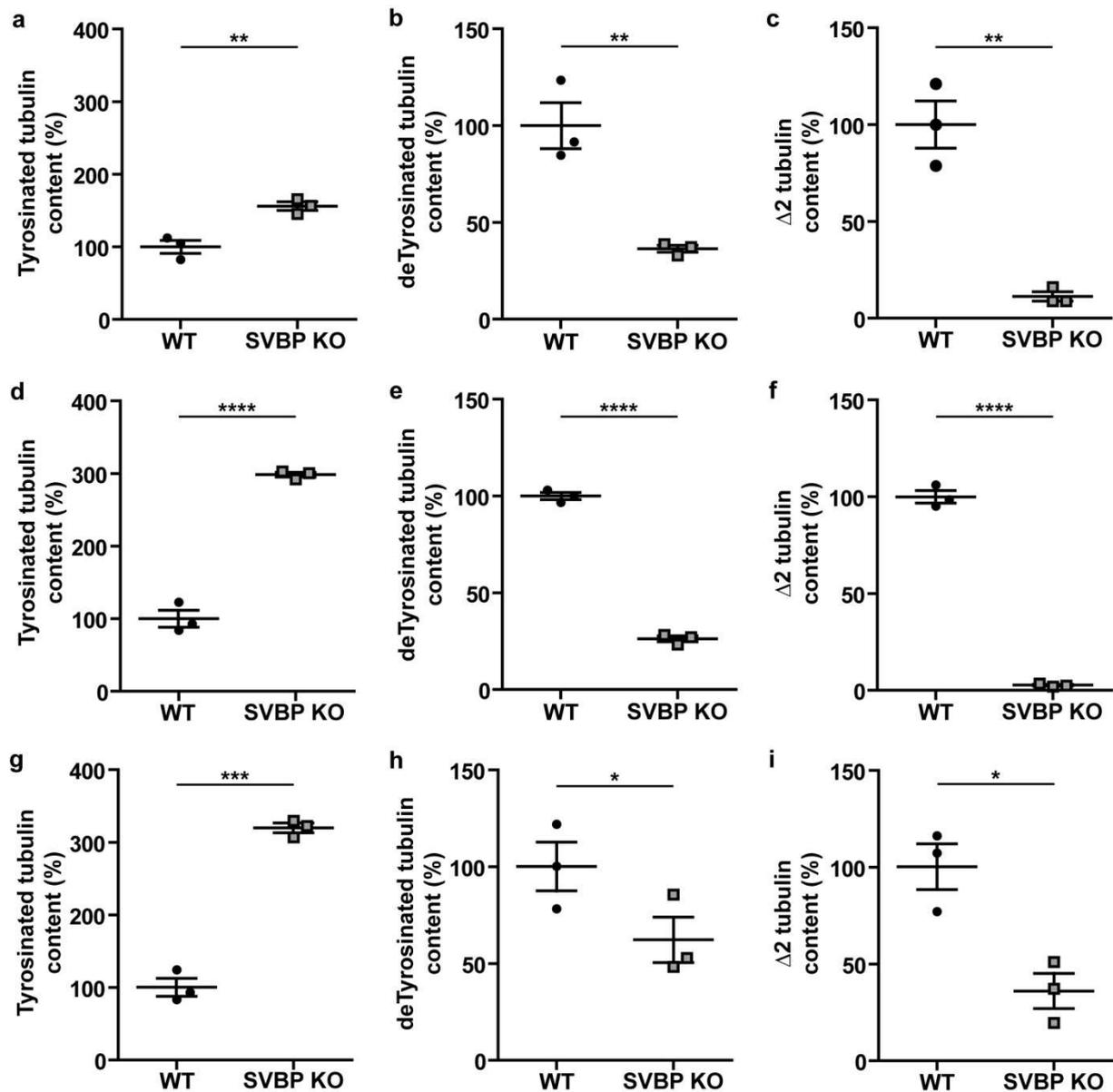


Figure S5. Comparison of α -tubulin modifications in wild-type and *Svbp* KO cultured neurons. Hippocampal neurons were cultured from E17.5 wild type or *Svbp* KO embryos. After 2 (a, b, c), 7 (d, e, f) or 17 days (g, h, i) of differentiation in vitro (2 DIV, 7 DIV or 17 DIV), protein extracts were prepared. Specific antibodies were used to detect tyrosinated, detyrosinated and $\Delta 2$ α -tubulin pools, and GAPDH staining was used to estimate amount of proteins in the extract. Triplicate immunoblots of the extracts from 3 different cultures were analyzed. Quantity in *Svbp* KO is expressed as percentage of quantity in wild-type (after correction using GAPDH signal) for (a, d, g) tyrosinated tubulin level, (b, e, h) detyrosinated tubulin level, and (c, f, i) $\Delta 2$ tubulin levels. Student *t* test two tails, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

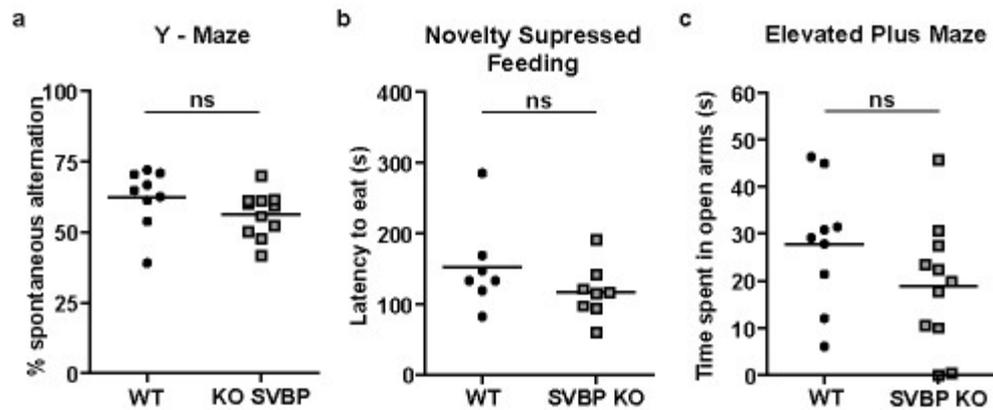


Figure S6. Behavioral analysis of *Svbp* KO mice. (a) Percentage of spontaneous alternation in Y-Maze (horizontal bar represents mean, student t-test, two-tails, $n = 9$ and 11 for wild-type (WT) and *Svbp* KO mice respectively, $p = 0.16$). (b) Latency to eat for the novelty suppressed feeding test (horizontal bar represents mean, Mann-Whitney test, $n = 7$ and 9 for WT and *Svbp* KO mice respectively, $p = 0.11$). (c) Time spent in open arms in the elevated plus maze test (the horizontal bar represents the average, Mann-Whitney test, $n = 9$ and 11 for WT and *Svbp* KO mice respectively, $p = 0.19$). ns, not significant.

Table S1: Phenotypes of affected individuals in families described in this study.

See separate excel file.

Name	Sequence	PCR size (bp)
tracrRNA	5' AAACAGCAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAA AGUGGCACCGAGUCGGUGC [U*U*U*]U3'	
SVBP-crRNA	5' [G*A*U*]UUUUCUUUCCGGGCAGGGUUUUUAGAGCUAUGCUGUUUUUG3'	
SVBP-for (F0)	5' CCAGCTTGGGATTCTGACAC3'	591
SVBP-rev (F0)	5' TTCTCGGGCCTTCTCATCTG3'	
WT-for	5' GATCCACCTGCCCGGAAA3'	170
WT-rev/ Δ 11-rev	5' TTTCTTCCAGCACCCCTCTCC3'	
Δ 11-for	5' CAAACCATGGATCCACGAAA3'	168
WT-rev/ Δ 11-rev	5' TTTCTTCCAGCACCCCTCTCC3'	

Table S2: Description of oligonucleotide used for generation and genotyping of mice. Note: * are for phosphothiorate and [] for 2'-O-methyl chemical modifications.

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Table S1: Phenotypes of affected individuals in families described in this study. -, not known or missing data. AED, Anti epilepsy drugs. Reported centiles are calculated based on growth charts available at www.rcpch.ac.uk/resources/uk-who-growth-charts-2-18-years and Freeman *et al* 1995 (PMID: 7639543).

	Family 1				Family 2		Family 3		Summary of patients from Iqbal <i>et al</i> 2019 (N=4)
	V-6 (proband)	V-7	IV-3	IV-4	II-2	II-3 (proband)	II-1	II-3 (proband)	
SVBP variant (NM_199342.3) and ClinVar accession ID	c.82C>T; p.Q28* (SCV000926436)				c.39_42del; p.K13Nfs*18 (SCV000926437)		c.82C>T; p.Q28*		All have c.82C>T; p.Q28*
Ethnicity (degree of parental consanguinity)	Pakistani (1st-cousin)				Kuwaiti (1st-cousin once removed)		Pakistani (first-cousin)		Syrian (2nd-cousin) and Pakistani (1st-cousin)
Gender	Female	Female	Female	Male	Male	Female	Male	Female	3 Female, 1 Male
Intellectual disability	Yes (moderate to severe)	Yes (severe)	Yes (moderate to severe)	Yes (moderate to severe)	Yes	Yes	Yes	Yes (severe)	4/4 (moderate to severe)
Speech	Little expressive language, better receptive language (few single words)	Little expressive language (few single words)	Few words	Little expressive language (few words - 3 word sentences)	Few words	Only a few words aged 6	Only 10 words aged 21 years	Delayed speech and language development	3 with delayed speech (1 too young for assessment)
Behavioural problem	Quiet	Loses temper, bangs head, cries, screams	-	-	-	-	-	Autistic behaviour	2 reported to be aggressive
Height	106.9 cm aged 4 years 10 months (50th centile), 122.1 cm aged 7 years 7 months (25th centile)	90.7 cm aged 3 years 4 months (2nd-9th centile), 131.2 cm aged 9 years and 10 months (9th-25th centile)	141.3 cm aged 45 years (<0.4th centile)	158.4 cm aged 39 years (0.4th centile)	-	79 cm aged 2 years (2nd centile)	Unable to stand-flexion deformity at hips and knees	96 cm age 3 years 6 months (25th centile)	<2 SD and -3.62 to -3.90 SD
Head circumference	48.5 cm aged 5 years 4 months (0.4th centile)	45.2 cm aged 3 years and 11 months (<0.4th centile)	56.0 cm aged 45 years (50th-75th centile)	55.5 cm aged 39 years (9th-25th centile)	49.7 cm aged 7 years and 10 months (0.4th centile)	44.5 cm aged 2 years (<0.4th centile); 47.5 cm at 6 years 6 months (<0.4th centile)	46 cm aged 3 years (<0.4th centile)	45 cm (<0.4th centile)	-2.63 to -6.43 SD
Spasticity	Yes (lower limb spasticity with brisk tendon reflexes)	Yes (increased tone in the lower limbs, with pathologically brisk reflexes and extensor plantar responses)	No (walks only with the use of a walking frame, but lower limb tone and reflexes are normal)	Yes (spastic paraparesis with brisk lower limb reflexes and extensor plantar responses; several operations to release contractures at his hips and ankles)	Yes (generalized hypertonia predominantly in both lower limbs, easily elicited reflexes, unsteady spastic gait)	Yes (generalized hypertonia predominantly in both lower limbs, ankle clonus, easily elicited reflexes, unsteady spastic gait and toe walking)	Yes (spastic diplegia and non-ambulant)	Yes (progressive spasticity with delayed gross and fine motor development)	No (4/4 with muscular hypotonia)
Gross motor development	Delayed (walked at > 24 months)	Delayed (walked at 24 months)	Delayed (walked at 7 years)	Delayed (walked at 5 years)	-	-	'Walks on knees'	Moderately delayed (walked at 14 months). Needs splints	Delayed (walked at 2-3 years)
Brain imaging results	MRI showed irregular ventricular margins and a thin corpus callosum (data not shown)	MRI showed dysgenic corpus callosum with dysmorphic ventricles; slight prominence of CSF spaces; normal cisterna magna, white matter, deep grey structures and calcification (data not shown)	MRI not available	MRI not available	-	MRI showed a thin corpus callosum, dilated ventricles with poor volume of white matter (Fig. 1d)	-	MRI detected a thin corpus callosum and reduced periventricular white matter bulk with mildly enlarged ventricles (Fig. 1e)	CT scan performed for 2/4 and normal brain structures reported (data not shown)
Seizures	-	Single episode of status epilepticus as a 2 year old; ongoing seizure disorder controlled by AEDs (resolved as teenager)	Seizures as teenager aged 13-14 years	Seizures as an infant, seizures when young, resolved as teenager.	-	No	-	-	-
Facial dysmorphism	Coarse facial features, prominent forehead, epicanthic folds, broad nasal bridge	Coarse facial features, low anterior hairline	Coarse facial features	Coarse facial features	No	No (just a right preauricular skin tag)	Coarse facial features	-	1/4
Digital abnormalities	Tapering fingers; short 5th metacarpals; very short 3rd and 4th toes	-	Short 3rd, 4th and 5th metacarpals; wasting of the intrinsic muscles of hands with clawing of the fingers	Short thumbs; marked muscle wasting of the small muscles of hands with clawing of his fingers	-	-	2-4 toe cutaneous syndactyly	-	-
Mirror movements	Yes (fingers)	Yes (fingers)	-	Yes	Yes (hand)	-	-	-	-
Other	Thick dry curly hair, low anterior hairline, iron deficiency anaemia, muscular ventricular septal defect, mongolian blue spot, long sighted	Thick dry curly hair, squint, iron deficiency, anaemia	Bicuspid aortic valve	Hypothyroidism	-	-	-	Hyperpigmentation of the skin in the form of small café au lait macules	3 with ataxia (1 too young for assessment); 1 with early closure of sutures; 2 with malformed chest (1 abnormal-shaped and enlarged chest and 1 mild pectus excavatum)