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2 **Deletion of VPS50 protein in mice brain impairs synaptic function and behavior**

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16

17 **Abstract.**

18 VPS50, is an accessory protein, involved in the synaptic and dense core vesicle acidification and
19 its alterations produce behavioral changes in *C.elegans*. Here, we produce the mosaic knock out
20 (mKO) of VPS50 using CRISPR/Cas9 system in both cortical cultured neurons and whole animals
21 to evaluate the effect of VPS50 in regulating mammalian brain function and behavior. While
22 mKO of VPS50 does not change the number of synaptic vesicles, it produces a mislocalization of
23 the V-ATPase pump that likely impact in vesicle acidification and vesicle content to impair
24 synaptic and neuronal activity in cultured neurons. In mice, mKO of VPS50 in the hippocampus,
25 alter synaptic transmission and plasticity, and generated robust cognitive impairments

26 associate to memory formation. We propose that VPS50 is an accessory protein that aids the
27 correct recruitment of the V-ATPase pump to synaptic vesicles, thus having a crucial role
28 controlling synaptic vesicle acidification and hence synaptic transmission.

29

30

31 **Introduction.**

32 Thousands of mutations have been implicated in developing neurodevelopmental disorders,
33 each showing different degrees of certainty and producing a myriad of phenotypes¹⁻⁶. Thus,
34 studying the specific gene mutations and their phenotype is crucial to finding common
35 pathways and potential therapies for these disorders. Among neurodevelopmental disorders,
36 autism spectrum disorders (ASD) are neurodevelopmental disorders characterized by deficits in
37 social interaction, repetitive behaviors, and anxiety⁷. These phenotypes are mainly produced
38 by genetic mutations that cause changes in brain wiring, structure, and function. More than
39 1300 mutations have been associated with ASD, depicting the complexity of its multifactorial
40 genetics and the phenotypes produced¹⁻⁶. Mutations that affect the function, filling, or
41 availability of synaptic vesicles have been strongly associated with the appearance of ASD
42 phenotypes⁸. For instance, mice carrying mutations in *Syn1*, a synaptic vesicle component
43 causing limited synaptic vesicle release, or *Nhe9*, a Na/H exchanger that causes hyper-
44 acidification of vesicles, show phenotypes associated with ASD⁹⁻¹¹. Recently, the function of
45 VPS50 in *C. elegans* controlling behavioral states and its expression in murine culture neurons
46 was described¹². VPS50 is an accessory protein widely expressed in the nervous system starting
47 early in embryonic life¹². Recent studies have demonstrated that VPS50 is associated with
48 some components of the Golgi-associated retrograde protein (GARP) complex, and more
49 specifically with the endosome-associated recycling protein (EARP) complex, suggesting a role
50 in endocytosis of synaptic vesicles by early endosomes^{13,14}. VPS50 physically interacts with the
51 protein VHA-15, a component of the V-ATPase complex pump responsible for acidifying both
52 dense core and synaptic vesicles, and its knockdown in cultured neurons shows a robust
53 decrease in synaptic vesicle acidification¹². In humans, a deletion spanning the *Vps50* human
54 gene, and the calcitonin receptor has been reported in an ASD patient¹⁵. More recently, two

55 individuals with homozygous loss of function mutations for *Vps50* have been described with a
56 severe neurodevelopmental disorder¹⁶. These findings underscore the potential role of VPS50
57 in ASD. However, no defined mechanisms have been identified that could explain the
58 phenotypes shown by these individuals.

59 Here we used the CRISPR/Cas9 system to produce the mosaic KO (mKO) of VPS50 in cultured
60 neurons and animals. We determined the effects on synaptic function, to later analyze mouse
61 behavior to determine VPS50's association to ASD, and in particular cognitive impairment.

62 Our findings provide insights into the role of VPS50 in synaptic function and behavior, as well as
63 elucidate the mechanisms through which VPS50 mutants can contribute to ASD phenotypes.
64 The brain mosaic VPS50 KO animal model will enable us to further investigate the cellular and
65 molecular mechanisms underlying the function of VPS50 and its implications in ASD.

66

67 **Materials and Methods**

68 **Animals and injections**

69 All animal procedures and experiments were performed according to the NIH and ARRIVE
70 guidelines and were approved by the animal ethics committee from Universidad Andrés Bello
71 (020/2018). Newborn Cas9 KI mice (C57BL/6J; JAX 026179) were cryoanesthetized in a cold
72 aluminum plate and injected with 1 μ L of concentrated AAV (1×10^{11} vg) in each cerebral
73 ventricle at a depth of 3 mm in the animal's head at 2/5 of the intersection between lambda
74 and the eye with a 10 μ L HAMILTON syringe (Hamilton, 7653-01) and a 32 G needle (Hamilton,
75 7803-04). After de injection, P0 mice were placed in a heating pad until they recovered their
76 color and temperature, then they were returned to their cage with the mother¹⁷⁻¹⁹. Control
77 mice were the same age as the injected ones. In the third week after birth, mice from both
78 conditions were weaned off and separated by sex in cages with a 12/12 light/dark cycle with
79 free access to food and water. A chip (p-chips, Pharmseq) was put in the tail of each animal for
80 easy tracking during behavioral testing.

81

82 **Neuronal Cultures.**

83 At P1 neonatal mice were quickly decapitated and dissected in ice cold HBSS to obtain cerebral
84 cortices as in ¹⁸⁻²¹. Cortices were minced and incubated for 20 min at 37C with papain
85 (Worthington, USA) for enzymatic digestion. Then cells were transferred to a 15ml tube
86 containing plating media (D-MEM supplemented with 10% Fetal bovine serum and 100 U/ml
87 penicillin/streptomycin (Life technologies 15070-063). Cells were resuspended by mechanical
88 agitation through fire-polished glass Pasteur pipettes of decreasing diameters. Cells were
89 counted and plated on freshly prepared poly-L-lysine-coated plates. 2 hours later plating media
90 was replaced with growth media (Neurobasal-A (Life technologies 1088802) supplemented with
91 B27 (Life technologies 17504044), 2 mM L-glutamine (Life technologies 25030-081), 100 U/ml
92 penicillin/streptomycin (Life technologies 15070-063)]. Half of the media was replaced every 3
93 days.

94

95 **AAV Production**

96 AAV viral particles containing sgRNA directed to VPS50, red fluorescent protein tdTomato, hSyn
97 promoter, and PHP.eB capsid ²² were obtained from HEK 293T cells and purified as described in
98 ^{18,19}. To produce concentrated AAV viral particles with the plasmid containing the VPS50 sgRNA
99 and the fluorescent protein tdTomato mediated by the hSyn promoter and with the PHP.eB
100 capsid ^{18,19,22}. In addition, AAVs were prepared using plasmids coding for Chr2 (Addgene
101 Cat#28017), GCamP7f (Addgene Cat#104488), and custom plasmids coding for eCas9 ²³, SyPhy
102 ²⁴, and pHoenix for synaptic vesicle acidification ²⁵. HEK 293T cells were grown to approximately
103 6×10^4 cells/cm² with DMEM 10% FBS. Cultures were transfected using PEI “MAX” reagent
104 (Polysciences, Cat 24765) with PHP.eB capsid plasmids, the vector with VPS50 sgRNA-tdTomato,
105 and the helper plasmid DF6. After 24 h of transfection, the media was exchanged for DMEM 1%
106 FBS. After 72h, media was collected from the plates and replaced with fresh DMEM 1% FBS. The
107 collected media was stored at 4°C. 120 h after transfection, the cells were detached from the
108 plate and transferred to 250 mL conical tubes, together with the collected media. They were
109 centrifuged for 10 min at 2000 g, and the supernatant was removed and saved for later use.
110 The pellet was resuspended in SAN digestion buffer (5 mL of 40 mM Tris, 500 mM NaCl and 2
111 mM MgCl₂ pH 8.0) containing 100U/mL of Salt Active Nuclease (Arcticzymes, USA) and

112 incubated at 37°C for 1 hour. The supernatant was precipitated using 8% PEG 8000 and 500mM
113 NaCl. It was incubated on ice for 2 h and centrifuged at 4000 g for 30 min in 250 mL bottles. The
114 supernatant was collected and resuspended with SAN digestion buffer. The solution was placed
115 in an iodixanol gradient and ultracentrifuged at 350,000g for 2.5h. The phase containing the
116 AAV was rescued and frozen at -80°C for later use.

117

118 **Brain sectioning and mounting**

119 Half of the brains were submerged to assess brain infection and left to fix for a minimum of 24h
120 in PBS CaMg + 4% PFA + 4% Sucrose into 30 mL flasks. After fixation, a Leica VT1000 vibratome
121 was used to cut 100 µm coronal sections. Slices were kept in PBS CaMg 1X and mounted using
122 Fluoromont G (EMS, Hatfield, PA) to preserve the fluorescence signal. Brain images were
123 captured with a Nikon Eclipse TE2000 epifluorescence microscope.

124

125 **Protein extraction and electrophoresis**

126 To carry out the immunodetection tests, proteins were extracted from the cortex and
127 hippocampus of mice brains. 5 to 50mg of tissue was ground in N-PER lysis buffer
128 (ThermoFisher Product No. 23225) with protease and phosphatase inhibitors (cOmplete™
129 Protease Inhibitor Cocktail 11697498001, Roche Diagnostics) until a homogeneous solution was
130 achieved and incubated for 10 min on ice. Then it was centrifuged at 10,000 g for 10 min at 4°C.
131 Following the manufacturer's recommendations, the supernatant with total proteins was
132 collected and quantified using the BCA method (Perkin-Elmer). For electrophoresis, the proteins
133 from the total extracts were denatured in loading buffer (NuPAGE LDS Sample buffer 4X;
134 NP0007), heating at 95°C for 10 min. Then 40 µg of the sample was loaded on a 6% SDS
135 acrylamide-bisacrylamide gel to visualize proteins larger than 100 kDa and 10% for proteins
136 smaller than 100 kDa. Electrophoresis was carried out at 80-150 V constant voltage per gel in
137 running buffer (25 mM Tris·Cl, 250 mM glycine, 0.1% SDS) in a minichamber (MiniPROTEAN
138 System, BIO-RAD).

139

140 **Western Blot**

141 After electrophoresis, the proteins were transferred to an activated PDVF membrane in transfer
142 buffer (250 mM glycine, 25 mM Tris-Cl, 0.1% SDS, 20% methanol) at 400 mA constant current
143 for 1.5 h. To verify protein transfer, the membrane was incubated with Ponceau red S (0.1%
144 Ponceau S, 5% acetic acid). The membrane was washed with TBS/Tween 20 0.05% until the
145 Ponceau red staining was removed. Next, the membrane was blocked for 1h to avoid non-
146 specific protein binding sites in a 0.05% TBS/Tween 20 solution with 5% skim milk. The
147 membrane was then incubated with the specific primary antibody VPS50 (Sigma, HPA026679-
148 100UL, Rabbit, 1/500 dilution) in 0.05% TBS/Tween 20 solution with 5% skim milk at 4°C
149 overnight. The membranes were washed with TBS/Tween 20 0.05% five times for 5 min each
150 time. Then the membrane was incubated with a second antibody directed against the first
151 antibody and coupled to horseradish peroxidase (HRP) in TBS/Tween 20 0.05% with 5% skim
152 milk at room temperature for 1 h. Then, the membrane was washed with TBS/Tween 20 0.05%
153 five times for 5 min each time. Detection was performed with chemiluminescence reagents
154 (SignalFire™ Elite ECL Reagent). Protein expression was normalized to β -Tubulin expression
155 (Abcam Cat: ab6046, 1/500).

156

157 **DNA and RNA extraction**

158 5 to 50mg of tissue was weighted for DNA and RNA extractions, and the Quick-DNA/RNA
159 Miniprep kit (Cat: D7001) was used. Briefly, the tissue was homogenized with dounces in lysis
160 buffer and centrifuged for 30s at 14,000g. The supernatant was collected and transferred to a
161 Zymo-Spin™ IICR column in a collection tube, centrifuged at 14,000g for 30s. The filtrate was
162 used for RNA extraction and the column for DNA extraction. For RNA extraction, the same
163 volume of 100% ethanol was added and homogenized, transferred to a Zymo-Spin™ IICR
164 column, and centrifuged at 14,000g for 30s. The column was then treated with DNase I, and
165 400 μ L DNA/RNA Prep Buffer was added, centrifuged, and washed twice with wash buffer. To
166 elute the RNA, 25 μ L of DNase/RNase-Free Water was added, incubated for 3 min, quantified,
167 and stored at -80°C for later use. For DNA extraction, 400 μ L DNA/RNA Prep Buffer was added
168 to the Zymo-Spin™ IICR column, centrifuged, and washed twice with wash buffer. To elute

169 the DNA, 50 μ L of DNase/RNase-Free Water was added, incubated for 5 min, quantified, and
170 stored at -80°C for later use.

171

172

173

174 **RT-qPCR**

175 RT-qPCR assays were carried out from 400 ng of extracted total RNA. To obtain complementary
176 DNA (cDNA), each sample was mixed with 0.25 μ g Oligo-dt (New England Biolab S1316S) in 10
177 μ L to be denatured at 75°C for 5 min and then quickly transferred to 4°C for 5 min. Reverse
178 transcription (RT) was performed in a final 20 μ L volume containing: 10 μ L denatured RNA, 100
179 U of M-MLV Reverse Transcriptase (NEB; M0253S), M-MLV RT buffer (NEB; B0253S) 1X, 20 U of
180 RNase Inhibitor (NEB; M0314S) and 0.5 mM of dNTPs (Biotechnology N557-0.5ML). The mixture
181 was incubated at 42°C for 1 hour, then at 95°C for 5 min, and the reaction was stopped at 4°C
182 and then diluted five times with nuclease-free water. The cDNA was quantified by real-time PCR
183 using 3 μ L of the diluted RT mixture. Using relative abundance by the ddCt method, using the
184 GAPDH gene as a loading control. Transcript detection was performed with specific primers for
185 VPS50 mRNA (Sense: TGTTACTTCTCCGAGGCAGG, Antisense: GCTCTCAAAGGACCAAGAT) and
186 GAPDH (Sense: ATGGTGAAGGTCGGTGTGAA, Antisense: CATTCTCGGCCTTGACTGTG).

187

188 **Calcium Imaging**

189 Cortical neurons control and VPS50 mKO were co-infected at 3 DIV with GCaMP7f (Addgene
190 Cat#104488). At 10 DIV, neurons were imaged using a Nikon Eclipse TE-2000 microscope
191 equipped with a Co2/temperature chamber (Tokai-Hit). Pictures were acquired every 30ms for
192 5 minutes. The frequency of spiking was calculated using fluorescence over time plots using
193 ImageJ.

194

195 **Electrophysiology cultured neurons**

196 Whole-cell patch-clamp recordings on infected cortical neurons were performed and analyzed
197 as previously described^{18,21,26}. The external solution contained (in mM) 150 NaCl, 5.4 KCl, 2.0

198 CaCl₂, 2.0 MgCl₂, 10 HEPES (pH 7.4), and 10 glucose. Patch electrodes (5–7 MΩ) were filled
199 with (in mM) 120 CsCl, 10 BAPTA, 10 HEPES (pH 7.4), 4 MgCl₂, and 2 ATP-Na₂. After the
200 formation of a high resistance seal and break-in (>1 GΩ), whole cell voltage and current signals
201 were recorded with an Axopatch 700B amplifier (Molecular Devices). Signals were low pass
202 filtered (5 kHz) and digitized (5–40 kHz) on a PC using pClamp 10 software. Cells were held at
203 –60 mV.

204 To analyze synaptic function, we recorded isolated AMPA-mediated synaptic currents using a
205 mixture of antagonists against NMDARs (20 μM d-APV) and GABAARs (2 μM bicuculline). In
206 some experiments, the sodium channel blocker TTX (500 nM) was added to the bath to record
207 miniature AMPA-mediated currents. For current-clamp analyses, cells were patched, and
208 spontaneous spiking of cells was recorded. Depolarization of neurons was performed using
209 square pulses or TTL activation of 488nm light to activate Channelrhodopsin channels.
210 MiniAnalysis software (Synaptosoft) was used to analyze synaptic events during the tests. The
211 frequency and amplitude of currents were automatically calculated and plotted.

212

213 **Hippocampal slice electrophysiology.**

214 Electrophysiological recording from hippocampal slices were conducted as previously described
215 ^{27,28}. Briefly, acute coronal hippocampal slices (400 μm thick) were prepared from control and
216 VPS50 mKO at postnatal day (P) 30 to P45. Brain slices were cut using a DKT vibratome in a
217 solution containing the following (in mM): 215 sucrose, 2.5 KCl, 26 NaHCO₃, 1.6 NaH₂PO₄, 1
218 CaCl₂, 4 MgCl₂, 4 MgSO₄, and 20 glucose. After thirty minutes recovery, slices were incubated
219 in an artificial CSF (ACSF) recording solution containing the following (in mM): 124 NaCl, 2.5 KCl,
220 26 NaHCO₃, 1 NaH₂PO₄, 2.5 CaCl₂, 1.3 MgSO₄, and 10 glucose equilibrated with 95% O₂/5%
221 CO₂, pH 7.4. Slices were incubated in this solution for 30min before recordings.

222 All experiments, except where indicated, were performed at 28 ± 1°C in a submersion-
223 type recording chamber perfused at 1–2 ml/min rate with ACSF supplemented with the GABA_A
224 receptor antagonist picrotoxin (PTX; 100 μM). Extracellular field potentials (fEPSPs) were
225 recorded with a patch pipette filled with 1mM NaCl and placed in the CA1 stratum radiatum.
226 Whole-cell voltage-clamp recordings (Multiclamp 700B Molecular Devices, USA) were made

227 from CA1 pyramidal neurons voltage-clamped at -60 mV using patch pipette electrodes (3–4 M)
228 containing the following intracellular solution (in mM): 131 Cs-gluconate, 8 NaCl, 1 CaCl₂, 10
229 EGTA, 10 glucose, 10 HEPES, pH 7.2, 292 mmol/kg osmolality.

230 fEPSPs and EPSCs were evoked by stimulating Schaffer collateral inputs with a
231 monopolar electrode filled with ACSF and positioned ~100–150 mm away from the recording
232 pipette. Miniature EPSCs (mEPSCs) were recorded at 32 ± 1°C in the continuous present of
233 tetrodotoxin (TTX, 500 nM) to block action potential dependent release, whereas spontaneous
234 EPSC (sEPSCs) were recorded in the absent of TTX. Short- term synaptic plasticity was induced
235 by two pulses (100 ms interstimulus interval) to calculate paired-pulse ratio (PPR) that was
236 defined as the ratio of the slope or amplitude of the second EPSP/EPSC to the slope or
237 amplitude of the first EPSP/EPSC, respectively. Long-term potentiation (LTP) was induced by 4
238 trains of 100 pulses at 100 Hz repeated four times, separated by 10 seconds. Reagents were
239 obtained from Sigma, Tocris and Ascent Scientific, prepared in stock solutions (water or DMSO)
240 and added to the ACSF as needed. Total DMSO in the ACSF was maintained less than 0.01%. For
241 whole cells experiments, series resistance (range, 8–12 MW) was monitored throughout the
242 experiment with a 5 mV, 80 ms voltage step, and cells that exhibited significant change in series
243 resistance (20%) were excluded from analysis.

244

245 **Behavioral analyses**

246 All behavioral tests on mice were carried out eight weeks after AAV injection and will be
247 conducted as previously described^{18,19,21,29}. Before each test, mice cages were transported to
248 the behavior room and habituated for 30 min in the dark. After completing a trial, the
249 equipment and devices used were cleaned with 70% ethanol. Tests were recorded and analyzed
250 with ANY-Maze software. Behavior tests were performed between 9:00 am and 6:00 pm. At the
251 end of the battery of behavioral tests, the animals were sacrificed for subsequent molecular
252 analyses.

253

254 **Contextual fear conditioning:** UGO-BASILE apparatus controlled by ANY-Maze was used. This
255 equipment consists of a sound attenuating box, fan, light (visible/IR), a speaker, a USB camera,

256 a single onboard controller, and a mouse cage. All trials were recorded, and all mice underwent
257 habituation, conditioning, and testing phase. Twenty-four hours after training, the animals
258 were tested for contextual memory. Each mouse was placed in the fear conditioning box,
259 allowed to explore for 5 min freely, and returned to its cage. The number of freezing episodes
260 and freezing time were registered.

261

262 **Barnes Maze:** A non-reflective gray circular platform (91 cm diameter) with 20 holes (5 cm
263 diameter) evenly distributed along the perimeter, with one hole containing a metal escape
264 tunnel, was used. Three exogenous visual cues (length/width ~30 cm) were used around the
265 platform: a black circle, a blue triangle, and a yellow square. The light was adjusted to 1000 lux
266 in the center of the platform. All animals underwent a phase of habituation, spatial acquisition,
267 and testing. On test day, the position of the escape tunnel was changed, and the animal was
268 brought in the start box to the center of the platform, left for 10 s, and sound reproduction was
269 started. The test ended at 90 seconds, or when the mouse found the escape tunnel. The
270 number of primary and total errors, primary and total latency, and total distance before finding
271 the gap were recorded. The number of visits to each hole was also measured to show
272 preference.

273

274 **Data analysis**

275 All values are presented as means \pm standard error (SE) for three or more independent
276 experiments. Statistical analyzes were performed using Student's t-test. Values of $p < 0.05$ are
277 considered statistically significant. All statistical analyzes were performed using Graphpad
278 Prism.

279

280

281 **Results**

282

283 **VPS-50 regulates synaptic vesicle acidification and V-ATPase pump localization in cortical**
284 **neurons.**

285 In *C. elegans*, VPS50 knock-out causes significant changes in behavior likely due to changes in
286 synaptic and dense core vesicle acidification¹². In mice, VPS50 is highly expressed in the central
287 nervous system and, its knock down in culture causes a deregulation of synaptic vesicle
288 acidification¹². To further investigate the role of VPS50 in regulating mammalian synaptic
289 function and behavior, we used CRISPR/Cas9 technology to get better insights into the
290 mechanisms and consequences of VPS50 knock-out in mouse brain function. Cortical neurons
291 from Cas9 KI animals were infected at 3 DIV with AAV coding for tdTomato as an infection
292 marker alone or together with sgRNAs targeting *Vps50*. Transduction efficiency reached >90%
293 of plated neuron in culture. Genomic DNA was extracted ten days after infection, and a
294 surveyor assay was performed to determine the efficiency of gene edition. A combination of
295 different sgRNAs targeting the *Vps50* genomic locus was tested, finding increased efficiency
296 when pair of sgRNA1-6 was used (Supplementary Figure 1A). We performed the following
297 experiments using sgRNA1-6 (hereafter VPS50 mKO) (Figure 1A). Edition of the locus causes a
298 ~70% reduction in VPS50 mRNA and protein levels in cortical neurons (Figure 1 B-D), confirming
299 an efficient mKO of VPS50 after gene editing. Locus-specific sequencing shows the resulting
300 edited genomic sequence caused by CRISPR/Cas9 (Supplementary Figure 1B). Importantly,
301 VPS50 KO neurons shows no significant difference in the total number of synaptic vesicles
302 assessed by electron microscopy (Figure 1E-F), however, we found a robust reduction in vesicle
303 acidification assessed by ratio-SyPhy probe³⁰ (Figure 1G), consistent with the idea that VPS50
304 regulate synaptic vesicle acidification¹².

305 As VPS50 is enriched in synaptic and dense core vesicles as a soluble protein in mouse brain
306 extracts¹², we next performed proximity ligation assays (PLA) to evaluate its approximate
307 location within the synapse. First, we tested in control neurons whether VPS50 was close to
308 the post-synaptic marker PSD95 or the presynaptic Synapsin1. We found that the PLA signal
309 was only observed in VPS50/Synapsin1 condition, confirming that VPS50 is near synaptic
310 vesicles (Figure 2A). As control, PLA reactions using Synapsin1/Synaptophysin and
311 PSD95/Synapsin1 were used (Supplementary Figure 2). As VPS50 co-fractionates with the V-
312 ATPase pump in mice tissue¹² and moreover, synaptic vesicle acidification is reduced in VPS50
313 mKO neurons (Figure 1D); it is possible that VPS50 might interact and/or help to localize the V-

314 ATPase V1 pump to synaptic vesicles to acidify them for neurotransmitter filling. To test this
315 possibility, first we use PLA to evaluate the proximity of VPS50 and V-ATPase pump in control
316 neurons. We observed PLA signal indicating proximity between VPS50 and V-ATPase pump
317 (Figure 2B). However, in VPS50 mKO neurons PLA signal is absent confirming the reduced
318 expression of VPS50 and, consequently, the null interaction between VPS50 and V-ATPase
319 pump (Figure 2B). Second, we evaluated whether the localization of V-ATPase pump was
320 disrupted in VPS50 mKO neurons. While PLA experiments for the V-ATPase pump and Synapsin
321 1 (Syn) show that they are in proximity in control neurons (Figure 2C; top), no signal was
322 observed in VPS50 mKO neurons (Figure 2C; bottom), strongly suggesting that knock-out of
323 VPS50 causes a mis-localization of the V-ATPase pump in synaptic vesicle. Altogether, these
324 results indicate that knockdown of VPS50 does not affect the total number of synaptic vesicles
325 but produces a mis-localization of the V-ATPase pump that likely impair synaptic vesicle
326 acidification and hereby synaptic function.

327

328 **VPS50 mKO impairs synaptic activity in cortical neurons.**

329 To further evaluate the effect of VPS50 mKO in synaptic function, neurons were infected at 3
330 DIV and miniature and spontaneous excitatory postsynaptic currents (mEPSC and sEPSC,
331 respectively) were recorded at 12-13 DIV. Compared to control neurons, VPS50 mKO neurons
332 show a strong reduction in both the amplitude and frequency of sEPCS (Figure 3A-C). While the
333 frequency and the amplitude of sEPSC does not change over 30-minute recordings in
334 controlneurons, VPS50 mKO neurons display a significant reduction in the amplitude of the
335 sEPSCs over time (Supplementary Figure 3A-C), consistent with the idea that vesicular content
336 might be reduced in VSP50 mKO neurons. Moreover, VPS50 mKO neurons show a drastic
337 reduction in the frequency but not in the amplitude of mEPSCs (Figure 3D-F), an effect that
338 could reflect an increase in the exocytosis of empty or partially filled vesicles. Such reduction in
339 the synaptic strength correlated with a decrease in the neuronal activity as the spontaneous
340 spike frequency is decreased in VPS50 mKO neurons compared to control neurons (Figure 3G,
341 H). In addition, calcium imaging using GCaMP7 shows that VPS50 mKO neurons have reduced
342 calcium event frequency, confirming deficient spiking (Supplementary Figure 3D-E). To further

343 evaluate if synaptic vesicle acidification is partly responsible for the reduction in vesicle content
344 and neuronal activity, we used pHoenix, a genetically encoded proton pump targeted to
345 synaptic vesicles²⁵. Spontaneous spiking of pHoenix infected control and VPS50 mKO neurons
346 was recorded for 1 min as baseline, and then 532nm light was used to activate pHoenix. During
347 the 2-minute activation period, we observed a partial recovery of spiking in VPS50 mKO
348 neurons close to control levels (Figure 3 I-J; blue line). Moreover, after stimulation we observed
349 no significant differences with control neurons (Figure 3K). Altogether, our results strongly
350 suggest that the reduction in synaptic activity of VPS50 mKO requires synaptic vesicle
351 acidification, a phenomenon that can be rescued by artificially acidifying synaptic vesicles.

352 **VPS50 mKO impacts synaptic function and memory formation.**

353 Once we have demonstrated that VPS50 mKO causes deficit in vesicle acidification and synaptic
354 function in cultured cortical neurons, next we aimed to evaluate whether VPS50 mKO in mice
355 might cause significant changes in behavior as suggested previously in *C.elegans*¹² and humans
356¹⁶. Toward this end, we used the CRISPR/Cas9 system to induce VPS50 mKO in the mouse entire
357 brain. We delivered high titer AAVs at postnatal day 1 (P1) intracerebroventricular in Cas9 KI
358 animals, packed using the PHP.eB capsid²² for its high efficiency in targeting the mouse brain.
359 Animals were injected with the same sgRNAs tested in culture together with tdTomato or
360 tdTomato alone as a fluorescence marker under the control of the human synapsin1 promoter.
361 First, post-mortem analyses of animals show high AAV infection in multiples brain areas (Figure
362 4A), including the cortex and the hippocampus. In these brain areas, VPS50 protein expression
363 is significantly reduced (Figure 4B), confirming an efficient mKO of VPS50 after gene editing in
364 mouse brain. Second, we monitored basal synaptic function at Schaffer collateral to CA1
365 synapses in acute hippocampal slices. A significant decrease in the frequency but not in the
366 amplitude of miniature excitatory postsynaptic currents (mEPSCs) was observed in VPS50 mKO
367 synapses (Figure 5A-C). Likewise, a strong reduction in the frequency and amplitude of
368 spontaneous EPSCs (sEPSCs) was also observed in the VPS50 mKO synapses (Figure 5D-F).
369 Moreover, input/output curves revealed a large decrease in the amplitudes of evoked EPSC at
370 all stimulus intensities tested in VPS50 mKO synapses (Figure 6A-B). Importantly, paired-pulse
371 facilitation remains unchanged (Figure 6C-D) suggesting that the decrease in evoked EPSC

372 amplitude cannot be accounted for changes in release probability, but could be due to a
373 decrease in vesicle content and/or vesicle refilling by change the level of acidification as
374 observed in cultured neurons (Figure 1G). To further evaluate whether the state of vesicle
375 content might be involved in this synaptic change, we evaluated the response of VPS50 mKO
376 synapses to high-frequency stimulation a condition in which one might detect the release of
377 partially filled vesicles. Under this conditions, VSP50 mKO synapses response was greatly
378 reduced compared to control synapses (Figure 6E-F), confirming that vesicle content is reduced
379 in VSP50 mKO synapses. Next, we investigated the impact of VSP50 mKO in long-term synaptic
380 plasticity (LTP) in the hippocampus, the cellular mechanism underlying learning and memory.
381 We found that the magnitude of LTP induced by high-frequency stimulation was reduce in mice
382 with disrupted VSP50 compared to control (Figure 6G-H), suggesting cognitive deficit in the
383 context of memory formation in VPS50 mKO mice.

384 Finally, we evaluated VPS50 mKO mice performance in Barnes maze and Fear conditioning
385 apparatus (Figure 6), two memory formation paradigms dependent on the hippocampus. First,
386 we analyzed if VPS50 mKO mice model had locomotor problems that could affect their
387 performance in behavioral testing. VPS50 mKO animals spent more time on the ramp in the
388 accelerated rotarod apparatus than Control animals (Figure 7A), indicating that brain mKO of
389 VPS50 have no impact on motor coordination. However, VPS50 mKO animals made a
390 significantly higher number of primary errors (holes checked before finding the escape hole) on
391 days 1 and 2 (Figure 7C), but behaved similar to control error numbers on days 3 and 4. We also
392 found that primary latency, the time the animal takes to find the escape hole, was significantly
393 affected in VPS50 mKO, but only on the first day (Figure 7D). Moreover, we found that VPS50
394 mKO animals spent significantly less time in the escape zone on days 1-3 compared to control
395 littermates (Figure 7E). Lastly, using the context-dependent fear conditioning paradigm, we
396 found that VSP50 mKO animals significantly decreased in freezing compared to control
397 littermates (Figure 7F). Altogether, these results indicate that VPS50 mKO mouse display
398 alteration in hippocampal synaptic function and memory deficits dependent on this brain area.

399

400 **Discussion.**

401 VPS50 is a conserved protein across multiple species including *C. elegans*, *M. musculus*, and
402 humans, indicating its fundamental importance in cellular function^{12,16}. In the cell, VPS50 is
403 associated to the EARP complex and more specifically to dense core and synaptic vesicles³¹.
404 Evidence from *C. elegans*, shows that VPS50 control locomotion behaviors and is associated to
405 the V-ATPase pump¹².

406 The V-ATPase pump is recruited to synaptic vesicles after recycling serving as an initial step for
407 neurotransmitters filling³². Studies using *C. Elegans* show that worm mutants for *unc-32*, the
408 homolog to the human V-ATPase pump, have severe neurotransmission deficits in
409 motoneurons due to the lack of acidification and hence filling of neurotransmitters in their
410 synaptic vesicles³³. Interestingly, the V-ATPase pump works by acidifying cellular
411 compartments broadly in the cell, but some specificity is given due to the differential formation
412 of the entire complex by different isoforms and proteins interactions³⁴.

413 Our findings reveal a critical role of VPS50 in regula synaptic function and behavior in mammals
414 through its involvement in synaptic vesicle acidification. Using CRISPR/Cas9 to produce the mKO
415 of VPS50, we discovered that VPS50 mKO neurons exhibit mislocalization of the V-ATPase
416 pump, lacking proximity to synaptic vesicles, without affecting the total number of synaptic
417 vesicles. Instead, this mislocalization affects vesicle acidification, thereby influencing vesicular
418 content and/or vesicle refilling. Consequently, the limited acidification of synaptic vesicles leads
419 to a significant reduction in neurotransmitter filling and a subsequent decline in synaptic
420 activity^{8,32,35}. These findings strongly indicate that VPS50 plays a crucial role in facilitating the
421 proper localization of the V-ATPase pump near synaptic vesicles to allow synaptic vesicle
422 acidification. However, it remains to be investigated whether by the mislocated v-ATP pump
423 the extent of vesicle filling is impacted.

424 Furthermore, our data demonstrates that the reduction of VPS50 impairs synaptic transmission
425 in cultured neurons where these deficiencies can be recovered by artificially acidifying synaptic
426 vesicles. *In vitro* electrophysiological recordings using hippocampal slices, show severe deficit in
427 LTP formation. The strong decrease in the frequency of synaptic events observed in VPS50 mKO
428 synapses suggests an increase in the exocytosis of empty or partially filled vesicle. Consistently,
429 significant effects were observed with high-frequency stimulation, a condition in which one

430 might detect the release of partially filled vesicles. These observations strongly support, that
431 the total content rather than vesicle refilling could be account for the synaptic deficit observed
432 at hippocampal VPS50 mKO synapse. Ultimately, these synaptic transmission defects impair
433 memory formation in animals, linking the deficits in synaptic vesicle acidification and/or
434 synaptic filling to complex cognitive behaviors such as learning and memory formation.

435 In summary, our data strongly support the role of VPS50 in regulating synaptic transmission by
436 facilitating the recruitment of the V-ATPase pump to synaptic vesicles, thereby enabling vesicle
437 acidification and modulating vesicle content. These functional alterations have significant
438 implications, as VPS50 mKO mice exhibit cognitive impairment. Future studies will investigate
439 additional complex behaviors to provide a comprehensive understanding of the behavioral
440 consequences associated with VPS50 mKO including its associated to ASD.

441

442

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452

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455

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553

554 **Figure legends.**

555

556 **Figure 1. VPS50 gene edition causes decrease in synaptic vesicle acidification but no change in**
557 **synaptic vesicle number.** (A) Surveyor assay of Control or VPS50 mKO cortical neurons. (B) RT-
558 qPCR to quantify relative mRNA expression of VPS50 mKO. (C-D) VPS50 protein expression in
559 VPS50 mKO and Control neurons. (E-F) Representative images of synaptic terminals by electron
560 microscopy and quantification of the number of synaptic vesicles in Control and VPS50 mKO
561 neurons (n=50 cells per condition). (G) Representative images of Ratio-SyPhy signal to
562 determine synaptic vesicle acidification in Control and VPS50 mKO neurons. Scale bar, 25um.
563 ***p<0.001.

564
565 **Figure 2. VPS50 mKO causes mislocalization of v-ATPase pump in cortical neurons.** (A) PLA to
566 determine pre- or post- synaptic localization of VPS50 in Control neurons using PSD95 or
567 Synapsin1 (Syn) antibodies. (B) Control and VPS50 mKO PLA to determine proximity of VPS50
568 and v-ATPase pump. (C) PLA to determine localization of v-ATPase pump in Control and VPS50
569 mKO neurons. Inset shows magnification of the selected areas. Scale bar, 25 um.

570
571 **Figure 3. VPS50 mKO neurons show a reduction in synaptic activity that can be recovered by**
572 **inducing synaptic vesicle acidification.** (A) Representative traces and (B-C) quantification of
573 Amplitude (B) and Frequency (C) of spontaneous AMPA-mediated EPSCs. (D)
574 Representative traces and (E-F) quantification of the frequency (E) and amplitude (F) of
575 miniature AMPA-mediated currents. (G) Current clamp of Control and VPS50 mKO neurons.
576 (H) Quantification of the frequency of spiking in current clamp. (I-K) Current clamp
577 representative traces of Control and VPS50 mKO neurons. Blue bars show the period
578 pHoenix is activated. (K) Quantification of the frequency of spiking of Control and VPS50
579 mKO neurons before, during and after stimulus with pHoenix to acidify synaptic vesicles. At
580 least 18 neurons from 3 independent experiments were analyzed. *** p<0.001.

581
582 **Figure 4. Systemic injection of AAV at P0 to produce VPS50 mKO.** (A) Representative image of
583 a coronal section of a mouse brain injected at P0 with AAV after 15 weeks. (B)
584 Representative western blot analyses for the detection of VPS50 expression in Control and
585 VPS50 mKO animals in both cortex and hippocampus. 2 animals are shown per condition. B-
586 Tubulin is used as loading control.

587

588 **Figure 5. Hippocampal spontaneous excitatory synaptic activity is impaired in**
589 **VSP50 mKO mouse.** (A) Representative traces of mEPSC in hippocampal slices of Control
590 and VPS50 mKO animals. (B-C) Quantification of the Frequency (B) and Amplitude (C) of
591 mEPSC. (D) Representative traces of sEPSC in hippocampal slices of Control and VPS50
592 mKO animals. (E-F) Quantification of the Frequency (E) and Amplitude (F) of mEPSC.
593 * $p < 0.05$, *** $p < 0.001$.

594

595 **Figure 6. Hippocampal synaptic function and plasticity are impaired in VSP50 mKO**
596 **mice.** Electrophysiological recording of Control and VPS50 mKO hippocampal slices. (A)
597 Representative traces of Input-output responses. (B) Input-output curves reveal a strong
598 reduction in the amplitude of evoked EPSC at all stimulus intensity tested. (C)
599 Representative traces and (D) quantification of paired-pulse facilitation at different inter-
600 stimulus interval. (E) Representative traces and quantification (F) of the response to high-
601 frequency stimulation. (G) Representative traces before and after LTP induction by high-
602 frequency stimulation. (H) Quantification of potentials during LTP induction. Number of
603 slices (s) or cells (c) and animals (a) are indicated in parenthesis. * $p < 0.05$.

604

605 **Figure 7. VPS50 mKO animals show impaired hippocampal memory formation.** Brain
606 wide Control and VPS50 mKO animals were subjected to behavioral testing. (A) Accelerated
607 rotarod apparatus. (B) Scheme of the Barnes maze memory paradigm where clues attached
608 to the wall, escape hole, and escape zone are shown. (C) Quantification of the numbers of
609 primary errors before finding the escape hole. (D) Quantification of the primary latency to
610 find the escape hole. (E) Quantification of the time spent in the escape zone. (F)
611 Quantification of freezing percentage time in the fear conditioning paradigm. * $p < 0.05$.

612

613 **Supplementary Figure 1.** Cortical neurons were infected at 3 DIV with AVV coding for
614 TdTomato as control and a combination of sgRNAs (1-6 / 2-4 / 1-4 / 2-6) targeting Vps50,
615 as shown in figure. 10 days later genomic DNA was extracted, and Surveyor assay
616 performed (A). Surveyor assay for sgRNA combinations (B) Vps50 locus specific sequencing
617 of edited genomic DNA using VPS50 mKO.

618

619 **Supplementary Figure 2.** Proximity ligation assay (PLA) assay controls. PLA was performed
620 using specific antibodies for (A) Synapsin1-Synaptophysin (pre-pre synaptic) or (B)
621 Synapsin1-PSD95 (pre-post synaptic), as shown in figure. PLA signal is only observed in
622 Synapsin1-Synaptophysin pair where the two proteins are in close proximity to each
623 other. Scale bar, 25 μ m.

624

625 **Supplementary Figure 3.** VPS50 mKO neurons show deficits in synaptic transmission. (A)
626 Representative traces of AMPA mediated EPSCs for 30 minutes of Control and VPS50 mKO
627 neurons. VPS50 mKO neurons show a robust reduction in both Amplitude (B) and Frequency
628 (C) of AMPA EPSCs. (D) Cortical neurons were co-transduced with GCaMP7 to measure
629 calcium events by changes in fluorescence over time. A significant reduction is observed in
630 VPS50 mKO neurons compared to control. Control n=1338; VPS50 mKO n=1256.
631 **p<0.01, ***p<0.001.

632

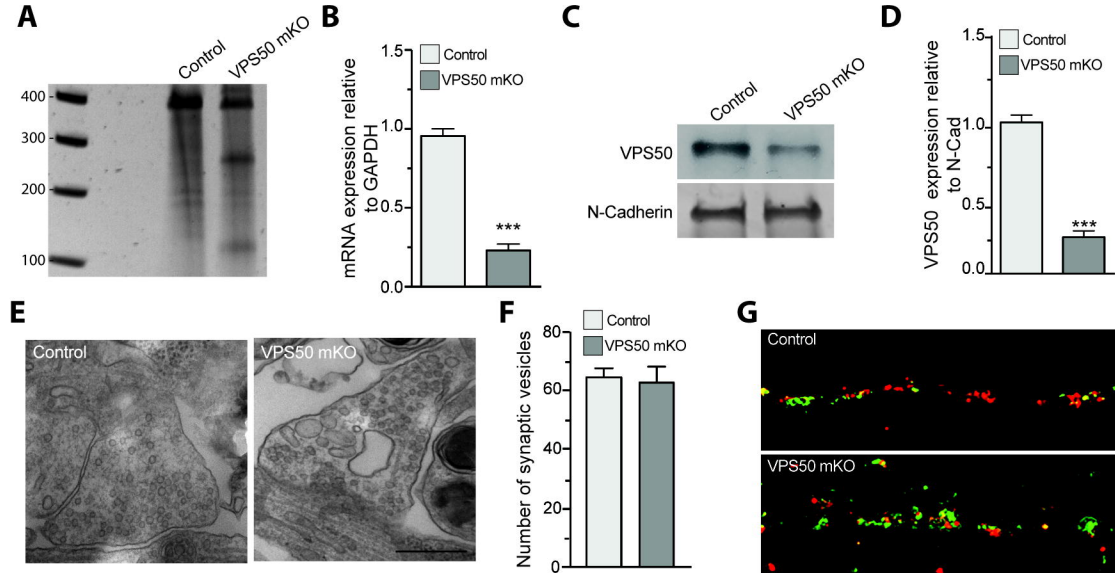
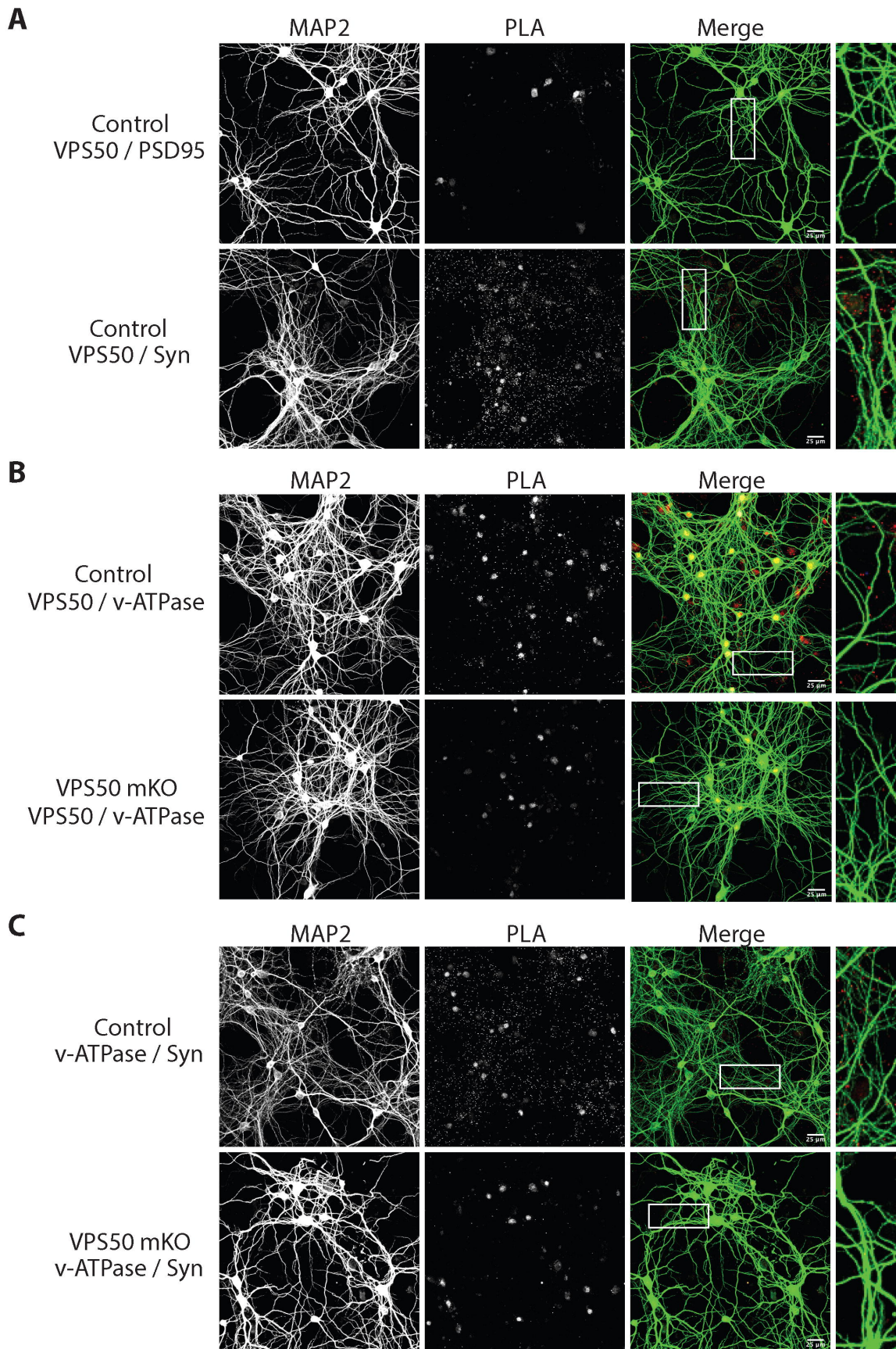
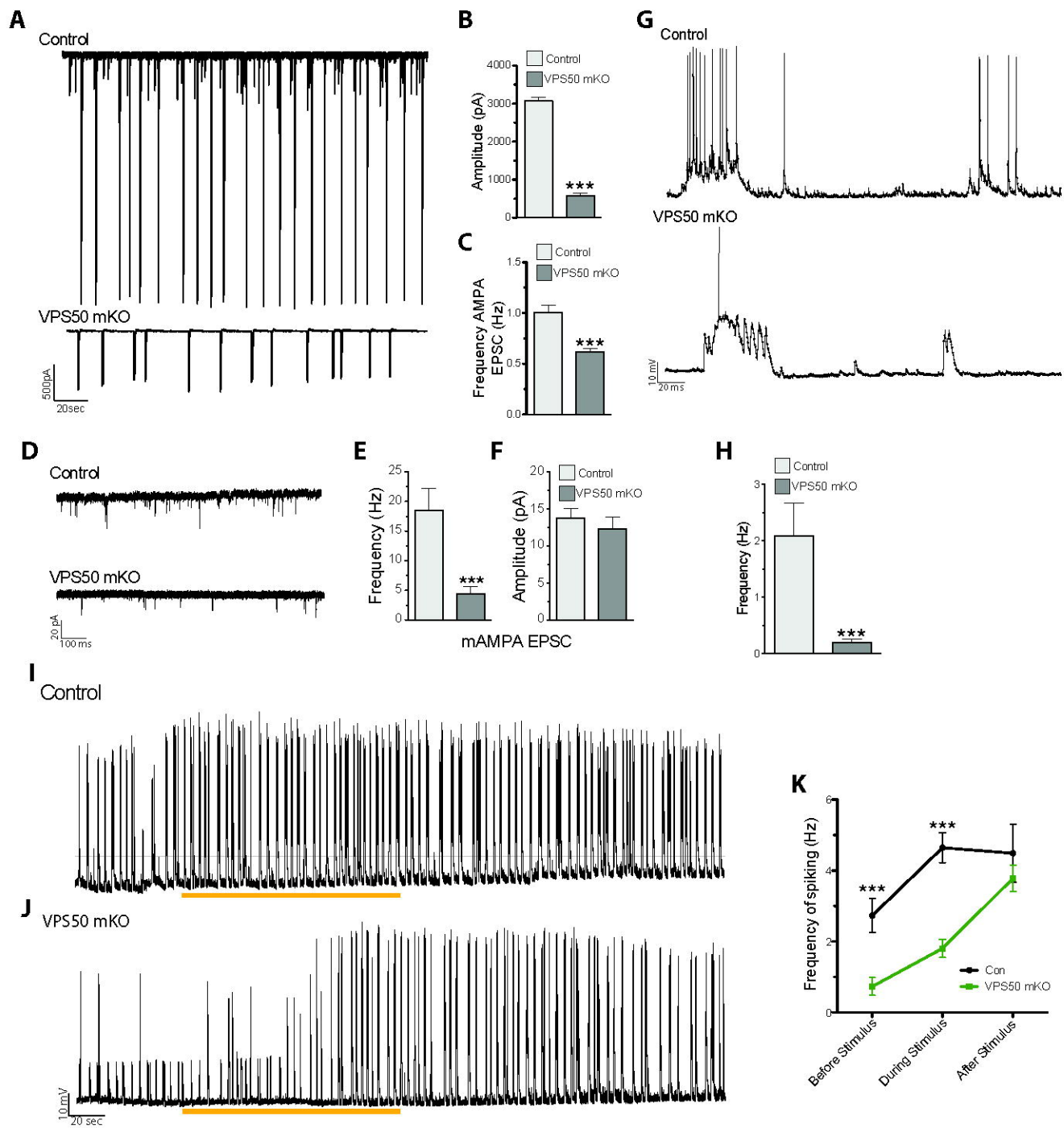
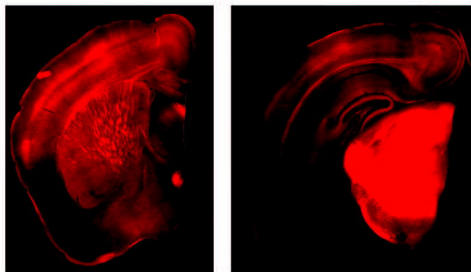
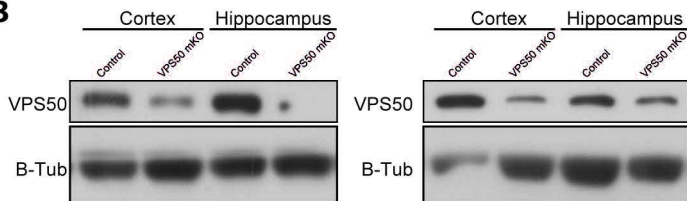


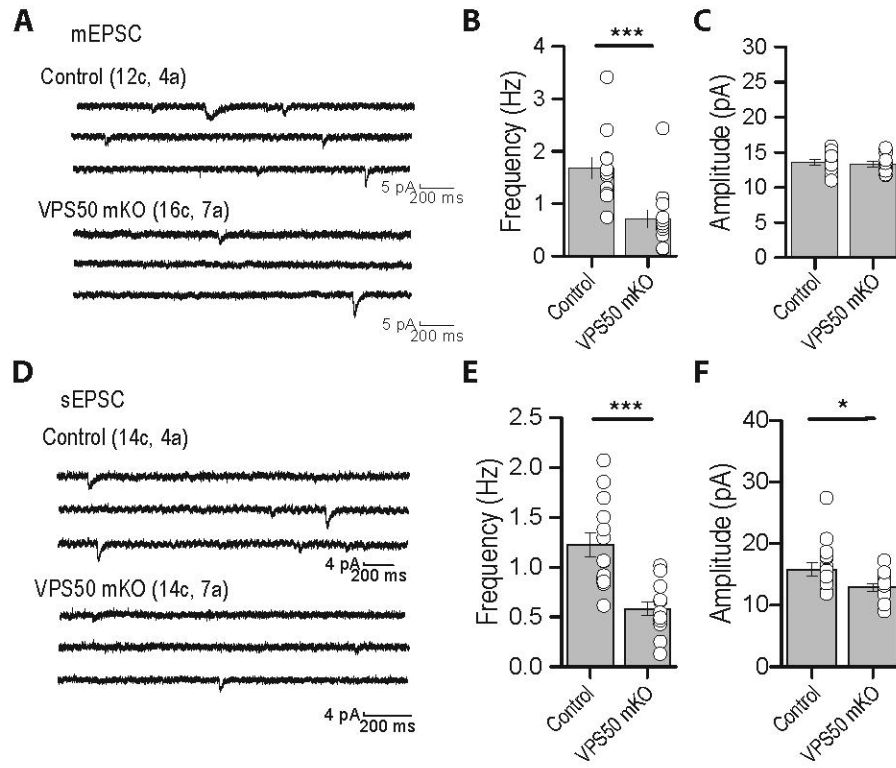
Figure 1. VPS50 gene editing causes decrease in synaptic vesicle acidification but no change in synaptic vesicle number. (A) Surveyor assay of Control or VPS50 mKO cortical neurons. (B) RT-qPCR to quantify relative mRNA expression of VPS50 mKO. (C-D) VPS50 protein expression in VPS50 mKO and Control neurons. (E-F) Representative images of synaptic terminals by electron microscopy and quantification of the number of synaptic vesicles in Control and VPS50 mKO neurons (n=50 cells per condition). (G) Representative images of Ratio-SyPhy signal to determine synaptic vesicle acidification in Control and VPS50 mKO neurons. Scale bar, 25 μ m. ***p<0.001.

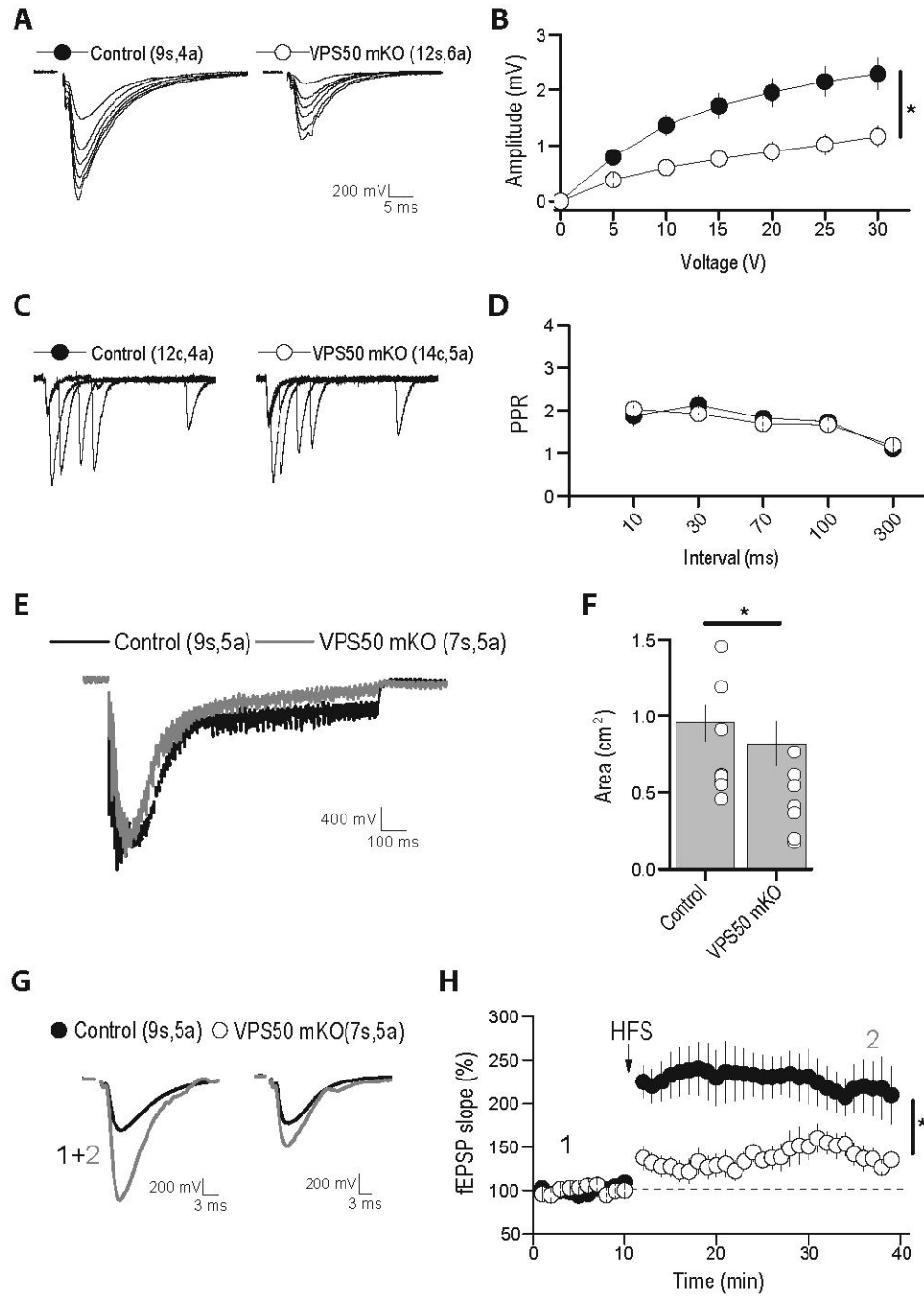


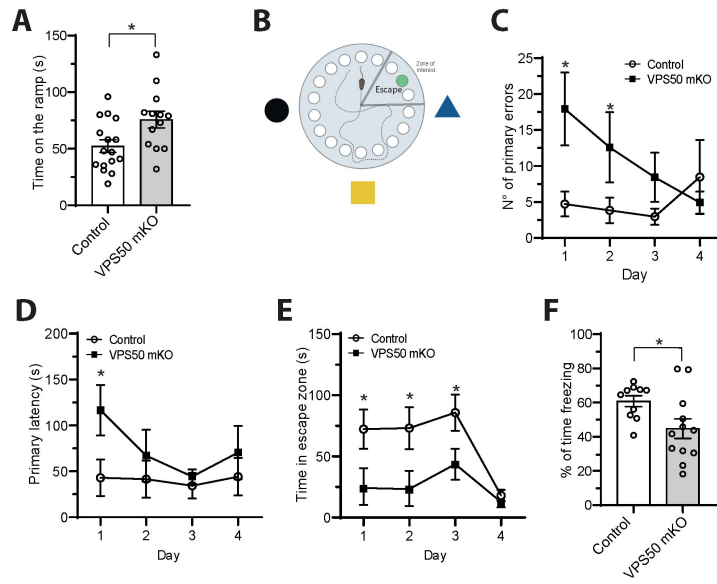


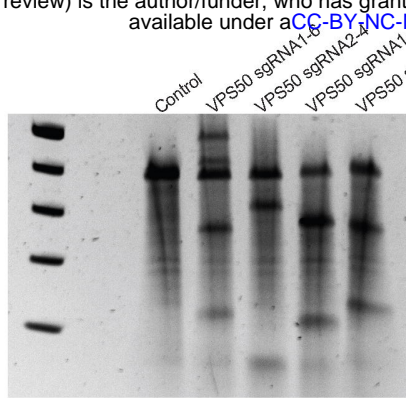
Ahumada et al., Figure 3.

A**B**









B

Control

GGAAAGGCTGACCCAGGCGGGCAGCTCCACGTGACGACTCACTAGGCTTCCCTGTGTTGTAGCCGGCTGTCTGGTGGGATTATGTGATTTGTTACTTCTCCGAGGCAGG
sgRNA6

VPS50 Exon 1

VPS50 EDITED sgRNA1

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GGAAAGGCTGACCCAGGCGG-----GGGATTATGTGATTTGTTACTTCTCCGAGGCAGG
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