

1 **Post-mitotic Prox1 expression controls the final specification of**
2 **cortical VIP interneuron subtypes**

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11 **Number of Figures:** 4

12 **Number of Supplementary Figures:** 4

13 **Summary**

14 Neuronal identity is controlled in multiple developmental steps by key transcription factors that
15 determine the unique properties of a cell. During embryogenesis, the transcription factor Prox1
16 has been shown to regulate VIP interneuron migration, survival, and as a result, circuit
17 integration. Here, we explore the role of Prox1 as a regulator of genetic programs that guide the
18 final specification of VIP interneuron subtypes in early post-natal life. Using in-vitro
19 electrophysiology we find that post-natal removal of Prox1 differentially affects the synaptic
20 integration of VIP bipolar and multipolar subtypes.

21 RNA sequencing reveals that one of the downstream targets of Prox1 is the postsynaptic
22 protein Elfn1, a constitutive regulator of presynaptic release probability. Genetic,
23 pharmacological and electrophysiological experiments demonstrate that knocking out Prox1
24 reduces Elfn1 function in VIP multipolar but not in bipolar cells. Thus, in addition to the activity-
25 dependent and contextual processes that finalize developmental trajectories, genetic programs
26 engaged by Prox1 control the differentiation and connectivity of VIP interneuron subtypes.

27 **Introduction**

28 Although cortical interneuron (IN) diversity begins at their place of birth within distinct embryonic
29 progenitor domains (Mi et al., 2018), single cell sequencing and manipulation experiments at
30 different developmental stages have suggested that INs undergo their final specification while
31 integrating into the developing circuit (De Marco García et al., 2011; Mayer et al., 2018). The
32 developmental mechanisms by which distinct types of INs acquire their mature characteristics
33 are only beginning to be revealed (Favuzzi et al., 2019). Interestingly, post-mitotic manipulations
34 have demonstrated a persistent requirement for key transcription factors (TF) in the final
35 specification and maintenance of pyramidal cell fate (De La Rossa et al., 2013). Whether similar
36 TF mechanisms exist for cortical INs remains unknown.

37

38 Cortical vasoactive intestinal peptide-expressing (VIP) INs are a diverse population (Tasic et al.,
39 2018). Even though they make up less than 5% of all neurons, VIP INs are critically important
40 for cortical circuit maturation and their malfunction has been implicated in neurodevelopmental
41 disorders (Batista-Brito et al., 2017; Mossner et al., 2020). The TF Prospero-related homeobox1
42 (Prox1) is expressed by the majority of INs derived from the caudal ganglionic eminence (CGE).
43 Its removal during embryonic development impairs VIP cell migration, cell survival, as well as
44 dendritic development and afferent connectivity of the Calretinin-expressing (CR+) VIP bipolar
45 subtype (Miyoshi et al., 2015). Importantly, Prox1 remains expressed in all VIP INs as they
46 integrate into the developing circuit, suggesting it may have a role in organizing not only their
47 early stages of development, but also their final specification. In addition to requiring Prox1, it
48 has been shown that CR+ VIP bipolar cells also require proper network activity to acquire their
49 characteristic axo-dendritic profile, as do Reelin- and Somatostatin (SST)- expressing INs (De
50 Marco García et al., 2011, 2015; Pan et al., 2018). Although Prox1 is also expressed in the
51 cholecystokinin-expressing (CCK+) VIP multipolar subtype, these cells do not show any
52 positional or morphological deficits following activity manipulations (De Marco García et al.,

53 2011). Taken together, these findings raise the possibility that during the final developmental
54 steps of VIP IN specification, cell-autonomous and activity-dependent genetic programs work in
55 tandem to guide the network integration of distinct VIP subtypes in a differential manner.

56

57 In this study, we assess the post-natal requirement of Prox1 for the final diversification of VIP IN
58 subtypes. To achieve this, we conditionally remove this TF during the first post-natal week,
59 using a *VIPCre* driver mouse line, and explore the synaptic integration of bipolar and multipolar
60 VIP cells using functional and RNA screening methods. Surprisingly, we find that Prox1 removal
61 impacts the two subtypes differentially. Consistent with previous findings, bipolar cells have
62 reduced synaptic excitation upon removal of Prox1. In contrast, multipolar cells show instead an
63 alteration in the short-term synaptic dynamics of their incoming excitation. Using transcriptomic
64 screening and pharmacological manipulations we demonstrate that a Prox1-dependent
65 engagement of the trans-synaptic protein Elfn1 selectively enables the synaptic facilitation
66 observed in multipolar cells.

67 **Results**

68 **1) Postnatal Prox1 removal leads to changes in presynaptic release probability onto VIP
69 multipolar but not bipolar cells**

70 Cell-specific synaptic wiring properties are a prominent feature of IN cell type diversity. Previous
71 research found that embryonic Prox1 removal leads to aberrant network integration of VIP
72 bipolar cells (Miyoshi et al., 2015) while the effect on multipolar cells is unknown. Therefore, we
73 first wanted to test whether postnatal loss of function of Prox1 (KO) affects the network
74 integration of bipolar, as well as multipolar VIP cells. To postnatally (~P3) remove Prox1, we
75 used a VIP knock-in mouse line that drives the expression of Cre from the endogenous peptide
76 locus (*VIPCre*), combined with a conditional *Prox1* allele. The *Prox1* coding region is flanked by
77 *loxP* sites and recombination shifts eGFP into frame (*Prox1eGFP*) to label all Prox1+ VIP cells
78 (Figure 1A). We first surveyed spontaneous excitatory postsynaptic currents (sEPSCs) in L2/3

79 control (*Prox1* heterozygote; GFP labeled) and *Prox1* KO VIP INs in acute brain slices for
80 alterations which would indicate connectivity changes. Indeed, we found that postnatal
81 expression of *Prox1* regulates incoming excitatory inputs onto both VIP subtypes, albeit with
82 subtype specific differences in time course and valence (Supplementary Figure 1).

83 To reveal the mechanism by which *Prox1* regulates the strength of excitatory inputs onto the
84 two VIP subtypes we further examined the short-term dynamics of evoked synaptic responses
85 onto control and *Prox1* KO cells at P17-21 (Figure 1B). Changes in the paired pulse ratio (PPR)
86 of the 2nd to 1st evoked EPSC amplitude implicate a presynaptic versus postsynaptic change,
87 and provide an indication of whether presynaptic release probability for a given synapse is low
88 (PPR > 1), moderate (PPR ~ 1), or high (PPR < 1). Two electrical stimuli were delivered at 50
89 Hz through a glass pipette placed close to the soma and proximal dendrites of the recorded
90 cells. We found that glutamatergic synapses onto control VIP bipolar cells show negligible
91 synaptic facilitation (mean PPR: 1.15 ± 0.13) and that *Prox1* KO does not affect the PPR
92 significantly (mean PPR 0.93 ± 0.15) (Figure 1C, D). On the other hand, excitatory inputs onto
93 VIP multipolar cells show more pronounced facilitation in the control condition (mean PPR: 1.51 ± 0.11)
94 and a notable reduction of the PPR upon removal of *Prox1* (mean PPR: 1.13 ± 0.1)
95 (Figure 1E, F). These results demonstrate that in control conditions the initial release probability
96 of glutamatergic synapses onto VIP multipolar cells is lower than onto bipolar cells, in line with
97 previous reports showing that L2/3 CR+ bipolar cells display short-term synaptic depression at
98 10Hz and no change at 50Hz (Caputi et al., 2009) (<https://portal.brain-map.org/explore/connectivity/synaptic-physiology>). Importantly, the data also shows that
99 postnatal removal of *Prox1* leads to an increase in initial release probability of excitatory
100 synapses onto multipolar, but not bipolar cells.

102 **2) Loss of *Prox1* leads to a downregulation of the trans-synaptic protein *Elfn1***

103 Having identified a Prox1-dependent suppression of synaptic release probability onto multipolar
104 cells, we hypothesized that this may occur through the regulation of genes encoding for synaptic
105 proteins. To identify such potential downstream targets of Prox1 we performed a RNA
106 sequencing screen on control and Prox1 KO cells, after fluorescence activated cell sorting
107 (FACS) of the GFP+ VIP cells at P8 and P12 (Figure 2A). Differential gene expression analysis
108 of the data identified several potential candidate genes (Figure 2B, C) (Supplementary Figure
109 2C, D), which were analysed for gene ontology (GO) enrichment. The GO analysis revealed that
110 most of the upregulated genes in Prox1 KO cells are associated with glial cell programs (Figure
111 2D). This result suggests that in control VIP cells, post-natal expression of Prox1 suppresses
112 those glial programs, directing the cell instead towards a neuronal fate. In line with this, as well
113 as with our functional findings, the most enriched downregulated genes are associated with
114 synapses and synapse-associated signalling (Figure 2D, E). Within those synapse-associated
115 programs we specifically looked for genes that could mediate trans-synaptic interactions
116 between the postsynaptic VIP and the presynaptic excitatory cell to regulate synaptic release.
117 One such gene was the Extracellular Leucine Rich Repeat and Fibronectin Type III Domain
118 Containing 1 (*Elfn1*) (Figure 2C), which creates the strongly facilitating excitatory synaptic inputs
119 of SST INs (Sylwestrak and Ghosh, 2012). Postsynaptic Elfn1 produces cell-autonomous
120 suppression of presynaptic glutamate release through its trans-synaptic recruitment of
121 metabotropic-glutamate receptor 7 (mGluR7) (Stachniak et al., 2019; Tomioka et al., 2014).
122 The RNA sequencing data showed that the level of *Elfn1* mRNA in VIP cells is high at both P8
123 and P12 (Supplementary Figure 3A). To assess if *Elfn1* is continuously expressed into
124 adulthood, we consulted published single-cell RNA sequencing data (Tasic et al., 2018) and
125 found that, in the adult cortex, almost all VIP cells express *Elfn1* (Supplementary Figure 3B).
126 Furthermore, this dataset also shows a persistent expression of *Prox1*, which suggests a
127 continued requirement for these two factors in the maintenance of cell function throughout life.

128 **3) Reduction in Elfn1 expression recapitulates the Prox1 KO phenotype in VIP multipolar**
129 **cells**

130 To test if Elfn1 is the downstream molecule responsible for the synaptic phenotype in Prox1 KO
131 multipolar VIP cells we used a compound mouse line that labeled VIP neurons (*VIPCre* x
132 *tdtomato* reporter; *Ai14*) in the background of a germline *Elfn1* KO allele (*Elfn1KO*) (Figure 3A).
133 We chose to compare VIP *tdtomato*+ cells from heterozygous *Elfn1KO* (Het Elfn1) animals to
134 those from wildtype (control) littermates, to match our findings in the RNA sequencing screen,
135 which showed a two-fold reduction of *Elfn1* in Prox1 KO cortex (Figure 2C). We found that
136 reducing Elfn1 expression does not affect the PPR significantly in VIP bipolar cells (control
137 mean PPR: 1.15 ± 0.14 ; Het Elfn1 mean PPR 0.98 ± 0.08) (Figure 3B, C). On the other hand,
138 excitatory inputs onto VIP multipolar cells showed a notable reduction in the PPR when Elfn1
139 levels are reduced (control mean PPR: 1.30 ± 0.13 ; Het Elfn1 mean PPR: 0.88 ± 0.08) (Figure
140 3D, E). Our results show that a decrease in Elfn1 expression recapitulates the effect of
141 knocking-out Prox1 in VIP multipolar cells but has no notable effect in bipolar cells. This finding
142 suggests that Prox1 is important for initiating and/or maintaining the expression of Elfn1 in
143 multipolar cells, which leads to facilitation of incoming excitatory synaptic responses.

144 **4) Prox1-dependent engagement of Elfn1 in VIP cells**

145 To directly test the relationship between Prox1 and the expression of Elfn1 in multipolar and
146 bipolar cells, we turned to a pharmacological agent, (RS)- α -Methylserine-O-phosphate (MSOP),
147 that acts as an antagonist for presynaptic mGluRs (including mGluR7), which are reported to
148 interact trans-synaptically with Elfn1 (Figure 3F) (Dunn et al., 2018). Application of MSOP
149 indirectly tests for the presence of Elfn1 in control and Prox1 KO VIP cells by blocking the
150 constitutive suppression of synaptic release that Elfn1 induces through mGluRs (Stachniak et
151 al., 2019). We therefore evoked excitatory synaptic events onto the two VIP subtypes, as

152 described above, and assessed changes in neurotransmitter release in response to MSOP. We
153 found that MSOP did not affect the release probability onto control (PPR: 1.11 ± 0.18 at
154 baseline vs. 1.04 ± 0.14 in MSOP) or Prox1 KO VIP bipolar cells (PPR: 0.95 ± 0.29 at baseline
155 vs. 0.88 ± 0.24 in MSOP) (Figure 3G, H). In contrast, MSOP markedly increased the initial
156 release probability of excitatory inputs onto control VIP multipolar cells and thus reduced the
157 paired pulse ratio (PPR: 1.43 ± 0.14 at baseline vs. 1.11 ± 0.09 in MSOP) (Figure 3I). However,
158 the effect of MSOP was absent in the Prox1 KO VIP multipolar cells (PPR: 1.37 ± 0.19 at
159 baseline vs. 1.23 ± 0.21 in MSOP) (Figure 3J).

160 In previous studies, both VIP bipolar and multipolar cells were shown to express *Elfn1* in the
161 adult cortex (Paul et al., 2017; Tasic et al., 2018). This mRNA data stands in apparent
162 contradiction to the VIP multipolar cell-selective effects of *Elfn1* we observe in our Prox1 loss-of-
163 function, *Elfn1* downregulation, and pharmacological experiments. We therefore hypothesized
164 that either only multipolar cells express *Elfn1* before P21, or that Prox1 selectively regulates
165 *Elfn1* expression in multipolar cells. To test these two possibilities, we collected and sectioned
166 brains from Prox1 conditional postnatal KO mice, (*Prox1fl* x *VIPCre* x *Ai14*) at P12 and
167 performed *in situ* hybridization for *Elfn1* mRNA and *CR* mRNA to distinguish putative bipolar
168 (*CR+*) and multipolar (*CR-*) VIP cells (Figure 4A, B). We then obtained images of whole cortices
169 and analyzed them using a custom-written MATLAB script (see Methods). We first assessed the
170 presence of *Elfn1* mRNA in control VIP cells at P12, which showed that, similarly to the adult
171 cortex (Paul et al., 2017; Tasic et al., 2018), *CR+* VIP cells express the gene at a higher level
172 than *CR-* VIP cells (Figure 4C, Supplementary Figure 4C). This finding negates the first
173 hypothesis regarding age-dependent selective expression of *Elfn1* in multipolar cells.
174 We subsequently compared *Elfn1* and *CR* expression between control and KO tissue and found
175 a clear reduction of both signals (Figure 4D, E), which is in line with the RNAseq results that
176 showed a downregulation of both genes (log2 ratio of -1.466 for *CR* and -1.065 for *Elfn1*).

177 Intriguingly, this reduction was seen for both *CR*+ (bipolar) VIP cells and *CR*- VIP ones (many of
178 which belong to the multipolar subtype) (Figure 4F, G, Supplementary Figure 4D, E). These
179 results suggest that Prox1 regulates *Elfn1* in both subtypes of VIP cells. Hence our second
180 hypothesis is also negated, leaving open other possible mechanisms that are discussed below.

181 **Discussion**

182 In the mammalian nervous system the TF Prox1 is known to regulate cell-cycle exit (Cid et al.,
183 2010; Kaltezioti et al., 2010) and cell fate determination (Iwano et al., 2012; Kaltezioti et al.,
184 2014) of neural precursor cells. Post-mitotic removal of Prox1 in embryonic CGE-derived
185 cortical INs results in a failure of *CR*+ VIP INs to migrate to the correct cortical layers, a
186 dramatic decrease in their numbers, and a subsequent reduction in the excitatory synaptic input
187 onto the remaining cells (Miyoshi et al., 2015). Postnatal removal of Prox1 circumvents cell
188 death and layer mis-targeting. Nevertheless, we find a continued requirement for the TF in the
189 regulation of the proper synaptic integration and final specification of VIP subtypes. Our results
190 demonstrate that post-natal expression of Prox1 is necessary for maintenance of expression
191 levels of the bipolar cell marker *CR*, and for both VIP bipolar and multipolar cells to connect
192 properly to their cortical networks. Furthermore, we show that Prox1 is necessary for synaptic
193 facilitation of excitatory inputs onto VIP multipolar cells and that it exerts this function by
194 regulating *Elfn1* expression. An emerging theme from single cell transcriptomics studies is that
195 cell adhesion molecules like *Elfn1* may determine the cell specific interactions that guide
196 connectivity profiles of IN subtypes (Paul et al., 2017). Accordingly, by regulating VIP subtype
197 specific *Elfn1* engagement, the TF Prox1 promotes and maintains functional diversity for VIP IN
198 subtypes.

199 The selective engagement of *Elfn1* in VIP multipolar cells leads to excitatory synaptic facilitation
200 through a reduction in the initial release probability. In SST INs this *Elfn1*-dependent facilitation

201 of excitatory inputs prevents rapid recruitment, thereby creating the characteristic “delayed”
202 firing of these INs (Sylwestrak and Ghosh, 2012) and biasing responsiveness towards high
203 frequency activity (Pouille and Scanziani, 2004; Stachniak et al., 2019). The same mechanism
204 would allow VIP multipolar cells to selectively tune to the high frequency activity characteristic of
205 cortico-cortical communication (Palmer et al., 2012). Thus, by regulating Elfn1, Prox1 may prime
206 these neurons to coordinate intra-cortical communication within the superficial layers of the
207 cortex. Interestingly, the SST INs do not express Prox1, therefore our results not only identify a
208 novel functional role for Elfn1 in VIP multipolar INs, but also reveal a novel regulatory pathway
209 for Elfn1 expression.

210 In contrast, short-term plasticity of excitatory inputs onto bipolar cells is unaffected by removal of
211 Prox1, by decreased Elfn1 expression, or by the pharmacological blockade of its synaptic
212 effects. These findings are surprising given that our results and published data show high levels
213 of *Elfn1* mRNA expression in bipolar VIP cells. Thus, it appears that in this VIP subtype, *Elfn1*
214 mRNA levels are uncoupled from Elfn1 function. An explanation for this discrepancy between
215 expression and function could be that the presynaptic excitatory terminals onto bipolar cells lack
216 mGluR7, despite the presence of Elfn1 protein. This could arise if distinct cell populations target
217 VIP bipolar and multipolar cells, which, for example, may receive different amounts of thalamo-
218 cortical and cortico-cortical inputs. The two subtypes tend to sit in different parts of L2/3, with
219 VIP multipolar cells often found close to the border to L1, while VIP bipolar cells usually are
220 located closer to L4 (He et al., 2016). In combination with their distinct dendritic architecture, this
221 could determine the origin of inputs the VIP subtypes receive (Sohn et al., 2016). Additionally,
222 the distinct glutamate receptor composition expressed by the two VIP subtypes (Paul et al.,
223 2017) could also point to cell selective synaptic wiring. Alternatively, the cell type specificity we
224 observe could be a combination of Prox1-dependent and independent mechanisms that would
225 regulate subtype specific protein expression, such as differential microRNA expression or

226 alternative splicing. Indeed, *Elfn1* can be regulated by microRNAs, and showed a 75% increase
227 in expression following deletion of the *mirg* microRNA cluster in an induced neuronal culture
228 system (Whipple et al., 2020).

229 Even though we find that Prox1 does not affect release probability onto bipolar cells, it clearly
230 plays a role in the cells' synaptic integration into the circuit. Previous work demonstrated a
231 critical role for electrical activity in the final specification of VIP bipolar cells (De Marco García et
232 al., 2011). This finding may well relate to the differential expression of excitatory synaptic
233 components such as the subunits of NMDA receptors. Published research has shown that adult
234 cortical bipolar VIP cells have high expression of the NR2B subunit (Paul et al., 2017). This
235 finding is intriguing given that the receptors containing this subunit are critical for the integration
236 of superficial Reelin-positive INs, via their activation by thalamocortical terminals (De Marco
237 García et al., 2015). Although we find no evidence for direct regulation of NR2B expression by
238 Prox1, the regulation of cofactors that control thalamocortical excitation through NR2B-
239 containing receptors could underlie the observed Prox1 KO phenotype in bipolar cells.

240 The final specification of neurons is a process that takes place after the cells' birth, as they start
241 integrating into the resident circuit. It is during this time of establishing connections that the
242 needs of the circuit, as defined by those of the animal, can instruct a neuron towards its mature
243 state, which includes the proper construction and function of inputs and outputs. In inhibitory INs
244 these input/output specificities vary considerably, not only between cardinal IN classes, but also
245 between the subtypes within a class (Huang and Paul, 2019). Our data provides evidence that
246 the continuous expression of the TF Prox1 is necessary for the final specification of bipolar and
247 multipolar VIP cells, allowing them to acquire diverging roles within the adult cortical network.

248 **Material and Methods**

249 **Mice**

250 All animal experiments were approved by the Cantonal Veterinary Office Zurich and followed
251 Swiss national regulations. Animal lines used in this study are: *VIPCre* (*Vip*^{tm1(cre)Zjh/J}) (Taniguchi
252 et al., 2011), *Ai14* (B6;129S6-Gt(ROSA)^{26Sortm14(CAG-tdTomato)Hze/J}) (Madisen et al., 2010), *Prox1fl*
253 (*Prox1*^{tm2Gco}) (Harvey et al., 2005), *Prox1eGFP* (*Prox1*^{tm1.1Fuma}) (Iwano et al., 2012) and *Elfn1KO*
254 (*Elfn1*^{tm1(KOMP)Vlcg}) (created by the Knock Out Mouse Project). The following compound lines
255 were created for this study: *VIPCre* x *Prox1eGFP* and *VIPCre* x *Ai14* x *Elfn1KO* for
256 electrophysiology experiments, *VIPCre* x *Ai14* x *Prox1fl* for *in situ* experiments. All crosses were
257 setup to produce both KO and Control animals in the same litter and littermate controls were
258 used throughout the study.

259 **Electrophysiology**

260 Whole-cell patch-clamp electrophysiological recordings were performed on GFP labelled VIP
261 neurons located in neocortical layers II–III of barrel cortex (approximately bregma -0.5 to -2.0
262 mm) in acute brain slices prepared from P17–P22 male and female mice. Briefly, animals were
263 decapitated and the brain was dissected out and transferred to cold cutting solution containing
264 (in mM): 75 sucrose, 87 NaCl, 25 NaHCO₃, 25 D-glucose, 2.5 KCl, 7 MgCl₂, and 1.25 NaH₂PO₄,
265 aerated with 95% O₂ / 5% CO₂. 300 µm slices were recovered in artificial cerebrospinal fluid
266 (ACSF) composed of (in mM): 128 NaCl, 3 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂,
267 and 10 glucose at 34°C for 15 min. Acute slices were perfused at a rate of 2–3 ml/min with
268 oxygenated recording ACSF at room temperature. Patch electrodes were made from
269 borosilicate glass (Harvard Apparatus) and had a resistance of 2-4 MΩ. The intracellular
270 solution contained (in mM): 126 cesium methanesulfonate, 4 CsCl, 10 HEPES, 20
271 phosphocreatine, 4 MgATP, 0.3 NaGTP, pH 7.3, 290 mOsm, with addition of 2.5 mg/mL of
272 biocytin.

273 Experiments were performed in voltage-clamp mode using the Axopatch 200B amplifier
274 (Molecular Devices). sEPSCs were recorded at a holding potential (V_h) = -65 mV, with a
275 sampling rate of 10 kHz and were filtered on-line at 2 kHz. Access resistance was monitored to
276 ensure the stability of recording conditions. Recordings with access resistance >40 M Ω , whole
277 cell capacitance <4 pF or holding current >200 pA were excluded. No compensation was made
278 for access resistance and no correction was made for the junction potential between the pipette
279 and the ACSF. Following a baseline stabilization period (3 min), synaptic currents recorded in
280 2x 3 min traces were low pass filtered at 400 Hz, then analyzed using clampfit event detection
281 template match. Evoked synaptic responses were recorded at $V_h = -70$ mV. Electrical
282 stimulation from a Digitimer isolated stimulator (DS2A Mk.II) was through a monopolar glass
283 pipette (2-4 M Ω) positioned in L2/3. The stimulating electrode was placed typically 50 - 150 μ m
284 from the recorded cell, parallel to the pial surface. Stimulation intensity and duration were
285 adjusted to produce stable evoked EPSC amplitudes. Stimulation intensities were larger for
286 bipolar cells (86 ± 1 V for 103 ± 6 μ s) than for multipolar cells (74 ± 2 V for 86 ± 6 μ s, $p = 3 \times 10^{-6}$,
287 $p = 0.06$), but did not differ between genotypes (multipolar prox1 control: 69 ± 3 V for 57 ± 5 μ s,
288 KO: 71 ± 4 V for 74 ± 10 μ s, $p = 0.6$, $p = 0.2$; bipolar prox1 control: 85 ± 2 V for 91 ± 9 μ s, KO: $84 \pm$
289 3 V for 88 ± 15 μ s, $p = 0.9$, $p = 0.9$; multipolar Elfn1 control: 77 ± 3 V for 114 ± 13 μ s, Het: 82 ± 3 V
290 for 125 ± 19 μ s, $p = 0.2$, $p = 0.6$; bipolar Elfn1 control: 86 ± 3 V for 113 ± 13 μ s, Het: 89 ± 2 V for
291 130 ± 15 μ s, $p = 0.3$, $p = 0.4$, t test). Paired pulse ratios (PPR) were calculated from an average
292 of 12 sweeps, after a 2-minute stable baseline was established. Bath applied compound MSOP
293 (100 μ M, pH 7.4) was dissolved in water.

294 **Electrophysiology data analysis**

295 Values are represented as mean \pm SEM. Number of measurements n/N indicates cells recorded
296 (n) from animals (N), typically using 1 cell per slice to recover biocytin-stained cell morphology.
297 Cell type was classified as bipolar vs. multipolar based on cell body morphology and number of

298 dendritic processes emanating from it, using both initial determination prior to patching and
299 post-hoc verification of recovered biocytin-labelled cells. Matched recordings were performed
300 with Prox1 control and Prox1 KO littermates on the same day, whenever possible. Statistical
301 testing was done in Matlab. Comparisons within conditions were made by two-tailed paired
302 Student's t-test, treatment versus baseline. Comparisons across conditions or between
303 genotypes were done with an unpaired t-test assuming unequal variance. To accommodate
304 skewed distributions in spontaneous synaptic frequency and amplitude, we performed
305 Kolmogorov-Smirnov non-parametric testing on event distributions and Wilcoxon non-
306 parametric testing on averages (mean frequency, median amplitude). For multiple comparisons,
307 a one-way or two-way ANOVA was done with a Bonferroni post hoc test. Statistical outcomes
308 are represented in figures as: n.s. $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

309 **Cell sorting and RNA sequencing**

310 EGFP-labeled cells were purified from the P8 and P12 cortices of control heterozygous and
311 Prox1 KO animals (*VIPCre x Prox1eGFP/+* and *VIPCre x Prox1eGFP/eGFP*) as described
312 (Hempel et al., 2007). Briefly, animals were anesthetized in 2% vol/vol isoflurane (~1.5min),
313 decapitated, the brain was extracted, the olfactory bulb and cerebellum were removed, and the
314 brain was cut into 400-um thick coronal sections using a vibratome (Leica VT1000S; Leica,
315 speed 5, vibration frequency 7-8), while in bubbling ice cold ACSF (recipe as above, with 1mM
316 CaCl₂ and 1mM MgCl₂) in the chamber. Sections were collected and transferred into a
317 protease digestion solution of ACSF (with Pronase (1mg ml-1), for 25 minutes and then
318 transferred into a quenching solution of ACSF (with 1%FBS). Microdissection of the cortex to
319 include the somatosensory areas was performed using a fluorescent dissection microscope
320 (Olympus, MVX 10). Dissected cortices were collected in a 15mL falcon tube containing 1.5mL
321 of ACSF sorting solution containing 1%FBS and DNase and cells were dissociated by gently
322 triturating ten times with a large, then medium, and finally small fire-polished Pasteur pipette

323 while avoiding the generation of bubbles. The cell suspension was then passed through a 50uM
324 filter (Sysmex CellTrics) before automated fluorescence-activated cell sorting (FACS) using the
325 MoFlo™ or FACSAria™ devices. A GFP negative littermate control cortex was also included as
326 a negative control for the FACS setup. Cells were collected into Arcturus Picopure extraction
327 buffer and immediately processed for RNA isolation using the Arcturus PicoPure Isolation Kit
328 (KIT0204). RNA quality and quantity was measured using Agilent High Sensitivity RNA
329 ScreenTape system (High Sensitivity RNA Screen Tape 5067-5579-5580-5581). All samples
330 had high quality scores between 6-8 RIN. 4-5 RNA samples of each genotype for each of the
331 two age groups (P8 and P12) were used to prepare 19 barcoded libraries.

332 The libraries were prepared using the Smart-seq2 protocol (Picelli et al., 2013). Briefly, total
333 RNA was placed in 4 μ l of lysis buffer (0.1% vol/vol Triton X-100, 2.5 mM dNTPs, 2.5 μ M oligo-
334 dT, 1 U/ μ l Promega RNasin Plus RNase inhibitor). Reverse transcription was performed
335 followed by cDNA amplification. The quality of the cDNAs was evaluated using an Agilent 2100
336 Bioanalyzer. The resulting cDNA (1 ng) was fragmented using Illumina Nextera XT according to
337 standard protocol. Nextera adapters containing Unique Dual Indices (UDI) were added by PCR.
338 The libraries were double-sided size, selected and quantified using an Agilent 4200 TapeStation
339 System.

340 TruSeq SR Cluster Kit HS4000 (Illumina, Inc, California, USA) was used for cluster generation
341 using 10 pM of pooled normalized libraries on the cBOT. Sequencing was performed on the
342 Illumina HiSeq 4000 single end 100 bp, using the TruSeq SBS Kit HS4000 (Illumina, Inc,
343 California, USA).

344 **RNA sequencing data analysis**

345 The raw reads were first cleaned by removing adapter sequences, trimming low quality ends,
346 and filtering reads with low quality (phred quality <20) using Trimmomatic (Version 0.33) (Bolger

347 et al., 2014). The read alignment was done with STAR (v2.5.3a)(Dobin et al., 2013) As
348 reference we used the Ensembl genome build GRCm38. with the gene annotations downloaded
349 on 2015-06-25 from Ensembl. The STAR alignment options were "--outFilterType BySJout --
350 outFilterMatchNmin 30 --outFilterMismatchNmax 10 --outFilterMismatchNoverLmax 0.05 --
351 alignSJDBoverhangMin 1 --alignSjoverhangMin 8 --alignIntronMax 1000000 --
352 alignMatesGapMax 1000000 --outFilterMultimapNmax 50".

353 Gene expression values were computed with the function featureCounts from the R package
354 Rsubread (v1.26.0) (Liao et al., 2013). The options for featureCounts were: min mapping quality
355 10 - min feature overlap 10bp - count multi-mapping reads - count only primary alignments -
356 count reads also if they overlap multiple genes. One sample was excluded from further analysis
357 based on quality control standards (Supplementary Figure 2A, B).

358 To detect differentially expressed genes we applied a count based negative binomial model
359 implemented in the software package EdgeR (R version: 3.6.0, EdgeR version: 3.26.1)
360 (Robinson et al., 2009). The differential expression was assessed using an exact test adapted
361 for over-dispersed data. Genes showing altered expression with adjusted (Benjamini and
362 Hochberg method) p-value <0.05 were considered differentially expressed.

363 A list of potential candidate genes at both P8 and P12 was generated by selecting genes that
364 had a $\log_{2}\text{ratio} \geq |0.5|$ and $p\text{-value} \leq 0.01$. This list was analysed for gene ontology (GO)
365 enrichment using g:Profiler (Raudvere et al., 2019) (<https://biit.cs.ut.ee/gprofiler/gost>).

366 ***In situ* experiments**

367 *VIPCre x Ai14 x Prox1fl* animals were sacrificed at P12. In short, the animals were deeply
368 anesthetized before transcardial perfusion with 1x PBS followed by ice-cold 4% PFA. The brains
369 were dissected and post-fixed in ice-cold 4% PFA for 1h before being placed in a 30% Sucrose
370 solution at 4°C for >24h for cryo-protection. The brains were embedded in OCT using a peel-

371 away mold and stored at -80°C. Coronal 20µm-thick brain sections containing barrel cortex
372 were cut and collected on-slide using a cryostat (Microm International, HM 560M) and the slides
373 were stored at -80°C until further processing.

374 *In situ* hybridization for *Elfn1* mRNA and *CR* mRNA was performed using the RNAscope kit
375 (RNAscope Intro Pack for Multiplex Fluorescent Reagent Kit v2- Mm, 323136). Briefly, the slides
376 were thawed, OCT residue was removed using 1x PBS (3x5min washes). The slides were then
377 baked for 30mins at 60°C, post-fixed for 30mins in 4% PFA, dehydrated in an Ethanol dilution
378 series (50%, 70%, 2x 100%) and incubated with RNAscope Hydrogen Peroxide for 10mins.
379 RNAscope 1x Target Retrieval Reagents was brought to the boil and the slides were
380 submerged for 2mins. Protease III was added to the slides and left to incubate for 45mins at
381 40°C. After the pre-treatment the probes (Mm-Calb2, 313641 and Mn-Elfn1, 449661-C3) were
382 hybridized to the slices for 2h at 40°C and the signal was amplified using branched DNA
383 amplification methods and visualized with Opal dyes (Opal 520 FP1487001KT and Opal690
384 FP1497001KT).

385 Following the RNAscope protocol the slices were immunostained to retrieve the tdtomato signal
386 that was lost during the RNAscope protocol. Slides were washed two times with 1x PBS for
387 5min before being blocked for 30mins using 10% normal donkey serum and 1% bovine serum
388 albumin (BSA) in 1xPBS. The primary antibody against tdtomato (goat anti tdTomato, SICGEN
389 Antibodies, AB8181-200) was diluted 1:700 in 1xPBS-1%BSA and left to incubate at 4°C
390 overnight followed by a 2h incubation of secondary antibody at room temperature (donkey anti
391 goat Cy3, Jackson Immuno Research 705-165-147, diluted 1:1000 in 1xPBS-1%BSA). Slides
392 were cover-slipped with Fluoromount-G with DAPI (00-4959-52) and imaged using a
393 Slidescanner (Zeiss, AxioScan Z1). Mosaic images of the whole cortex were taken using a 20x
394 objective.

395 ***In situ* data analysis**

396 Image analysis was performed using custom written MATLAB codes. A two-dimensional
397 difference of Gaussian feature enhancement algorithm was used to improve the VIP-tdtomato
398 images, followed by Otsu thresholding to get an initial segmentation of neuronal cell bodies. To
399 ensure an accurate representation of the cell body, segmentation of VIP INs was finalized using
400 active contour segmentation (Kass et al., 1988) (MATLAB functions fspecial, conv2, graythresh
401 and activecontour were used for segmentation). “

402 During preprocessing of the *CR* and *Elfn1* images background subtraction was performed using
403 a disk-shaped structuring element (MATLAB functions fspecial and imfilter were used for
404 background subtraction). Subsequently, fluorescent intensity levels of both *CR* and *Elfn1* were
405 measure within each of the segmented VIP IN cell bodies. Layers 1-6, as well as the
406 somatosensory region, were segmented manually using the DAPI channel as reference
407 (Supplementary Figure 4A, B) (MATLAB functions bwlabel, bwconncomp, regionprops were
408 used for manual segmentation). To define whether a VIP cell is *CR*+ we followed a Bayesian
409 approach by assuming 80% of VIP cells are *CR*+ (Kubota et al., 2011). Hence, once mean CR
410 intensity per cell was calculated for each image, the population was thresholded such that
411 approximately 80% of total VIP cells are considered CR positive. Statistical analysis of the data
412 was done using the Mann-Whitney U test.

413 **Data and Code availability**

414 Data and custom written codes are available upon request.

415 **Author Contribution**

416 Conceptualization & Methodology T.J.S., R.K. and T.K.; Formal Analysis T.J.S., R.K. and
417 A.Ö.A.; Investigation T.J.S., R.K. and O.H.; Writing-Original Draft, R.K.; Writing-Review &
418 Editing, T.J.S., R.K., O.H., A.Ö.A. and T.K.; Supervision & Funding Acquisition, T.K.

419 **Acknowledgments**

420 We would like to thank G. Fishell for helpful discussions and for supporting the initiation of this
421 project in his lab, while at NYU Medical Center, by providing access to the Prox1fl and
422 Prox1eGFP mice. We would also like to thank L. Ibrahim for her comments and efforts on this
423 project. Furthermore, we thank F. Matsuzaki for his generous gift of the conditional Prox1eGFP
424 mouse line (<http://www2.clst.riken.jp/arg/mutant%20mice%20list.html> CDB0482K). We are
425 grateful to C. Aquino and L. Opitz at the Functional Genomics Center Zurich (FGCZ) who
426 performed the sequencing of the extracted mRNA and data analysis. Slidescanner imaging was
427 performed with equipment maintained by the Center for Microscopy and Image Analysis (ZMB),
428 University of Zurich. This work was supported by grants from the European Research Council
429 (ERC, 679175, T.K), the Swiss National Science Foundation (SNSF, 31003A_170037, T.K),
430 Fond zur Förderung des Akademischen Nachwuchs of the UZH Alumni (T.K) and the Swiss
431 Foundation for Excellence in Biomedical Research (R.K and T.K). O.H was supported by an
432 EMBO post-doctoral fellowship.

433 **Additional Information**

434 Supplementary Figures S1-S4

435 The authors declare no competing interests

436 **References**

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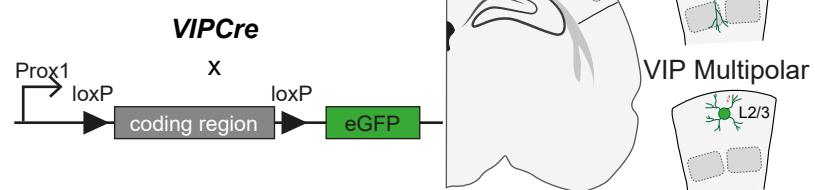
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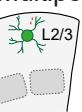
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B P17-21

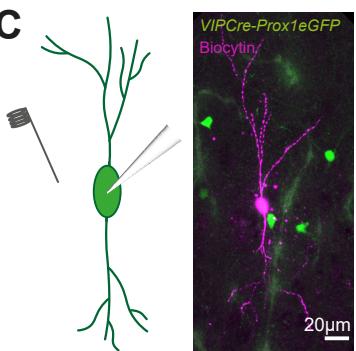
Postnatal KO of Prox1



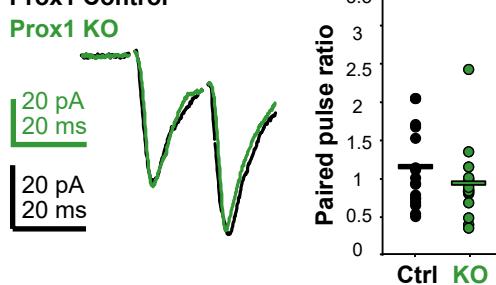
VIP Multipolar



VIP Bipolar evoked excitatory synaptic response

C

VIP Bipolar

D

VIP Multipolar evoked excitatory synaptic responses

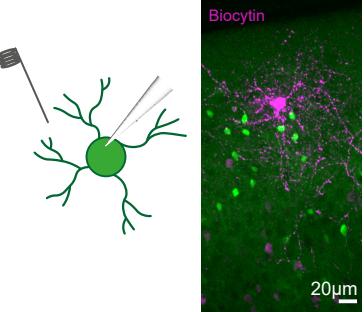
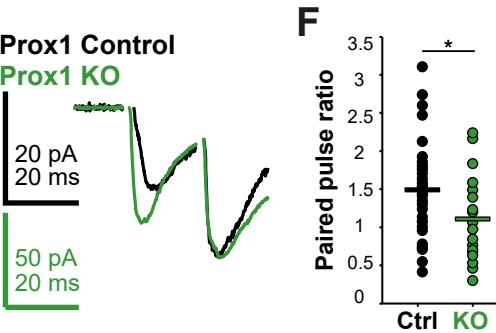
E**F**

Figure 1: Postnatal Prox1 removal leads to changes in presynaptic release probability onto VIP multipolar but not bipolar cells

(A) Visual representation of Prox1 conditional knock-out strategy. VIPCre turns on postnatally and removes part of the coding region of the Prox1 locus shifting eGFP into frame and allowing for the visualization of the control (Prox1 het) and Prox1 KO cells.

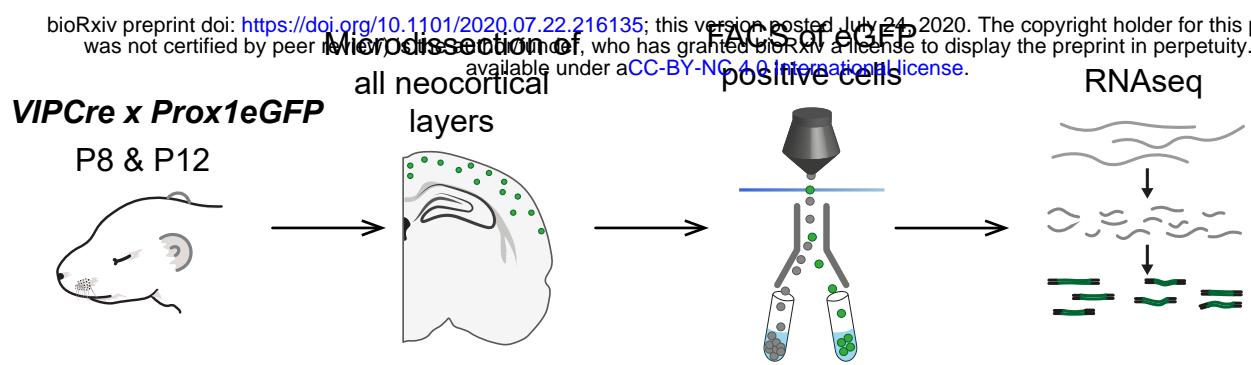
(B) Schematic representation of the experiment. L2/3 multipolar and bipolar VIP cells were recorded in the somatosensory barrel cortex at P17-21 of acute brain slices from control and Prox1 KO animals.

(C) & (E) Left: schematic representation for probing the evoked excitatory synaptic responses onto control and Prox1 KO VIP cells. The stimulating electrode is shown close to the soma and proximal dendrites of the eGFP-positive cells, which are recorded in whole-cell patch clamp mode. Middle: the recorded cells were filled with biocytin and their morphology revealed post-hoc. Right: examples of a pair of evoked synaptic responses for control and KO cells, which are overlaid and scaled to the second response.

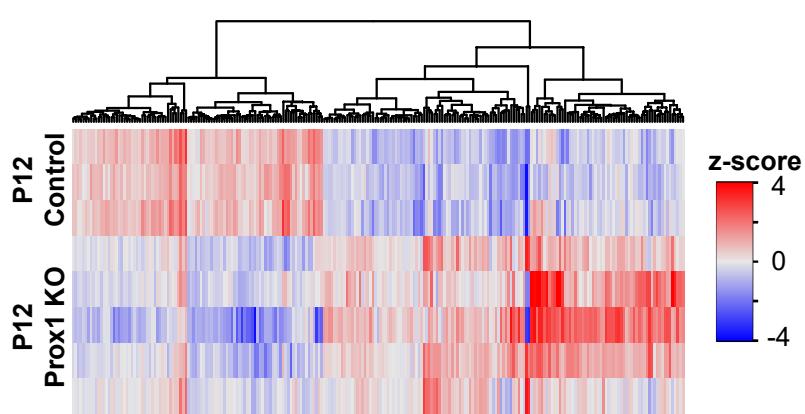
(D) All data points for paired pulse ratio (PPR; 2nd/1st response) for control (n/N=18/11) and KO (n/N=13/11) bipolar VIP cells, $p = 0.3$, $t = 1.1$.

(F) All data points for paired pulse ratio of control (n/N=33/18) and KO (n/N=19/14) multipolar VIP cells, $p = 0.03$, $t = 2.17$. Statistics: t-test.

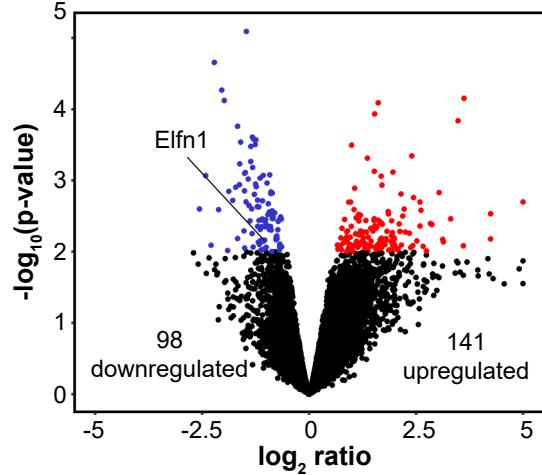
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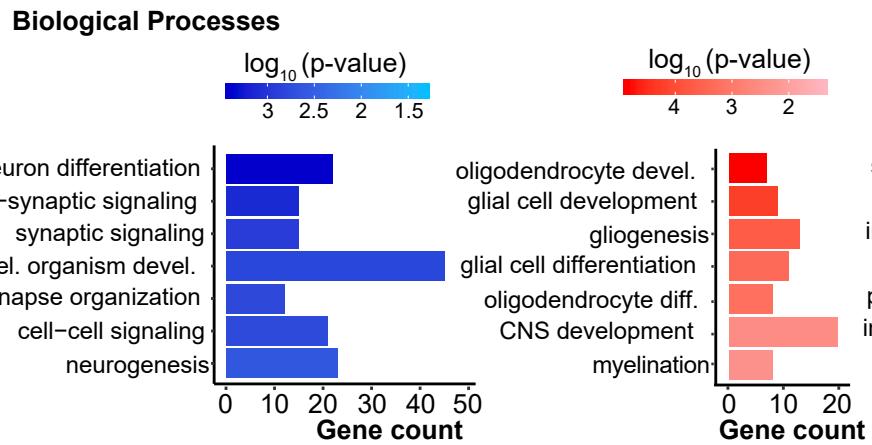
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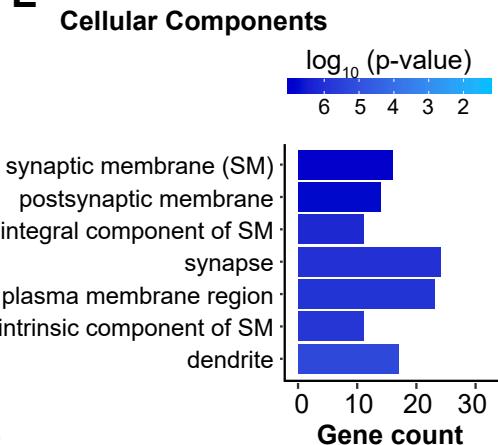


Figure 2: Postnatal removal of Prox1 from VIP cells leads to transcriptomic changes in synaptic proteins

(A) Schematic of experimental workflow. VIPCre-Prox1eGFP control and KO cells were sorted using FACS at P8 and P12 and bulk RNA sequencing was performed

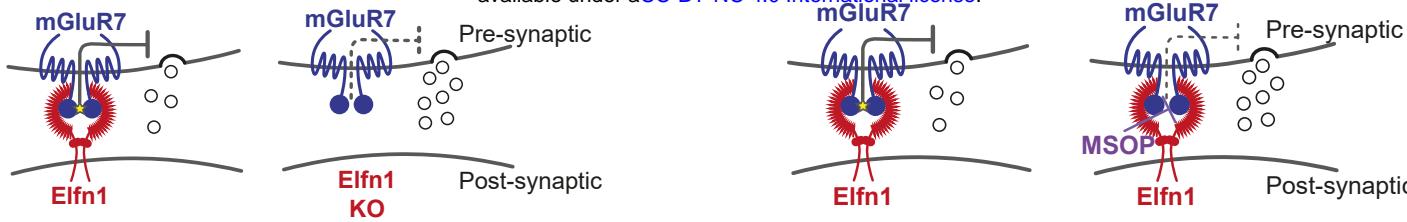
(B) Heat map showing the clustering according to function of up- (red) and downregulated (blue) genes at P12.

(C) Volcano Plot highlighting the differentially expressed candidate genes at P12. They were selected based on $\log_2 \text{ratio} \geq 0.5$ and $p\text{-value} \leq 0.01$

(D) GO: Biological Processes enrichment analysis of the candidate genes at P12. The down-regulated genes are depicted in blue and the up-regulated gene in red.

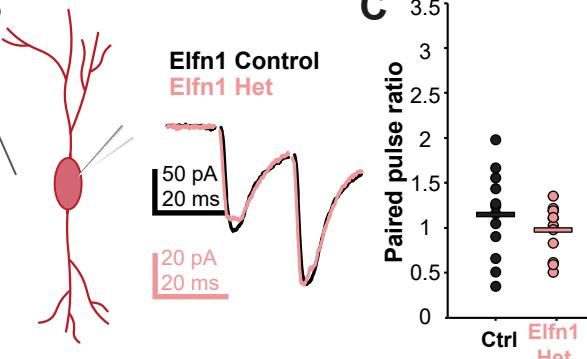
(E) GO: Cellular Components (CC) enrichment analysis of the candidate genes at P12. The down-regulated genes are depicted in blue. The up-regulated genes did not show any clustering in the GO:CC

A



VIP Bipolar (VIPC^{Cre} x *Ai14* x *Elfn1KO*)

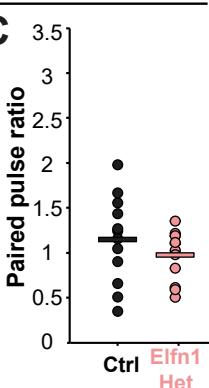
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VIP Bipolar (VIPC^{Cre} x *Prox1eGFP*)

VIP Bipolar (VIPC^{Cre} x *Prox1eGFP*)

C

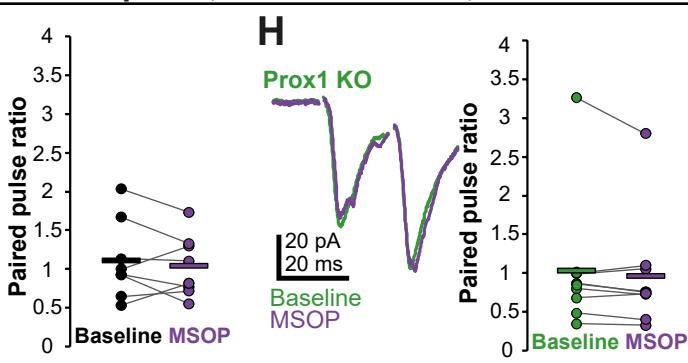


Prox1 Control



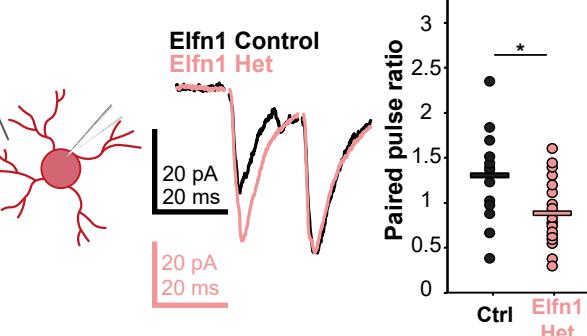
VIP Bipolar (VIPC^{Cre} x *Prox1eGFP*)

H



VIP Multipolar (VIPC^{Cre} x *Ai14* x *Elfn1KO*)

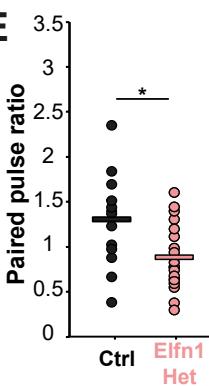
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VIP Multipolar (VIPC^{Cre} x *Prox1eGFP*)

VIP Multipolar (VIPC^{Cre} x *Prox1eGFP*)

I



Prox1 Control



J

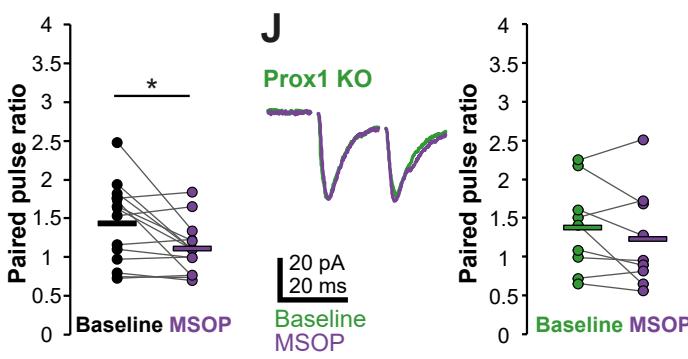


Figure 3: Elfn1 contributes to synaptic facilitation onto VIP+ multipolar, but not bipolar cells.

(A) A compound mouse line labeling VIP cells was combined on the background of an *Elfn1* germline knock-out. The presence of Elfn1 increases initial release probability of glutamate. Animals heterozygous (het) and wildtype (wt) for the germline removal were used as experimental and control group respectively.

(B)/(D) Left: schematic representation of the evoked paired-pulse protocol, with the extracellular stimulating and intracellular recording electrodes shown. Right: example of a pair of synaptic responses evoked onto VIP bipolar and multipolar cells after stimulation (scaled to 2nd evoked response).

(C) All data points for paired pulse ratio (PPR) of Elfn1 Control (n/N=12/4) and Het (n/N=12/4) bipolar VIP cells, $p = 0.3$, $t = 1.06$.

(E) All data points for PPR of Elfn1 Control (n/N=14/6) and Het (n/N=20/8) multipolar VIP cells, $p = 0.008$, $t = 2.84$. Statistics: t-test

(F) Testing for the effect of MSOP, a presynaptic mGluR blocker and hence Elfn1 function, on evoked excitatory responses onto control (Het) and Prox1 KO cells.

(G-J) Left: example of a pair of synaptic responses evoked onto VIP bipolar and multipolar cells after stimulation (scaled to 2nd evoked response). Right: all data points for PPR are plotted.

(G) PPR of bipolar Prox1 Control cells (n/N=8/8) under baseline conditions and with MSOP, $p = 0.5$, $t = 0.74$.

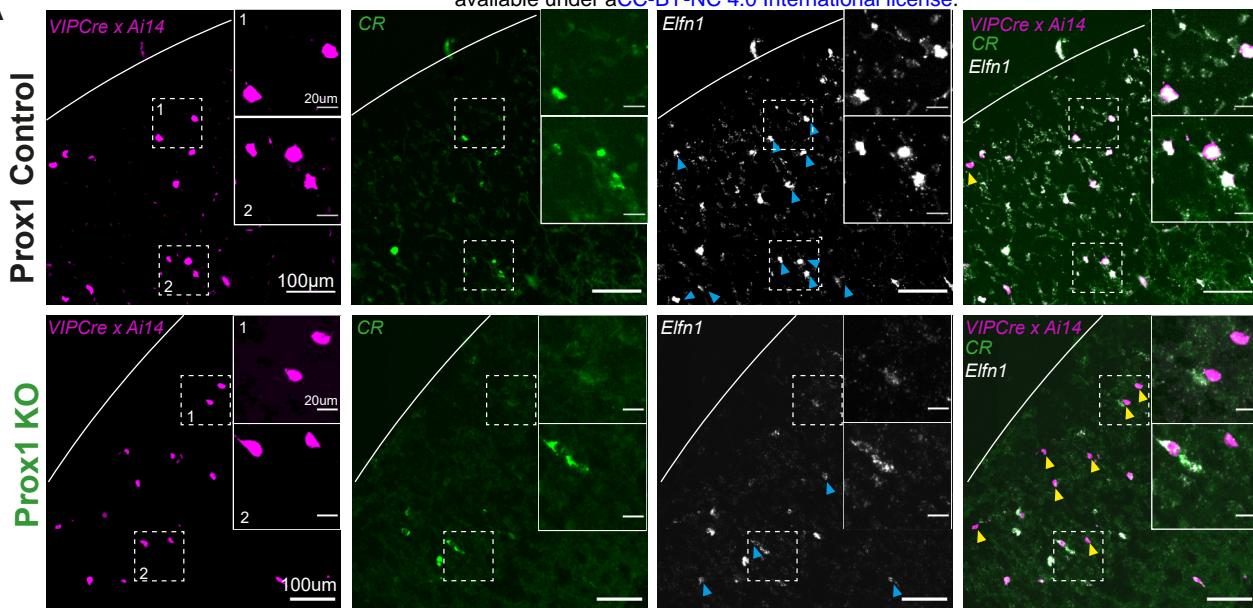
(H) PPR of bipolar Prox1 KO cells (n/N=9/9) under baseline conditions and with MSOP, $p = 0.2$, $t = 1.26$.

(I) PPR of multipolar Prox1 Control cells (n/N=14/9) under baseline conditions and with MSOP, $p = 0.02$, $t = 2.74$.

(J) PPR of multipolar Prox1 KO cells (n/N=9/8) under baseline conditions and with MSOP, $p = 0.2$, $t = 1.33$.

Statistics: paired t-test

A



B

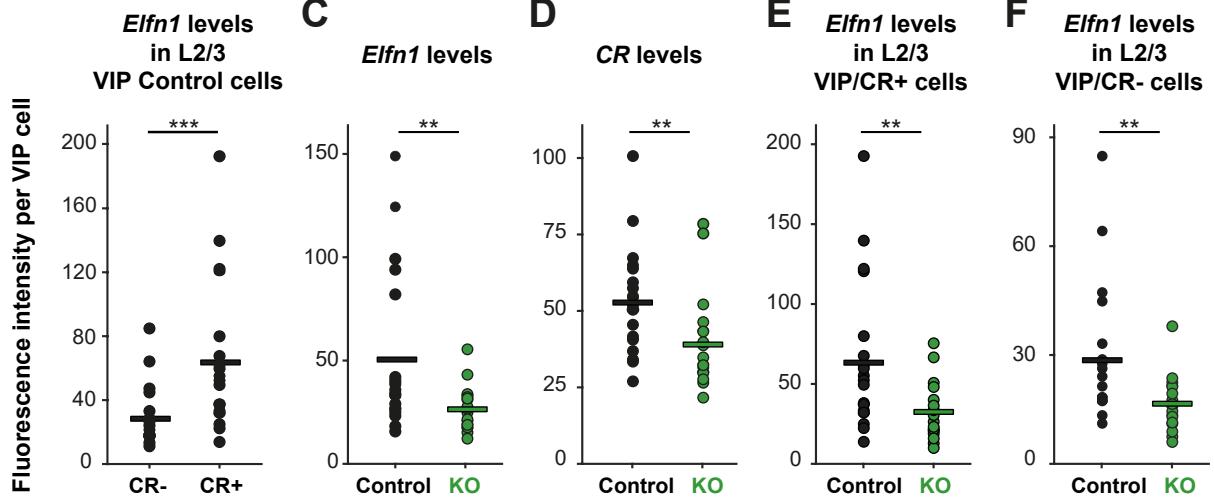


Figure 4: Prox1 regulates *Elfn1* expression in all VIP interneurons

(A) Examples of *Elfn1* and *CR* *in-situ* labelling in L2/3 tdtomato positive VIP cells in control (top) and Prox1 KO (bottom) tissue at P12. The insets show the close up of the dashed-line boxes including *CR* positive (*CR+*) and *CR* negative (*CR-*) VIP cells. The white parabolic line indicates the pia. Yellow arrows highlight VIP positive *Elfn1* negative cells. Blue arrows highlight *Elfn1* positive VIP cells. Note: there are also *Elfn1* positive cells that are not VIP positive likely corresponding to somatostatin cells.

(B) *Elfn1* levels in VIP multipolar (*CR-*) and bipolar (*CR+*) cells in L2/3 of the somatosensory cortex, $p=0.001$ (N=3 animals, n=19 sections/images).

(C) *Elfn1* levels in VIP cells in all layers and cortical areas in control (N/n=3/19) and Prox1 KO tissue (N/n=2/18), $p=0.004$.

(D) *CR* levels in VIP cells in all layers and cortical areas in control (N/n=3/19) and Prox1 KO tissue (N/n=2/18), $p=0.004$.

(E) *Elfn1* levels in VIP bipolar cells in L2/3 of the somatosensory cortex in control (N/n=3/19) and Prox1 KO (N/n=2/18) tissue, $p=0.004$.

(F) *Elfn1* levels in VIP multipolar cells in L2/3 of the somatosensory cortex in control (N/n=3/19) and Prox1 KO (N/n=2/18) tissue, $p=0.006$. Statistics: Mann-Whitney-U-Test.