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1 **Antibody-directed extracellular proximity biotinylation reveals Contactin-1 regulates axo-**
2 **axonic innervation of axon initial segments.**

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28 **ABSTRACT**

29 Axon initial segment (AIS) cell surface proteins mediate key biological processes in
30 neurons including action potential initiation and axo-axonic synapse formation. However, few
31 AIS cell surface proteins have been identified. Here, we used antibody-directed proximity
32 biotinylation to define the cell surface proteins in close proximity to the AIS cell adhesion
33 molecule Neurofascin. To determine the distributions of the identified proteins, we used
34 CRISPR-mediated genome editing for insertion of epitope tags in the endogenous proteins. We
35 found Contactin-1 (Cntn1) among the previously unknown AIS proteins we identified. Cntn1 is
36 enriched at the AIS through interactions with Neurofascin and NrCAM. We further show that
37 Cntn1 contributes to assembly of the AIS-extracellular matrix, and is required for AIS axo-axonic
38 innervation by inhibitory basket cells in the cerebellum and inhibitory chandelier cells in the
39 cortex.

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41 **MAIN**

42 The axon initial segment (AIS) is essential for proper neuronal and brain circuit function.
43 AIS integrate synaptic inputs, generate and modulate axonal action potentials, and regulate the
44 trafficking of proteins, vesicles, and organelles to maintain neuronal polarity. These functions
45 depend on a tightly regulated network of scaffolding and cytoskeletal proteins that serve as an
46 organizing platform for ion channels and cell adhesion molecules (CAMs) ^{1,2}. However, the AIS
47 proteins that have been described likely represent only a small fraction of the overall AIS
48 proteome since the molecular mechanisms involved in many AIS-associated processes remain
49 poorly defined.

50 Recently, proximity-dependent biotinylation (PDB) approaches have emerged as robust
51 experimental strategies to define the molecular composition of organelles and subcellular
52 domains ³. PDB is particularly attractive to identify AIS proteomes since the AIS is very
53 detergent insoluble and refractory to more traditional proteomic approaches like
54 immunoprecipitation (IP) mass-spectrometry. Streptavidin pulldown of biotinylated AIS
55 proteins allows for the use of much stronger solubilizing detergents. We previously used one
56 PDB approach (BioID) to discover new AIS proteins ⁴; we targeted the biotin ligase BirA* to the
57 AIS by fusing it to a variety of known AIS cytoskeleton-associated proteins. These experiments
58 identified known and some new cytoplasmic AIS proteins, including Mical3 and Septins.
59 However, our experiments were strongly biased towards cytoplasmic proteins and recovered
60 very few membrane and cell surface proteins. Some PDB approaches have successfully
61 captured cell surface proteins. For example, Li et al. (2020) ⁵ used an extracellular, membrane
62 tethered horseradish peroxidase (HRP) to identify cell surface proteins that function as

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63 regulators of neuronal wiring; their transgenic approach revealed the cell surface proteome of
64 *Drosophila* olfactory projection neurons. Shuster et al. ⁶ used the same approach in mice, but
65 restricted the expression of the membrane tethered HRP to Purkinje neurons to reveal their cell
66 surface proteome. However, neither of these approaches was designed to interrogate
67 subcellular domains. As an alternative approach, Takano et al. (2020) ⁷ used a split PDB
68 strategy (Split-TurboID) to elucidate the cell surface proteome of astrocyte-neuron synapses.
69 Their experiments revealed transcellular interactions between neuronal NrCAM and astrocytic
70 NrCAM that stabilize the structure and function of inhibitory synapses.

71 To overcome some of the limitations of intracellular PDB for identification of AIS cell
72 surface proteins, we used Selective Proteomic Proximity Labeling Assay Using Tyramide
73 (SPPLAT) ^{8,9}; the approach has also been called Biotinylation by Antibody Recognition (BAR) ¹⁰.
74 Our application of this strategy uses highly specific primary antibodies against the extracellular
75 domain of the AIS-enriched CAM Neurofascin (Nfasc) to direct HRP conjugated secondary
76 antibodies to the AIS. Addition of biotin-tyramide and hydrogen peroxide generates biotin
77 phenoxy radicals that biotinylate membrane proteins within a range of ~250 nanometers of the
78 peroxidase ¹¹. We performed this labeling at multiple timepoints throughout neuronal
79 development *in vitro* on live neurons. We identified all previously reported AIS extracellular,
80 and membrane cell adhesion and recognition molecules. In addition, we found many novel
81 membrane proteins that were reproducibly in proximity to Nfasc, with different temporal
82 enrichment profiles. We further investigated a subset of these using CRISPR-mediated
83 endogenous gene tagging. Among these, we identified Contactin-1 (Cntn1) as a new, *bona fide*
84 AIS CAM recruited to the AIS through interaction with the AIS CAMs Nfasc and NrCAM.

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85 Remarkably, loss of Cntn1 severely impaired inhibitory axo-axonic innervation of the AIS in both
86 cerebellar Purkinje neurons and cortical pyramidal neurons. Thus, using antibody directed
87 extracellular proximity biotinylation, we identified Cntn1 as a new AIS protein that regulates
88 axo-axonic innervation of the AIS.

89

90 ***Proximity Biotinylation at the AIS Membrane***

91 We reasoned the Nfasc proximity proteome could be used to help define the AIS cell
92 surface proteome since Nfasc is highly enriched at the AIS. Therefore, we adapted the
93 SPPLAT/BAR method^{8,10} for use with live, unpermeabilized neurons; we avoided fixation to
94 maximize protein recovery and subsequent mass spectrometry. We labeled rat hippocampal
95 neurons in culture with highly specific and validated chicken primary antibodies against the
96 ectodomain of Nfasc¹², since its 186 kDa isoform (NF186) is highly enriched at the AIS (Fig. 1a,
97 b), with lower concentrations along the distal axon, at growth cones, and in the soma¹³. After
98 live labeling with the anti-Nfasc primary antibody, HRP-conjugated anti-chicken secondary
99 antibodies were used to label the anti-Nfasc primary antibody. The Nfasc-localized HRP
100 generates the reactive biotin phenoxyl from biotin tyramide (biotin phenol), resulting in the
101 addition of tyrosine residues to proteins in proximity to Nfasc with a range of several hundred
102 nm^{9,11}. As with other PDB methods, nonspecifically and endogenously biotinylated proteins, as
103 well as non-specific protein background adsorbing to solid phase surfaces during the
104 enrichment steps and prior to the mass spectrometry analysis, must be excluded. The omission
105 of the primary antibody serves as a simple and straightforward negative control. Without
106 fixation or detergents, the membrane-impermeability of biotin-phenoxyl restricts the

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107 biotinylation reaction to the extracellular surface. Hereafter, we refer to this method as Nfasc-
108 BAR.

109 We found that Nfasc-BAR resulted in AIS-enriched streptavidin labeling that colocalized
110 with NF186 (Fig. 1b); this pattern was not seen when the anti-Nfasc antibody was omitted. The
111 amount of biotinylation also depends on the duration of the labeling reaction (Fig. S1a). For the
112 experiments described below, we used a reaction time of 5 minutes (Fig. S1b). To identify
113 biotinylated AIS proteins, we then solubilized neuronal membranes using a strong solubilization
114 buffer, purified biotinylated proteins using streptavidin-conjugated magnetic beads, and finally
115 identified the biotinylated proteins using mass spectrometry (Fig. 1a). To confirm the
116 reproducibility and robustness of our approach we performed surface proximity labeling in
117 parallel using rabbit polyclonal antibodies targeting the ectodomain of NrCAM (Fig. S1c),
118 another AnkG-binding CAM found at the AIS. Proximity biotinylation directed by NrCAM
119 antibodies strongly labeled the AIS (Fig. S1c). Importantly, the resulting mass spectrometry
120 datasets confirmed the robustness of the strategy since Nfasc-BAR and NrCAM-BAR proximity
121 proteomes were highly concordant (Fig. S1d; supplemental Table 1).

122

123 ***NF186 proximity proteomes across neuronal development***

124 The maturation of axons includes the enrichment of proteins that mediate key functions
125 or developmental mechanisms. For example, toward the end of the first week *in vitro*, the
126 scaffolding protein AnkyrinG (AnkG) localizes to the proximal axon; this enrichment precedes
127 and is necessary for the subsequent recruitment of Nfasc, and voltage-gated Na⁺ (Nav) and K⁺
128 (Kv) channels to the AIS^{14,15}. NF186 enrichment at the AIS and along distal axons also increases

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129 during development (Fig. 2a). To determine the extracellular Nfasc proximity proteome and
130 how it changes during development (both before and after AIS formation), we performed
131 Nfasc-BAR on primary hippocampal neurons at five different timepoints (supplemental Table 2):
132 from day *in vitro* 4 (DIV4; prior to AIS formation) to DIV28 after establishment of the AIS-
133 associated ECM (Fig. S2); all experiments were performed three times independently for each
134 developmental timepoint with 2 million neurons per experiment. To compare Nfasc cell surface
135 proximity proteomes from cultures of different ages, we normalized peptide spectral match
136 (PSM) counts to total spectral counts in the set of endogenously biotinylated carboxylases
137 detected (Fig. S2), as a correction factor for differences in total protein amount used in
138 individual pulldowns (see methods). We found that as neurons, the AIS, and axons develop, the
139 cell surface Nfasc proximity-proteome changes, with an increasing number of proteins
140 displaying significant changes in fold enrichment (Fig. 2, supplemental Table 2). Consistent with
141 a developing and maturing AIS and increasing levels of overall Nfasc, volcano plots (Fig. 2a and
142 supplemental table 3) show the enrichment of proteins identified using Nfasc-BAR compared to
143 controls at the various developmental time points. We used a cutoff of $\log_2(\text{Nfasc PSMs}/\text{Ctrl PSMs})$
144 or $\log_2[\text{fold change (FC)}] > 2$ (vertical dotted line) with a significance cutoff of $p < 0.05$
145 (horizontal dotted line).

146 To visualize the increase in proteins identified using Nfasc-BAR across development and
147 to select candidates to focus on, we identified 285 proteins that satisfied two filtering criteria
148 for at least one of the five timepoints: (1) normalized PSMs > 10 and (2) $\log_2(\text{Nfasc PSMs}/\text{Ctrl PSMs})$
149 or $\log_2(\text{FC}) > 2$ (Fig. S2; Supplemental Table 2). Among these 285 proteins there were a
150 variety of protein expression profiles (Fig. 2b). Although present, relatively few proteins

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151 showed a reduction in the cell surface Nfasc proximity proteome. Most proteins identified in
152 the Nfasc proximity proteome increased in abundance (Fig. 2c; only the 100 candidates with the
153 largest increase across all time points are shown). We also plotted the \log_2FC at each time
154 point for the 100 proteins with the largest fold change (DIV 4 only had 63 proteins with \log_2FC
155 >2) (Fig. S3). The results for each protein were highly reproducible at each time point and
156 consistently revealed similar sets of cell surface proteins. Cytoplasmic AIS proteins such as
157 AnkG, $\beta 4$ spectrin, and TRIM46 were conspicuously absent consistent with our experimental
158 design to restrict the biotinylation to cell surface proteins.

159

160 ***The Nfasc-BAR proximity proteome includes AIS enriched proteins***

161 Among the cell surface proteins that passed our selection criteria (Fig. S2), we found all
162 known AIS membrane proteins and AIS enriched extracellular matrix molecules with the
163 notable exception of ion channels (Fig. 3a). Why might that be the case since ion channels are
164 known to be highly enriched at the AIS? The number of PSMs recovered for any protein
165 depends on: 1) the number of available extracellular tyrosine residues (Nfasc-BAR-mediated
166 biotinylation occurs on tyrosine residues); 2) the amount and local membrane density of the
167 protein; and 3) the proximity of the protein to the biotinylation source. Since ion channels are
168 highly enriched at the AIS and are in close proximity to Nfasc, their absence from our data set
169 likely reflects the small number of extracellular tyrosine residues found in ion channels and a
170 topology that has extracellular residues very close to the membrane. For example, the AIS-
171 enriched K^+ channel subunit KCNQ3 has four extracellular regions comprising 45 amino acids,
172 with two of those being tyrosine (Fig. 3b); we did not detect any KCNQ3 peptides in our

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173 experiments. Similarly, Nav1.2 (encoded by *Scn2a*), the main voltage-gated Na⁺ channel
174 expressed at AIS in DIV14 hippocampal neurons, has an average of one tyrosine per
175 extracellular domain and many are very close to or immediately adjacent to the membrane. In
176 contrast to ion channels, cell adhesion molecules like Nfasc and Contactin-1 (*Cntn1*) have large
177 extracellular domains with many tyrosine residues (Fig. 3b). Thus, the low number of
178 extracellular tyrosines found in ion channels and the proximity of these residues to the
179 membrane may make them difficult to biotinylate using Nfasc-BAR; other membrane or cell
180 surface proteins with few or inaccessible tyrosine residues may also be poorly represented in
181 our data set.

182 In contrast to ion channels, an analysis of the 201 proteins identified at DIV14 showed
183 no correlation between the number of PSMs for a protein and the number of extracellular
184 tyrosines (Figs. 3c, d). This suggests a much stronger dependence of PSM number on proximity
185 and protein abundance. As an estimate for both proximity and abundance, we calculated the
186 ratio of extracellular tyrosines to PSM count for all 201 proteins identified at DIV14. Thus, a
187 lower ratio suggests greater abundance of protein and closer proximity to the HRP-dependent
188 biotinylation source (Fig. 3e). This analysis shows many candidates with low extracellular
189 tyrosine/PSM ratios that were also previously reported to be directly or indirectly linked to
190 Nfasc, including PlxnA4, Ncam1, L1CAM, and NrCAM (Fig. 3e)^{16,17}.

191

192 ***Tagging of endogenous membrane proteins***

193 Our results across 5 developmental timepoints yielded an NF186 proximity proteome
194 (Fig. 2a); filtering based on fold-enrichment and number of PSMs recovered resulted in 285

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195 candidate cell surface proteins in close proximity to NF186 (Fig. S2). Among these, Nfasc-BAR
196 successfully identified the 13 previously reported AIS-enriched cell surface proteins (excluding
197 ion channels; Fig. 3a). However, it is unlikely that the remaining 272 proteins are also enriched
198 at the AIS since NF186 is present in lower densities in the soma, axons, and at growth cones¹³,
199 some of the proteins were identified before the AIS forms or is mature (e.g., DIV4), and the
200 range of SPPLAT/BAR is ~200-300 nm^{9,11}. Thus, proteins identified by Nfasc-BAR may be in
201 proximity to NF186 but not enriched at the AIS. We previously used antibodies to validate the
202 AIS proteomes we identified using BioID⁴. However, antibodies are frequently non-specific and
203 for reasons that are unclear, many antibodies that label AIS are not against their claimed
204 targets¹⁸⁻²⁰. Therefore, to circumvent some of the challenges associated with antibodies and to
205 look for AIS- and axon-enriched proteins, we performed CRISPR-mediated epitope tagging of
206 endogenous proteins^{19,21-23}. We selected 23 different candidates (Fig. 4a) identified using
207 Nfasc-BAR based on 1) the high fold-enrichment compared to control BAR, 2) the high number
208 of PSMs recovered, and 3) the estimate of proximity to the biotin source (Figs. 2 and 3).
209 Included in these 23 candidates were four cell adhesion molecules previously reported at the
210 AIS: Nfasc, NrCAM, L1CAM, and Cntn2²⁴⁻²⁶. To endogenously label these 23 cell surface
211 proteins, we generated two adeno-associated viruses (AAV) to transduce cultured DIV 0 rat
212 hippocampal neurons with 1) Cas9 and 2) a gene specific single guide RNA (sgRNA), a sgRNA
213 that recognizes donor recognition sites (DRS) flanking spaghetti monster fluorescent protein
214 with V5 tags (smFP-V5), and smFP-V5 (Fig. 4b). The gene specific sgRNAs were targeted to the
215 last exon of each gene of interest allowing for the insertion of smFP-V5 in the last exon. 2
216 weeks after transduction, neurons were fixed and immunostained for β 4 spectrin to label the

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217 AIS, and V5 to detect the endogenously tagged cell surface protein. Since we targeted the last
218 exon (C-terminus) of each protein resulting in premature termination of the protein, it is
219 possible the addition of the smFP-V5 disrupted the normal localization of the cell surface
220 protein. However, C-terminal tagging of endogenous Nfasc, NrCAM, L1CAM, and Cntn2 all
221 resulted in AIS labeling as previously reported²⁵⁻²⁷ (Figs. 4c-f). The candidates we tested
222 labeled AIS, axons, and dendrites (Figs. 4 and S4). For example, endogenous tagging of Ncam1
223 revealed uniform surface labeling in somatodendritic, AIS, and axonal domains (Fig. 4g), while
224 Ptpns and Tenm4 showed preferential labeling of AIS and axons (Figs. 4h, i); endogenous
225 labeling of Adgrl3 strongly labeled dendrites and spines (Fig. 4j). Among all the candidates we
226 tested that had not previously been reported at the AIS, we found that endogenous tagging of
227 Cntn1 showed the strongest labeling at the AIS (Fig. 4k).

228

229 ***Cntn1 is a bona fide AIS cell surface protein***

230 Cntn1 is a glycosylphosphatidyl inositol (GPI)-anchored cell adhesion molecule widely
231 expressed throughout the nervous system in both neurons and glia²⁸. It has essential roles in
232 forming the axoglial junctions flanking nodes of Ranvier where it forms a complex together with
233 axonal Caspr and the glial 155 KDa splice variant of Nfasc (NF155)^{29,30}. Cntn1-null mice die in
234 the 3rd postnatal week, emphasizing the importance of Cntn1 to normal function. Cntn1 was
235 also reported at nodes of Ranvier, although its function there is unknown³¹; detection of nodal
236 or paranodal Cntn1 requires different fixation and treatment conditions³², suggesting that in
237 some subcellular domains Cntn1 may engage in protein-protein interactions that preclude
238 immunostaining. With this in mind, our efforts to immunolabel Cntn1 at AIS in control mouse

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239 brain failed. Nevertheless, we performed immunostaining *in vitro* using a goat-polyclonal anti-
240 Cntn1 antibody in control and Cntn1-deficient neurons. We disrupted endogenous Cntn1
241 expression in cultured hippocampal neurons using AAV to express Cas9 and three control or
242 three *Cntn1* specific sgRNAs (Fig. 5a). Whereas neurons transduced with Cas9 and the control
243 sgRNAs had Cntn1 and AnkG immunolabeling at the AIS (Fig. 5b, arrowheads), neurons
244 transduced with the *Cntn1* sgRNAs lost both the perisomatic and AIS Cntn1 immunoreactivity
245 (Fig. 5c, arrowhead). Thus, immunostaining of cultured hippocampal neurons reveals AIS
246 Cntn1. These results also demonstrate the specificity of the Cntn1 antibody. However, loss of
247 AIS Cntn1 had no effect on AIS AnkG (Fig. 5c, arrowhead), or clustering of Nfasc or Na⁺ channels
248 (data not shown).

249 Transduction of DIV10 cultured hippocampal neurons using AAV to express myc-tagged
250 Cntn1 (Fig. 5d, e) also showed Cntn1-myc enriched at the AIS that colocalized with β 4 spectrin
251 at DIV14 (Fig. 5e, arrowheads). Similarly, retro-orbital injection of AAV Cntn1-myc in 13-week
252 old mice, showed strong AIS enrichment of Cntn1-myc in transduced cortical neurons four
253 weeks after injection (Fig. 5d, f, arrowheads). Finally, we performed *in vivo* AAV-dependent and
254 CRISPR-mediated tagging of endogenous Cntn1 using smFP-V5 (Fig. 5g). As with cultured
255 neurons (Fig. 4k), we found the sgRNA targeting *Cntn1* resulted in V5 labeling of cortical neuron
256 AISs (Fig. 5h, arrowheads). Together, these results show that Cntn1 is a *bona fide* AIS cell
257 surface protein.

258

259 ***Cntn1 is localized at the AIS through binding to L1-family cell adhesion molecules***

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260 Since Cntn1 is a GPI-anchored cell surface protein, we reasoned that it must be
261 recruited to the AIS through interactions with a co-receptor or some other AIS transmembrane
262 protein. Cntn1 has 6 N-terminal immunoglobulin-like (Ig-like) and 4 C-terminal fibronectin III
263 (FNIII) domains (Fig. 6a). To determine how Cntn1 is clustered at the AIS, we generated myc-
264 tagged Cntn1 with various internal deletions of these domains. We found the N-terminus and
265 first four Ig-like domains of Cntn1 are required for its AIS clustering (Fig. 6b). In contrast,
266 deletion of the last two Ig domains or any of the FNIII domains did not affect recruitment of
267 Cntn1 to the AIS (Fig. 6c).

268 What membrane protein recruits Cntn1 to the AIS? Biochemical and cell biological
269 studies suggest that Cntn1 interacts with members of the AnkG-binding L1 family of cell
270 adhesion molecules including Nfasc, L1CAM, and NrCAM^{33,34}. Although all three of these CAMs
271 are enriched at the AIS, L1CAM is also found at high levels along the distal axon (Figs. 4c-e). To
272 determine if the AIS-enriched Nfasc or NrCAM recruits Cntn1 to the AIS, we generated sgRNAs
273 vectors (Fig. 5a) to delete Nfasc and NrCAM from neurons. Surprisingly, removal of Nfasc or
274 NrCAM alone had no effect on the clustering of Cntn1 at the AIS (Figs. 6d, e). However,
275 simultaneous deletion of both Nfasc and NrCAM blocked the AIS clustering of Cntn1 (Figs. 6d,
276 arrowhead; e). These results suggest that the AIS enriched CAMs Nfasc and NrCAM
277 redundantly recruit Cntn1 to the AIS through its first four Ig-like domains.

278

279 ***Cntn1 helps assemble the AIS extracellular matrix***

280 We found Tenascin-R (Tnr) in our Nfasc-BAR proximity proteome (Fig. 3a). Tnr is an
281 extracellular matrix molecule and a known Cntn1 interactor³³. Immunostaining of cultured

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282 hippocampal neurons using antibodies against Tnr showed that it strongly labeled the AIS and
283 colocalizes with Nfasc (Fig. 6f). Previous studies showed that Nfasc regulates the AIS
284 recruitment of the extracellular matrix molecule Brevican¹⁵. To determine if Nfasc or Cntn1
285 also regulate the AIS clustering of extracellular Tnr, we disrupted expression of *Nfasc* and
286 *Cntn1*; sgRNA vectors (Fig. 5a) targeting *Nfasc* and *Cntn1* were highly efficient (Figs. 5c and 6g).
287 Although control sgRNAs had no effect on AIS Tnr, loss of both Nfasc and Cntn1 significantly
288 reduced Tnr's AIS enrichment (Fig. 6g, h). Thus, Nfasc and Cntn1 both contribute to the
289 assembly or stabilization of the Tnr-containing AIS extracellular matrix.

290

291 ***Cntn1 regulates the assembly of pinceau synapses in the cerebellum.***

292 Purkinje neuron AISs are innervated by inhibitory basket cell interneurons that
293 powerfully modulate neuronal excitability³⁵. Basket cells form a stereotypical 'pinceau'
294 synapse (Fig. 7a), with presynaptic terminals at the AIS highly enriched in Kv1 K⁺ channels and
295 PSD-95, among other proteins³⁶. The loss of the AIS scaffolding protein AnkG disrupts pinceau
296 synapses and the AIS clustering of Nfasc, suggesting that their assembly requires AnkG-
297 dependent clustering of CAMs like Nfasc and NrCAM³⁷. Since AIS enrichment of Cntn1 also
298 depends on these CAMs, we wondered if Cntn1 plays important roles in cerebellar pinceau
299 synapse assembly. Therefore, we examined pinceau synapse formation in P18 *Cntn1*^{-/-} mice;
300 *Cntn1*^{-/-} are very sick and typically die before 3 weeks of age³⁰. Immunostaining of Purkinje
301 neurons using antibodies against Nfasc, Kv1.2, and PSD95 showed stereotypical enrichment and
302 clustering of these proteins at the AIS of control heterozygote *Cntn1*^{-/+} mice, but *Cntn1*^{-/-}
303 mice had profoundly disrupted pinceau synapse formation (Fig. 7b, arrows) with significantly

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304 reduced Kv1.2 and PSD95 intensity at the pinceau synapse (Fig. 7c). Thus, *Cntn1* is required for
305 proper assembly of pinceau synapses at the AIS of cerebellar Purkinje neurons.

306

307 ***Cntn1* regulates axo-axonic innervation of pyramidal neurons by chandelier cells**

308 In the cortex and hippocampus, axo-axonic synapses are formed between chandelier
309 cells (ChCs, also known as axo-axonic cells) and the AIS of pyramidal neurons (PyNs). ChCs are
310 derived from progenitors in the ventral region of the medial ganglionic eminence. They have a
311 unique axonal arbor consisting of multiple arrays of short, vertically oriented terminals of
312 presynaptic boutons called cartridges, and each of these cartridges selectively innervates
313 neighboring PyN AISs. These ChCs powerfully reduce PyN output by inhibiting AIS excitability;
314 this inhibition can subsequently modulate brain circuit function and behavior³⁸⁻⁴⁰. However,
315 the mechanisms that control the precise innervation of PyN AISs by ChCs remains incompletely
316 understood, with ankyrin-interacting L1CAM so far being the only CAM known to be required
317 for ChC/PyN AIS innervation⁴¹; since L1CAM is found throughout the axon and not just at the
318 AIS, additional mechanisms must exist to allow for precise AIS innervation. To determine if
319 *Cntn1* regulates ChC/PyN AIS innervation, we performed in utero electroporation (IUE) using
320 control or *Cntn1*-targeting sgRNA- and smFP-HA-expressing plasmids in *Nkx2.1-CreER;Rosa26-*
321 *loxPSTOPloxP-tdTomato (Ai9)* pregnant mice at embryonic day 15.5 (E15.5) (Fig. 8a). This
322 timing of IUE results in disruption of the *Cntn1* gene in layer II/III PyNs. At E18.5 tamoxifen was
323 administered to the pregnant mother to induce expression of tdTomato red fluorescence
324 protein (RFP) in a sparse group of layer II ChCs (Fig. 8b). We collected brains from P17 mice and
325 analyzed the innervation and assembly of inhibitory synapses on PyN AISs by immunostaining

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326 for AnkG or β 4 spectrin to label AISs, and antibodies to gephyrin and VGAT to label post- and
327 pre-synaptic compartments of GABAergic synapses, respectively. We found that transfection
328 with Cntn1 sgRNA significantly reduced the percentage of PyN AISs innervated by single RFP-
329 positive ChCs in layer II/III of the somatosensory cortex, as compared to control sgRNA (Figs. 8c,
330 arrowheads, and d). We found that Cntn1-deficient neurons also had significantly fewer
331 inhibitory synapses along their AIS as indicated by gephyrin (Figs. 8e and f) and VGAT puncta
332 (Figs. 8g and h). Together, these results show that Cntn1 is required for efficient ChC/PyN AIS
333 innervation and consequently, the proper assembly of AIS axo-axonic inhibitory synapses.

334

335 **DISCUSSION**

336 AIS properties essential for brain function include: 1) high densities of ion channels, 2)
337 mechanisms to regulate neuronal polarity, and 3) precise innervation by inhibitory
338 interneurons. The molecular mechanisms regulating these properties all converge on the AIS
339 scaffolding protein AnkG^{37,41-43}. However, the distinct proximal mechanisms regulating these
340 AIS properties remain poorly understood, highlighting the need to define the composition of
341 the AIS in much greater detail. BioID-dependent cytoplasmic proximity biotinylation and
342 differential mass spectrometry have partially elucidated AIS proteomes^{4,44}, but they are clearly
343 deficient in proteins involved in transient interactions, posttranslational modifications, and cell
344 surface proteins mediating extra- and intercellular interactions. Thus, experimental approaches
345 that address these deficiencies are desperately needed.

346 We aimed to use Nfasc-BAR to identify AIS cell surface proteins. The results were highly
347 reproducible at each developmental time point *in vitro*, but showed changing profiles of cell

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348 surface proteins during development. As expected, our data sets included known AIS cell
349 surface proteins, and consisted almost exclusively of cell surface proteins whose expression
350 levels increased with neuronal maturation. The strategy is highly flexible and can be used with
351 other AIS specific antibodies including those targeting cytoplasmic epitopes after detergent
352 solubilization; the strategy can also be applied to other neuronal compartments including
353 synapses, dendrites, and growth cones so long as highly specific and validated antibodies are
354 used.

355 Since Nfasc is highly enriched at the AIS, we expected AIS-enriched membrane proteins
356 to be over-represented in our data set. However, Nfasc is also found at lower densities in
357 somatodendritic and distal axonal domains, and their total membrane area exceeds that of the
358 AIS. Thus, despite the high density of AIS Nfasc, the total pool of Nfasc in non-AIS membrane is
359 likely much greater. HRP-mediated biotinylation is very efficient and has a range ~25 times
360 greater than BioID or APEX¹¹. Thus, the proteins we recovered are more accurately described
361 as an Nfasc surface proximity proteome, with a subset of those proteins also being found at the
362 AIS.

363 Given the promiscuity of Nfasc-BAR, the biggest challenge in our experiments was to
364 determine which proteins to focus on and how to validate their presence or enrichment at the
365 AIS. To this end, we narrowed our analysis using stringent filtering criteria including fold-
366 enrichment, significance of that enrichment, and a minimum number of PSMs recovered. We
367 also estimated the relative proximity to Nfasc based on the ratio of extracellular tyrosine
368 residues to the PSMs recovered. Nfasc-BAR and the filtering criteria used here may
369 underestimate proximity or miss AIS proteins that were not well biotinylated (e.g. ion

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370 channels). Nevertheless, among the proteins recovered and that satisfied the filtering criteria,
371 our use of endogenous gene tagging revealed several that were present or even enriched at the
372 AIS, but that had not been previously described as AIS proteins. In particular, we found that
373 Cntn1 is highly enriched at the AIS. We validated its enrichment there by endogenous gene
374 tagging *in vitro* and *in vivo*, by expression of exogenous epitope-tagged Cntn1 at the AIS *in vitro*
375 and *in vivo*, and by immunostaining of cultured hippocampal neurons using Cntn1 antibodies
376 whose specificity was confirmed using CRISPR-mediated gene disruption. Other candidate AIS
377 proteins (e.g. Tenm4 and Ptprs) will require additional studies to further validate and confirm
378 they are *bona fide* AIS proteins. Additional endogenous gene tagging may reveal more AIS
379 membrane proteins since we only tested a small subset of the Nfasc proximity proteome.

380 The use of numerous methods to confirm that Cntn1 is a *bona fide* AIS protein is
381 important since relying on antibody staining alone can lead to incorrect assignment of a protein
382 being enriched at the AIS¹⁸⁻²⁰. Methods allowing CRISPR-mediated endogenous gene tagging
383 are a significant advance to validate protein localization without the confound of off-target
384 antibodies or mislocalization due to over-expression²¹⁻²³. However, the method of tag
385 insertion we used also disrupts coding regions in the last exon of the proteins analyzed, and
386 some proteins that depend on their C-terminal amino acids may be mislocalized. Thus, failure
387 of a protein to localize to the AIS after endogenous gene tagging should not be considered a
388 definitive criterion for exclusion as an AIS protein.

389 Cntn1 is a GPI-anchored cell adhesion molecule that has been studied in the nervous
390 system mainly in the context of its role in axon-glia interactions as an essential component of
391 the paranodal axoglial junction formed between axons and myelinating glia³⁰. There, Cntn1

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392 participates in cis interactions in the axon with Caspr1 (Contactin ASsociated PRotein 1) and
393 trans interactions with the glial 155 kD form of Nfasc (NF155)^{29,45}. Cntn1 can engage in diverse
394 interactions with many cell adhesion and extracellular matrix molecules including Caspr1,
395 members of the L1 family of cell adhesion molecules (Nfasc, NrCAM and L1CAM), Tnr, Tnc, and
396 receptor tyrosine phosphatase β ^{28,46,47}. Our experiments show that Cntn1's AIS localization
397 requires its first four Ig-like domains and redundant interactions with either Nfasc or NrCAM
398 (Fig. S5a), since only simultaneous deletion of both disrupts Cntn1's AIS localization. In
399 addition, Cntn1 helps assemble the AIS extracellular matrix since its loss affects Tnr
400 recruitment. Future experiments may also reveal roles for Cntn1 in association with other AIS
401 extracellular matrix molecules or membrane proteins including Tnc and Na⁺ channel β subunits
402 ⁴⁸. The importance of Cntn1 in humans is highlighted by the observation that a pathogenic
403 variant of *CNTN1* caused lethal severe fetal akinesia syndrome ⁴⁹.

404 ChCs and basket cells precisely innervate cortical PyNs and Purkinje neurons,
405 respectively, to regulate AIS excitability. For example, Dudok et al.³⁸ and Schneider-Mizell et al.
406 ³⁹ showed that a variety of behaviors including pupil dilation, locomotion, and whisking can
407 synchronously activate populations of ChCs to inhibit PyNs through GABAergic synapses.
408 Together, these observations highlight the central role played by ChCs in modulating brain
409 states and behavior. Similarly, pinceau synapses provide strong inhibitory control over Purkinje
410 neuron output, but this is due to ephaptic inhibition rather than chemical inhibition ⁵⁰. Despite
411 their importance, the molecular mechanisms responsible for the precise innervation and
412 maintenance of AIS axo-axonic synapses are incompletely understood.

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413 Loss of AnkG from Purkinje neurons disrupts pinceau synapse formation and has been
414 attributed to mislocalization of AIS NF186; however, loss of ankyrin-binding NrCAM, L1CAM, or
415 CHL1 did not affect pinceau synapse assembly³⁷. These observations are consistent with our
416 findings that *Cntn1* also regulates pinceau synapse assembly (Fig. S5b), since loss of AnkG
417 affects both NF186 and NrCAM localization⁵¹. We report here that *Cntn1*'s AIS localization can
418 be independently directed by both of these CAMs (Fig. 6d). This result is consistent with the
419 observation that the specific deletion of NF186 alone in adults does not disrupt pinceau
420 synapse maintenance⁵², suggesting that *Cntn1* can also partner with other AIS CAMs (e.g.,
421 NrCAM) for synapse maintenance. Since *Cntn1* *-/-* mice die by P21, we cannot rule out the
422 possibility that pinceau synapses fail to develop due to widespread developmental defects.
423 Future studies utilizing Purkinje neuron specific deletion of *Cntn1* will be required to more
424 precisely define *Cntn1*'s role in pinceau synapse assembly and maintenance.

425 Previously, Tai et al.⁴¹ reported a small RNAi screen of 14 candidate cell adhesion
426 molecules to identify regulators of PyN AIS innervation by ChCs. The candidates screened
427 included *Nfasc*, NrCAM, and all previously reported AIS CAMs (e.g. *Cntn2*). Loss of *Nfasc*,
428 NrCAM, or *Cntn2* alone had no effect on ChC/PyN AIS innervation; but the impact of
429 simultaneous loss/depletion of *Nfasc* and NrCAM remains to be tested. Importantly, among the
430 candidates screened, only loss of L1CAM significantly reduced PyN AIS synaptic innervation by
431 ChCs, despite the fact that L1CAM is found not only at the AIS, but along the entire axon²⁵.
432 This suggests that L1CAM may cooperate in cis with other membrane or adhesion molecules
433 like *Cntn1* for precise innervation of the AIS by ChCs (Fig. S5c). In addition to L1CAM, Hayano et
434 al.⁵³ reported the cell adhesion molecule *Igsf11* functions both pre- and post-synaptically in

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435 layer 2/3 of cortex to regulate assembly of PyN/ChC axo-axonic synapses. However, we did not
436 find Igsf11 in our Nfasc-BAR results at any time point; although this may reflect Igsf11 having
437 relatively few extracellular tyrosines. Alternatively, there may be differences between neurons
438 *in vitro* and *in vivo*, or even between types of neurons.

439 One limitation of this study is that the proximity biotinylation experiments were
440 performed on cultured neurons. AIS cell surface proteins whose localization depends on the
441 native brain environment may not be represented. For example, AIS GABAergic synapses may
442 form *in vitro*, but they are rare, making it difficult to identify presynaptic receptor(s) for Cntn1
443 and L1CAM using Nfasc-BAR. Future experiments using extracellular split TurboID⁵⁴, where
444 PyNs and ChCs each express one half of TurboID may help to identify the pre-synaptic receptor
445 found on ChCs. Alternatively, developing *in situ* Nfasc-BAR for use with brain tissue may reveal
446 additional AIS cell surface proteins and their receptors.

447 In summary, we used extracellular proximity biotinylation to identify Cntn1 as a new AIS
448 adhesion molecule. Cntn1 is restricted to the AIS through its binding to AnkG-localized Nfasc or
449 NrCAM (Fig. S5a). In Purkinje neurons, Cntn1 is required for AIS innervation by basket cells (Fig.
450 S5b). In PyNs, Cntn1 functions together with L1CAM to regulate the AIS-specific targeting and
451 developmental assembly of PyN/ChC axo-axonic synapses (Fig. S5c). Thus, our results suggest a
452 model where axo-axonic innervation of diverse neuron types converges on AIS-enriched Cntn1.

453

454

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455 **METHODS**

456 ***Animals***

457 Timed pregnant Sprague-Dawley rats were obtained from Charles River Laboratories.
458 Rats were euthanized for embryo collection at E18. Brains were collected from *Cntn1* $-/-$ and $+/-$
459 mice at P17 (catalog #Jax:034216, RRID:IMSR_JAX:034216). The *Cntn1* mice were maintained at
460 the Weizmann Institute of Science, Rehovot, Israel. P0 ICR mice were used for intraventricular
461 injection of AAV for overexpression of *Cntn1*-Myc. Transgenic Cas9 mice (catalog #Jax: 027650,
462 RRID: IMSR_JAX:027650) were used for intraventricular injection of AAV to perform tagging of
463 endogenous *Cntn1*. *Nkx2-1*^{tm1.1(Cre/ERT2)zjh/J} and B6;129S6-Gt(*Rosa*)26Sor^{tm9(CAG-tdTomato)Hze/J} were
464 a gift from Dr. Z.J. Huang⁵⁵. Swiss Webster mice were purchased from Charles River (Cat#
465 CRL:24; RRID: IMSR_CRL:24). All experiments were performed in compliance with the National
466 Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by
467 the Baylor College of Medicine, the Cold Spring Harbor, and the Weizmann Institute of Sciences
468 Institutional Animal Care and Use Committees.

469

470 ***Cell Culture***

471 Primary cultures of hippocampal neurons were obtained from E18 Sprague-Dawley rat
472 embryos. Hippocampi were dissected and dissociated. For imaging, neurons were plated onto
473 Poly-D-Lysine (Sigma) and laminin-coated glass coverslips (Life Technologies) at a density of
474 $\sim 1.25 \times 10^4$ cells/cm². For mass spectrometry, neurons were plated onto Poly-D-Lysine and
475 laminin-coated 10 cm dishes at a density of $\sim 2.5 \times 10^4$ cells/cm². Hippocampal neurons were
476 maintained in Neurobasal medium (Life Technologies) containing 1% Glutamax (Life

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477 Technologies), 1% penicillin and streptomycin (Life Technologies), and 2% B27 supplement (Life
478 Technologies) in an incubator at 37°C with 5% CO₂.

479

480 ***Biotinylation by Antibody Recognition***

481 Cultured rat primary neurons (~2 × 10⁶ primary hippocampal neurons for each
482 condition) were biotinylated at each of 5 different timepoints. Cells were live labeled by
483 incubating with primary antibodies diluted in culture media for 1 hr at 37°C, then washed with
484 neurobasal media and incubated in culture media alone for 1 hr at 37°C. Cells were incubated
485 with horseradish peroxidase (HRP)-labeled secondary antibodies diluted in culture media for 30
486 min at 37°C, then washed with PBS. Biotin tyramide (Perkin Elmer Cat# NEL749A001KT) was
487 diluted 1:500 in a dilution buffer containing H₂O₂, and applied to cells for 5 min at 4°C. Cells
488 were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% sodium
489 deoxycholate, 0.1% SDS, and 1% NP-40). Biotinylated proteins were isolated using streptavidin
490 magnetic sepharose beads (GE Healthcare Cat# 28-9857-38) overnight at 4°C and then washed
491 seven times in RIPA buffer. Control cells were labeled and processed using the same steps
492 except for the omission of primary antibodies.

493

494 ***Mass Spectrometry.***

495 Sample-incubated streptavidin magnetic sepharose beads were resuspended in 5 mM
496 DTT in 100 mM NH₄HCO₃ and incubated for 30 min at room temperature. After this,
497 iodoacetamide was added to a final concentration of 7.5 mM and samples incubated for 30
498 additional minutes. In all, 0.5 µg of sequencing grade trypsin (Promega) was added to each

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499 sample and incubated at 37°C overnight. Supernatants of the beads were recovered, and beads
500 digested again using 0.5 µg trypsin in 100 mM NH₄HCO₃ for 2 h. Peptides from both consecutive
501 digestions were recovered by solid phase extraction using C18 ZipTips (Millipore, Cat#
502 ZTC18S096), and resuspended in 0.1% formic acid for analysis by liquid chromatography-mass
503 spectrometry (LC-MS/MS). Peptides resulting from trypsinization were analyzed on a QExactive
504 Plus (Thermo Scientific), connected to a NanoAcquity™ Ultra Performance UPLC system
505 (Waters). A 15-cm EasySpray C18 column (Thermo Scientific) was used to resolve peptides (90-
506 min gradient with 0.1% formic acid in water as mobile phase A and 0.1% formic acid in
507 acetonitrile as mobile phase B). MS was operated in data-dependent mode to automatically
508 switch between MS and MS/MS. The top ten precursor ions with a charge state of 2⁺ or higher
509 were fragmented by high-energy collisional dissociation. Peak lists were generated using PAVA
510 software ⁵⁶. All generated peak lists were searched against the rat subset of the UniProt
511 database (UniprotKB 2017.11.01) using Protein Prospector ⁵⁷. The database search was
512 performed with the following parameters: a mass tolerance of 20 ppm for pre- cursor masses;
513 30 ppm for MS/MS, cysteine carbamidomethylation as a fixed modification and acetylation of
514 the N terminus of the protein, pyroglutamate formation from N-terminal glutamine, and
515 oxidation of methionine as variable modifications. All spectra identified as matches to peptides
516 of a given protein were reported, and the number of spectra (Peptide Spectral Matches, PSMs)
517 used for label free quantitation of protein abundance in the samples.

518

519 ***Antibodies***

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520 The following antibodies were used for *in vitro* biotinylation (dilutions for each antibody
521 are indicated in parentheses): chicken polyclonal antibody anti-neurofascin (1:1000; R&D
522 Systems Cat# AF3235, RRID:AB_10890736), rabbit polyclonal anti-NrCAM (1:1000; Abcam Cat#
523 ab24344, RRID:AB_448024), anti-chicken HRP-labeled secondary antibody (1:2000; Aves Labs
524 Cat# H-1004, RRID:AB_2313517), anti-rabbit HRP-secondary antibody (1:2000; Jackson
525 ImmunoResearch Labs Cat# 111-035-003, RRID:AB_2313567).

526 The following antibodies were used for immunofluorescence studies (dilutions for each
527 antibody are indicated in parentheses): chicken polyclonal anti-neurofascin (1:500; R&D
528 Systems Cat# AF3235, RRID:AB_10890736), chicken polyclonal anti-MAP2 (1:1000; EnCor
529 Biotechnology Cat# CPCA-MAP2, RRID:AB_2138173), mouse monoclonal anti-Ankyrin-G (1:500;
530 NeuroMab N106/36, RRID:AB_10673030), mouse monoclonal anti-Tenascin-R (1:250; R&D
531 Systems Cat# MAB1624, RRID:AB_2207001), rabbit polyclonal anti-NrCAM (1:250; Abcam Cat#
532 ab24344, RRID:AB_448024), rabbit polyclonal anti- β 4 spectrin (1:500, Rasband lab,
533 RRID:AB_2315634), rabbit polyclonal anti-Kv1.2 (1:250, James Trimmer, University of California,
534 Davis, RRID:AB_2756300), mouse monoclonal anti-PSD-95 (1:250; Antibodies Incorporated Cat#
535 75-028, RRID:AB_2292909), mouse monoclonal anti-Tuj1 (1:700; BioLegend Cat# 801202,
536 RRID:AB_10063408), goat polyclonal anti-Cntn1 (1:500; R&D Systems Cat# AF904,
537 RRID:AB_2292070), mouse monoclonal anti-Myc (1:2000; MBL International Corporation Cat#
538 M192, PRID: AB_11160947), rat monoclonal anti-HA (1:500; Millipore Sigma Cat# 11867423001,
539 RRID: AB_390918), mouse monoclonal anti-V5 (1:500; Invitrogen Cat# R960CUS, RRID:
540 AB_159298). Anti-RFP (guinea pig pAb, 1:1000, Synaptic systems 390 005), anti-gephyrin

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541 (mouse mAb IgG1, 1:500, Synaptic Systems 147 011), and anti-VGAT (guinea pig pAb, 1:500,
542 Synaptic Systems 131 004).

543 The following secondary antibodies were used: Alexa Fluor 555 goat anti-rat (1:1000,
544 Thermo Fisher Scientific A-11006), Alexa Fluor Plus 555 goat anti-rabbit (1:1000, Thermo Fisher
545 Scientific A32732), Alexa Fluor 647 goat anti-rabbit (1:1000, Thermo Fisher Scientific A-21244),
546 Alexa Fluor 555 goat anti-guinea pig (1:1000, Thermo Fisher Scientific A-21435), Alexa Fluor 647
547 goat anti-guinea pig (1:1000, Thermo Fisher Scientific A-21450), Alexa Fluor 647 goat anti-
548 mouse IgG1 (1:1000, Thermo Fisher Scientific A-21240), Alexa Fluor 488 goat anti-mouse IgG2a
549 (1:1000, Thermo Fisher Scientific A-21131), Aminomethylcoumarin (AMCA) anti-chicken IgY
550 (1:1000 Jackson Immunoresearch labs 103-155-155), Alexa Fluor 488 anti-chicken IgY, (1:1000
551 Jackson Immunoresearch labs 103-545-155), Alexa Fluor 488 anti-mouse IgG (1:1000 Thermo
552 Fisher Scientific A11029), Alexa Fluor 594 anti-rabbit (1:1000 Thermo Fisher Scientific A11034),
553 Alexa Fluor 594 anti-mouse IgG (1:1000 Thermo Fisher Scientific A32742). Streptavidin Alexa
554 Fluor 594 conjugates were purchased from Thermo Fisher Scientific (1:5000; S11227). Hoechst
555 fluorescent reagent (1:100,000; Thermo Fisher Scientific Cat# H3569, RRID:AB_2651133) was
556 used to label nuclei.

557

558 ***In Utero* Electroporation and Tamoxifen Induction**

559 To manipulate Cntn1 expression in pyramidal neurons (PyNs) and sparsely label
560 chandelier cells (ChCs) in the same neocortical layer, ventricular zone-directed *in utero*
561 electroporation targeting neocortical PyN progenitors was performed in *Nkx2.1-CreER;Rosa26-*
562 *loxpSTOPloxp-tdTomato (Ai9)* embryos. Specifically, Swiss Webster females were bred with

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563 *Nkx2.1-CreER^{+/-};Ai9^{+/+}* males. Pregnant females at 15.5 days of gestation were anesthetized, the
564 uterine horns were exposed, and approximately 1 μ l of plasmid solution (0.75 μ g/ μ l pCAG-
565 1BP-NLS-Cas9-1BP-NLS + 1.5 μ g/ μ l pAAV-3x-sgRNA-smFP (with control or target specific sgRNAs;
566 see STAR Methods) was injected manually into the lateral ventricle of the embryos using a
567 beveled glass micropipette (Drummond Scientific). After injection, five square 50 ms pulses of 45
568 V with 950 ms intervals were delivered across the uterus with two 5 mm electrode paddles (BTX,
569 45-0489) positioned on either side of the head (BTX, ECM830). After electroporation, the uterine
570 horns were placed back in the abdominal cavity of the pregnant dam and the wound was
571 surgically sutured. Tamoxifen (3 mg/30 g of body weight) was administered to the pregnant dam
572 by oral gavage at 18.5 days of gestation to induce CreER activity and excision of the STOP cassette,
573 resulting in tdTomato red fluorescent protein expression in a sparse population of nascent
574 neocortical ChCs in their offspring. Pups were euthanized at postnatal day 17.

575

576 ***Immunofluorescence labeling***

577 Cultured rat primary hippocampal neurons were fixed in 4% paraformaldehyde (PFA, pH
578 7.2) for 15 minutes at 4°C. Acutely dissected brains were drop fixed in 4% paraformaldehyde
579 (PFA, pH 7.2) for 60 minutes at 4°C. Brains were then equilibrated overnight in 20% and 30%
580 sucrose in 0.1 M PB overnight at 4°C. Brains were then sectioned at 12-25 μ m and mounted on
581 coverslips. Fixed neurons and brain sections were permeabilized and blocked with 10% normal
582 goat serum in 0.1 M PM with 0.3% Triton X-100 (PBTGS) for one hour. Cells and sections were
583 then incubated in primary antibodies diluted in PBTGS overnight at room temperature or 4°C.
584 Tissues and cells were then washed three times using PBTGS for 5 min. each. Fluorescent

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585 secondary antibodies were then diluted in PBTGS and added to cells and tissues for one hour.
586 Coverslips were then washed once using PBTGS, 0.1 M PB, and finally 0.05 M PB for 5 min.
587 each. Coverslips were then mounted using Vectashield plus (vector labs) anti-fade mounting
588 media.

589 For immunostaining of electroporated neonatal brains, P17 mice were deeply
590 anesthetized with isoflurane and perfused transcardially with PBS and 4% paraformaldehyde
591 (PFA) in 0.1 M phosphate buffer. Brains were post-fixed in 4% PFA in 0.1 M phosphate buffer
592 overnight at 4°C and then cryoprotected with 30% sucrose in PBS. 50 µm thick coronal sections
593 were subsequently generated using a Vibratome (Leica VT1000S). For gephyrin and VGAT
594 immunostaining, brain slices were subjected to mild antigen retrieval with 10 mM citrate buffer
595 for 30 minutes at 60°C. Subsequently, brain slices were blocked and permeabilized with 10%
596 normal goat serum (NGS) and 0.3% Triton X-100 in PBS at RT for 30 minutes and then incubated
597 with primary antibodies diluted in 2% NGS and 0.3% Triton X-100 in PBS overnight at 4°C.
598 Fluorescent secondary antibodies diluted in 2% NGS and 0.3% Triton X-100 in PBS were applied
599 for 2 h at RT the following day. Sections were then washed three times with PBS for 20 min per
600 wash and mounted with Fluoromount-G (Southern Biotech).

601

602 ***Plasmid construction***

603 The sgRNAs and the homology-independent donor templates were generated following
604 strategies similar to those described previously^{19,22,23,58}. Briefly, the U6 promoter and scaffold
605 sequences were PCR amplified from pMJ117 and pMJ179 (gifts from Jonathan Weissman,

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606 Addgene plasmid #85997 and #85996). The smFP-V5 (a gift from Loren Looger, Addgene
607 plasmid #59758) was used as a knock-in donor.

608 The knockout constructs expressing three independent sgRNAs and a smFP-HA marker
609 (pAAV-3x-gRNA-smFP) were generated as follows: the U6 promoter and scaffold sequences
610 were PCR amplified from pMJ114, pMJ117, and pMJ179 (gifts from Jonathan Weissman,
611 Addgene plasmid # 85995, #85997, and #85996). Human Synapsin1 promoter and smFP-HA
612 were PCR amplified from pAAV-hSyn-EGFP (a gift from Bryan Roth, Addgene Plasmid #50465)
613 and pCAG_smFP-HA (a gift from Loren Looger, Addgene plasmid #59759), respectively. The
614 plasmid PX552 (a gift from Feng Zhang, Addgene plasmid #60958) was digested with a *NotI*
615 restriction enzyme (NEB) and used as a plasmid backbone. DNA fragments were ligated
616 together using an In-Fusion Snap Assembly Master Mix (Takara). The sgRNA sequences for
617 knock-in and knockout are listed in the supplemental materials table. The AAV-SpCas9 plasmid
618 (a gift from Feng Zhang, Addgene plasmid #60957) was modified by removing the HA tag.

619 Cntn1 constructs were generated in both pcDNA3 and AAV backbones. pcDNA3 was
620 digested with *EcoRI* restriction enzyme (NEB) and pAAV-hSyn-EGFP (a gift from Bryan Roth,
621 Addgene Plasmid #50465) was digested with *BamHI* and *XhoI* restriction enzymes (NEB) and
622 used as plasmid backbones. Full-length and truncated Cntn1 was PCR amplified from rat
623 contactin-myc³⁴ and ligated together using an In-Fusion Snap Assembly Master Mix (Takara).
624 All DNA constructs were verified by sequencing (Genewiz and plasmidsaurus).

625

626 ***Adeno-associated virus (AAV) production***

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627 Small scale AAV cell-lysates were produced using the AAVpro Purification Kit (All Serotypes)
628 (Takara) with slight modifications. Briefly, HEK293T cells were triple-transfected with AAV
629 plasmid, helper plasmid (Agilent Technologies, Cat # 240071), and serotype PHP.S or PHP.eB
630 plasmids (a gift from Viviana Gradinaru, Addgene plasmids #103002 and #103005) with PEI Max
631 (Polysciences, Cat # 24765). The medium was changed the next day of transfection and cells
632 were incubated for 3 days after transfection. HEK cells were then collected and lysed with the
633 AAV Extraction Solution A plus. The extracted solution was centrifuged at 10,000 x *g* for 10 min
634 to remove debris and mixed with Extraction Solution B. This small scale AAV solution was stored
635 at 4°C and used for neuronal transduction into cultured neurons. AAV vectors for *in vivo*
636 transduction were produced by the Baylor College of Medicine Neuroconnectivity Core or in
637 our lab following the strategies described previously⁵⁹.

638

639 ***Viral transduction of neurons***

640 For viral transduction of cultured neurons, 10 µl of AAV-Cas9 and 10 µl of AAV-sgRNA and
641 donor, or AAV-3x-sgRNA-smFP, were added into a well of a 12-well plate at 0-1 DIV. The
642 medium was replaced 2 days after infection. For viral transduction of neurons *in vivo*, AAV
643 vectors were injected into the lateral ventricles of neonatal mice as described previously⁶⁰.
644 Briefly, P0 to P2 pups were anesthetized on ice and 1-2 µl of AAV vectors were bilaterally
645 injected. The pups were placed in a heated cage until the animals recovered and then returned
646 to their mother. For transduction of neurons in adult, viruses were injected retro-orbitally in 13
647 week-old C57Bl/6J mice. Tissues were collected 4 weeks after infection.

648

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649 ***Confocal Image Acquisition and Analysis of ChC/PyN AIS Innervation and GABAergic Synapse***

650 ***Density at PyN AISs***

651 For analysis of ChC/PyN AIS percent innervation, images of coronal brain slices (50 μm thick) were
652 acquired using an LSM 800 confocal laser-scanning microscope (Zeiss) with a 63x oil-immersion
653 objective and sequential acquisition settings applied at a resolution of 1024x1024 pixels. 200 μm
654 x 200 μm images of single RFP+ ChCs and neighboring GFP+ electroporated PyNs in layer II of the
655 somatosensory cortex were collected using a z series of 30-36 images with a depth interval of 1
656 μm . ChC/PyN AIS percent innervation was calculated by dividing the number of GFP+ PyNs
657 innervated at their AIS by a single RFP+ ChC by the total number of GFP+ PyNs in the entire 200
658 μm x 200 μm image z stack. Representative maximum projection images were generated from
659 10 z-series images with a depth interval of 1 μm . To quantify the average density of gephyrin or
660 VGAT puncta per μm on the AIS of PyNs, 90 μm x 90 μm images were acquired at a resolution of
661 1280x1280 pixels using a z-series of 40-60 images with a depth interval of 0.37 μm . The number
662 of gephyrin or VGAT puncta overlapping with AnkG+ or β 4-spectrin+ PyN AISs in individual z-
663 plane images was manually counted and AIS lengths were measured using Zeiss Zen (Blue Edition)
664 imaging software. Gephyrin or VGAT puncta density at the AIS was then calculated by dividing
665 the number of PyN AIS gephyrin or VGAT puncta by the length of the AIS. Representative
666 maximum projection images of PyN AIS GABAergic synapses visualized via gephyrin or VGAT
667 immunostaining were generated using a z-series of 10 images with a depth interval of 0.37 μm .

668

669 ***Image Acquisition***

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670 Images of immunofluorescence were captured using an Axio-imager Z2 microscope fitted with
671 an apotome attachment for structured illumination (Carl Zeiss MicroImaging) and a Nikon
672 Eclipse Ni2. 20X (0.8 NA), 40X (0.95 NA), and 63X (1.4 NA) objectives were used. Images were
673 taken using Zen 3.2 (Zeiss) or NIS-Elements (Nikon). For measurements of AIS streptavidin
674 fluorescence intensity, 20 neurons per timepoint per replicate were imaged and line scans were
675 drawn using Zen 3.2 software. Images were exported to Fiji, Adobe Photoshop, and Adobe
676 illustrator for figure presentation. Some figures were generated using Biorender.

677

678 ***Statistical Analysis and Quantification***

679 Unpaired, two-tailed Student's *t*-test was used for all statistical analyses unless otherwise
680 indicated. Data were analyzed using Microsoft Excel and GraphPad Prism. All error bars are
681 \pm SEM unless otherwise indicated. PSMs were normalized using the formula $PSM_{Norm} = PSM_{DIVX} * Csum_{Max} / Csum_{DIVX}$.
682 $Csum_{Max}$ / $Csum_{DIVX}$. PSM_{DIVX} is the raw PSM count for a candidate protein for a particular
683 replicate at the specified timepoint; $Csum_{Max}$ is the maximum sum of seven endogenously
684 biotinylated carboxylases Acaca, Acacb, Pc, Pcca, Pccb, Mccc1, Mccc2 for an individual replicate
685 across all 15 replicates; $Csum_{DIVX}$ is the sum of seven endogenously biotinylated carboxylases
686 Acaca, Acacb, Pc, Pcca, Pccb, Mccc1, Mccc2 for an individual replicate at the specified timepoint
687 DIVX. Heatmaps were generated using GraphPad Prism. Candidates were rank-ordered by the
688 slope of the linear regression of their \log_2 fold enrichment over time. The number of
689 extracellular tyrosines for proteins whose \log_2 fold change was > 2 and that had at least ten
690 PSMs were counted using a script that extracted protein topology from the Uniprot database

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691 (www.uniprot.org). The tyrosine-PSM proximity plot was generated using Python

692 (www.python.org). Colors were added in Adobe Illustrator.

693

694 ***Extended materials***

695 A detailed list of all materials including all gRNA sequences, antibodies, plasmids,

696 sources, etc. is provided in the supplemental extended materials file.

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701

702 **AUTHOR CONTRIBUTIONS**

703 Conceptualization methodology, validation, investigation, visualization, and writing— reviewing
704 and editing: Y.O. and B.C.L.; Investigation, analysis, and writing – reviewing and editing: S.G.;
705 Investigation and analysis: J.M.R., Y.E-E., and F.B.; Investigation, data curation: J.O-P.;
706 Resources, supervision, funding acquisition, and writing – reviewing and editing: A.L.B., E.P.,
707 and L.V.A.; Conceptualization, methodology, data curation, writing— original draft and editing,
708 project administration, and funding acquisition: M.N.R.

709

710 **COMPETING INTERESTS**

711 The authors declare no competing interests.

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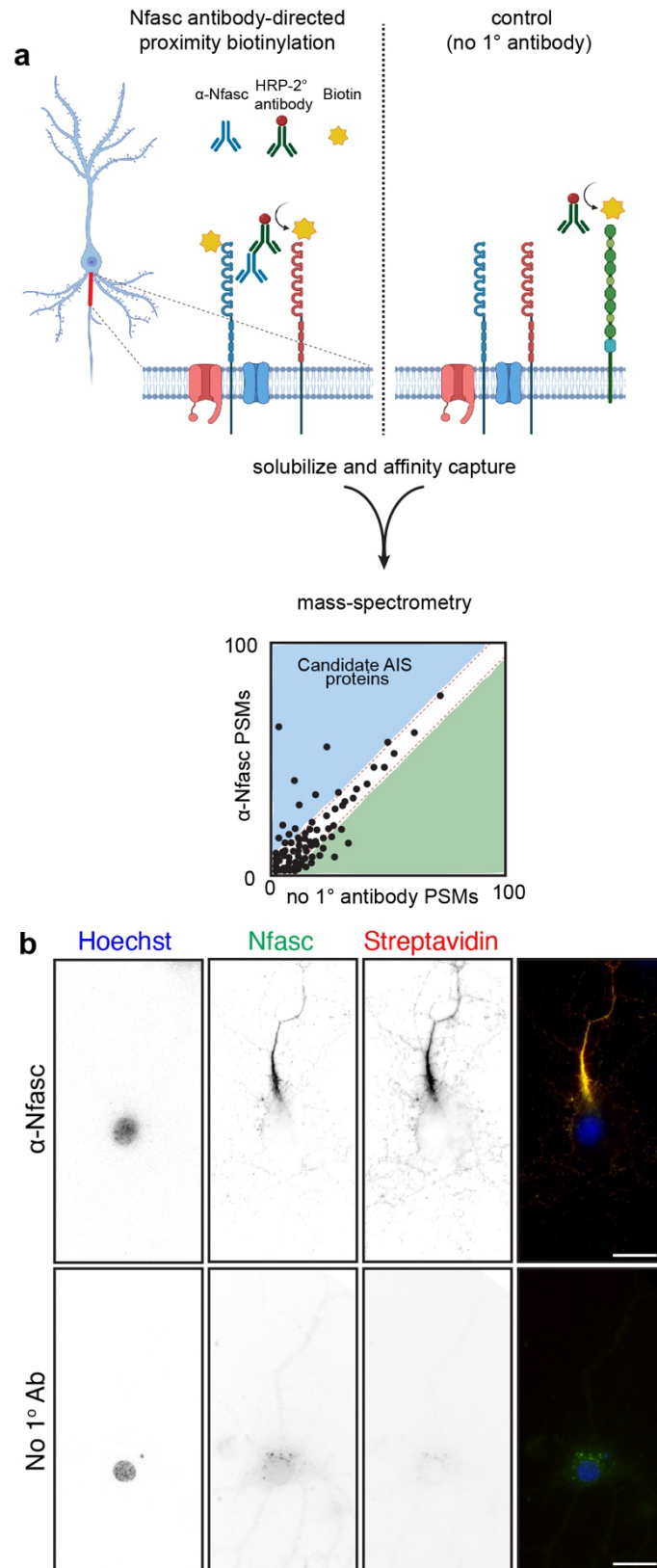
713 **DATA AVAILABILITY STATEMENT**

714 All mass spectrometry data sets from experiments included here are deposited at PRIDE
715 (Proteomics Identifications Database).

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717 FIGURES

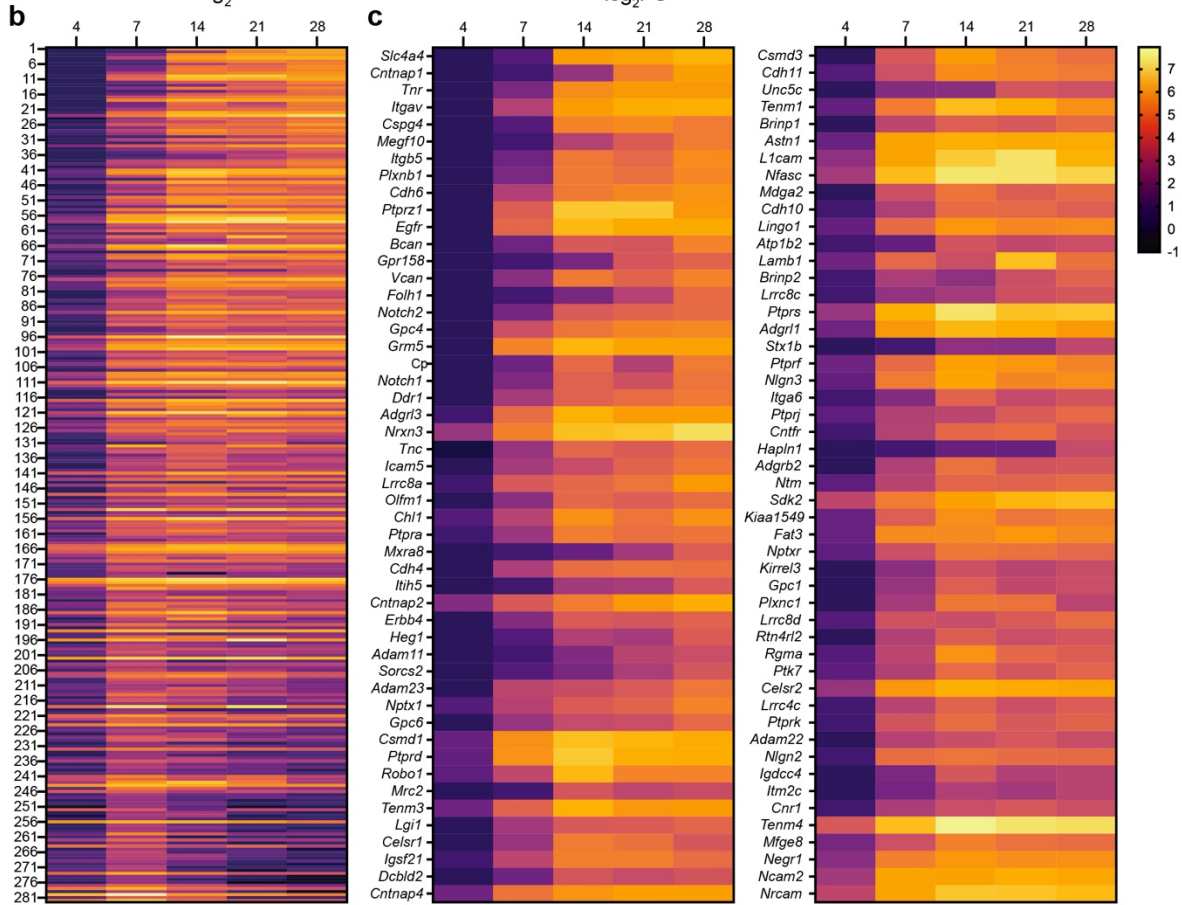
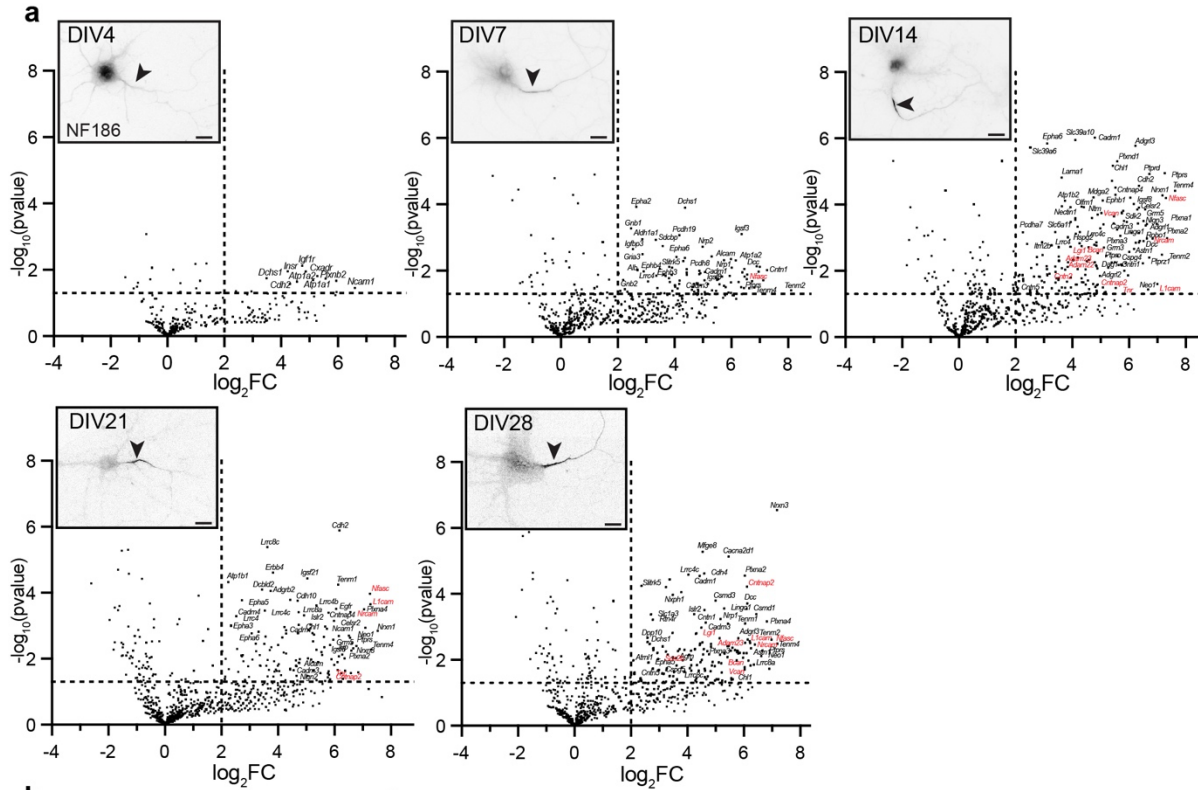


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719 **Figure 1. Proximity-dependent biotinylation using Nfasc antibodies. a,** Illustration of the
720 antibody-directed proximity biotinylation strategy. Anti-Nfasc antibodies bind to Nfasc, while
721 HRP-conjugated secondary antibodies bind to the Nfasc antibodies. Addition of biotin phenol
722 (biotin tyramide) in an H₂O₂ containing diluent results HRP-mediated conversion of the biotin
723 phenol to an active radical biotin phenoxy that covalently adds the tyramide biotin to
724 extracellular tyrosine residues. Omission of the primary anti-Nfasc serves as a control. After
725 stringent solubilization and affinity capture by streptavidin-conjugated magnetic beads.
726 Biotinylated proteins are then identified by mass spectrometry. **b,** Fluorescence imaging of
727 DIV14 rat hippocampal neurons labeled by Nfasc-BAR or a control condition (no primary Ab).
728 Nfasc fluorescence (green) enrichment defines the AIS. Biotinylated proteins were detected
729 using Alexa594-conjugated streptavidin. Scale bars, 20 μm.

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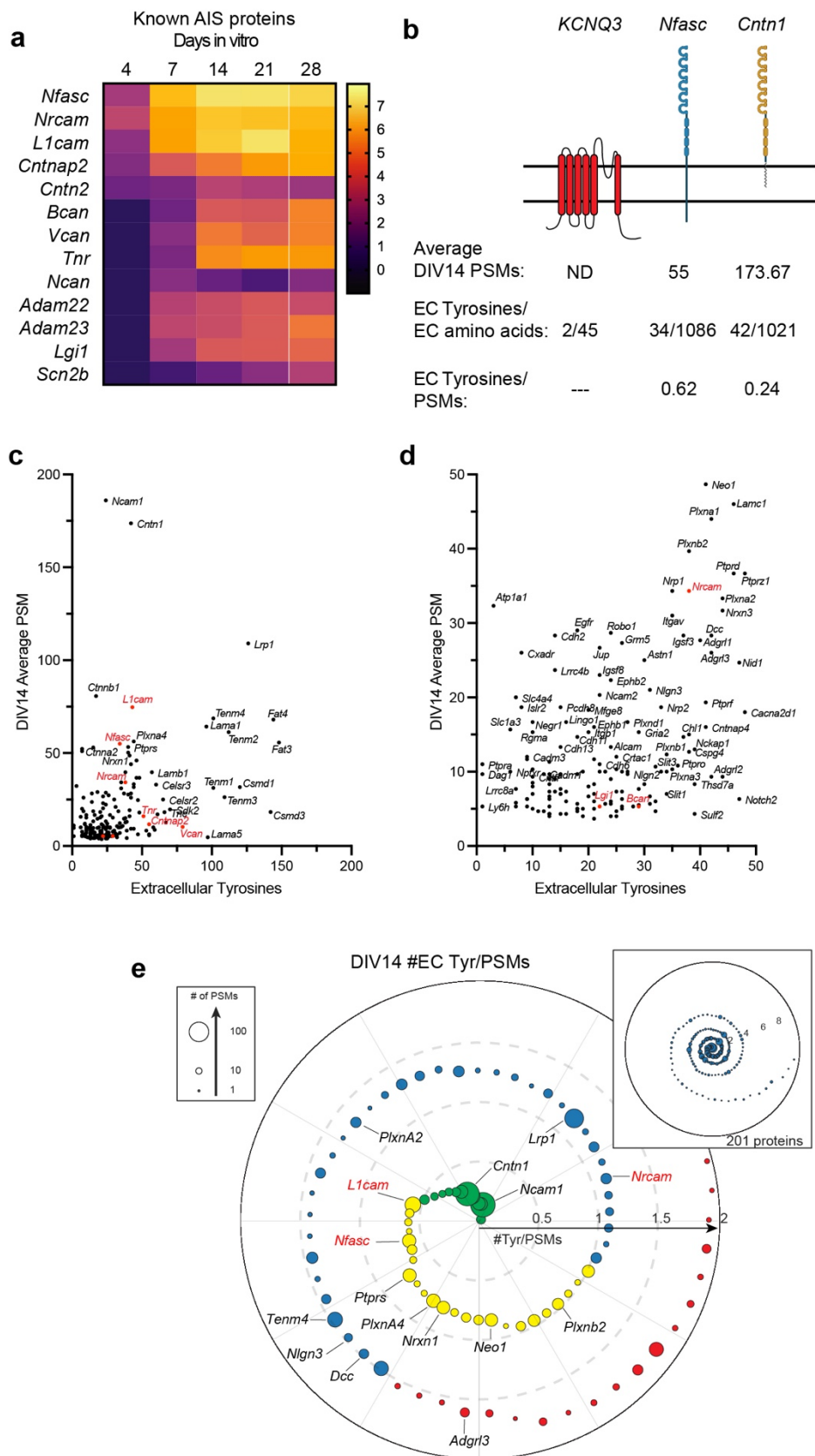


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Figure 2. NF186 proximity proteomes across neuronal development. a, Volcano plots showing the \log_2 -fold changes of proteins versus the statistical significance $-\log_{10}(\text{pvalue})$ identified using Nfasc-directed proximity biotinylation (N=3). $p < 0.05$ was used as a cutoff for significance (horizontal dashed line). Some identified proteins are indicated (corresponding gene names listed), with those previously reported as AIS cell surface proteins in red, respectively. Inset images show immunofluorescence labeling of NF186 at different timepoints of hippocampal neuron development *in vitro*. Lower panels: magnified images show the Nfasc-labeled AIS at each time point. Scale bars, 20 μm . **b,** Heatmaps showing \log_2 -fold changes at each timepoint for all 285 proteins that satisfied two filtering criteria [(1) normalized PSMs > 10 ; (2) $\log_2\text{FC}$ (Nfasc/Ctrl) > 2] for at least one of five timepoints, rank-ordered by the slope of the linear regression of their \log_2 fold enrichment over time. **c,** Expanded heat map showing gene names for the proteins (1-50 and 51-100) with the largest rate of increase in PSM count (B). Data shown are from N = 3 replicates for each timepoint (see Figure S2).

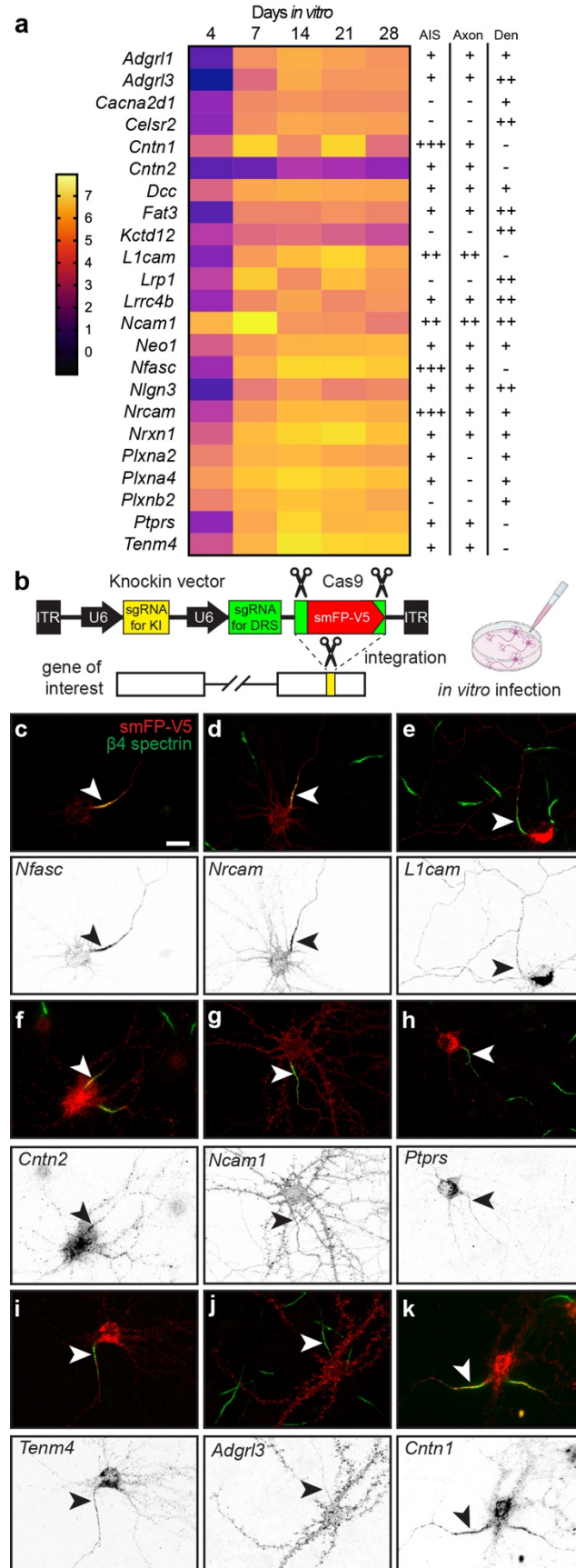
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748 **Figure 3. Nfasc-BAR identifies known AIS membrane and membrane-associated proteins. a,**
749 Known AIS membrane and membrane associated proteins and their \log_2FC (Nfasc/Ctrl). **b,**
750 Illustration of membrane topology, average PSMs (at DIV14), and the number of extracellular
751 tyrosine residues for three different AIS and membrane proteins. Figure generated using
752 Biorender. **c, d,** Scatter plot of the number of peptide spectral matches (PSMs) for each
753 biotinylated protein identified by mass spectrometry as compared to the number of tyrosine
754 residues present in each protein's extracellular domain, shown at different scales. Proteins in
755 red were previously reported at the AIS. **e,** Proximity plot showing biotinylated proteins (at
756 DIV14) ordered by extracellular (EC) tyrosine/PSM ratio. The plot is an estimate of abundance
757 and proximity to the HRP secondary antibody bound to the Nfasc primary antibody. Each
758 protein is represented by a circle with size proportional to the number of PSMs identified for
759 that protein. Proteins analyzed in subsequent experiments are indicated by their gene names.
760 EC Tyrosine/PSM ratio: 0-0.5 green, 0.5-1.0 yellow, 1.0-1.5 blue, 1.5-2.0 red.
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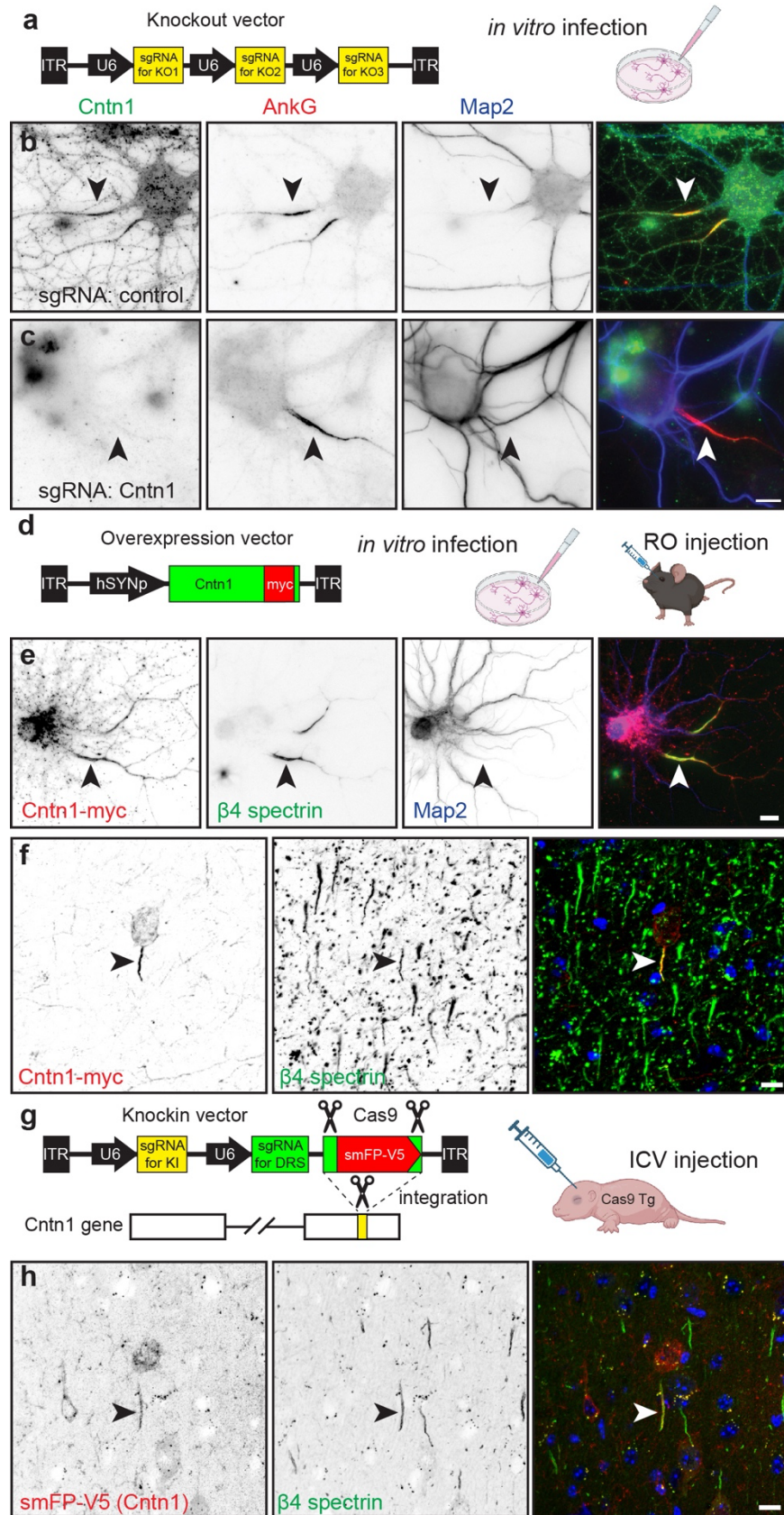
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763 **Figure 4. Tagging of endogenous membrane proteins. a**, Proteins whose distribution was
764 tested using endogenous protein tagging. The heatmap shows the increase in expression level
765 is shown as a function of days *in vitro* (see also Figure 2c). The presence of the tagged protein
766 in AIS, axon, and dendrite is indicated. **b**, Schematic of the knock-in vector for *in vitro* CRISPR-
767 mediated endogenous gene tagging. DRS, donor recognition sites. **c-k**, Examples of smFP-V5
768 tagged proteins (red) enriched at the AIS (c, d, f, k), the axon (e, g), dendrites (j), or in multiple
769 domains (e, g, h, i). AIS are labeled for β 4 spectrin (green) and are indicated by an arrowhead.
770 Scale bar, 20 μ m.
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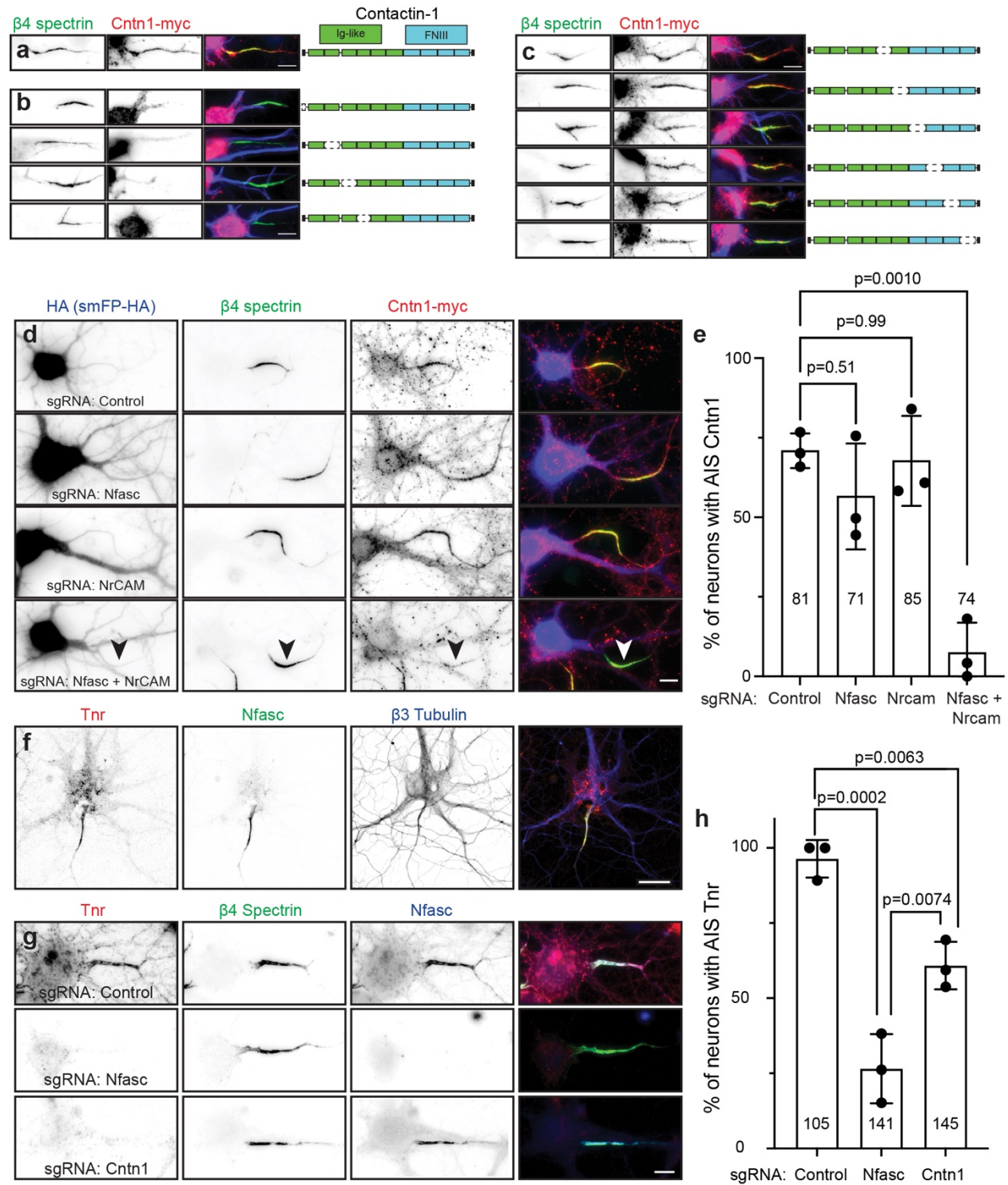
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774 **Figure 5. Cntn1 is a bona fide AIS protein. a**, Schematic of the knockout vector including 3
775 sgRNAs targeting the gene of interesting. The AAV generated using this vector were used for *in*
776 *vitro* transduction of neurons. **b, c**, Immunostaining for Cntn1 (green), AnkG (red) and Map2
777 (blue) after transduction with AAV to Cas9 and control (b) or Cntn1 (c) sgRNAs. Neurons
778 transduced with the Cntn1 sgRNAs lacked AIS Cntn1, but retained robust AnkG at the AIS. AIS
779 are indicated by the arrowheads. Scale bar, 10 μ m. **d**, Schematic of the Cntn1-myc
780 overexpression vector used for *in vitro* and *in vivo* infection of neurons. **e**, Transduction of
781 cultured hippocampal neurons using AAV to express Myc-tagged Cntn1. Cntn1-myc (red) is
782 enriched at the AIS (arrowhead) where it colocalizes with β 4 spectrin (green). The
783 somatodendritic domain is identified using antibodies against Map2 (blue). Scale bar, 10 μ m. **f**,
784 *In vivo* transduction of cortical neurons using AAV to express Myc-tagged Cntn1. Cntn1-myc
785 (red) is enriched at the AIS (arrowhead) where it colocalizes with β 4 spectrin (green). Nuclei are
786 labeled using Hoechst dye (blue). Scale bar, 10 μ m. **g**, Schematic of the knock-in vector for *in*
787 *vivo* CRISPR-mediated endogenous tagging of Cntn1. DRS, donor recognition sites. AAV were
788 delivered by intracerebroventricular (ICV) injection at P0. **h**, *In vivo* transduction of cortical
789 neurons for CRISPR-dependent genome editing to tag endogenous Cntn1 using smFP-V5 (red).
790 The smFP-V5 tagged Cntn1 colocalizes with β 4 spectrin (green) at the AIS (arrowhead). Nuclei
791 are labeled using Hoechst dye (blue). Scale bar, 10 μ m.

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795 **Figure 6. Cntn1 is recruited to the AIS through interactions with AnkG-binding L1-family cell**
796 **adhesion molecules. a,** Cntn1-myc (red) is targeted to the AIS and colocalizes with β 4 spectrin
797 (green). Cntn1 consists of 6 N-terminal Immunoglobulin (Ig)-like domains and 4 C-terminal
798 Fibronectin type III (FNIII) domains. Scale bar, 10 μ m. **b,** Cntn1-myc with N-terminal and
799 internal deletions of the first 4 Ig-like domains fail to localize at the AIS. Scale bar, 10 μ m. **c,**
800 Cntn1-myc localization to the AIS does not depend on the last 2 Ig-like domains or any FNIII
801 domain. Scale bar, 10 μ m. **d,** Cultured hippocampal neurons transduced with AAV to express
802 Cas9 and control, Nfasc, NrCAM, or Nfasc+NrCAM gRNAs. Neurons were labeled using
803 antibodies against HA as a transduction marker (to label the spaghetti monster fluorescent
804 protein tagged with HA, smFP-HA; blue), β 4 spectrin (green), and Cntn1-myc (red). Scale bar,
805 10 μ m. **e,** Quantification of the percentage of transduced neurons with AIS Cntn1-myc. N= 3
806 independent experiments. Ordinary one-way ANOVA with Tukey's multiple comparisons test.
807 Error bars, \pm SEM. The total number of neurons analyzed is also indicated. **f,** Immunostaining of
808 cultured hippocampal neurons using antibodies against Tenascin R (Tnr; red), β 4 spectrin
809 (green), and β 3 Tubulin (blue). Scale bar, 25 μ m. **g,** Cultured hippocampal neurons transduced
810 with AAV to express Cas9 and control, Nfasc, or Cntn1 gRNAs. Neurons were labeled using
811 antibodies against Tnr (red), β 4 spectrin (green), and Nfasc (blue). Scale bar, 10 μ m. **h,**
812 Quantification of the percentage of transduced neurons with AIS Tnr. N= 3 independent
813 experiments. Ordinary one-way ANOVA with Tukey's multiple comparisons test. Error bars,
814 \pm SEM. The total number of neurons analyzed is also indicated.

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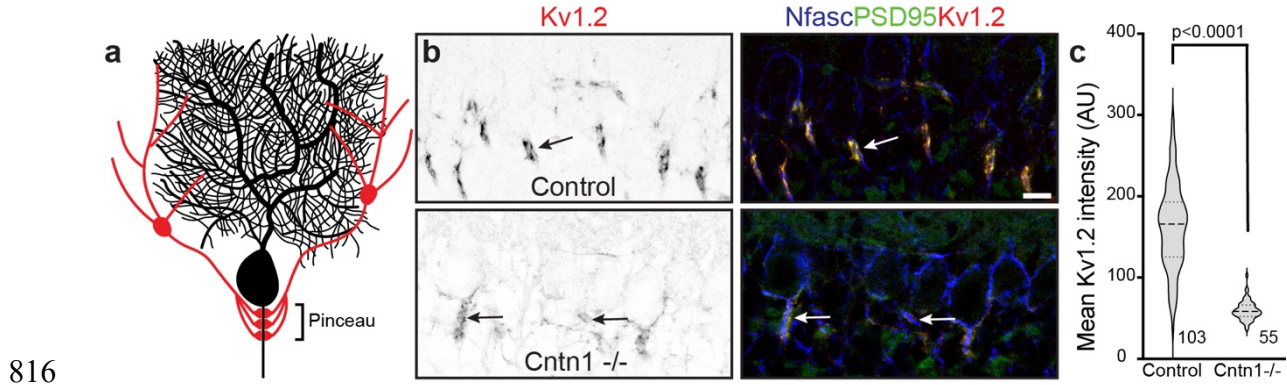
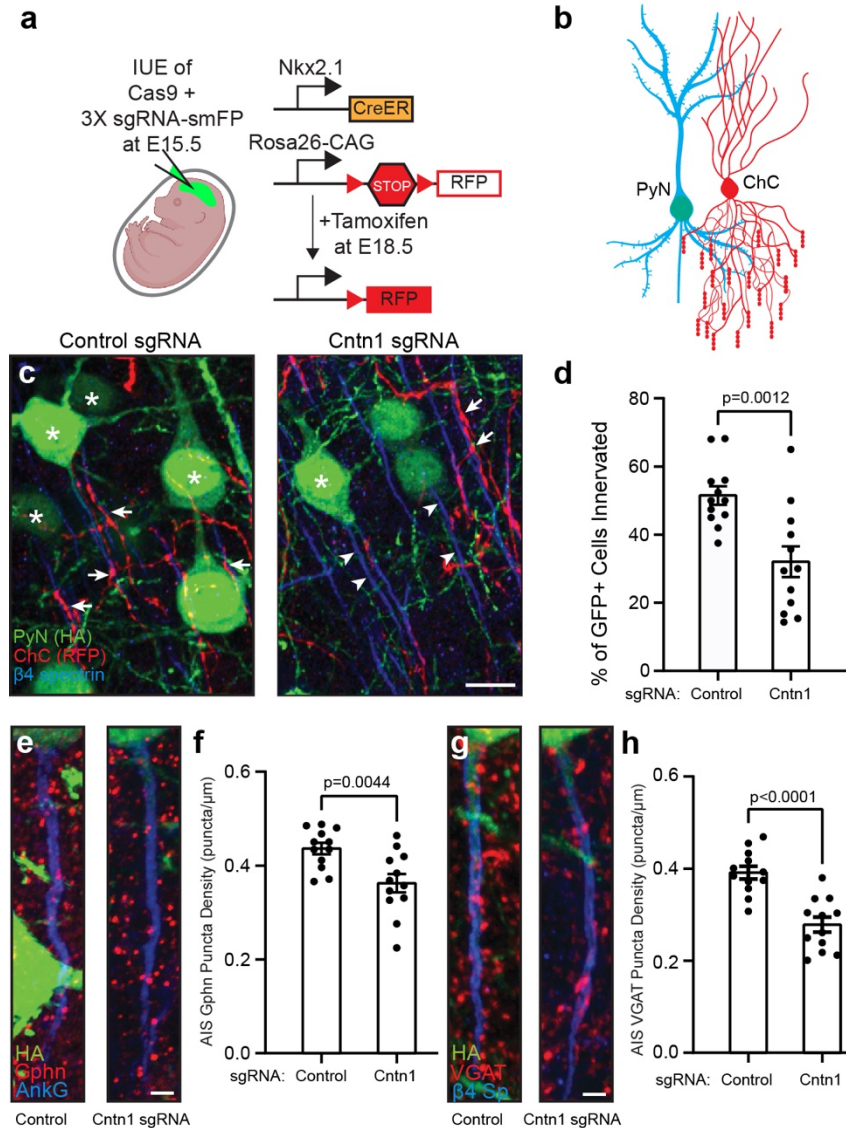


Figure 7. Cntn1 is required for axo-axonic innervation of Purkinje neuron AIS. **a**, Illustration of a Purkinje neuron (black) with a basket cell (red) forming the cerebellar pinceau on the AIS. **b**, Immunostaining of P17 cerebellar pinceau in control and *Cntn1*^{-/-} mouse brain using antibodies against Kv1.2 (red) and PSD95 (green) to label the pinceau, and Nfasc (blue) to label the Purkinje neuron AIS. Scale bar, 10 μ m. **c**, Violin plot of the mean Kv1.2 intensity of the cerebellar pinceau in control and *Cntn1*^{-/-} mice. N=3 control and 2 *Cntn1*^{-/-} mice. The number of pinceau analyzed is indicated.

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828 **Figure 8. Pyramidal neuron Cntn1 is important for AIS synaptic innervation by ChCs. a,**

829 Illustration of the knockout and labeling strategy for PyN and ChCs. PyNs are electroporated at

830 E15.5 using plasmids to express Cas9 and 3X sgRNA-smFP (HA tag) to delete expression on Cntn1.

831 ChCs are labeled by expression of red fluorescent protein (RFP) using inducible Cre (CreER) in

832 *Nkx2.1-CreER* mice at E18.5. **b,** Illustration of ChC (red) innervation of PyN (blue/green) AIS. **c,**

833 Representative images of PyNs innervated at their AIS by ChC cartridges (red) in layer II of the

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834 somatosensory cortex from *Nkx2.1-CreER;Ai9* mice co-electroporated at E15.5 with a plasmid
835 expressing Cas9 and a plasmid expressing smFP-HA and a control sgRNA or *Cntn1* sgRNA; mice
836 were sacrificed at P17. AISs and PyNs are visualized by immunostaining for β 4 spectrin (blue) and
837 HA (green), respectively. Stars in C indicate HA+ PyNs and arrows indicate ChC innervation of PyN
838 AISs. Arrowheads in C indicate AIS of transfected with *Cntn1* sgRNA and that lack innervation by
839 ChC cartridges. Scale bar, 10 μ m. **d**, Quantification of the percentage of HA+ PyNs innervated by
840 single RFP+ ChCs at P17. 12 ChCs and 15–66 HA+ PyNs per ChC from 3 animals were analyzed for
841 each condition. Data are mean \pm SEM. **e, f**, Representative images of HA+ PyN AISs from *Nkx2.1-*
842 *CreER;Ai9* mice electroporated at E15.5 with plasmids indicated in (a) and sacrificed at P17.
843 Inhibitory synapses are visualized by immunostaining for the GABAergic postsynaptic marker
844 gephyrin (Gphn; red; **e**) or the GABAergic presynaptic marker VGAT (red; **g**). AISs (blue) are
845 visualized by immunostaining for AnkG in **e** and β 4 spectrin in **g**. Scale bars, 2 μ m. **f, h**,
846 Quantification of the average number of gephyrin (**f**) or VGAT (**h**) puncta per μ m of HA+ PyN AIS
847 at P17. 23-40 AISs from 4 fields of view from 3 animals were analyzed for each condition. Data
848 are mean \pm SEM.

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851 **Extended Data:**

852 **1. Supplemental Figures S1-S5**

853 **2. Supplemental extended materials file**, detailed listing of all reagents, plasmids, gRNA

854 sequences, antibodies, sources, etc.

855 **3. Supplemental Table 1**, Nfasc-BAR and NrCAM-BAR proximity proteomes comparison

856 (included as excel file)

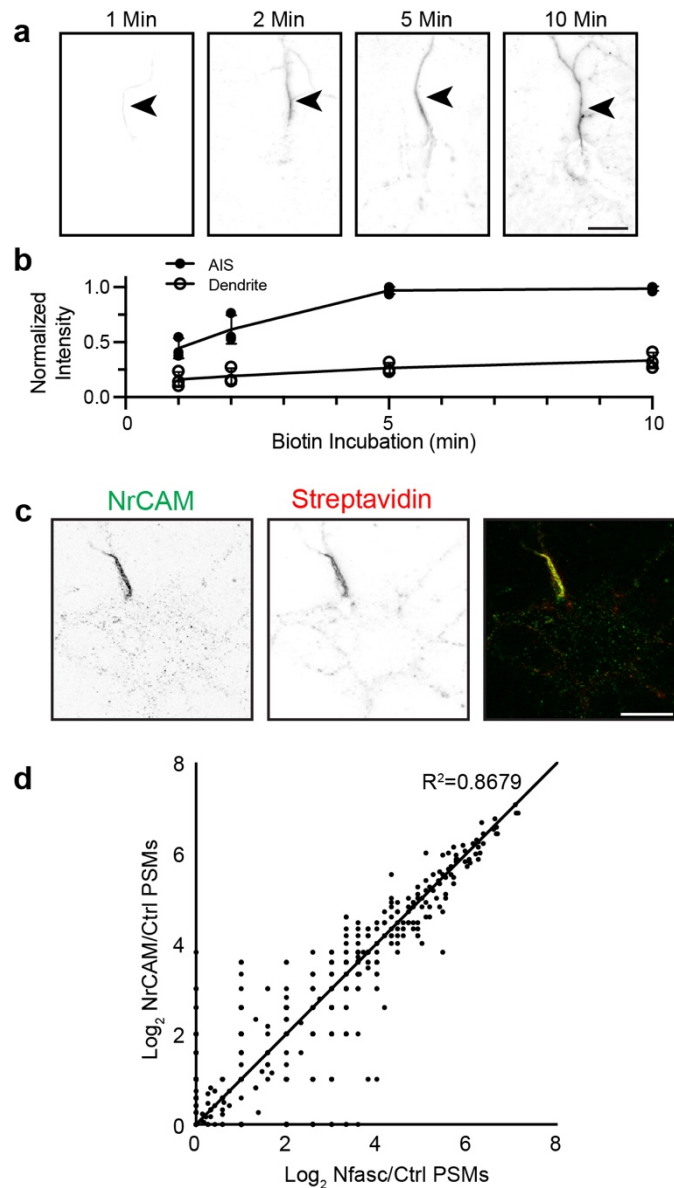
857 **4. Supplemental Table 2**, Nfasc-BAR proximity proteomes at DIV 4, 7, 14, 21, and 28

858 (included as excel file).

859 **5. Supplemental Table 3**, Nfasc-BAR fold change and p-values for all identified proteins

860 (included as excel file).

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861

862 **Figure S1. BAR-Nfasc and BAR-NrCAM are highly concordant. a, b,** Biotinylation of the AIS

863 (arrowhead) as a function of biotin-tyramide incubation time. N = 3 independent experiments.

864 Error bars, \pm SEM. Scale bar, 20 μm . **c,** Fluorescence imaging of DIV14 rat hippocampal neurons

865 labeled by NrCAM-BAR. NrCAM immunofluorescence (green) enrichment defines the AIS.

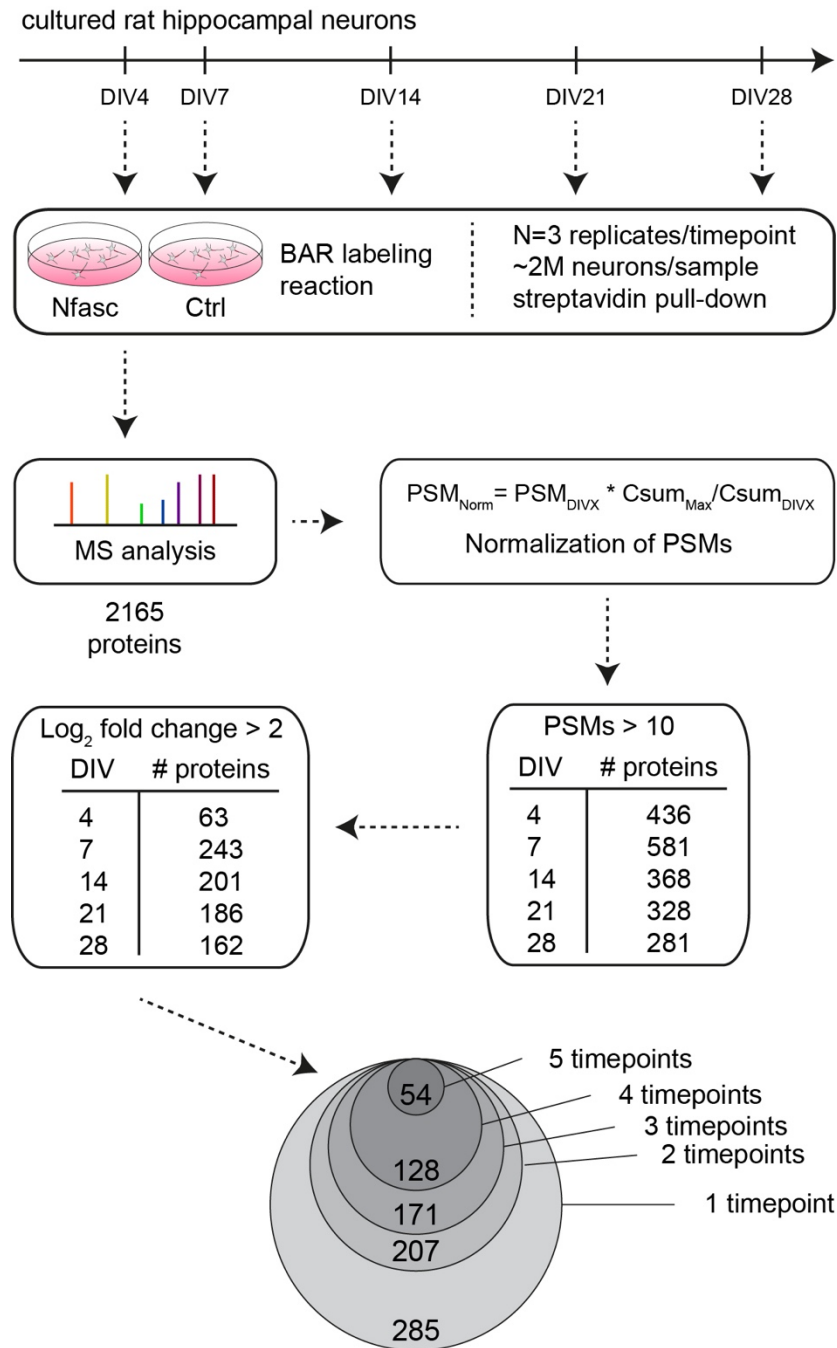
866 Biotinylated proteins were detected using Alexa594-conjugated streptavidin. Scale bar, 20 μm .

867 **d,** Scatter plot of the number of peptide spectral matches (PSMs) for each biotinylated protein

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868 identified by mass spectrometry using either Nfasc-BAR or NrCAM-BAR. Data were highly
869 concordant since most proteins identified fell on or close to the solid line representing equal
870 enrichment in both BAR conditions.
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873 **Figure S2. Workflow of Nfasc-BAR labeling and normalization of PSMs.** Nfasc-BAR labeling was

874 performed in triplicate for each of five timepoints. Mass spectrometry analysis resulted in a

875 dataset of 2165 proteins. To compare across timepoints, PSMs were normalized using the

876 formula $PSM_{i, Norm, DivX} = PSM_{i, DivX} * SPSM_{Carboxylases}^{DivY} / SPSM_{Carboxylases}^{DivX}$, where *i* is a particular

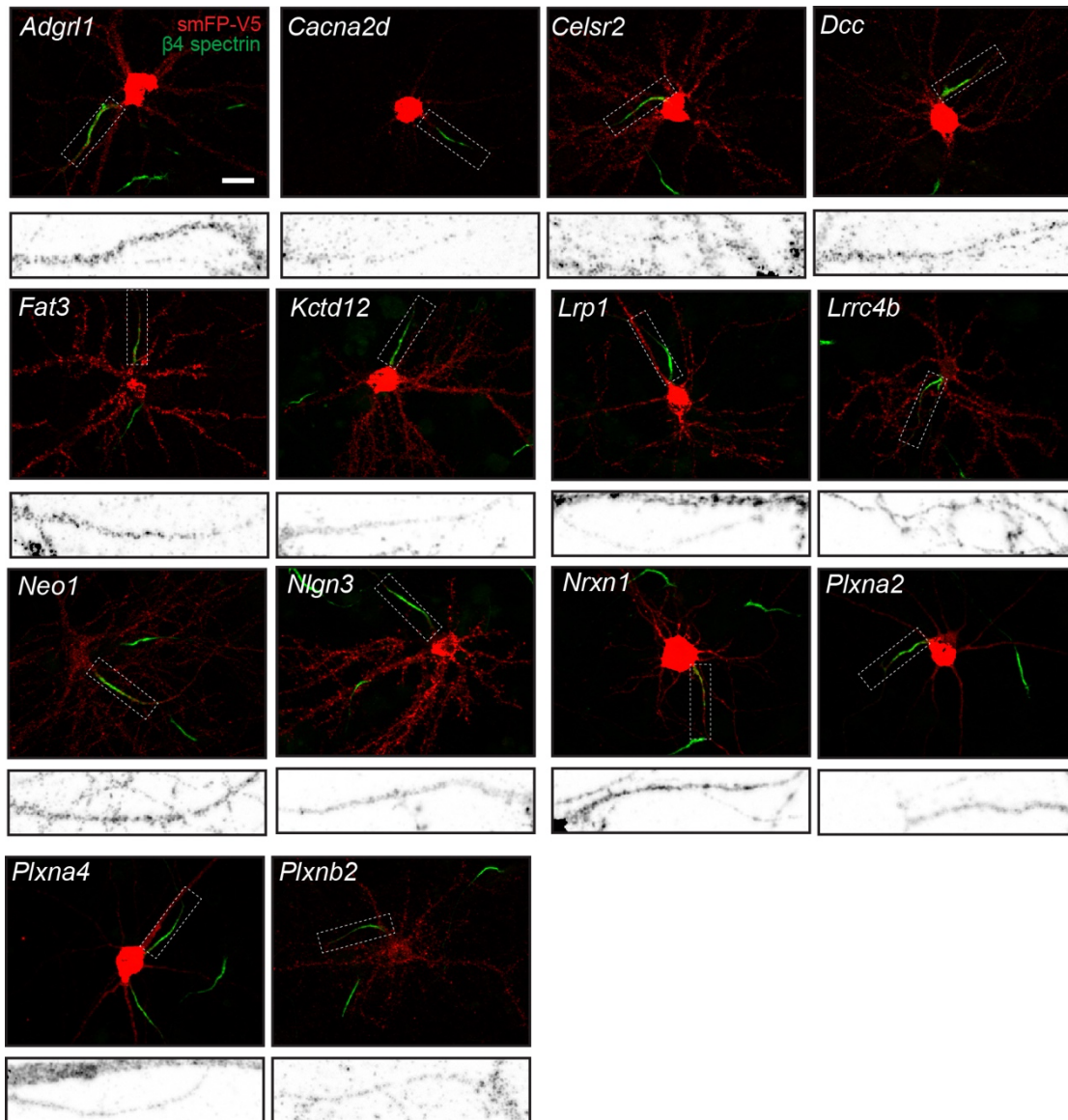
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877 protein on the set, X is a Div time point, Y is the Div time point with the maximum value for
878 summed carboxylase PSMs. Candidates were distinguished from background by filtering based
879 on the number of PSMs identified and the fold change. Tables show the number of proteins
880 that satisfied one or both criteria at a given timepoint. Circles show the number of proteins that
881 satisfied both criteria for 1-5 timepoints.
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884 **Figure S3. Fold-enrichment for the top 100 most enriched proteins identified using Nfasc-BAR**
885 **during *in vitro* neuron development. a-e, Log₂ fold-change (log₂(FC)) for the top 100 most**
886 **enriched proteins identified at DIV 4 (a), 7 (b), 14 (c), 21 (d) and 28 (e). Known AIS proteins are**
887 **indicated in red. Error bars, ±SEM.**
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892 **Figure S4. Tagging of endogenous membrane proteins.** Examples of smFP-V5 tagged

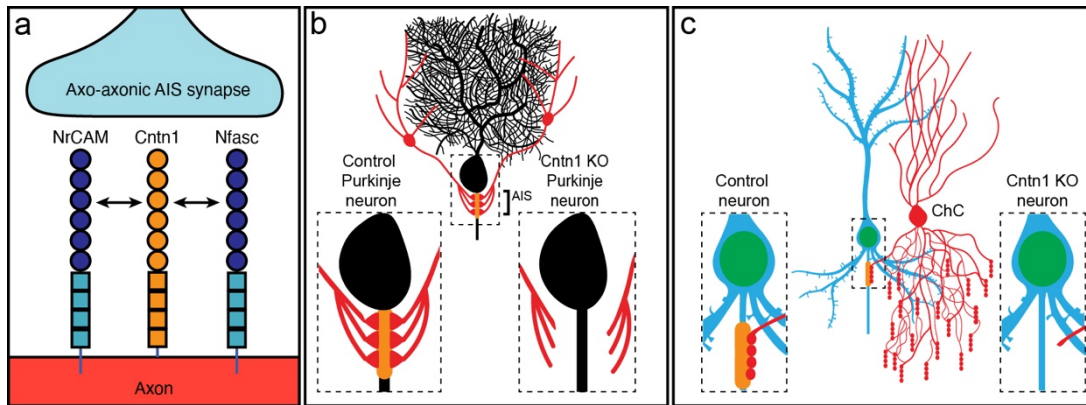
893 proteins (red). AIS are labeled for $\beta 4$ spectrin (green). The inset boxed regions

894 correspond to the tagged protein at the AIS. Scale bar, 20 μm .

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899 **Figure S5. Summary of results.** a, Cntn1 interacts with and is redundantly recruited to the AIS
900 through interactions with both Nrcam and Nfasc. b, Loss of AIS Cntn1 (orange at AIS) from
901 cerebellar Purkinje neurons disrupts basket cell innervation of the AIS and formation of pinneau
902 synapses. c, Loss of AIS Cntn1 (orange) from Pyramidal neurons results in reduced innervation
903 of AIS by Chandelier cells (ChC) and reduced numbers of AIS inhibitory synapses.

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