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2 **Title**

3 Subsynaptic positioning of AMPARs by LRRTM2 controls synaptic strength
4

5 **Authors**

6 A.M. Ramsey^{1,2†}, A.H. Tang^{1,2,4†}, T.A. LeGates^{1,2§}, X.Z. Gou⁴, B.E. Carbone^{5||},
7 S.M. Thompson^{1,2,3}, T. Biederer⁵, and T.A. Blanpied^{*1,2}
8

9 **Affiliations**

10 ¹Department of Physiology, ²Program in Neuroscience, ³Department of Psychiatry,
11 University of Maryland School of Medicine, Baltimore, MD, USA.

12 ⁴CAS Key Laboratory of Brain Function and Disease and Hefei National Laboratory for
13 Physical Sciences at the Microscale, School of Life Sciences, Division of Life Sciences
14 and Medicine, University of Science and Technology of China, Hefei, China.

15 ⁵Department of Neurology, Yale School of Medicine, New Haven, CT.

16 §Present address: Department of Biological Sciences, University of Maryland, Baltimore
17 County, Baltimore, MD, USA.

18 ||Present address: Ohio State University College of Medicine, Columbus, OH, USA.

19 † These authors contributed equally.

20 * Corresponding author
21

22 **Abstract**

23 Recent evidence suggests that nanoorganization of proteins within synapses may control the
24 strength of communication between neurons in the brain. The unique subsynaptic
25 distribution of glutamate receptors, which cluster in nanoalignment with presynaptic sites
26 of glutamate release, supports this idea. However, testing it has been difficult because
27 mechanisms controlling subsynaptic organization remain unknown. Reasoning that
28 transcellular interactions could position AMPA receptors, we targeted a key transsynaptic
29 adhesion molecule implicated in controlling AMPAR number, LRRTM2, using engineered,
30 rapid proteolysis. Severing the LRRTM2 extracellular domain led quickly to nanoscale de-
31 clustering of AMPARs away from release sites, not prompting their escape from synapses
32 until much later. This rapid remodeling of AMPAR position produced significant deficits
33 in evoked, but not spontaneous, postsynaptic receptor activation. These results dissociate
34 receptor numbers from their nanopositioning in determination of synaptic function, and
35 support the novel concept that adhesion molecules acutely position AMPA receptors to
36 dynamically control synaptic strength.
37

38 **MAIN TEXT**

39
40 **Introduction**

41 The complex neural processes of information encoding, storage, and retrieval are
42 enabled by fine regulation of synaptic strength. It is well established that the number of
43 AMPA-type glutamate receptors (AMPARs) within a single synapse is a key property
44 determining the amplitude of the excitatory postsynaptic current (EPSC) response to
45 neurotransmitter release (1–3). However, several constraints appear to limit AMPAR
46 activation following glutamate release (4–6), suggesting that factors beyond receptor
47 number also control synapse strength. Among the most critical is that a variety of modeling
48 approaches suggest AMPARs even ~90 nm from the site of glutamate release open with
49 only about half the likelihood of those close to the site of vesicle fusion (7, 8), due to the
50 low affinity and rapid desensitization of receptors (9–11). This greatly diminishes the
51 expected EPSC, a prediction in line with experimental results suggesting that glutamate
52 release from a single vesicle is not sufficient to maximize postsynaptic receptor activation
53 (12, 13). Unfortunately, it has been difficult to test whether such distance-dependence plays
54 a physiological role in neurons because the mechanisms that determine the precise
55 positioning of receptors across from sites of release are not known.

56 Discerning these mechanisms is complex because AMPARs and a number of
57 scaffolding molecules involved in their synaptic retention, most notably PSD-95, are non-
58 homogeneously distributed within individual PSDs, forming nanometer-scale clusters (14–
59 17). Similarly, within the presynaptic active zone (AZ), molecules critical for vesicle
60 priming and Ca²⁺ channel recruitment such as the Rab3 Interacting Molecule (RIM) and
61 Munc13 are clustered into ~100 nm nanodomains (18). These AZ nanodomains are widely
62 conserved across many synapse types (19) and are thought to govern vesicle positioning
63 and establish sites in the AZ where action potentials drive synaptic vesicle exocytosis with
64 highest probability (20, 21). Critically, in mammalian brain, presynaptic sites of glutamate
65 exocytosis as marked by RIM nanoclusters are aligned with postsynaptic nanoclusters of
66 AMPARs across the cleft in an organization referred to as a nanocolumn (20, 22, 23). If
67 receptor distance to the site of neurotransmitter exocytosis regulates receptor activation,
68 then this aligned organization likely enhances basal excitatory synaptic transmission, and
69 its disruption would reduce synaptic strength. This is important to determine, since
70 modulation of transsynaptic alignment then would open a number of different mechanisms
71 of synaptic plasticity (24).

72 It remains unclear how subsynaptic alignment of receptor clusters with release sites
73 is created or maintained. Though many models have been proposed (5), perhaps the most
74 parsimonious idea is that cleft-resident synaptic cell adhesion molecules (CAMs) link pre-
75 and postsynaptic nanodomains through their transsynaptic binding interactions. The most
76 prominent candidates to test for this role are the neuroligin (NL) and Leucine Rich Repeat
77 Transmembrane (LRRTM) families that are postsynaptic partners of presynaptic neurexins
78 and that bind PSD-95 (25). However, such tests are complicated because CAM families are
79 large, the roles they play are diverse, and the family members exhibit substantial redundancy
80 upon knockout (26, 27). Indeed, disruption of postsynaptic NL by expression of dominant
81 negative mutants or prolonged incubation with interfering peptides does in fact alter
82 receptor alignment with RIM (28), providing support for the idea. However, these extended
83 treatments also prompt a complex set of other effects including altering synapse numbers,
84 presynaptic vesicle release probability, and frequency of spontaneous transmission (28–31).

85 The LRRTM family are strong candidates to mediate transsynaptic alignment. A key
86 abundant family member in hippocampus, LRRTM2, binds postsynaptic PSD-95 through a

87 C-terminal motif (32, 33) and the presynaptic Neurexin-Heparan sulfate complex through
88 10 extracellular LRR repeats (34). LRRTM2 has been found to be important for evoked
89 AMPAR-mediated, though not NMDAR-mediated, synaptic transmission independent of
90 synaptogenesis (35). Furthermore, the extracellular domain of LRRTM2 alone is sufficient
91 to rescue AMPAR-mediated synaptic transmission following LRRTM1,2 double knockout,
92 a mechanism proposed to be achieved by the anchoring of AMPARs in the PSD (35, 36).
93 LRRTM2 within synapses also forms nanoscale clusters of similar size to scaffold, receptor,
94 and release machinery nanodomains (37). Thus, we hypothesized that LRRTM2 coordinates
95 positioning of receptors relative to evoked release sites.

96 Long-term manipulations can prompt substantial reorganization of synapses which
97 makes deducing the native state difficult. To test the role of LRRTM2 while avoiding such
98 effects, we used acute extracellular proteolysis of an engineered cleavage site to disrupt its
99 extracellular interactions within seconds, thus uncoupling it from the postsynaptic
100 membrane while avoiding complications of genetic compensation. With this approach, we
101 discovered that LRRTM2 acutely controls the fine positioning of AMPARs relative to the
102 site of release. The repositioning of AMPARs following loss of the LRRTM2 extracellular
103 domain leads to reduction in the amplitude of evoked but not spontaneous responses.
104 Further, the basal distribution of LRRTM2 is in nanoscale register with both RIM and
105 AMPAR nanodomains. Together, these data suggest that postsynaptic LRRTM2 establishes
106 a transcellular, structural linkage mediating nanocolumn alignment of AMPARs with
107 preferential sites of evoked neurotransmitter release and provide strong evidence that
108 AMPAR organization within the synapse is critical for the strength of basal synaptic
109 transmission.

110 Results

111 Acute and specific cleavage of the LRRTM2 extracellular domain

112 To test the role of LRRTM2 extracellular interactions in synapse structure and
113 function independent of synaptogenesis and genetic compensation, we adapted a previous
114 approach (38) and inserted the short recognition sequence for the endoprotease thrombin
115 (LVPRGS) at an extracellular, juxtamembrane position within human LRRTM2 (Fig. 1A).
116 To visualize the molecule and enable live-cell measurement of its cleavage in neurons, we
117 appended EGFP to the N-terminus, and used this to replace endogenous LRRTM2 following
118 knockdown with published shRNA targeting sequences. We denote the molecule GFP-Thr-
119 LRRTM2*, where * indicates the human sequence designed to be resistant to the shRNA
120 (33, 39).
121

122 These modifications of LRRTM2 did not appear to disrupt its function. When
123 expressed in cultured rat hippocampal neurons, GFP-Thr-LRRTM2* clustered avidly in
124 small puncta that colocalized nearly exclusively with synapses immunolabeled for PSD-95
125 and RIM1/2 (Fig. 1B-C), though some puncta appeared in the dendritic shaft apart from
126 synapses. In addition, when expressed in HEK cells, GFP-Thr-LRRTM2* trafficked
127 strongly to the plasma membrane and retained the synaptogenic ability of wild type
128 LRRTM2 to cluster presynaptic markers in the axons of co-cultured wild type neurons (Fig
129 1D, Supplementary Fig. 1A).
130

131 Though knockdown of LRRTM2 was successful (Supplementary Fig. 2), typical
132 rescue strategies can still result in overexpression. Since overexpression of LRRTM2 in
133 cultured neurons increases excitatory synapse density (33, 39), we tested for functional
134 effects of LRRTM2 overexpression by measuring synapse density via PSD-95
135 immunolabeling. As expected, expression of GFP-Thr-LRRTM2* without concurrent
136 knockdown resulted in a ~1.3-fold increase in PSD-95 puncta compared to controls

137 expressing cytosolic mCerulean3 alone (Fig. 1E). However, puncta density was unchanged
138 following knockdown of endogenous LRRTM2 and replacement with GFP-Thr-LRRTM2*.
139 Similarly, compared to mCerulean transfected neurons, spine numbers were increased by
140 GFP-Thr-LRRTM2* overexpression, but were unchanged following knockdown and
141 replacement (Fig. 1F). Overexpression also resulted in an increase in spine length though
142 not spine area, but we found no changes in either measure with the knockdown-replacement
143 approach (Fig. 1G-H). The replacement strategy also minimized non-synaptic localization
144 of GFP-Thr-LRRTM2*, which was enriched much more specifically in synapses when
145 endogenous LRRTM2 was knocked-down as judged by the levels of thrombin-sensitive
146 extrasynaptic fluorescence (Supplementary Fig. 1B). These data suggest that GFP-Thr-
147 LRRTM2* incorporates readily into excitatory synapses without disrupting synaptogenesis
148 and with minimal effects of overexpression.

149 Next, we tested whether thrombin successfully cleaved GFP-Thr-LRRTM2* in
150 synapses, particularly at low working concentrations to avoid potential effects which could
151 be mediated via PAR receptors (40). Following baseline measurements of EGFP
152 fluorescence, we bath-applied thrombin at 10 units/mL. This prompted the rapid and robust
153 loss of GFP fluorescence from puncta in dendritic spines (Fig. 1I, below). Thrombin
154 application to neurons expressing GFP-LRRTM2* (with no thrombin recognition sequence)
155 resulted in no decrease in fluorescence, indicating the loss was due to cleavage of the
156 extracellular domain (ECD) and not non-specific effects of thrombin (Fig. 1I, above). The
157 LRRTM2 ECD was lost with a time constant of $\tau = 11.08$ seconds (95% C.I 10.74 to 11.43
158 sec; Fig. 1J), surprisingly rapid given its presumed interactions within the synaptic cleft.
159 Incubations in thrombin for up to 1 hour showed sustained loss of GFP-Thr-LRRTM2* (Fig
160 1K; fractional fluorescence remaining; 0.09 ± 0.02 compared to baseline; mean \pm SEM),
161 but no loss of the LRRTM2 ECD in GFP-LRRTM2* transfected neurons (Fig 1K; fraction
162 remaining; 0.96 ± 0.05 , compared to baseline). The rate of cleavage is likely limited by the
163 speeds of perfusion and proteolysis, but regardless suggests that LRRTM2 ECD interactions
164 are insufficient to immobilize it for substantial periods within the synaptic cleft. In addition,
165 the quick action and extensive loss of fluorescence confirms that GFP-Thr-LRRTM2* was
166 trafficked to the cell surface as expected, and suggests that LRRTM2 is only minimally
167 retained intracellularly at steady state in these neurons. Overall, these results demonstrate
168 that expressed GFP-Thr-LRRTM2* localizes appropriately to excitatory synapses, retains
169 its synaptogenic activity, induces no observable morphological changes in spines, and can
170 be proteolytically cleaved acutely and specifically on demand.

171 **No rapid loss of AMPARs following removal of the LRRTM2 extracellular domain**

172 The role of LRRTM2 in synaptogenesis has been well studied, but its functions in
173 established synapses have been explored in far less detail. The four C-terminal amino acids
174 in its intracellular domain form a PDZ-binding motif which is thought to play a role in the
175 recruitment of PSD-95 in developing synapses (33), through interactions with the first and
176 second PDZ domains of PSD-95 (32). We considered whether maintenance of PSD-95 at
177 established synapses depends on stable LRRTM2 extracellular interactions. To test this, we
178 co-transfected neurons with GFP-Thr-LRRTM2* and PSD95*-mCherry (15) (here, * also
179 denotes resistance to co-expressed shRNA) and measured their fluorescence intensity over
180 the course of a 30 min thrombin application. Strikingly, despite a large and sustained
181 reduction in the GFP-Thr-LRRTM2* fluorescence (fraction remaining: 0.15 ± 0.07 , Fig.
182 2A-C), PSD95*-mCherry fluorescence at synapses remained unchanged (fraction
183 remaining: 0.94 ± 0.05 , Fig. 2A-C). Immunocytochemical analysis of synaptic PSD-95
184 content after thrombin cleavage (discussed below) confirmed this result. Thus, the
185

186 interactions of LRRTM2 within the synaptic cleft are not necessary for the retention of PSD-
187 95 in established synapses.

188 LRRTM2 is important for both establishing the number of GluA1-containing
189 synapses as well as basal synaptic content of GluA1 and GluA2 (33, 36, 41). In neurons at
190 rest, many AMPARs continuously diffuse within the synapse and exchange between
191 synaptic and extrasynaptic domains on a time scale of seconds to minutes (42), but the
192 mechanisms that counteract diffusion and enrich them in the PSD are incompletely
193 understood. Extracellular interactions in the synaptic cleft may be important, and it is
194 conceivable that the ECD interactions of LRRTM2 could assist in the stabilization of both
195 LRRTM2 and additionally GluA1-containing AMPARs in established synapses. To
196 visualize synaptic AMPAR content during live imaging before and after cleavage of
197 LRRTM2, we utilized super-ecliptic pHluorin (SEP)-tagged GluA1 and GluA2, as
198 previously described (43). We expressed these receptors along with a version of LRRTM2*
199 in which the GFP was replaced with the smaller α -bungarotoxin recognition sequence (44)
200 (BRS-Thr-LRRTM2*) which retained its synaptogenic activity (see Fig 1D, Supplementary
201 Fig. 1) and allowed us to select the wavelength of the labeled α -bungarotoxin. Alexa-647
202 conjugated to α -bungarotoxin was applied to live cells, resulting in synapse-specific
203 labeling and visualization of the LRRTM2 ECD in co-transfected neurons. We predicted
204 that if the LRRTM2 ECD interacts directly or indirectly with the GluA1 extracellular
205 domain, its acute loss would reduce SEP-GluA1,2 content in synapses. As with GFP-Thr-
206 LRRTM2*, thrombin application produced a rapid and dramatic loss of α -bungarotoxin-
207 Alexa-647 fluorescence (fraction remaining: 0.10 ± 0.02 , Fig. 2D-F) indicating cleavage
208 and dispersal of the LRRTM2 ECD. However, SEP-GluA1,2 fluorescence colocalized with
209 LRRTM2 puncta did not decrease even after 10 or 30 min (fraction remaining: 0.96 ± 0.03 ,
210 Fig. 2D-F), suggesting no changes in the number of receptors present within the PSD.
211 Furthermore, neurons expressing SEP-GluA1/2 along with the cleavable or non-cleavable
212 versions of LRRTM2*, showed no difference in the SEP-GluA1,2 synaptic cluster
213 localization density as measured by dSTORM after a 10-min thrombin treatment (Mann-
214 Whitney Test, $p = 0.85$; data discussed below, Supplementary Fig. 3). These data suggest
215 that the LRRTM2 ECD is not required for the synaptic retention of AMPARs within a time
216 frame of 30 minutes, during which many receptors exchange in and out of the synapse (45).

217 This result was surprising because conditional knockout of LRRTM1 and 2 leads to
218 a reduction in AMPAR content and EPSC amplitude at established synapses (35). One
219 major difference between the conditional deletion and the acute cleavage is the vastly
220 differing time scales of the two approaches. To test whether the prolonged loss of the
221 LRRTM2 ECD affects AMPAR retention in spines, we performed live-cell imaging for up
222 to 2 hours post-cleavage. In fact, synaptic AMPAR content remained almost completely
223 unchanged for at least 60 min after LRRTM2 cleavage. Only after this, a slow decline set
224 in, and 2 hours after cleavage there was a $23.55\% \pm 0.08\%$ reduction in AMPAR content
225 compared to non-cleavable controls (Supplementary Fig. 4). To examine longer time points,
226 we fixed transfected neurons 24 hours after thrombin treatment and stained for surface SEP-
227 GluA1,2. Compared to controls expressing non-cleavable BRS-LRRTM2*, neurons that
228 underwent LRRTM2 cleavage displayed much weaker surface SEP-GluA1,2 expression
229 (Supp. Fig. 4). These data corroborate the previously reported idea that LRRTM2 is
230 important for AMPAR stability in synapses (35), but show that this effect plays out only
231 over extended periods without the LRRTM2 ECD.

232 Another possible role of LRRTM2 may be to instruct organization of presynaptic
233 release machinery. To test this, we transfected cultured hippocampal neurons with GFP-
234 Thr-LRRTM2* along with soluble Cerulean3 to identify transfected spines following
235 elimination of the EGFP fluorescence post-cleavage. Then, following live-cell cleavage of

236 LRRTM2 with 10 U/mL thrombin for 10 minutes, cells were fixed, permeabilized, and
237 stained for endogenous PSD-95 and the critical presynaptic scaffolding molecule RIM1/2.
238 Despite near complete elimination of EGFP fluorescence at transfected spines we observed
239 no changes in endogenous RIM1/2 content (fraction remaining: 1.06 ± 0.06 , compared to
240 vehicle, Fig. 2G-I). These data suggest that LRRTM2 is not necessary for the retention of
241 RIM in the active zone. Analysis of PSD-95 staining intensity further confirmed that
242 cleavage of LRRTM2 in established synapses did not change PSD-95 content (fraction
243 remaining; 1.02 ± 0.06 , compared to vehicle, Fig. 2G-I) supporting our earlier observations
244 during live imaging. Taking these data together, we conclude that although the LRRTM2
245 ECD is quickly lost after thrombin cleavage in established synapses, its acute removal does
246 not rapidly lead to loss of other key molecules, including AMPARs.

247 248 **LRRTM2 is enriched within the trans-synaptic nanocolumn**

249 Growing evidence indicates that different CAMs possess unique and distinct
250 organizations within excitatory synapses (37). For instance, both SynCAM1 and Neurexin-
251 1 are enriched in a small number of subsynaptic ensembles, but the nanoclusters of
252 SynCAM-1 are often found near or around the border of the synapse (46, 47) whereas
253 Neurexin-1 nanoclusters tend to occur just slightly off-center within the PSD (48). How
254 these distributions subservise particular functions is not known. LRRTM2 forms tight clusters
255 in the postsynaptic density (37). Its enrichment within these nanoclusters is notably tighter
256 than Neuroligin-1, which more homogeneously distributes through the synapse (37), but
257 neither the location nor function of LRRTM2 nanoclusters is known. We hypothesized that
258 LRRTM2 may link pre- and postsynaptic nanodomains, and therefore predicted that it is
259 enriched with other proteins found within the trans-synaptic nanocolumn (20).

260 To test whether LRRTM2 formed subsynaptic clusters within excitatory PSDs, we
261 performed two-color 3D dSTORM in our LRRTM2 knockdown-replacement system using
262 an anti-GFP antibody (Fig. 3A). Maps of the local density at each molecular location (Fig.
263 3B) showed that LRRTM2 is non-uniformly organized within the PSD, forming
264 nanodomains of similar size and number to those previously reported for receptors and
265 scaffolding molecules (14, 15, 37). To quantify the degree to which LRRTM2 was self-
266 clustered, we calculated an autocorrelation measurement and found that LRRTM2 was
267 robustly organized into clusters with a ~ 100 nm diameter within synapses (Fig. 3C).

268 To test how LRRTM2 was organized relative to nanocolumn-resident molecules, we
269 measured the subsynaptic organization of GFP-Thr-LRRTM2* relative to endogenous
270 PSD-95 (Fig. 3D-E) and RIM1/2 (Fig. 3F-G) using an enrichment assay reported previously
271 (20). LRRTM2 was tightly enriched within PSD-95 nanodomains (enrichment index: 1.37
272 ± 0.13 , Fig. 3E) and aligned with RIM1/2 nanodomains across the synaptic cleft (enrichment
273 index: $1.56 \pm 0.21\%$, Fig. 3G). To analyze the LRRTM2 distribution with respect to that of
274 RIM1/2 or PSD-95 without requiring the identification of nanoclusters of either protein, we
275 measured the cross-correlation between LRRTM2 and RIM1/2 or PSD-95 density
276 distributions (Sup. Fig. 3). This demonstrated that the distribution of LRRTM2 and PSD-95
277 as well as LRRTM2 and RIM1/2 are highly similar to one another. To illustrate this
278 similarity, we compared the distribution of LRRTM2 to a probe without nanocolumns
279 enrichment. We used an engineered single pass transmembrane protein called SEP-TM
280 containing an N-terminal extracellular super-ecliptic pHluorin (SEP) appended to the
281 transmembrane domain from PDGFR (49). SEP-TM traffics avidly to the plasma membrane
282 (Supplementary Fig. 5), but we predicted that it would not be enriched within the
283 nanocolumn because it lacks relevant binding via its N- or C-terminus. While LRRTM2 was
284 tightly aligned across the cleft from RIM nanodomains, SEP-TM was not (enrichment
285 index: 0.83 ± 0.12 , Fig. 3G-H, Supplementary Fig. 6). This suggests that the subsynaptic

286 positioning of LRRTM2 is actively determined by protein-protein interactions rather than
287 arising as a general feature of transmembrane proteins in the dense synaptic environment.
288 The distribution of LRRTM2 within the synapse, tightly clustered and colocalized with
289 transsynaptically aligned protein nanodomains, suggests that it acts within the nanocolumn.
290

291 **LRRTM2 is critical for AMPAR enrichment with preferential sites of evoked** 292 **neurotransmitter release**

293 Expression of LRRTM2 with mutations that disrupt its interaction with presynaptic
294 neurexin decreases content of expressed mutant LRRTM2 at synapses and leads to lower
295 synaptic AMPAR content and reduced AMPAR-mediated EPSCs (35, 41). However, it
296 remains unclear whether LRRTM2 exerts ongoing control of synaptic transmission in
297 established synapses. Given the location of LRRTM2 within the nanocolumn, we reasoned
298 that its extracellular interactions may contribute to the nanoscale alignment of AMPARs to
299 RIM nanodomains. To test this, we took advantage of the acute nature of the protease
300 cleavage approach, which avoids the complications of compensation by other CAMs during
301 the prolonged periods required for molecular expression.

302 Since we wanted to assure that we measured receptors only in cells where we
303 manipulated LRRTM2 rather than nearby untransfected synapses, we first used two-color
304 3D dSTORM to measure the distribution of SEP-tagged AMPARs co-transfected with
305 LRRTM2. Our previous work demonstrated that endogenous receptors are enriched in ~80
306 nm nanodomains aligned with surprising precision to presynaptic RIM nanodomains (20).
307 As expected, SEP-GluA1/2 AMPARs in neurons co-transfected with BRS-Thr-LRRTM2*
308 but treated only with vehicle formed nanodomains of ~80 nm, as judged by the
309 autocorrelation of their distributions (Supplementary Fig. 7A,B). These were strongly
310 enriched with RIM nanodomains across the cleft, decaying in enrichment over
311 approximately 80 nm (Fig 4A, Supplementary Fig. 7C).

312 In these neurons, we applied either vehicle (aCSF) or thrombin to test the acute
313 regulation of AMPAR organization by LRRTM2's extracellular interactions. Remarkably,
314 brief treatment with thrombin dramatically reduced the density of AMPARs directly across
315 from RIM nanodomains (to $37.7 \pm 9.1\%$ of control enrichment index, Fig. 4A). Of note,
316 RIM1/2 density across from detectable AMPAR nanodomains was unchanged ($97.7 \pm$
317 12.2% of control, Fig. 4B), suggesting a strictly postsynaptic nanoscale re-organization of
318 AMPAR patterning within the PSD, but also that the position of the detected AMPAR
319 nanodomains relative to RIM1/2 nanoclusters were largely unchanged. Since we could not
320 yet rule out that this was caused by specific or off-target effects of thrombin, we repeated
321 the experiment except that neurons were transfected with either BRS-Thr-LRRTM2* or
322 BRS-LRRTM2*, and both conditions received brief treatment with thrombin. Consistent
323 with the prior result, thrombin treatment to neurons expressing the cleavable, but not the
324 non-cleavable LRRTM2 resulted in a large reduction in AMPAR density across from RIM
325 nanodomains ($36.5 \pm 27.3\%$ of control enrichment index, Fig. 4C, Supplementary Fig. 7D),
326 confirming that the effect is specific to the cleavage of the LRRTM2 ECD. Furthermore,
327 RIM density across from these AMPAR nanodomains was again unaffected ($92.5 \pm 30.6\%$
328 of control, Fig. 4D). To visualize this AMPAR de-enrichment another way, we calculated a
329 2D view of these data by aligning all receptor map data to the peak of their corresponding
330 RIM1/2 nanocluster, producing a histogram of receptor density across from the RIM1/2
331 nanocluster peak averaged over all measured nanoclusters from many synapses (Fig. 4E).
332 This output thus represents the average distribution of receptors arrayed in the synaptic
333 membrane facing a vesicle that might fuse at the center of a RIM nanodomain. Following
334 thrombin treatment, the peak of this receptor array is diminished, and receptors are dispersed
335 so that they are much less concentrated directly in line with the RIM nanodomain. Together,

336 the rapid effect of thrombin in these experiments demonstrates that LRRTM2 via its
337 extracellular domain is actively involved in the nanoscale organization of AMPARs within
338 established synapses.

339 Changes in either the density or arrangement of these AMPAR nanodomains could
340 have produced changes to the enrichment measurements. To further discriminate how
341 AMPAR organization changed upon LRRTM2 cleavage, we assayed a number of properties
342 that these AMPAR nanoclusters exhibited. To assess the effect on RIM-AMPA alignment
343 without explicitly identifying nanoclusters of either protein, we measured the cross-
344 correlation between AMPAR and RIM1/2 density distributions from the same neurons
345 transfected with BRS-Thr-LRRTM2* or BRS-LRRTM2*. This measure showed a
346 reduction following LRRTM2 cleavage ($63.8 \pm 1.3\%$ of control, Fig. 4F), indicating that
347 their relative density distributions became less similar, consistent with the above. Receptor
348 nanoclusters were $67.5 \pm 0.7\%$ the volume of control receptor nanoclusters (Fig. 4G), and
349 $28.3 \pm 0.8\%$ less numerous (Fig. 4H), while RIM1/2 nanocluster number and volume were
350 not altered ($94 \pm 10.9\%$, $98 \pm 5.7\%$, respectively; Fig. 4G,H). Furthermore, the summary
351 enrichment data (an average of the data within 50 nm of the opposite nanocluster)
352 demonstrated a significant decrease following LRRTM2 cleavage for AMPARs, but not
353 RIM1/2 (Fig. 4I). Taken together, these data are consistent with a model of a postsynaptic
354 nanodomain-specific de-enrichment of AMPARs near RIM nanodomains. These data
355 provide strong evidence that while not acutely required for controlling AMPAR number in
356 the PSD (Fig. 2D-F), LRRTM2's ECD is critical for ongoing coordination of AMPAR
357 density across from RIM nanodomains.

358 **Numerical model to predict effects of LRRTM2 loss on synaptic transmission**

359 These effects offer a unique opportunity to explore how changes in the nanoscale
360 spatial organization of AMPARs in the PSD could alter receptor activation and synaptic
361 transmission. To address this theoretically, we predicted the magnitude of the potential
362 effect using a model based on prior work. Prior models of glutamate release and diffusion
363 along with receptor opening kinetics have established that AMPAR open probability
364 decreases characteristically as their distance to the site of release increases (50, 51).
365 Beginning with this simplification enabled us to estimate the relative synaptic response after
366 release given different receptor distributions within the PSD without explicitly simulating
367 glutamate diffusion or receptor kinetics.

368 We generated simulated receptor maps based on several key synapse features
369 obtained from our measurements and the literature (Fig. 5A,B and Supplemental Fig 6A).
370 When subjected to our spatial analysis, this basal arrangement of simulated receptor
371 positions recapitulated the autocorrelation (Supplementary Fig. 8B) and well reflected both
372 the relative change in the enrichment profile (Supplementary Fig. 8C) and the relative
373 change in the enrichment index (Supplementary Fig. 8D) for receptors as measured in our
374 cultured neurons. To deduce how many AMPARs would need to leave the nanodomain to
375 result in de-enrichment to the same degree as observed after cleavage of LRRTM2 (Fig. 4F,
376 4M), we removed different numbers receptors from the modeled nanodomain, placed them
377 randomly within the PSD, and then compared the enrichment profile of the redistributed
378 synapse to that of the original modeled synapse. A loss of ~16 AMPARs from the modeled
379 nanodomain resulted in a ~60% reduction in density within the nanodomain and well
380 recapitulated the experimentally observed decrease in enrichment (Supplementary Fig.
381 6C,D).

382
383 Increasing evidence suggests that evoked and spontaneous transmission involve
384 separable presynaptic structures (52) and activate separated groups of receptors (53), but
385 their potential differential dependence on receptor nano-organization is not known. To

386 model the impact of receptor redistribution on these different release modes, randomized
387 release positions were constrained either to the nanodomain or the PSD as a whole to inform
388 our predictions about evoked and spontaneous release, respectively (Fig. 5A, (20)). Then,
389 by indexing the AMPAR peak open probability as function of distance from the vesicle
390 fusion site (as in Tang et al., 2016, shown schematically in Fig. 5C, 20), we calculated the
391 mean summed peak open probability of all receptors in response to single glutamate release
392 events before and after receptor redistribution. EPSC kinetics are not captured in such a
393 model, but the mean summed peak open probability successfully indicated that release
394 events closer to receptor nanodomains produce larger predicted responses (Fig. 5D)
395 consistent with results observed from Haas et al. (2018; (28)).

396 To simulate the receptor reorganization observed after acute cleavage of LRRTM2,
397 we removed the portion of the receptors from the nanodomain determined from the
398 modeling above (16 of 27) and placed them randomly into the PSD outside the nanodomain.
399 This manipulation substantially reduced the predicted response to release constrained to the
400 receptor nanodomain ($59.4 \pm 0.1\%$ of control; Fig. 5E, left). Thus, the model predicts that
401 evoked transmission at “average” synapses would be reduced by roughly 60% after
402 LRRTM2 cleavage. Strikingly, despite this strong effect, the response to release events
403 occurring at randomized positions across the AZ was essentially unaltered ($96.3\% \pm 0.1\%$
404 of control; Fig. 5E, right). Interestingly, variability of response amplitude for spontaneous
405 release was reduced upon redistribution (CV 33.03% for baseline parameters and 20.31%
406 after redistribution), suggesting that heterogeneity of receptor density across the face of an
407 individual PSD contributes to the response CV at that synapse.

408 This difference between the decrement of response to release at a nanodomain or
409 across the synapse persisted over a range of parameters. For instance, we found that as PSD
410 size increases, the proportional effect of receptor redistribution grows larger (Fig. 5F),
411 suggesting that nano-alignment may be most critical for maximizing postsynaptic
412 responsivity at large synapses. Similarly, positioning a greater fraction of receptors within
413 the nanodomain resulted in a greater reduction in mean summed peak open probability upon
414 redistribution (Fig. 5G) but again this only affected release constrained to the nanodomain,
415 and no differences were observed in the mean of the responses with release constrained to
416 the PSD.

417 A key parameter in the model is the decay profile in receptor open probability as a
418 function of distance from the release site, here modeled by default as $Po(d) = 0.42e^{-d/88}$
419 adapted from previous work (20, 50). To test whether the outcomes were robust to changes
420 in this parameter, we varied this decay constant by 50% in either direction. This altered the
421 magnitude of influence as expected but did not qualitatively affect the outcome. When the
422 decay rate was decreased ($-d/44$) making receptors less sensitive to release position, the
423 mean summed peak open probability after nanodomain release events was elevated (not
424 shown) yet was still strongly reduced upon redistribution (Fig. 5H). Conversely, when the
425 rate of decay was increased ($-d/132$), responses were lower but also strongly reduced after
426 redistribution. Note that for all values of the decay constant, in response to the modeled
427 spontaneous release events, the mean summed peak open probability was essentially
428 unchanged by redistribution of receptors (Fig. 5H).

429 Together, these simulations most generally suggest that receptor distribution within
430 a PSD strongly influences the amplitude of evoked but not average spontaneous
431 neurotransmission at that synapse. Specifically, they predict that following LRRTM2
432 cleavage, the amplitude of evoked EPSCs but not spontaneous mEPSCs should decrease
433 substantially.

434 **LRRTM2 is critical for basal strength of evoked, but not spontaneous transmission**

436 To assess these predictions of functional effects of AMPARs nanostructural
437 remodeling within the PSD following LRRTM2 cleavage, we performed patch-clamp
438 recordings from cultured hippocampal neurons while stimulating nearby cells to evoke
439 synaptic responses (Fig. 6A, Supplementary Fig. 9A). In untransfected neurons, thrombin
440 application had no effect on EPSC amplitude ($98.8 \pm 7.7\%$ after 10 min, $n = 9$, Fig. 6B),
441 confirming a lack of non-specific or endogenous effects of the protease. Similarly, in cells
442 transfected with LRRTM2 knockdown and rescued with the non-cleavable GFP-
443 LRRTM2*, EPSCs were unaffected ($95.5 \pm 5.8\%$, $n = 9$, Fig 6B). However, in cells
444 transfected with GFP-Thr-LRRTM2*, acute cleavage of the LRRTM2 ECD resulted in a
445 $45.3\% \pm 7.6\%$ reduction in EPSC amplitude ($n = 12$, Fig. 6B), consistent with our modeling
446 results.

447 Deficits in presynaptic release probability could have contributed to the decreased
448 EPSC amplitude. To test this, we calculated the paired-pulse ratio of responses to stimuli
449 50 ms apart. Thrombin treatment had no effect on the paired-pulse ratio across GFP-Thr-
450 LRRTM2*, GFP-LRRTM2*, and untransfected neurons (Fig. 6C), as well as compared to
451 their own baselines. These data suggest changes in release probability did not drive the
452 changes in the evoked response amplitude after LRRTM2 cleavage.

453 To test the effect of LRRTM2 cleavage on the postsynaptic response to spontaneous
454 release of glutamate, we measured miniature EPSCs (mEPSCs; Fig 6D-I) from neurons
455 expressing GFP-LRRTM2* or GFP-Thr-LRRTM2*. Thrombin application did not change
456 mEPSC amplitude in cells transfected with non-cleavable GFP-LRRTM2*. Neurons
457 transfected with cleavable GFP-Thr-LRRTM2* also showed no changes to mEPSC
458 amplitude following thrombin application ($98.5 \pm 3.7\%$ of control, Fig. 6F, Supplementary
459 Fig. 9B), consistent with the model's prediction. Nevertheless, the coefficient of variation
460 of mEPSC amplitude was smaller after thrombin exposure in neurons expressing GFP-Thr-
461 LRRTM2* vs GFP-LRRTM2* (Supplementary Fig 9G), as expected based on modeling
462 (Fig 5E). Together, these results suggest that the number of synaptic AMPARs was not
463 changed upon the acute loss of the LRRTM2 ECD, consistent with results in Fig. 2 that
464 synaptic content of both surface AMPARs and PSD-95 was unchanged within 30 minutes
465 following LRRTM2 cleavage.

466 To further test if loss of the LRRTM2 ECD alters presynaptic mechanisms, we
467 quantified mEPSC frequency. Neurons transfected with the non-cleavable LRRTM2
468 showed no changes in frequency after 10 minutes of thrombin exposure ($91.7 \pm 19.4\%$
469 of control, Fig. 6G, Supplementary Fig. 9C), consistent with the lack of nonspecific or
470 endogenous protease effects. Interestingly, neurons transfected with GFP-Thr-LRRTM2*
471 also showed no changes following 10 minutes of thrombin treatment (Fig. 6G), further
472 strengthening the idea that the LRRTM2 extracellular domain does not acutely regulate
473 presynaptic release probability.

474 It was previously shown that extracellular cleavage of neuroligin-1 (NL-1) reduced
475 evoked EPSC amplitude without changing in mEPSC amplitude, and this was attributed to
476 a reduction of presynaptic release probability (38). As NL-1 and LRRTM2 share
477 presynaptic Nr1 as a ligand, we examined whether NL-1 cleavage also resulted in
478 reorganization of transsynaptic alignment. Interestingly, the acute cleavage of NL-1 did not
479 change the relative nano-alignment of RIM and AMPARs (Supplementary Fig. 10). This
480 further distinguishes the unique role of LRRTM2 in maintaining synapse nanoarchitecture.

481 Numerical models of glutamate diffusion and AMPAR kinetics have demonstrated
482 that glutamate release away from AMPARs delays the opening of these channels by
483 decreasing the number of immediately doubly-bound receptors, leaving many in a singly-
484 bound state and slowing the rise of the EPSC as the concentration of glutamate in the cleft
485 quickly equilibrates (54). Unfortunately, the measured variability in EPSC kinetics in our

486 approach (which did not stimulate single presynaptic neurons for analysis) appeared to be
487 dominated by variation in axonal conduction and other presynaptic factors, precluding
488 interpretation of EPSC kinetics. However, we tested whether mEPSC kinetics were
489 disrupted after LRRTM2 cleavage. We quantified both normalized and raw 10-90% rise
490 time (Fig. 6H, Supplementary Fig. 9C) and 90-10% decay times (Fig. 6I, Supplementary
491 Fig. 9D) of mEPSCs from neurons transfected with either GFP-LRRTM2* and GFP-Thr-
492 LRRTM2* before and after the application of thrombin. Representative averaged traces
493 (Fig. 6E) and group quantification, however, demonstrated no change in either the rise time
494 ($101.8 \pm 8.9\%$ of control, Fig. 6H) or the decay time ($100 \pm 4.2\%$ of control, Fig. 6I). These
495 results in combination with the raw and normalized miniature amplitudes (Supplementary
496 Fig. 6F, Supplementary Fig. 7B), which are also sensitive to changes in single channel
497 kinetics, suggest no population changes in AMPAR single-channel kinetics following the
498 cleavage of the LRRTM2 ECD. These results are consistent with our numerical model of
499 AMPAR position within the PSD (Fig. 5), where the average distance of AMPARs to the
500 modeled spontaneous release positions across the AZ was unchanged upon AMPAR
501 redistribution.

502 Taking these data together, we conclude that the LRRTM2 extracellular domain is
503 required for close positioning of AMPARs to sites of evoked vesicle fusion, and that this
504 distribution of receptors preferentially enhances evoked, but not spontaneous postsynaptic
505 response amplitude.

506 Discussion

507 We used acute proteolysis of the LRRTM2 extracellular domain to test the idea that
508 transsynaptic interactions in the synaptic cleft control molecular organization and function
509 of established synapses, independent of synapse formation and on rapid time scales. We
510 found that acute cleavage of LRRTM2 quickly led to dispersal of its extracellular domain
511 from synapses and prompted a strong reduction in the strength of evoked but not
512 spontaneous synaptic transmission. Based on several lines of evidence, we conclude that
513 this reduction in transmission arose from nanoscale redistribution of AMPARs within the
514 synapse away from sites of glutamate release. First, LRRTM2 is concentrated in the
515 synaptic nanocolumn, heavily enriched in nanoscale subdomains containing PSD-95 and
516 AMPARs, and aligned transsynaptically with RIM nanodomains in the active zone. Second,
517 after cleavage of LRRTM2, AMPARs became less densely enriched across from RIM
518 nanodomains, even though total AMPAR content in synapses as measured by
519 immunostaining or live imaging was unchanged for at least 30 minutes. Third, despite
520 marked alteration of AMPAR distribution, the acute disruption of LRRTM2 did not grossly
521 alter synapse number, structure, or molecular content, and notably the total synaptic content
522 of PSD-95 also did not change within this period of interest. Fourth, presynaptic function
523 was unaltered, as judged by fully intact spontaneous release and evoked paired-pulse
524 response ratio. Finally, numerical modeling of AMPAR activation based on our
525 nanostructural measurements of AMPAR position well predicted the degree of reduction in
526 evoked transmission, while also providing a mechanism for the lack of effect on the
527 amplitude of spontaneous EPSCs. Together, these findings provide support for a model in
528 which the nanoscale patterning of AMPARs is dynamically controlled by interactions of
529 LRRTM2 with cleft proteins enriched within the nanocolumn, and that this organization can
530 dramatically enhance AMPAR activation during evoked synaptic transmission.

531 The close correspondence between our measurements and the predictions from
532 numerical modeling provide experimental support for the longstanding notion that receptor
533 distribution within synapses affects synaptic strength (5, 20, 55). Modeling indicates that
534 the combination of the sharp decay of glutamate concentration away from the site of fusion
535

536 with the rapid kinetics of AMPAR activation and desensitization necessitate that AMPARs
537 positioned closest to the site of glutamate exocytosis contribute proportionally most to
538 EPSCs (8, 50). The quick relaxation of EPSC amplitude towards a reduced steady state that
539 we observed following LRRTM cleavage indicates that this mechanism likely plays a role
540 in maintaining basal synaptic strength. Mechanisms that maintain synaptic strength absent
541 the active induction of plasticity are not clear, but have been postulated to involve adhesion
542 systems (56). Taken most broadly, our findings suggest that mechanisms of synaptic
543 maintenance may be divided into those which establish the molecular constituent list
544 including AMPAR number, and those which facilitate appropriate nanostructural
545 organization. It is interesting to consider whether synapses of limited molecular complexity
546 may adopt a somewhat disorganized configuration “by default,” whereas the presence of
547 LRRTM2 enables organization into a configuration of higher synaptic potency.

548 There are a number of means by which LRRTM2 may organize AMPARs. Single-
549 cell knockout of LRRTM1 and 2 in young adult mice reduced AMPAR-mediated evoked
550 transmission and destabilized AMPARs as measured by photoactivation without affecting
551 synapse number, release probability, or NMDAR-mediated transmission (35, 36),
552 suggesting that in established synapses, LRRTMs may serve as anchors for AMPARs in the
553 PSD. However, the role of LRRTM1 in this process appears limited, since LRRTM1
554 knockdown alone has no effects on evoked or spontaneous EPSC amplitudes, but substantial
555 impact on mEPSC frequency, spine density, and synaptic vesicle distribution (57) its
556 function may principally be limited to presynaptic roles following synaptogenesis. These
557 findings strongly suggest that LRRTM2 plays a unique role for AMPAR retention.
558 However, disruption of LRRTM2 did not lead to loss of AMPARs from the synapses within
559 30 to 60 minutes, even though it eventually produced large changes in AMPAR stability in
560 spines. Thus, LRRTM2 alone may not be sufficient to fulfill the functional role of “slots”
561 hypothesized to anchor AMPARs (58, 59). Further, we cannot rule out that the
562 transmembrane and intracellular domains of LRRTM2 that remain after thrombin cleavage
563 may contribute to the synaptic retention of AMPARs. This would be intriguing
564 intramolecular segregation of function within LRRTM2 for overall retention vs positioning
565 of AMPARs. However, GPI-anchored LRRTM2 ECD fully rescues the deficit in LTP
566 during conditional deletion of LRRTM1 and 2, suggesting a specific role for the LRRTM2
567 extracellular domain.

568 At the subsynaptic scale, the patterned distribution of AMPARs is generally thought
569 to be stabilized by a combination of factors in spite of continuous receptor diffusion in the
570 plasma membrane (15, 60, 61): a heterogenous affinity landscape in the synapse created by
571 the distribution of direct AMPAR binding partners (62), and an array of steric obstacles
572 which creates macromolecular crowding and hinders their motion within the dense synaptic
573 environment (49, 63). We suspect both these mechanisms may be involved in how
574 LRRTM2 controls the AMPAR pattern. There is some evidence that the LRRTM2 ECD can
575 interact directly with AMPARs (33, 36) (but see (64)), and LRRTM2 through its interaction
576 with PSD-95 might dynamically organize intracellular scaffolds (32, 33). At the same time,
577 its loss may trigger reorganization or even loss of synapse-resident proteins which could
578 alter the steric hindrance experienced by receptors in the cleft by their large extracellular
579 domains or in the substantially denser PSD by their smaller intracellular domains. In
580 addition, partitioning of the PSD via liquid-liquid phase separation is being actively
581 investigated as a potential organizing mechanism of synaptic nanostructures (65). Due to
582 multivalent interactions facilitated by LRRTM2 in the synaptic cleft, its presence could
583 serve to establish a nanoscale, phase-separated synaptic subdomain into which AMPARs
584 partition, and which would be disturbed by the cleavage of the LRRTM2 ECD (though we
585 do not know of evidence that LRRTM2 is cleaved endogenously). Overall, regardless of the

586 mechanism, our data indicate that the nanoscale organization of AMPARs is both modulated
587 by the LRRTM2 ECD and capable of rapid reorganization.

588 These observations are particularly interesting given that strong evidence also
589 implicates LRRTM2 in LTP (36). Conditional deletion of LRRTM1 and 2 in mature mice
590 reduces LTP in vivo, and expression of the LRRTM2 ECD alone, but not LRRTM4, is
591 sufficient to rescue these deficits (35), clearly consistent with our observation that acute loss
592 of the ECD regulates synaptic strength. Similarly, point mutations to the LRRTM2 ECD
593 which disrupt presynaptic neurexin binding fail to completely rescue LTP (35), suggesting
594 that neurexin binding may help explain how LRRTM2 specifically organizes AMPARs with
595 respect to active zone nanodomains. LRRTM2 has been proposed as an anchor that
596 stabilizes AMPARs during LTP induction (35, 36). Our work extends this by implying
597 synaptic nanostructure shaped by LRRTM2 may play several roles in functional plasticity.
598 Most simply, LRRTM2-augmented AMPAR activation may lower the threshold of activity
599 needed to trigger plasticity. Existing LRRTM2 nanodomains could also facilitate the
600 stabilization of recently exocytosed or otherwise labile receptors (3). Similarly, it is
601 conceivable that LRRTM2 could nucleate new nanoclusters added during LTP (66, 67),
602 though activity-dependent trafficking of LRRTM2 remains uninvestigated. In addition, our
603 prior observation that chemical LTP induction “sharpens” the AMPAR distribution under
604 RIM nanodomains (20) may suggest further that graded levels of AMPAR organizational
605 tuning could be facilitated by LRRTM2. Most broadly, it is a natural extension of our
606 findings here to suggest that behavioral or disease-relevant plasticity mechanisms, even
607 regardless of the potential involvement of LRRTM2, may regulate synaptic strength not
608 only by regulating AMPAR number but through controlling synapse nanostructure.

609 Surprisingly, we found the average postsynaptic response to spontaneous release
610 was rather insensitive to AMPAR nano-organization, though it remains to be seen if this
611 holds for all synapse geometries (e.g. small synapses). Our model and others predict that
612 different forms of release may produce different postsynaptic responses depending on the
613 subsynaptic distribution of release sites. There is indeed evidence that mEPSCs as well as
614 univesicular EPSCs evoked in the presence of Sr^{2+} differ in CV and amplitude from AP-
615 evoked EPSCs in the same neurons (68, 69), and our findings provide novel experimental
616 support for the idea. However, one shortcoming in previous work as well as our own is that
617 due to the large variation in synaptic potency even on single neurons, precise measures of
618 both mEPSCs and evoked quantal size from the same *synapses* not merely the same cells
619 will be needed for thorough experimental validation of these predictions. Such differences
620 may be important though, because while the roles of spontaneous synaptic transmission
621 remain unclear, it has been suggested to stabilize the basal structure and function of the
622 postsynapse (52, 70, 71). Local activity driven by spontaneous neurotransmitter release is
623 also important for restricting the lateral mobility of AMPARs, helping to trap them in the
624 PSD (72). Implicating transcellular mechanisms in the distinct regulation of evoked and
625 spontaneous transmission further distinguishes these two forms of transmission already
626 known to operate with heterogeneity at different active zones (73).

627 The effects of disrupting LRRTM2 and NL-1 differ in several ways both
628 electrophysiologically and molecularly. Perhaps most dramatically, our experiments
629 showed that acute manipulation of LRRTM2 but not NL-1 quickly prompted
630 disorganization of AMPARs, whereas in similar experiments, proteolytic cleavage of NL-1
631 rapidly altered synaptic neurexin content and reduced presynaptic release probability (38).
632 Thus, even adhesion molecules that share binding partners may play unexpectedly divergent
633 roles in maintaining organization of synaptic molecular complexes. More broadly, these
634 results suggest that many specific aspects of synapse structure and function are maintained
635 by unique subsets of the diverse cell adhesion systems present within single synaptic clefts.

636 Indeed, growing evidence demonstrates that synaptic CAMs themselves are found in
637 distinct subsynaptic patterns (5, 37, 46, 48). Clearer understanding of CAM organization
638 within synapses will provide insight into their contribution to synapse nanoarchitecture and
639 to their cooperative or even competitive functional roles.

641 **Materials and Methods**

642 **Plasmids**

643 All LRRTM2 plasmids were generated based on FCK-shLRRTM2 and pBOS-GFP-
644 hLRRTM2-FL described previously (33). For insertion of the thrombin cleavage site, the
645 sequence coding the four Ser's (S386-S389) was replaced with a sequence coding the
646 cleavage site (LVPRGS) with a flexible linker (GGGGS) on each side. For knockdown-
647 rescue experiments in neurons, the H1 promoter and sh-LRRTM2 sequences from FCK-
648 shLRRTM2 were subcloned into the pBOS-GFP-hLRRTM2-FL around the MluI site with
649 IVA cloning (74). For BRS-LRRTM2, GFP sequence was replaced with a sequence coding
650 the α -bungarotoxin-binding sequence (WRYYESSLEPYPD; (44)). GFP-Neuroigin1 and
651 GFP-Neuroigin1-Thr were kind gifts from Michael Ehlers (38).

652 **Co-culture synaptogenesis assay**

653 Co-culture assays were performed as described (75). Briefly, neurons were dissociated from
654 embryonic day 18 Sprague-Dawley rat hippocampi, plated at a density of 60,000 cells on
655 12 mm cover glasses pre-coated with 1 mg/ml poly-L-lysine (Sigma, P1274), and treated at
656 2 div for 24 with Ara-C (2 μ M) to prevent glial growth. Neurons were cultured in
657 Neurobasal medium (Invitrogen 21103-049) with 3% B27 (Invitrogen, 17504-001) and 1%
658 Glutamax (Invitrogen, 35050-061) and incubated at 37°C in 5% CO₂. When neurons
659 reached 7 div, 70% confluent HEK293 cells were transfected using polyethylenimine in 6
660 well dishes at approximately 0.4 picomol plasmid per 9.5 cm² well (76). Transfected
661 HEK293 cells were suspended 24 h later and seeded onto 8 div neurons at a density of 5,000
662 HEK cells per 12 mm cover glass. Ara-C was added at 2 μ M upon seeding to prevent HEK
663 cell overgrowth. After 48 h, co-cultures were fixed on 10 div with 4% PFA, 4% sucrose in
664 PBS, stained with primary antibodies diluted in 3% fetal bovine serum (FBS) and 0.01%
665 Triton-X 100 in PBS, and incubated overnight at 4°C. Secondary antibodies were diluted in
666 3% FBS and applied for 4 hours at 4°C. Neuronal cultures were stained with mouse
667 monoclonal antibodies against Bassoon (AssayDesigns Cat# VAM-PS003F, RRID:
668 AB_2313991; 1:500) and BTX-Alexa-647. Secondary immunostaining was performed with
669 Alexa dye-conjugated antibodies. Coverslips were mounted with Aqua/PolyMount (Fisher,
670 NC9439247). Confocal microscopy was performed on a Leica TCS SP8. Images were
671 acquired with an ACS APO 63x oil lens with 1.3 NA, using the same settings for each
672 condition. During image acquisition and analysis, the researcher was blind to the condition.
673 Images were analyzed using a custom written ImageJ script available upon request.

674 **Hippocampal Culture and Transfections**

675 All experimental protocols were approved by the University of Maryland School of
676 Medicine Institutional Animal Care and Use Committee or the Institutional Animal Care
677 and Use Committees at the University of Science and Technology of China (USTC) and the
678 Chinese Academy of Sciences (CAS). Dissociated hippocampal neurons from E18 SD rats
679 of both sexes were prepared as described previously (77). Neurons were transfected on DIV
680 7-10 with Lipofectamine 2000 and experiments were performed at least 7 days after the
681 transfection (DIV 14–21). All experiments were repeated on 3 or more separate cultures
682 unless otherwise specified.

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Immunocytochemistry

Neurons were fixed in 4% paraformaldehyde, 4% sucrose in phosphate buffered saline (PBS) for 10 min at room temperature and processed for immunofluorescence with standard procedures as described previously (20). Primary antibodies were: rabbit anti-RIM1/2 (Synaptic System #140203, 1:500), mouse anti-PSD-95 (NeuroMab clone K28/43, 1:200), chicken anti-GFP (Chemicon ab13970, 1:200). Secondary antibodies were from Jackson ImmunoResearch (West Grove, PA), either already conjugated with Alexa 647 or unconjugated that we labelled with Cy3b (GE Healthcare). Labeling with the anti-GFP antibody was performed after fixation but prior to overt permeabilization.

Live cell α -bungarotoxin (BTX) recognition sequence (BRS) labeling with α -bungarotoxin conjugated to Alexa-647 was performed prior to fixation described above. Coverslips were inverted on 50 μ l droplets of BTX-Alexa-647 (Thermofisher B35450, 1:100) in aCSF containing 2 mM Ca^{2+} and 2 mM Mg^{2+} and covered for 5 minutes at room temperature (21-24°C). Then, coverslips would be placed into a small weigh boat filled with aCSF, and gently agitated, removing and replacing the aCSF twice before mounting in the microscope imaging chamber.

For LRRTM2 and PSD-95 immunocytochemistry, neurons were fixed in 2% paraformaldehyde, 4% sucrose in cytoskeleton buffer (10 mM MES pH 6.8, 138 mM KCl, 3 mM MgCl_2 , 2 mM EGTA, 320 mM sucrose) for 8 minutes at room temperature. Coverslips were then washed 3 times for 5 minutes each with PBS/Gly. Cells were permeabilized with 0.3% TritonX-100 (TX-100) in PBS/Gly for 20 minutes at room temperature, then washed once in PBS/Gly with 0.1% TX-100 for 5 minutes. Blocking was performed with a solution containing 3% BSA, 5% goat serum, 5% donkey serum, and 0.1% TX-100 for 1 hour and 15 minutes. Coverslips were inverted and incubated with primary antibodies (α LRRTM2, IgG1A mouse, NeuroMab N209C/35.3, 1:10; α PSD-95, IgG2A, 1:80, stored in 50% glycerol) diluted in a 1:1 dilution of the blocking media and PBS/Gly overnight in a humidity chamber at 4°C. Coverslips were then washed 3x in PBS/Gly containing 0.1% TX-100 for 5 minutes. Secondaries ($\text{G}\alpha\text{M}$ IgG1A Alexa-647, Jackson, 115-605-205, Lot 143997, 1:200; $\text{D}\alpha\text{M}$ IgG2A, Jackson, Cat 20257, Lot 14C0225 1:200) were diluted in a 1:1 dilution of the blocking media and PBS/Gly and coverslips were inverted on secondary in a humidity chamber at room temperature for 1 hour. Then coverslips were washed 3x with PBS/Gly for 5 minutes. Cells were postfixed with 4% PFA, 4% sucrose in PBS for 15 minutes, then washed 3x with PBS/Gly for 5 minutes.

All imaging except for dSTORM and HEK co-culture assay was performed on an Andor Dragonfly spinning disk confocal on either an Olympus IX81 or a Nikon Ti2 microscope. In each case, a 60x/1.45 NA oil immersion objective and Zyla sCMOS camera were utilized, with image format of 103 nm/pixel. Except where indicated, experiments were conducted at room temperature (22-24°C). Otherwise, for imaging in culture medium, a stage-top incubator and objective heater (Tokai Hit) maintained the sample temperature at 37°C and CO₂ at 5%.

Proteolytic cleavage

Thrombin from bovine plasma (Sigma, T4648-1KU, Lot # SLBV3604) was diluted in the imaging buffer (aCSF; 2 mM Ca^{2+} , 2 mM Mg^{2+}) at 100 Units per mL such that when added to the bath by pipette the final concentration became 10 Units per mL. Thrombin was added drop-wise away from the objective into the media containing cells in the imaging chamber at 24°C. Thrombin was stored at -20°C with a volume of at least 600 μ l, and only underwent 1 freeze-thaw cycle. For imaging at 0.003 Hz, cells were maintained at 24°C. Z-stacks were taken every 5 minutes, and a maximum intensity projection was used for analysis. For

736 imaging at 20 Hz, cells were kept on the objective at 24°C. Imaging was performed with
737 488 nm excitation during continuous acquisition at 20 Hz. Binning (2x2) permitted the
738 identification of modestly expressing synaptic puncta at lowest possible laser power to
739 prevent photobleaching. Exposure was 50 ms per frame. Data was smoothed with a sliding
740 average window with a bin length of 3 frames (150 ms). For all experiments, chamber was
741 thoroughly washed with deionized water and 70% ethanol accompanied by physical
742 scrubbing in order to completely remove residual thrombin that can adhere to the plastics
743 and rubber of the imaging chamber and O-ring. All synaptic ROI measurements were
744 background subtracted and normalized to an average of each synapses own baseline.
745

746 **Quantification of protein retention at synapses**

747 *Live (30 minute)*. Cells were co-transfected with GFP-Thr-LRRTM2* or BRS-Thr-
748 LRRTM2* and PSD95-mCherry* or SEP-GluA1,2, respectively. For PSD-95 experiments,
749 multiposition z-stacks were acquired every five min. For analysis, maximum intensity
750 projections were calculated. ROIs of a fixed size (15 pixels) were drawn around synaptic
751 puncta containing both LRRTM2 and PSD95-mCherry* or SEP-GluA1/2 fluorescence.
752 Integrated intensity was measured, background subtracted (an average of multiple ROIs
753 across the field of view), then normalized to an average of the ROIs pre-thrombin baseline.
754 Only spines that remained within the ROI for the duration of imaging were included.

755 *Live (2 hour)*. Cells were co-transfected with SEP-GluA1,2 and BRS-LRRTM2* or BRS-
756 Thr-LRRTM2*. Multiposition z-stacks were taken every 15 or 20 minutes. For analysis,
757 maximum intensity projections were calculated. ROIs of a fixed size (25 pixels) were drawn
758 around synaptic puncta containing both LRRTM2 and SEP-GluA1,2 fluorescence.
759 Integrated intensity was measured, background subtracted (an average of multiple ROIs
760 across the field of view), then normalized to an average of the ROIs pre-thrombin baseline.
761 Only spines that remained within the ROI for the duration of imaging were included.

762 *Fixed*. Cells were co-transfected with GFP-Thr-LRRTM2* and mCerulean3. Cells were
763 immunostained for PSD-95 and RIM1/2 as described above. All regions were acquired with
764 the same imaging parameters on the Dragonfly confocal. For analysis, background
765 subtracted (values taken from an average of multiple background regions across the field)
766 integrated intensity within an ROI of a fixed size (15 pixels). Values were additionally
767 normalized to the median intensity in the field which helped to normalize potential
768 differences in any region to region variability in staining intensity. Normalization to median
769 intensity did not appear to be skewed by artefactual puncta as these were avoided during
770 acquisition or occupied a very small fraction of total pixels in the field.

771 *24-hour post-thrombin*. Cells were co-transfected with SEP-GluA1,2 and BRS-LRRTM2*
772 or BRS-Thr-LRRTM2*. Both groups were treated with thrombin for 10 minutes and then
773 returned to culture media. Then, 24 hours later cells were fixed and stained for GFP and the
774 BRS-tagged LRRTM2 ECD as described above. All regions were acquired with the same
775 imaging parameters on the Dragonfly confocal spinning-disk (Andor). For analysis,
776 maximum intensity projections were calculated. ROIs of a fixed size (15 pixels) were drawn
777 around α GFP puncta. Integrated intensity was measured, routinely background subtracted
778 (an average of multiple ROIs across the field of view).
779

780 **Quantification of PSD-95 puncta density**

781 Cells were transfected with cytosolic mCerulean3 alone or paired with either pBOS-
782 shLRRTM2 (tgctatttactgactcde;(33)), GFP-Thr-LRRTM2* (this paper), or pBOS-GFP-
783 Thr-LRRTM2 (this paper). Cells were then fixed and immunostained for PSD-95 (described
784 above). mCerulean fluorescence was used to demarcate the dendrites of transfected cells.
785 Using mCerulean fluorescence alone, in order to remain blinded to the transfection

786 condition, up to the first 6 transfected cells were selected for imaging and further analysis
787 in order to reduce bias. Regions were chosen at least ~75 μm from the soma when dealing
788 with a clear primary dendrite to avoid volume effects. Distance was calculated by drawing
789 a line in ImageJ (total pixel number x pixel size). All images were thresholded the same.
790 Each punctum had to consist of at least 4 suprathreshold pixels. Experimenter was blind to
791 the condition during image analysis.

792 **Quantification of spine morphology**

793 Cells (DIV 4-6) were transfected with mCerulean3 alone or mCerulean3 paired with GFP-
794 Thr-LRRTM2*. Cells were imaged at DIV 14-16. Maximum intensity projections of the
795 confocal stacks were analyzed in ImageJ by an observer blinded to conditions. Analysis
796 between groups were always performed within the same culture. For spine length, a line
797 was drawn from the edge of the spine head to the edge of the dendrite, parallel to the long
798 axis of the spine (total pixel number x pixel size). For spine area, an ROI was drawn around
799 the spine head. The images were thresholded based on intensity and area was measured in
800 ImageJ. Experimenter was blind to the condition during image analysis.

801 **Colocalization analysis**

802 Cells were transfected with GFP-Thr-LRRTM2*. Synapses were picked based on
803 colocalization with dendritic spines. Five consecutive spine-resident, GFP-positive puncta
804 were selected randomly from at least 4 separate dendritic regions per cell when possible.
805 When few branches were present, selection of dendritic regions of interest were as evenly
806 distributed throughout the image as possible. The data represent the number of those
807 randomly selected GFP-positive puncta which also contained at least 4 suprathreshold
808 pixels of PSD-95 or RIM1/2 staining. Analysis was performed using ImageJ. Experimenter
809 was blind during data analysis.

810 **3D-STORM imaging**

811 Imaging was performed essentially as described (20) on an Olympus IX81 ZDC2 inverted
812 microscope with a 100 \times /1.49 TIRF oil-immersion objective. Excitation light was reflected
813 to the sample via a 405/488/561/638 quad-band polychroic (Chroma) with an incident angle
814 near but less than the critical angle. The typical incident power out of objective was ~30
815 mW for 647 nm and ~60 mW for 561 nm. Emission was passed through an adaptive optics
816 device (MicAO, Imagine Optic) which corrected the aberrations and introduced astigmatism
817 for 3D imaging. A Photometrics DV2 was insert before an iXon+ 897 EM-CCD camera
818 (Andor) for simultaneous collection of the red and far-red emissions. All hardware was
819 controlled via iQ software (Andor), except the MicAO which was controlled via
820 Micromanager. Z stability was maintained by the Olympus ZDC2 feedback positioning
821 system. Imaging of NL1 experiments was carried out on a Nikon ECLIPSE Ti2 inverted
822 microscope equipped with a perfect focusing system and an 100 \times /1.49 TIRF oil-immersion
823 objective controlled with NIS-Elements AR 4.30.02 software; emission was collected with
824 a CMOS camera (ORCA-Flash4.0, Hamamatsu); localization detection, calibration and
825 drift correction were done using the NIS-Elements AR analysis 4.40.00 software. Lateral
826 drift was corrected with a cross-correlation drift correction approach²⁴. Samples were
827 imaged in a STORM imaging buffer freshly made before experiments containing 50 mM
828 Tris, 10 mM NaCl, 10% glucose, 0.5mg/ml glucose oxidase (Sigma), 40 $\mu\text{g}/\text{ml}$ catalase
829 (Sigma), and 0.1M cysteamine (Sigma). TetraSpeck beads (100 nm; Invitrogen)
830 immobilized within a thin layer of 4% agarose on a coverslip were localized across a z-stack
831 with 30-nm steps to get the 3D calibration and correct alignment between the two channels
832
833
834

835 as described previously. The average deviation of the bead localizations after correction was
836 <15 nm in x/y directions and 40–50 nm in z.

837 **Single-molecule localization and analysis of synaptic clusters**

838 All data analysis was performed offline using custom routines in MATLAB (Mathworks).
839 The lateral (x, y) and axial (z) coordinates of single fluorophores were determined from the
840 centroid position and ellipticity of the fitted elliptical 2D Gaussian function to a 7×7 pixel
841 array (pixel size 160 nm) surrounding the peak. Poorly localized peaks were removed with
842 a set of rejection criteria including an x–y precision <10 nm, fitting R² > 0.6, and comprising
843 >200 photons, and the shape of peaks²⁴. For peaks lasting for more than one frames, only
844 the localizations in the first frame were included in further analysis.

845 Synapses were identified as a juxtaposed pair of localization clusters of synaptic
846 proteins and only those with clear pre- and postsynaptic components were selected for
847 further analysis. A DB-SCAN filter was applied to the selected synaptic localizations with
848 MATLAB function ‘DBSCAN.m’ created by S. M. K. Heris to define the boundaries of
849 synaptic clusters. Only those localizations with a minimum of 60 localizations (MinPts =
850 60) within a radius of 5 times mean nearest neighboring distance (epsilon = 5 x MNND ≈
851 100–120 nm) were considered within the synaptic cluster. The cluster boundaries were
852 defined by an alpha-shape with $\alpha = 150$ nm.
853

854 **Nanocluster detection and protein enrichment analysis**

855 Nanoclusters within synaptic clusters were automatically identified based on local densities
856 defined as the number of localizations within a certain distance (d) from each localization.
857 To account for the variation in localization density across different synaptic clusters, we
858 defined d as 2.5 x MNND instead of a fixed value (78). The threshold of local density for
859 nanocluster detection was defined as Mean(LD0) + 4 x Std(LD0), where LD0 is the local
860 density of a randomized cluster with the same overall density as the synaptic cluster. The
861 threshold we used represented the 99.95% confidence that the measured density differs from
862 chance.
863

864 All localizations above the threshold were then ranked based on their local densities
865 in a descending order and assigned each localization sequentially as the peak of a new
866 nanocluster or a part of an existing nanocluster based on whether it was further enough from
867 peaks of all existing nanoclusters. The localization with highest local density, if above the
868 threshold, was defined as the peak of the first nanocluster. The second-highest-density
869 localization would be considered as the peak of another potential nanocluster if the distance
870 between the first peak to this potential second peak was larger than the defined cutoff
871 distance; otherwise, the second localization was considered a part of the first nanocluster.
872 The minimum peak-to-peak distance was set as 80 nm, which is about the average size of
873 synaptic nanoclusters (15, 20, 79). Then, each potential nanocluster was further divided into
874 sub-clusters based on the point-to-point distance with a cutoff of 2 x MNND using
875 MATLAB function 'clusterdata', and only the sub-cluster having the original peak
876 localization of this potential nanocluster was selected. Finally, the sub-clusters had to
877 include at least 4 localizations to be accepted as a nanocluster.

878 The enrichment analysis is based on the prediction that if the pre- and postsynaptic
879 nanoclusters align across the cleft, the presence of a nanocluster on one side will predict a
880 higher local protein density around its projected point on the other side. The synaptic cluster
881 pair was first translated to overlap with each other based on their general shape without bias
882 towards local densities (20, 78). The enrichment was then quantified as the average local
883 density of protein A over the distance from the projected peak of a protein B nanocluster.
884 In case of a positive alignment, this curve would start from a local density significantly

885 higher than the average at the small distance and then decay to the average. More details
886 and the defined MATLAB function for nanocluster detection and protein enrichment
887 analysis could be found in Chen, et al., 2020. Experimenter was blind during image analysis.
888

889 **Automatic enface projection and averaging of synapses**

890 A plane parallel to the cleft was defined by fitting all localizations after the translation (least
891 square of the normal distance to the plane). The two-dimensional enface projection was
892 achieved with calculation of the projected coordinates of all localizations along the fitted
893 plane. To avoid the potential dilution of local density after the collapse of one dimension,
894 maximal projection of 3D local density was made to generate the density map of projected
895 cluster. To visualize the enface distribution of both RIM1/2 and PSD-95 around PSD-95
896 nanoclusters, we averaged both normalized density maps centered around the projected
897 peaks of PSD-95 nanoclusters. Meanwhile, to avoid any artifact created by the bordering
898 effect, all values outside the synaptic cluster were replaced with 1 before averaging was
899 performed (78).
900

901 **Numerical model to estimate peak open probability of AMPARs**

902 We used a constrained deterministic approach to test how different AMPAR organizations
903 could impact the peak open probability of AMPARs at individual synapses based on key
904 biological measurements. The calculation of $\sum[\text{peak open probability}]$, also denoted
905 as $\sum[\text{peak } p(o)]$, is adapted from previous stochastic modeling (20, 50), where the
906 probability of channel opening characteristically decays as a function of the distance to the
907 position of glutamate exocytosis. This relationship has been modeled here as $Po(r) = 0.42e^{-r/88}$, as described previously (20).
908

909 AMPAR positions were randomly generated in MATLAB using `cirrdnPJ.m` which
910 creates points within a circle of a specified size, essentially building a map of randomized
911 AMPAR positions with 2D coordinates. We considered this the modeled PSD area, and this
912 area was determined by values taken from prior EM work (80). The number of points to be
913 generated within the PSD area was taken from prior work (81). Using a separate loop of
914 `cirrdnPJ.m`, another smaller radius could be specified within the larger PSD area, in which
915 points were randomly generated. We considered this the modeled nanodomain, and it
916 contained the average number of AMPARs suggested to form these nanodomains (79).
917

918 These modeled AMPAR organizations containing a single nanodomain were
919 examined using our spatial analysis. The autocorrelation measurement, as described
920 previously (20) for both biological and modeled localization data, was used to measure the
921 size of these modeled subsynaptic clusters. The detected size of the modeled nanodomain
922 was similar to the subsynaptic organizations observed in the biological data where the
923 profile decays back to 1 at ~ 80 nm indicating the size of the modeled nanodomain. Of
924 course, AMPAR nanodomains found in biological synapses can range in number impacting
925 the amplitude of this measurement due to the increased frequency of the signal, and multiple
926 nanodomains in one synapse will show a larger amplitude when measured by the
927 autocorrelation. Since we only model a single nanodomain within the synapse, the
928 autocorrelation correctly demonstrates a lower amplitude than the measured biological data.

929 These modeled AMPAR organizations were examined using our enrichment
930 analysis (20, 78). This analysis measures the density of points as a function of distance
931 beginning at a specified position and moving out radially at determined step sizes (distance
932 in nm). Using the previously described baseline parameters, and in agreement with the
933 autocorrelation, the enrichment analysis successfully demonstrated that these modeled
934 AMPAR positions show subsynaptic enrichment decaying to the average synaptic density
by ~ 80 nm, and this measure was expectedly sensitive to the number of AMPARs included

935 in the nanodomain. Then, the sensitivity of the threshold-based nanocluster detection
936 algorithm, which can detect the number of points included in a subsynaptic cluster was
937 adjusted until it successfully indicated that on average ~ 27 AMPARs were in-nanodomain.
938 In order to reflect the redistribution of AMPARs within synapses observed in the biological
939 data by dSTORM, some number of AMPARs had to be removed from the nanodomain, but
940 not lost from the PSD (Fig. 2, Fig. 6), which is referred to here as ‘redistribution.’ The
941 specific mechanisms driving AMPAR position after this redistribution remain unclear, for
942 instance, whether AMPARs are specifically excluded following LRRTM2 cleavage has not
943 been determined. To reflect this in our model, AMPARs were simply placed back randomly
944 into the modeled PSD, thus not specifically excluded from the nanodomain area after
945 redistribution. Then using the enrichment profile and enrichment index as readouts of this
946 reorganization, AMPARs were redistributed using this approach until the modeled
947 enrichment index and modeled enrichment profile closely approximated the difference in
948 the measured enrichment index and measured enrichment profile of AMPARs in biological
949 synapses compared to their respective controls. Then, using the nanocluster detection
950 algorithm adjusted to detect the modeled ‘ground truth’ number of AMPARs previously,
951 we redistributed AMPARs and quantified how many AMPARs were still considered to be
952 ‘in nanodomain’ after redistribution. Interestingly, some nanodomains were no longer
953 detectable given the magnitude of reorganization, which is consistent with our observations
954 in Fig. 4I.

955 The open probability of AMPARs is thought to critically depend on the distance to
956 the site of glutamate exocytosis. Release position has been thought to occur in spatially
957 distinct subregions of the active zone given different modes of neurotransmitter release, thus
958 influencing this key parameter. To understand how different constraints on release position
959 in the active zone impact AMPAR open probability, we modeled two modes of release,
960 again using `cirrdnPJ.m` to randomize AMPAR positions within a specified area. Release
961 constrained to the nanodomain is referred to here as ‘evoked release,’ as it is thought to
962 occur over a smaller fraction of the PSD and aligned with postsynaptic AMPAR
963 nanodomains. We refer to release over the entire area of the PSD as ‘spontaneous release’,
964 as it does not demonstrate a similar constraint in release position distribution determined by
965 live-imaging of vesicle fusion events (20).

966 Then, using the baseline parameters as a starting point, and our modeled
967 redistribution of AMPARs given the data from Fig. 4F,M (as described above), we
968 calculated the open probability of each AMPAR in the modeled PSD. This was done by
969 indexing the distance of each AMPAR to the modeled vesicle release position. Then by
970 summing these probabilities across every AMPAR in the modeled PSD, we could estimate
971 the number of AMPARs on average that would generally be expected to open in response
972 to spontaneous or evoked release. This informed the interpretations of the electrophysiology
973 from neurons that underwent LRRTM2 cleavage and subsequent AMPAR redistribution.
974 To test how these various spatial parameters (‘PSD area’, ‘proportion of AMPARs in the
975 nanodomain’, and the ‘decay constant of the decay profile’) used in the model influenced
976 AMPAR open probability, we kept the baseline parameters constant except for the
977 parameter being tested and repeated the redistribution of AMPAR positions described above
978 over a reasonable biological range.

980 Electrophysiology

981 Whole-cell recordings were made on neurons from DIV13-17 with 5-8 M Ω pipettes filled
982 with an internal solution that contained (in mM): 130 CsMeSO₃, 6 NaCl, 10 HEPES, 1
983 MgCl₂, 2 BAPTA-K, 0.2 CaCl₂, 3 Mg-ATP, 0.3 Tris-GTP, pH 7.3 with CsOH, 290-295
984 mOsm. Neurons were held at -70 mV at which the GABAergic current were minimal. The

bath solution consisted of (in mM): 130 NaCl, 2.5 KCl, 1 NaH₂PO₄, 10 HEPES, X CaCl₂, 4-X MgSO₄, and 10 Glucose. Lower [Ca²⁺]_o (X = 0.5-1) was used for eEPSC recordings to reduce the recurrent activity, while for mEPSC recordings normal [Ca²⁺]_o (X = 2) combined with TTX (1 μM) and picrotoxin (50 μM) was used. Evoked EPSCs were elicited with 1-ms extracellular field stimuli through a bipolar electrode made from θ-shape glass pipette with opening of 2 to 3 μm. The stimulation electrode was held a few μm above the cells and moved around to locate at a position where single-peak, monosynaptic currents were reliably evoked. The paired stimuli with a 50 ms interval were delivered every 10 s. For miniature EPSCs, glass pipettes were pulled to have a resistance of 3-6 MΩ. An internal solution containing 130 mM K-gluconate, 5mM KCl, 2 mM MgCl₆-H₂O, 10 mM HEPES, 4 mM Mg-ATP, 0.3 mM Na₂-GTP, 10 mM Na₂-phosphocreatine, and 1 mM EGTA was used to record at room temperature (21-24°C). The series resistances were monitored and data with changes of >20% were discarded. The capacitance and input resistance were not significantly different between different groups of neurons. Data were collected with MultiClamp 700B amplifiers (Molecular Devices) and digitized at 5 kHz with Digidata 1440 and Clampex 10 software (Molecular Devices). mEPSCs were detected by fitting to a variable amplitude template using pClamp 10 analysis software. Experimenter was blinded to condition during data analysis.

Statistical analysis

We used two-tailed Student's T or Mann-Whitney rank sum tests for comparisons between 2 groups. We used a one-way ANOVA with posthoc Dunnett's Test for multiple comparisons of three groups. Data are presented as means ± SEM except otherwise noted. Significance levels displayed as follows: n.s., not significant, $p > 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. These tests were performed in Prism 8.2.0 (GraphPad).

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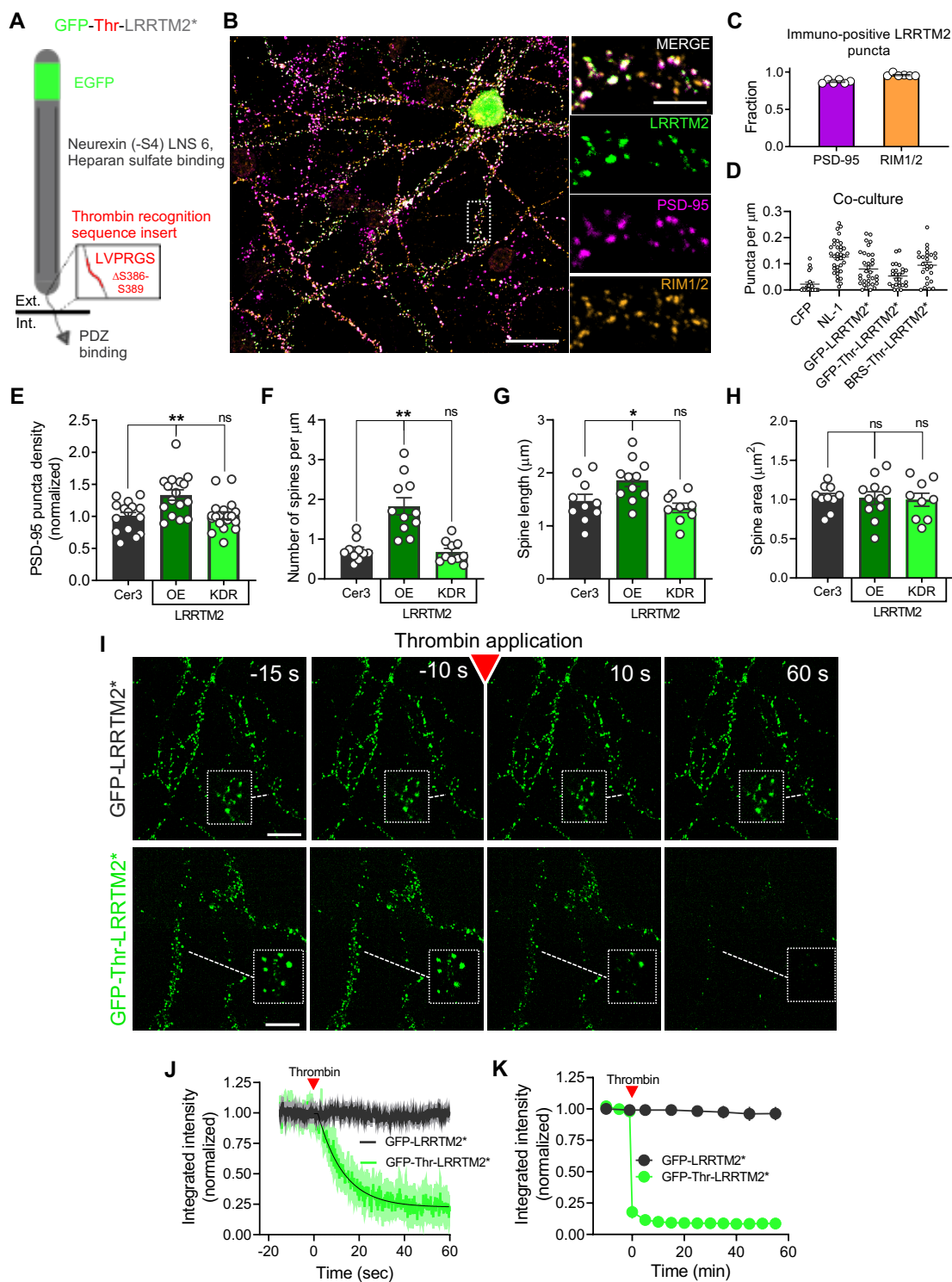


Fig. 1 Acute and specific cleavage of the LRRTM2 extracellular domain. (A) Schematic demonstrating the juxtamembrane insertion of the thrombin recognition sequence (38) and the N-terminal GFP* denotes co-packaging of an shRNA (33) that targets endogenous LRRTM2 expressed in the same vector as GFP-Thr-LRRTM2. (B) Expression of GFP-Thr-LRRTM2* in cultured hippocampal neurons and

immunostaining of endogenous PSD-95 and RIM1/2 visualized by confocal microscopy. Scale bar, left: 30 μm , right: 10 μm . **(C)** Quantification of the colocalization between expressed GFP-Thr-LRRTM2*, RIM1/2, and PSD-95. ($n = 120$ synapses/6 neurons/2 independent cultures per condition). **(D)** Quantification of Bassoon recruitment by LRRTM2 in an HEK-neuron coculture synaptogenesis assay alongside positive (CFP-NL1) and negative (CFP alone) controls. CFP alone ($n = 30$ cells/2 independent cultures), CFP-NL1 ($n = 34/2$), BRS-Thr-LRRTM2* ($n = 24/2$), GFP-Thr-LRRTM2* ($n = 25/2$), GFP-LRRTM2* ($n = 32/2$). **(E)** Quantification of PSD-95 puncta density in neurons expressing GFP-Thr-LRRTM2* (KDR, $n = 19$ neurons/3 independent cultures), GFP-Thr-LRRTM2 (OE, $n = 16/3$), or cytosolic mCerulean3 (Cer3, $n = 15/3$). **(F)** Quantification of spine density. ($n = 10$ neurons/3 independent cultures per condition). **(G)** Quantification of spine length. ($n = 10/3$). **(H)** Quantification of spine area. ($n = 10/3$). **(I)** Representative images from a confocal time series of GFP-Thr-LRRTM2* cleavage following thrombin application (red arrow, 10 units ml^{-1}). Scale bar: 10 μm . **(J)** Quantification of GFP-Thr-LRRTM2* ($n = 100$ synapses/5 neurons/3 independent cultures) and GFP-LRRTM2* ($n = 40/2/2$) cleavage. **(K)** Quantification of GFP-LRRTM2* ($n = 100/5/2$) or GFP-Thr-LRRTM2* ($n = 120/6/2$) for up to 60 minutes post thrombin exposure. One-way ANOVA with posthoc Dunnett's Test was used in E-H. Data are presented as mean \pm SEM, * $p \leq 0.05$, ** $p \leq 0.01$.

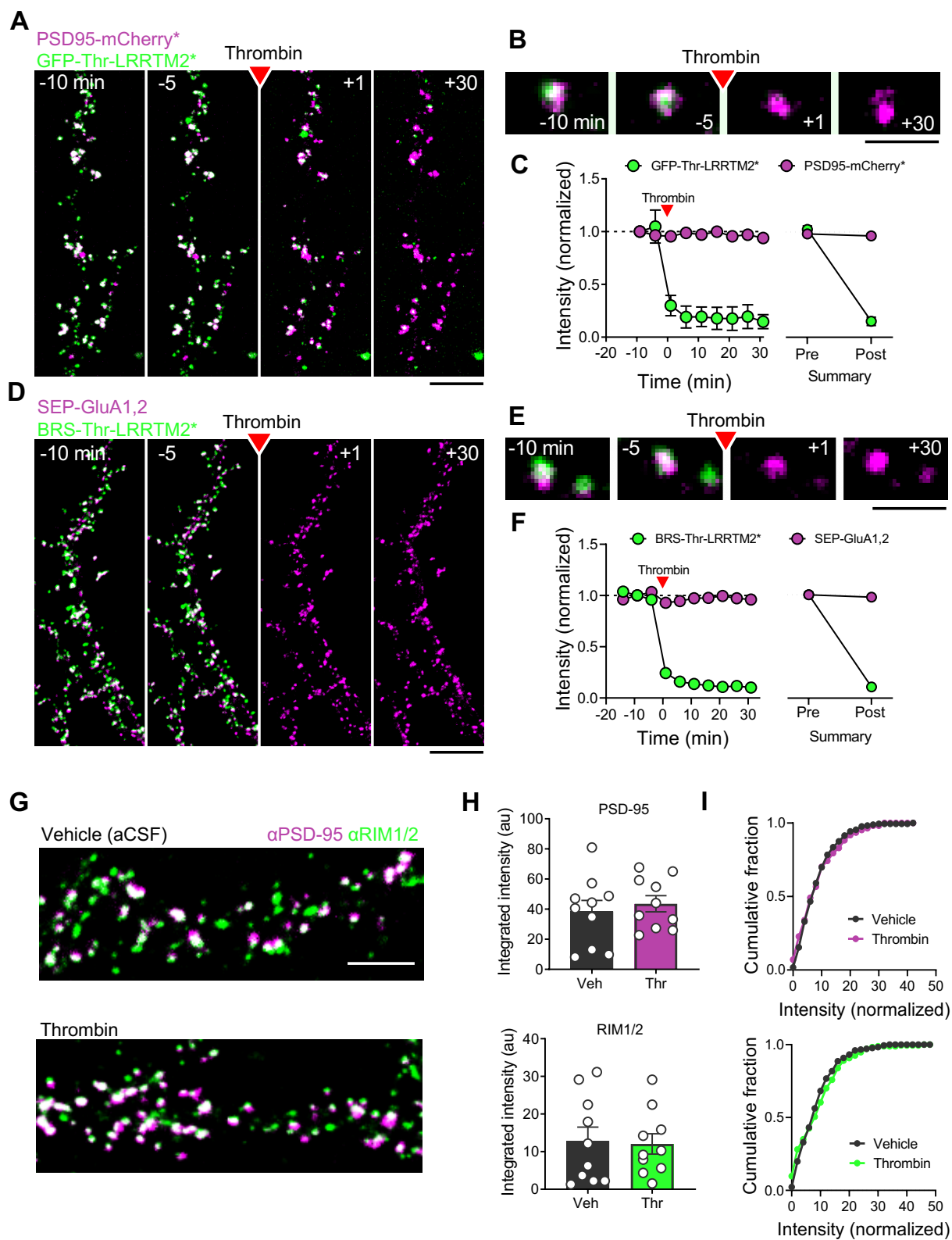


Fig. 2 No rapid loss of AMPARs following removal of the LRRTM2 extracellular domain. (A) Representative images of neuronal dendrites co-expressing GFP-Thr-LRRTM2* and PSD95-mCherry*.

Red arrow indicates the bath application of thrombin (10 units ml^{-1}). Scale bar: $10 \mu\text{m}$. **(B)** Enlarged view. Scale bar: $2 \mu\text{m}$. **(C)** Left, quantification of fluorescence intensity of both GFP-Thr-LRRTM2* and PSD95-mCherry*. Right, summary of baseline measurements compared to 30' post thrombin application. ($n = 14$ neurons/3 independent cultures). **(D)** Representative images of neuronal dendrites co-expressing BRS-Thr-LRRTM2* and SEP-GluA1/2. Red arrow indicates the bath application of thrombin (10 units ml^{-1}). Scale bar: $10 \mu\text{m}$. **(E)** Enlarged view. Scale bar: $2 \mu\text{m}$. **(F)** Left, quantification of fluorescence intensity of both BRS-Thr-LRRTM2* labeled with α -bungarotoxin conjugated to Alexa-647 and SEP-GluA1/2 over time normalized to their respective baseline. Right, summary of baseline measurements compared to 30' post thrombin application. ($n = 11$ neurons/3 independent cultures). **(G)** Representative images of immunocytochemical staining of endogenous RIM1/2 and PSD-95 from cultured hippocampal neurons expressing GFP-Thr-LRRTM2* and mCerulean3 treated with either vehicle (aCSF, above; $n = 173$ synapses/9 neurons/3 independent cultures) or thrombin (below, 10 units ml^{-1} for 10 minutes; $n = 176/9/3$). Scale bar: $5 \mu\text{m}$. **(H)** Quantification of synaptic staining intensity for PSD-95 (above) and RIM1/2 (below). **(I)** Cumulative distribution of synaptic staining intensities for cells treated with vehicle (aCSF, grey) or thrombin (magenta for PSD-95 and green for RIM1/2). Data are presented as mean \pm SEM.

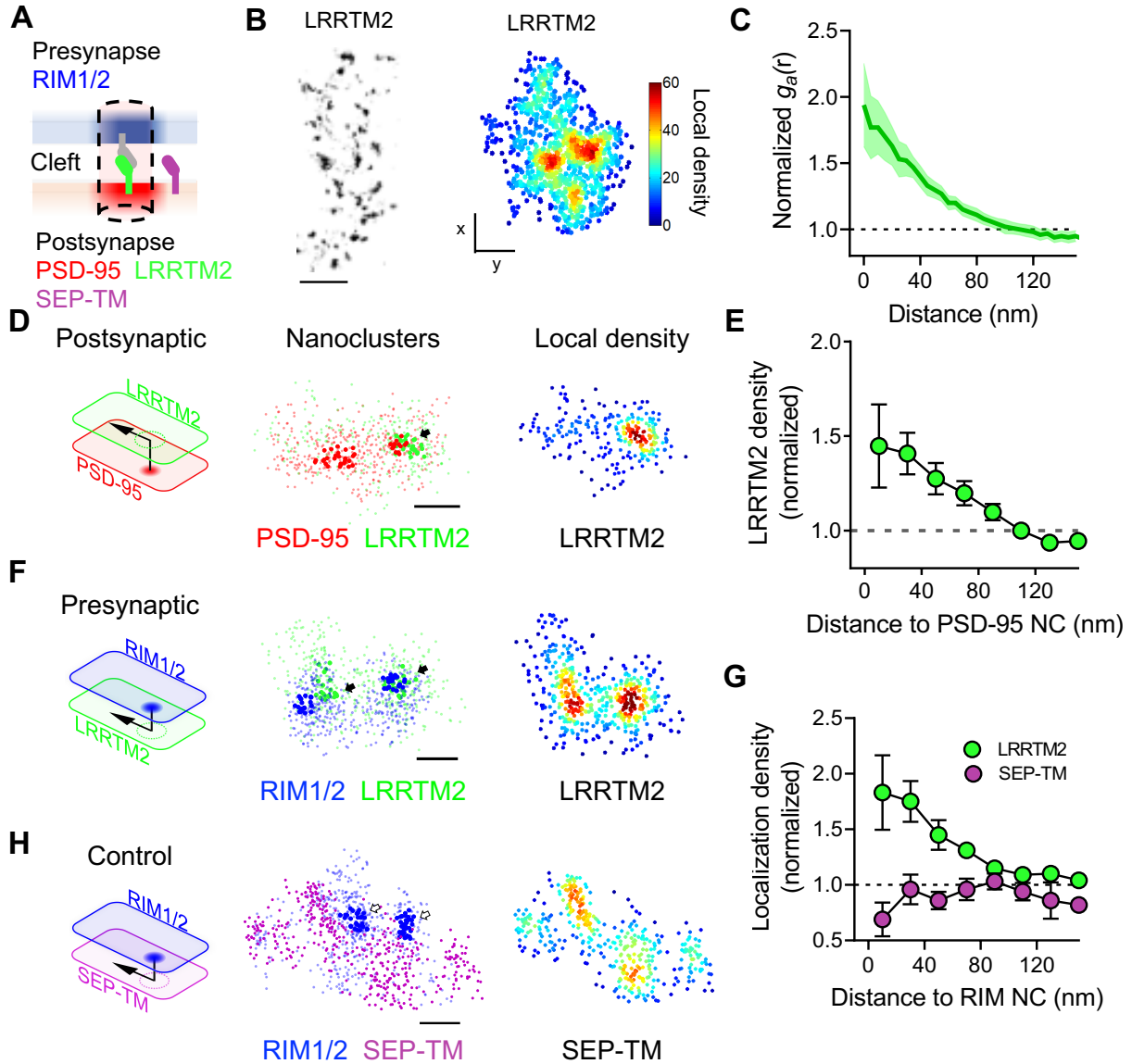


Fig. 3 LRRTM2 is enriched within the trans-synaptic nanocolumn. (A) Schematic demonstrating the trans-synaptic nanoscale organization of LRRTM2 relative to RIM and PSD-95. (B) Left, 3D dSTORM reconstruction of a dendrite from a neuron expressing GFP-LRRTM2*. Scale bar: 2 μ m. Right, 3D dSTORM reconstructions of an individual synapse with localizations color-coded by local density (5x nearest neighbor distance (NND)). Scale bar: 100 nm. (C) Auto-correlation of LRRTM2 synaptic clusters. (D) Schematic demonstrating the measurement of 3D co-enrichment between protein pairs (LRRTM2 green, PSD-95 red). Middle, left, en face view of the localized positions of PSD-95 (red) and LRRTM2 (green) with detected nanoclusters indicated in bold. Middle, right, the same LRRTM2 localizations coded by their local density (5x NND). Scale bar: 100 nm. (E) Quantification of LRRTM2 density as a function of the distance to the PSD-95 nanocluster center. (F) LRRTM2 cross-enrichment with RIM1/2 as displayed in D-E. (G) Quantification LRRTM2 cross-enrichment with RIM1/2 nanoclusters. ($n = 176$ nanoclusters/16 neurons/5 independent cultures). (H) Cross-enrichment of a diffuse target, SEP-TM, across from presynaptic RIM1/2 nanoclusters displayed as in D-E. Scale bar: 100 nm. ($n = 85/9/3$). (I) Quantification of SEP-TM density as a function of the distance to the RIM1/2 nanocluster center. Data are presented as mean \pm SEM.

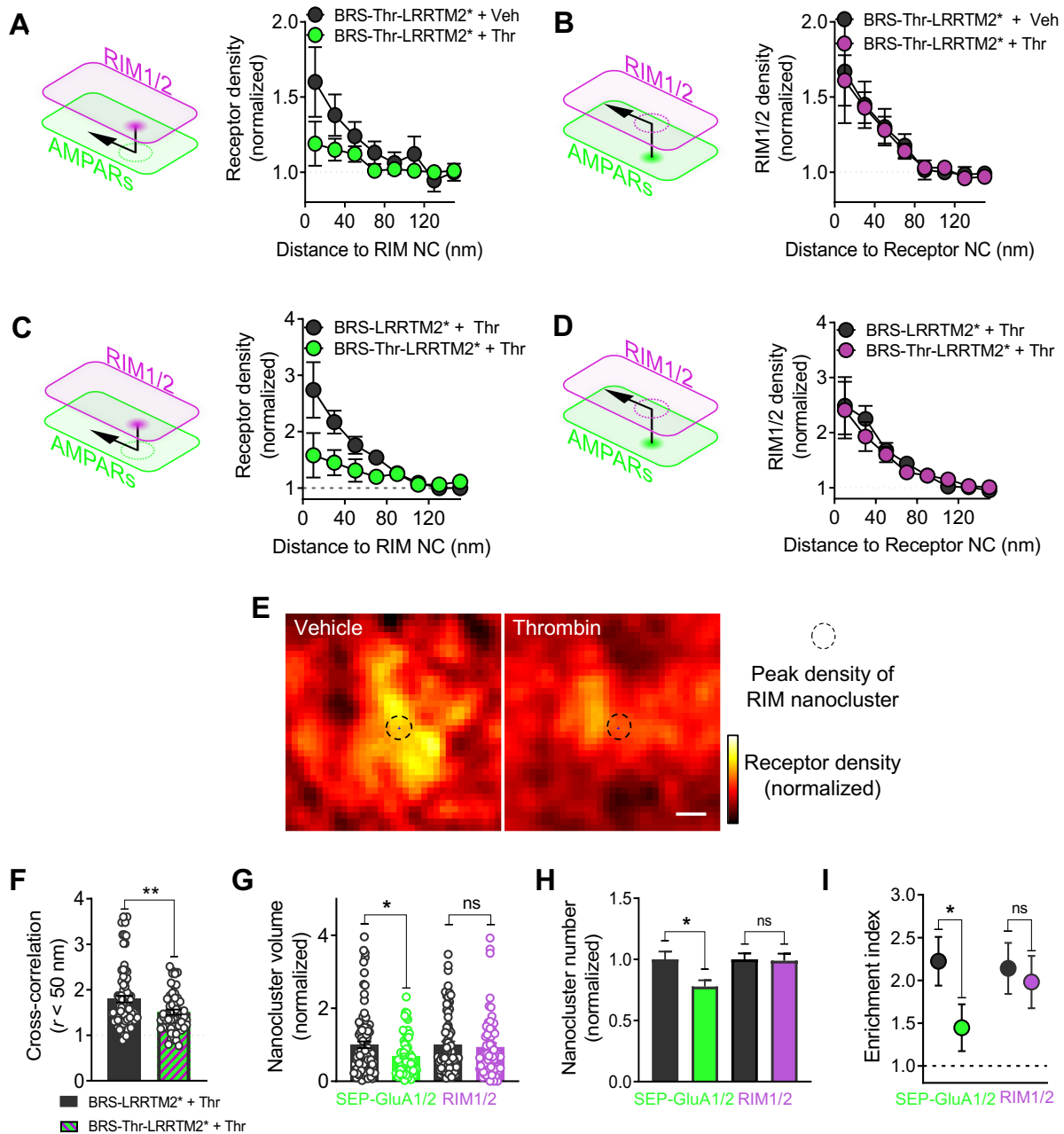


Fig. 4 LRRTM2 is critical for AMPAR enrichment across from preferential sites of evoked neurotransmitter release. (A) Schematic demonstrating the measurement of AMPAR localization density across from RIM1/2 nanoclusters (left). Quantification of AMPAR enrichment from neurons co-expressing BRS-Thr-LRRTM2* and SEP-GluA1/2 following treatment with thrombin (10 minutes, green) or vehicle (black). (B) RIM1/2 density across from AMPAR nanoclusters from neurons in A. (C) Quantification of AMPAR enrichment from neurons co-expressing either BRS-Thr-LRRTM2* (cleavable, green; $n = 95$ nanoclusters/11 neurons/3 independent cultures) or BRS-LRRTM2* (non-cleavable, black; 127/15/3) and SEP-GluA1/2 following treatment with thrombin (10 minutes). (D) RIM1/2 density across from AMPAR nanoclusters as displayed in B. Quantification (cleavable, magenta; $n = 90/11/3$) of BRS-LRRTM2* (non-cleavable, black; $n = 103/15/3$) and AMPARs following treatment with thrombin (10 minutes). (E) Representation of AMPAR density across from RIM1/2 peak density averaged across many synapses. Scale

bar: 50 nm. **(F)** Paired cross correlation $g_c(r) < 50$ nm of synaptic protein pairs of SEP-GluA1/2 and RIM1/2 from thrombin treated neurons expressing BRS-LRRTM2* (black) or BRS-Thr-LRRTM2* (green). **(G)** Volume of AMPAR nanoclusters. **(H)** Number of detected AMPAR nanoclusters. **(I)** Enrichment indices ($g_r < 50$ nm) for AMPARs across from RIM1/2 nanoclusters (grey, green, left) and RIM1/2 across from AMPAR nanoclusters (grey, magenta, right). Data are presented as mean \pm SEM, , * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Mann Whitney rank-sum test was performed for F-I.

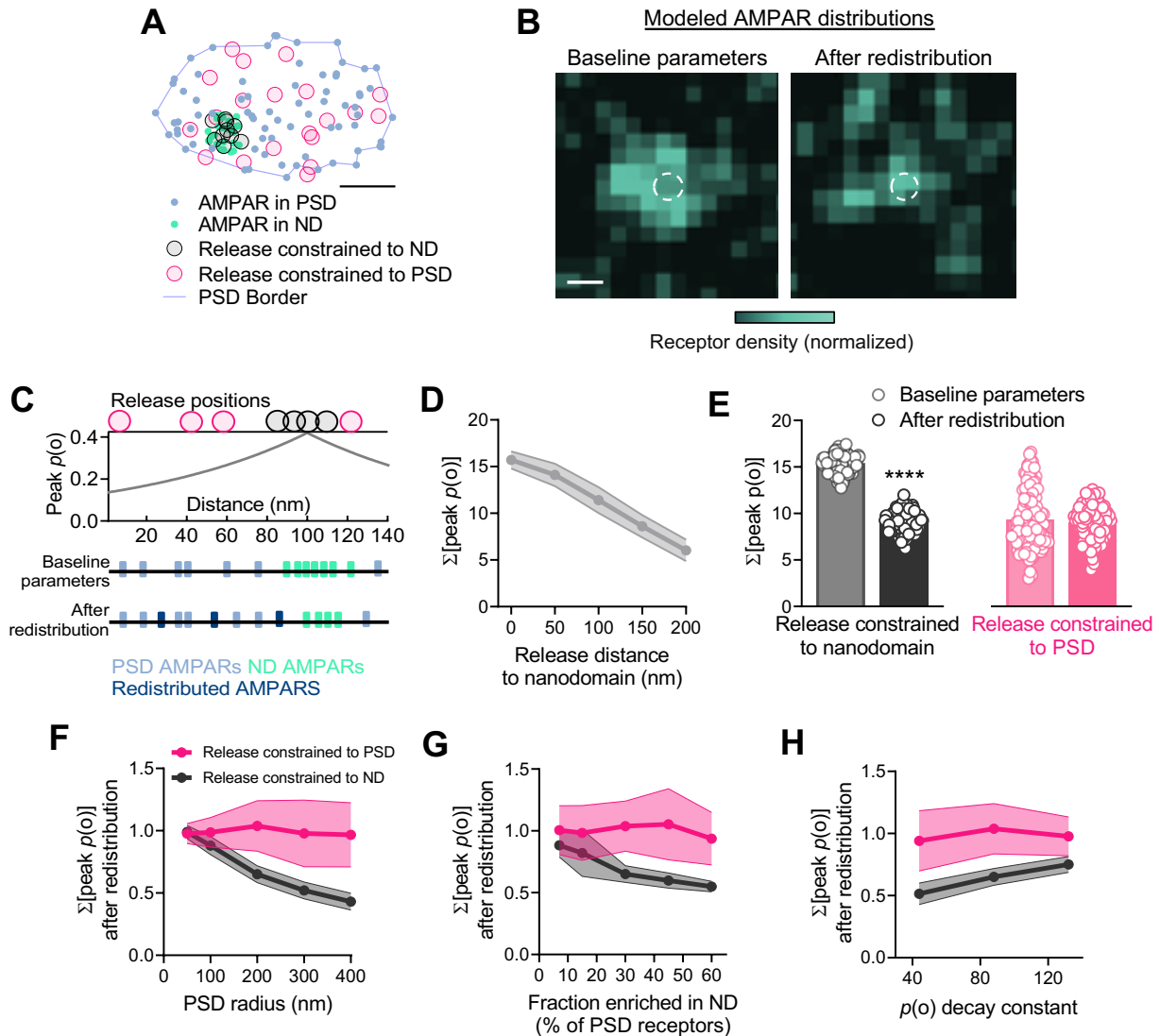


Fig. 5 Numerical model to predict the effects of LRRTM2 loss on synapse function. (A) Example of the distribution of randomized AMPAR positions within the PSD and nanodomain and the distribution of randomized vesicle release positions, where release is constrained to either the boundary of the nanodomain (black) or PSD (magenta). Scale bar: 100 nm. (B) Representative density histograms of individual modeled receptor distributions. (C) Schematic demonstrating the calculation of peak open probability of all AMPARs within the PSD given a randomized release position constrained as described in a. (D) Calculation of the summed peak open probability of AMPARs as the release position is offset from the nanodomain. (E) Calculation of the summed peak open probability of AMPARs for release positions constrained to the nanodomain (left) and release positions constrained to the PSD (right) using the modeled nanodomain parameters (grey, black) and redistribution of AMPARs (pink, magenta). (F) Calculation of the summed peak open probability of AMPARs as PSD diameter is adjusted. (G) Calculation of the summed peak open probability of AMPARs as the proportion of AMPARs included in the nanodomain is adjusted. (H) Calculation of the summed peak open probability of AMPARs from as the peak open probability as a function of distance decay constant is adjusted $\pm 50\%$. Data in F-H are normalized to the baseline parameters condition. Data are presented as mean \pm SEM. For all modeled data $n = 100$ randomizations/bin or 100 randomizations/condition. Mann Whitney rank-sum test was performed for E, **** $p \leq 0.0001$.

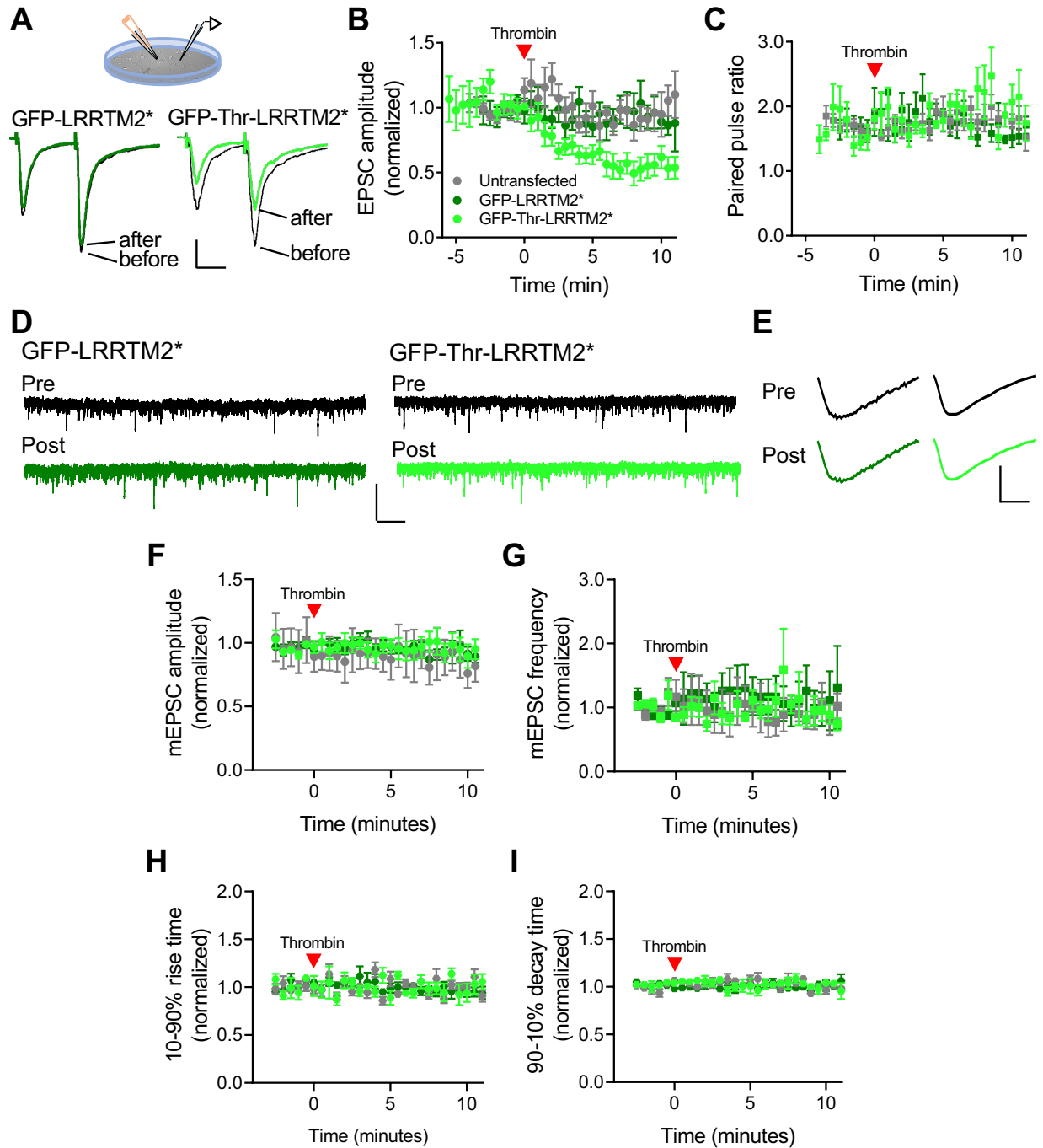


Fig. 6 LRRTM2 is critical for basal strength of evoked but not spontaneous transmission. (A) Schematic of whole-cell patch clamp recordings from cultured hippocampal neurons with bipolar electrode stimulation to evoke synaptic currents. Below, averaged traces of evoked synaptic events. Neurons transfected with GFP-Thr-LRRTM2* (light green, cleavable, post-thrombin), GFP-LRRTM2* (dark green, non-cleavable, post-thrombin), where black indicates their respective baselines. Scale bar: 100 pA, 20 ms. (B) Quantification of evoked synaptic current amplitudes normalized to their baseline measurements over time from neurons expressing GFP-LRRTM2* (dark green; n = 9 neurons/3 independent cultures), GFP-Thr-LRRTM2* (light green; n = 12/3), or untransfected neurons (grey; n = 9/3). (C) Quantification of the paired-pulse ratio. (D) Representative traces of miniature EPSC (mEPSC) recordings from cultured

hippocampal neurons transfected with GFP-LRRTM2* (dark green) or GFP-Thr-LRRTM2*(light green) before and after the application of thrombin. Scale bar: 35 pA, 2 s. **(E)** Averaged traces of miniature synaptic currents. GFP-Thr-LRRTM2* (light green) and GFP-LRRTM2* (dark green). Scale bar: 5 pA, 2 ms. **(F)** Quantification of miniature synaptic current amplitudes from GFP-LRRTM2* (n = 8/3), GFP-Thr-LRRTM2* (n = 8/3), or untransfected neurons (n = 5/3) before and after the application of thrombin. **(G)** Quantification of miniature frequency before and after the application of thrombin. **(H)** Quantification of the 10-90% rise times of mEPSC events over time, before and after the application of thrombin. **(I)** Quantification of the 90-10% decay time of mEPSC events over time, before and after the application of thrombin. Data are presented as mean \pm SEM.