Progressive overfilling of readily releasable pool underlies short-term facilitation at recurrent excitatory synapses in layer 2/3 of the rat prefrontal cortex

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Abstract

Short-term facilitation of recurrent excitatory synapses within the cortical network has been proposed to support persistent activity during working memory tasks, yet the underlying mechanisms remain poorly understood. We characterized short-term plasticity at the local excitatory synapses in layer 2/3 of the rat medial prefrontal cortex and studied its presynaptic mechanisms. Low-frequency stimulation induced slowly developing facilitation, whereas high-frequency stimulation initially induced strong depression followed by rapid facilitation. This non-monotonic delayed facilitation after a brief depression resulted from a high vesicular fusion probability and slow activation of Ca^{2+}-dependent vesicle replenishment, which led to the overfilling of release sites beyond their basal occupancy. Pharmacological and gene knockdown (KD) experiments revealed that the facilitation was mediated by phospholipase C/diacylglycerol signaling and synaptotagmin 7 (Syt7). Notably, Syt7 KD abolished facilitation and slowed the refilling rate of vesicles with high fusion probability. Furthermore, Syt7 deficiency in layer 2/3 pyramidal neurons impaired the acquisition of trace fear memory and reduced c-Fos activity. In conclusion, Ca^{2+}- and Syt7-dependent overfilling of release sites mediates synaptic facilitation at layer 2/3 recurrent excitatory synapses and contributes to temporal associative learning.

Introduction

Synapses in the mammalian central nervous system undergo activity-dependent modulation of synaptic weight during repetitive stimulation, known as short-term synaptic plasticity (STP). STP is mediated by presynaptic mechanisms that regulate the release probability (p_r), size of the readily releasable pool (RRP), and kinetics of vesicle replenishment of RRP (Zucker and Regehr, 2002; Jackman and Regehr, 2017; Neher and Brose, 2018). Short-term depression has been observed at synapses with a high baseline p_r, primarily because of the rapid depletion of releasable vesicles during high-frequency stimulation (HFS) (Lin et al., 2022). Conversely, vesicle depletion is less pronounced at synapses with a low baseline p_r, and cumulative increases in p_r during HFS are considered to mediate short-term facilitation (Rozov et al., 2001; Pan and Zucker, 2009; Aldahabi et al., 2024). Central synapses exhibit a wide spectrum of STP depending on different combinations of presynaptic factors mediating short-term depression and facilitation.

Previously, facilitation was modeled as an increase in p_r after an action potential (AP) and its slow decay during the inter-spike interval (ISI), resulting in a cumulative increase in p_r during HFS (Markram et al., 1998; Dittman et al., 2000). Recent studies have suggested that the number of docking (or release) sites in an active zone is limited and only partially occupied by releasable vesicles in the resting states (Miki et al., 2016; Pulido and Marty, 2018; Malagon et al., 2020; Lin et al., 2022). Therefore, p_r is determined by the vesicular fusion probability (p_v) and release site occupancy (p_{occ}). However, distinguishing the individual contributions of p_v and p_{occ} to p_r is challenging (Silva et al., 2021; Neher, 2024). It is also unclear whether facilitation is mediated by activity-dependent increases in the p_v, p_{occ}, or both.

64 Two recent models for vesicle priming and release view the 'priming' as a two-step reversible
65 process that leads to a release-ready state. The release-ready vesicles could be vesicles in a
66 tightly docked state (TS) or those occupying specialized docking sites (DS), and are supplied
67 from vesicles in a loosely docked state (LS) or those occupying a distinct replacement sites
68 (RS), which are called LS/TS model (Aldahabi et al., 2024; Neher, 2024) and RS/DS model (Miki
69 et al., 2016; Silva et al., 2021), respectively. Common to both models is the assumption that
70 release-ready vesicles constitute only a fraction of all docked vesicles under resting conditions
71 and that this fraction is up- and down-regulated by synaptic activity.
72 Synaptotagmin-7 (Syt7) was recently found to play a crucial role in facilitation at different
73 types of central synapses (Jackman et al., 2016; Chen et al., 2017; Martinetti et al., 2022). In
74 addition to its role in facilitation, Syt7 accelerates refilling of the RRP after depletion and
75 mediates asynchronous release (Bacaj et al., 2013; Liu et al., 2014; Jackman et al., 2016;
76 Tawfik et al., 2021). Syt7-dependent synaptic facilitation is interpreted as an activity-
77 dependent increase in p_v (Turecek et al., 2016; Jackman and Regehr, 2017; Turecek et al., 2017;
78 Norman et al., 2023). However, it is unclear whether Syt7 contributes to facilitation through
79 a Ca^{2+}-dependent increase in p_{occ} (i.e., overfilling). Overfilling has been proposed as a
80 mechanism supporting facilitation at the Drosophila neuromuscular junction (Kobbersmed et
81 al., 2020), yet whether this process also occurs at the mammalian central synapses mediated
82 by Syt7 has not been studied.
83 Each of layer 2/3 (L2/3) and layer 5 (L5) of neocortex displays intralaminar excitatory synapses
84 between pyramidal cells comprising a recurrent network (Holmgren et al., 2003; Thomson
85 and Lamy, 2007). Short-term facilitation at recurrent excitatory synapses in a cortical network

86 may play a crucial role in maintaining working memory (Mongillo et al., 2008; Mongillo et al.,
87 2012; Hansel and Mato, 2013). Although L2/3 pyramidal cells (PCs) represent task-relevant
88 information during a delay period in working memory tasks (Fujisawa et al., 2008; Ozdemir et
89 al., 2020), most studies on local excitatory recurrent synapses in the neocortex have focused
90 on L5 exhibiting short-term depression (Reyes and Sakmann, 1999; Hempel et al., 2000; Yoon
91 et al., 2020). Further, the synaptic properties of excitatory synapses in L2/3 remain poorly
92 understood.
93 In the present study, we found that HFS of local excitatory synapses in L2/3 of the medial
94 prefrontal cortex (mPFC) induced initial strong depression followed by delayed facilitation,
95 whereas low-frequency stimulation induced slow monotonous facilitation. This unique form
96 of STP could be explained by 1) high p_v of RRP vesicles at rest and throughout a train
97 stimulation; 2) the low resting vesicular occupancy of RRP and its activity-dependent increase.
98 These conditions led to delayed facilitation through progressive overfilling of release sites
99 during HFS with high p_v vesicles beyond the resting occupancy. Syt7 knockdown (KD) in L2/3
100 PCs completely abolished these synaptic enhancements by inhibiting the Ca^{2+}-dependent
101 increase in release site occupancy. Finally, behavioral tests for trace fear conditioning showed
102 that Syt7 KD impaired the acquisition of trace fear memory and reduced c-Fos expression in
103 the mPFC. Collectively, these results support that Ca^{2+}- and Syt7-dependent overfilling of
104 release sites mediates synaptic facilitation at L2/3 recurrent excitatory synapses and
105 contributes to temporal associative learning.
106

107 ## Results

108 *Frequency dependence of STP at local excitatory synapses in L2/3 of the prelimbic cortex*

109 We examined STP profiles at recurrent excitatory synapses between L2/3 PCs and at synapses
110 from L2/3-PCs to fast-spiking interneurons (FSINs) in the prelimbic area of the rat mPFC. The
111 PCs and FSINs were identified based on their distinct intrinsic properties and morphologies
112 (***Figure 1—figure supplement 1***). To stimulate presynaptic PCs, L2/3 PCs were transfected
113 with a plasmid encoding oChIEF using *in utero* electroporation (IUE; ***Figure 1A***; Lee et al.,
114 2024). Photo-stimulation of the oChIEF-expressing cell soma at 40 Hz consistently evoked
115 single APs with high fidelity throughout the 600-pulse trains (***Figure 1—figure supplement***
116 ***2A-B***), which was consistent with little use-dependent inactivation of oChIEF (Lin et al., 2009;
117 Hass and Glickfeld, 2016).
118 Given that IUE transfected approximately 10-20% of L2/3 PCs (***Figure 1A***), we made a whole-
119 cell patch on a non-transfected L2/3 PC or an FSIN (***Figure 1C***). To stimulate a minimal number
120 of excitatory axon fibers, EPSCs were evoked using minimal optical stimulation (***Figure 1—***
121 ***figure supplement 3A***; 470 nm, 3-4 µm in radius and 3-5 ms in duration). EPSCs evoked by
122 minimal stimulation were examined to determine the uniformity of the kinetic properties
123 (***Figure 1—figure supplement 3B-C***). Moreover, we reassessed the kinetics of EPSCs during
124 trains *post hoc* to ensure uniformity. EPSCs were evoked by 20-pulse trains at 4 different
125 frequencies (5, 10, 20, and 40 Hz) to cover the range of firing rates of PCs observed *in vivo*
126 during working memory tasks (Baeg et al., 2003). Excitatory synapses from PCs onto both
127 other PCs and FSINs (PC–PC and PC–FSIN) showed strong (~2-fold enhancement) and lasting

128 (several seconds) short-term facilitation at 20 Hz and lower frequencies (***Figure 1D-E***). The
129 PC-PC synapses exhibited stronger facilitation than did the PC-FSIN synapses.
130 Steady-state EPSCs at the PC-PC synapses were not significantly different from 5 to 20 Hz
131 (***Figure 1F***). Frequency invariance at the PC-PC synapses suggests that Ca^{2+}-dependent
132 synaptic facilitation may counteract vesicle depletion in a frequency-dependent manner
133 (Turecek et al., 2017; Lin et al., 2022). Despite facilitation at all frequencies, the paired pulse
134 ratio (PPR) rapidly decreased as the ISI decreased (***Figure 1G, I***). At 40 Hz stimulation, both
135 PC-PC and PC-FSIN synapses initially underwent strong depression then followed by
136 facilitation in a few stimuli (***Figure 1Dd, Ed***). Such delayed facilitation after paired pulse
137 depression (PPD) has not been previously observed in central synapses except for a slight
138 increase in EPSC at the cerebellar synapses under artificially depolarized conditions (Pulido
139 and Marty, 2018).
140 We confirmed that optically evoked EPSCs were strictly dependent on AP generation (***Figure***
141 ***1—figure supplement 4A***), suggesting that EPSCs are evoked by optical stimulation of
142 presynaptic axon fibers rather than by direct depolarization of presynaptic boutons.
143 Additionally, dual patch-clamp techniques showed that the STP of optically evoked EPSCs did
144 not differ from the electrically evoked STP of EPSCs at the PC-FSIN synapses (***Figure 1—figure***
145 ***supplement 4B***). Finally, we confirmed that AMPA receptor (AMPAR) desensitization was not
146 responsible for PPD at 40 Hz, ruling out the possible effects of postsynaptic factors on STP
147 (***Figure 1—figure supplement 5A***), and that AMPAR saturation was not significant during
148 facilitation (***Figure 1—figure supplement 5B***).
149

150 *Delayed facilitation results from slow activation of Ca^{2+}-dependent vesicle replenishment at a*
151 *constantly high vesicular fusion probability*

152 The strong PPD at 40 Hz suggests a high vesicular fusion probability (p_v) and likely tight
153 coupling of the releasable vesicles to a Ca^{2+} source. To test this hypothesis, we examined the
154 effects of EGTA, a slow calcium chelator, on synaptic transmission at the PC-PC synapses. The
155 baseline EPSC amplitude ($EPSC_1$) was not affected by incubation of the slice in the bath
156 solution containing 50 µM EGTA-AM for longer than 20 min, supporting tight coupling.
157 Moreover, the PPR was decreased to 0.19, and subsequent facilitation slowed but was still
158 distinct (***Figure 2A-B***). These findings indicate that the initial p_v should be higher than 0.81
159 considering vesicle recruitment during ISI. The effects of EGTA-AM were confirmed in the
160 same cell by measuring the baseline EPSC and PPR before and after applying EGTA-AM to the
161 bath. $EPSC_1$ remained unchanged, while PPR was reduced by half (***Figure 2—figure***
162 ***supplement 1***).
163 Notably, the application of EGTA-AM markedly slowed facilitation at 40 Hz (***Figure 2A, C***). The
164 frequency invariance of steady-state EPSCs (***Figure 1F***) and the slower facilitation at 40 Hz in
165 the presence of EGTA (***Figure 2A***) imply a Ca^{2+}-dependent increase in the refilling rate of the
166 RRP and/or an increase in the p_v of reluctant vesicles in light of previous studies (Hosoi et al.,
167 2007; Liu et al., 2014; Ritzau-Jost et al., 2014; Turecek et al., 2016; Ritzau-Jost et al., 2018;
168 Kusick et al., 2020). Moreover, facilitation was accelerated at higher stimulation frequencies.
169 The plot of the rate constant for the increase in EPSCs (denoted as $\mathbf{k}_{STF}$, the rate constant for
170 the development of short-term facilitation) as a function of the stimulation frequency (f_{stim})

171 revealed a linear relationship (***Figure 2C***). $\mathbf{k}_{STF}$ in the presence of EGTA-AM was distinctly
172 below this relationship, supporting the Ca^{2+}-dependent acceleration of facilitation.
173 $\mathbf{k}_{STF}$ was also accelerated when the stimulation frequency was increased from 5 Hz to 40 Hz
174 (***Figure 2D***; 1.27 ± 0.18/s to 24.27 ± 3.44/s). Notably, the second 40-Hz train stimulation led
175 to strong PPD, followed by accelerated facilitation (***Figure 2D-E***). The strong PPD suggests that
176 the vesicular fusion probability kept high during the 5-Hz stimulation, arguing against the
177 possibility for an increase in p_v. Both two-step sequential priming models, LS/TS and RS/DS
178 models, are suitable to interpret our data and we will refer to the different populations of
179 vesicles as TS and LS vesicles, respectively (Taschenberger et al., 2016; Neher and Brose, 2018;
180 Lin et al., 2022; Neher, 2024). Within this framework, vesicles are released from TS with high
181 p_v both in the resting state and during facilitation, and the delayed facilitation is seen as a
182 result of Ca^{2+}-dependent progressive increase in the occupancy of TS vesicles on docking sites.
183

184 *Presynaptic calcium measurements at axonal boutons of L2/3 pyramidal cells*

185 To test contribution of Ca^{2+} channel regulation to STP (Dobrunz et al., 1997; Jackman and
186 Regehr, 2017), we estimated total amount of Ca^{2+} increments during 40 Hz AP trains at axonal
187 boutons of L2/3 PCs in the mPFC. To this end, we measured AP-evoked Ca^{2+} transients (AP-
188 CaTs) using two-dye ratiometry techniques, and estimated calcium binding ratio from AP-CaTs
189 (***Figure 2—figure supplement 2A-C***). Measuring the amplitudes and decay time constants of
190 AP-CaTs at different concentrations of Fluo-5F (150, 250 or 500 µM), and plotting them as a
191 function of calcium binding ratio of Fluo-5F (κ_B), we estimated the amplitude of AP-CaTs (A_{Ca}

192 = 1.16 μM), Ca^{2+} decay time constant (τ_c = 43 ms) in the absence exogenous Ca^{2+} buffer, and
193 endogenous static Ca^{2+} binding ratio (κ_S = 96.4 or 77.3 estimated from ***Figure 2—figure***
194 ***supplement 2D-E***, respectively; *See Materials and Methods*). The resting [Ca^{2+}] ([Ca^{2+}]$_{rest}$) was
195 measured as 50 nM (***Figure 2—figure supplement 2F***). Estimating the total Ca^{2+} increments
196 for the first four AP-CaTs in a 40 Hz train according to Equation 2 (*Materials and Methods*),
197 we found no evidence for inactivation or facilitation of calcium influx during a 40 Hz train
198 (***Figure 2—figure supplement 2G-I***), arguing against the possibility that Ca^{2+} channel
199 inactivation and facilitation contribute to strong PPD and delayed facilitation during a 40 Hz
200 train.
201

202 *Release sites have low baseline occupancy and these are increased during facilitation and post-*
203 *tetanic augmentation*

204 Given that p_v was greater than 0.8, the two-fold increase in EPSC during 20 pulse train
205 stimulations could not be explained by an increase in p_v. Recent studies have shown that
206 vesicle release sites (N) are limited in number and are partially occupied at rest (Miki et al.,
207 2016; Pulido and Marty, 2018; Malagon et al., 2020; Lin et al., 2022). Under such a high p_v,
208 two-fold synaptic facilitation should be attributed to an increase in p_{occ}. The vesicle
209 replenishment rate is accelerated during HFS in a Ca^{2+}-dependent manner (***Figure 2A-C***; Hosoi
210 et al., 2007). This may lead to the overfilling of release sites beyond their basal vesicular
211 occupancy, which in turn may increase EPSC under high p_v. To test this hypothesis, we
212 determined the quantal parameters at the L2/3 local excitatory synapses. We first measured

213 the quantal size from asynchronous release events after replacing extracellular Ca^{2+} with Sr^{2+},
214 as previously described (Yoon et al., 2020) (***Figure 3—figure supplement 1A-B***). The mean
215 and coefficient of variance of the quantal sizes were 24 pA and 0.25 at PC-PC synapses,
216 respectively, and were 30 pA and 0.31 at PC-FSIN synapses (***Figure 3—figure supplement 1C***).
217 Next, we performed a variance-mean (V-M) analysis of phasic EPSCs at excitatory synapses
218 during train stimulation at 5 Hz and 40 Hz (Scheuss et al., 2002). Applying multiple-probability
219 fluctuation analysis (MPFA) using intra-site quantal variability of 0.2 (Valera et al., 2012), the
220 V-M plot was best fitted by the number of release sites (N) of 5.3 and the quantal size (q) of
221 25 pA at the PC-PC synapses (***Figure 3A***). The p_r for $EPSC_1$ was calculated as 0.32. We
222 performed the same analysis for the PC-FSIN synapses and found N = 5.3, q = 35 pA, and p_r
223 for $EPSC_1$ = 0.31 (***Figure 3B***). Our estimate for N was smaller but comparable to the estimate
224 (6.9) at excitatory synapses in L2/3 of the mouse barrel cortex (Holler et al., 2021).
225 The p_r estimate was clearly smaller than the possible minimum value for p_v (0.81) estimated
226 from the PPR data (***Figure 2B***). Given that p_r is a product of p_{occ} and p_v (Neher, 2024), a p_r
227 smaller than p_v indicates the partial occupancy of release sites in the resting state. If synaptic
228 facilitation were mediated by the recruitment of new release sites, it would be accompanied
229 by an increase in the variance of augmented EPSCs (Valera et al., 2012; Kobbersmed et al.,
230 2020). However, the augmented EPSCs during facilitation followed the same parabola in the
231 V-M plot (***Figure 3A-B***). Therefore, facilitation appears to be mediated by the progressive
232 overfilling of a finite number of release sites beyond the basal occupancy. In the next section,
233 we show that p_v is close to unity based on high double failure rate upon paired pulse
234 stimulation (***Figure 3—figure supplement 2***) and little effect of extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$)

235 elevation on baseline EPSC amplitudes (***Figure 3—figure supplement 3***). Assuming this, the
236 baseline p_r at the first pulse estimated from the fitted parabolic curve can be largely regarded
237 as a baseline p_{occ} (c.a. 30%), and the two-fold increase in EPSCs during facilitation can be
238 attributed to an increase of up to 60% in p_{occ}.
239 We investigated the time course of recovery from facilitation and post-tetanic augmentation
240 (PTA) by applying dual-train stimulations (30 pulses at 40 Hz) separated by different inter-
241 burst intervals (IBIs; ***Figure 3C-D***). The initial EPSCs in the second train were enhanced by more
242 than twofold, and followed by a strong PPD, suggesting that fusion probability stays high in
243 the IBIs and thus synaptic transmission is carried by TS vesicles. The decay time course of PTA
244 was characterized by two phases (initially fast and later slow; ***Figure 3E***), which may reflect a
245 two-phase Ca^{2+} decay or two distinct recovery processes that occur sequentially. The variance
246 of potentiated EPSCs measured at IBIs of 0.1, 0.2, and 0.5 s was not different from that of
247 EPSCs at the peak of facilitation (filled circles, ***Figure 3A-B***). Given that the same RRP mediates
248 both facilitation and augmentation and that post-tetanic EPSCs is carried by TS vesicles similar
249 to the baseline EPSCs, this plot suggests that PTA, similar to short-term facilitation, is
250 mediated by an increase in the TS vesicle occupancy.
251

252 *High double failure rate and little effect of $[Ca^{2+}]_o$ elevation on baseline EPSC amplitudes*
253 *suggest that vesicular fusion probability is close to one*

254 The vesicle dynamics of STP has been explained using a simple refilling model (Hosoi et al.,
255 2007; Neher and Sakaba, 2008). This model assumes that the vesicles are reversibly docked

256 to a limited number of release sites (N) with forward and reverse rate constants (denoted as
257 k_1 and b_1, respectively). Under the framework of the simple refilling model (see *Materials and*
258 *Methods*), the rate constant for the refilling of the RRP after depletion by the first pulse is the
259 sum of the baseline k_1 and b_1. This is estimated as 23/s from the plot of PPR as a function of
260 ISIs (***Figure 3—figure supplement 2A***). From the baseline occupancy of 0.3 and $p_{occ} = k_1 / (k_1$
261 $+ b_1)$, we estimated k_1 to be 6.9/s.
262 When paired APs were applied with an ISI of 20 ms, the failure rate at 1st pulse, $P(F_1)$, was
263 10.6%, and the probability of two consecutive failures, $P(F_1, F_2)$, was 6.2% (***Figure 3—figure***
264 ***supplement 2A***). From the equation $P(F_1) = (1 – p_r)^N$, p_r was calculated as 0.312 when N = 6,
265 comparable to p_r (= 0.32) estimated from the V-M analysis (***Figure 3A***). We calculated $P(F_1, F_2)$
266 under the framework of a simple refilling model, with p_v and k_1 set as free parameters and
267 other parameters set according to the following relationships: $P(F_1) = (1 – p_r)^N$, $p_r = p_v \cdot p_{occ}$,
268 and $p_{occ} = k_1 / (k_1 + b_1)$. The calculated values for $P(F_1, F_2)$ and their difference from the
269 observed value (6.2%) are shown in the plane of k_1 *vs*. p_v in ***Figure 3—figure supplement 2B-***
270 ***C***. Given that k_1 was greater than 5/s, the difference between the calculated and observed
271 values of $P(F_1,F_2)$ was minimal when $p_v = 1$ (***Figure 3—figure supplement 2C***).
272 To find optimal values for k_1 and p_v that best explain observed probabilities shown in ***Figure***
273 ***3—figure supplement 2Ac***, a cost function was implemented as the sum of squared errors
274 between observed and predicted probability values for the four combinations of success and
275 failure of 1st and 2nd EPSCs as described in *Materials and Methods*. The minimum of the cost
276 function (5.63×10^{-5}) was found at $k_1 = 5.21$/s and $p_v = 0.999$, which predicted $P_{11} = 41.5\%$, P_{10}

277 = 47.9%, P_{01} = 4.93%, and P_{00} = 5.67% (subscript 1 and 0 denote success and failure,
278 respectively).
279 To further validate that baseline p_v is near to unity, we examined EPSC changes following
280 elevation of $[Ca^{2+}]_o$ from 1.3 to 2.5 mM. According to the fourth-power relationship between
281 synaptic responses and $[Ca^{2+}]_o$ (Equation 3 in (Dodge Jr and Rahamimoff, 1967)), a 3.24-fold
282 increase in the EPSC amplitude was expected. However, the EPSC amplitude increased only
283 1.23-fold in average, a change that was not statistically significant (***Figure 3—figure***
284 ***supplement 3A-B***). This small response suggests that p_v is already saturated at rest, limiting
285 the dynamic range for further enhancement through increased calcium influx. Recent
286 morphological and functional studies revealed that elevation of $[Ca^{2+}]_o$ induces an increase in
287 the number of TS or docked vesicles to a similar extent as our observation (Kusick et al., 2020;
288 Lin et al., 2025), raising a possibility that an increase in p_{occ} is responsible for the 1.23-fold
289 increase in EPSC at high $[Ca^{2+}]_o$. A slight but significant reduction in PPR was observed under
290 high $[Ca^{2+}]_o$ too. An increase in p_{occ} is thought to be associated with that in the baseline vesicle
291 refilling rate. While PPR is always reduced by an increase in p_v, the effects of refilling rate to
292 PPR is complicated. For example, PPR can be reduced by both a decrease (***Figure 2—figure***
293 ***supplement 1***) and an increase (Lin et al., 2025) in the refilling rate induced by EGTA-AM and
294 PDBu, respectively. Thus, the slight reduction in PPR is not contradictory to the possible
295 contribution of p_{occ} to the high $[Ca^{2+}]_o$ effects. Notably, when slices were pre-incubated with
296 50 µM EGTA-AM, elevating extracellular $[Ca^{2+}]$ from 1.3 to 2.5 mM produced no significant
297 change in either baseline EPSC amplitude or PPR (***Figure 3—figure supplement 3C-D***),

298 supporting that the modest Ca^{2+}-dependence of baseline EPSCs and PPR in the absence of
299 EGTA is primarily mediated by a change in p_{occ} rather than p_v.
300

301 *Pharmacological manipulations reveal specific molecular identities underlying vesicle loading*
302 *processes*

303 Our results suggest that the baseline occupancy of TS vesicles is low and that the activity-
304 dependent progressive overfilling of docking sites with TS vesicles is responsible for short-
305 term facilitation and augmentation. To elucidate the specific molecular link between synaptic
306 enhancement and Ca^{2+}-dependent overfilling, we examined the effects of several drugs
307 known to affect vesicle dynamics. We first examined the roles of phospholipase C (PLC) and
308 diacylglycerol (DAG) by applying 5 μM U73122, a PLC inhibitor, or 20 μM 1-oleoyl-2-acetyl-sn-
309 glycerol (OAG), a DAG analog. The STP at 40 Hz and PTA at 0.5-s interval were examined before
310 and after applying each drug to the bath. For both drugs, the facilitation and augmentation
311 were reduced (***Figure 4A-B***). U73122 did not affect the basal EPSC amplitude ($EPSC_1$) but
312 resulted in a stronger PPD, slower facilitation, and lower augmentation (***Figure 4A***). These
313 effects of U73122 suggest the involvement of Ca^{2+}-induced PLC activation in progressive
314 overfilling. In contrast, OAG increased $EPSC_1$ while maintaining a pronounced PPD (***Figure 4B***),
315 indicating an increase in the TS vesicle pool size. Meanwhile, OAG slowed $\mathbf{k}_{STF}$ and reduced
316 augmentation, indicating occlusion of activity-dependent synaptic facilitation. Phorbol esters
317 and DAG are thought to accelerate priming through Munc13 activation (Rhee et al., 2002;
318 Taschenberger et al., 2016; Aldahabi et al., 2022); they also shorten the bridges that interlink

319 docked vesicles with the active zone (AZ) membrane (Papantoniou et al., 2023). Collectively,
320 these suggest that PLC- and DAG-mediated signaling play a role in the overfilling of docking
321 sites underlying short-term facilitation and augmentation.
322 Although the acceleration of Ca^{2+}-dependent vesicle refilling supports rapid facilitation at 40
323 Hz, the later part of the delayed facilitation underwent a slight depression, which was more
324 pronounced during the second burst (***Figure 3D and Figure 4***). Sustained synaptic
325 transmission is limited by the spatial availability of docking sites because it is undermined by
326 accumulation of exocytic remainings in the presynaptic AZ during HFS (Neher, 2010; Haucke
327 et al., 2011). To verify this, we examined the effects of dynasore (100 μM), a specific inhibitor
328 of endocytosis (Macia et al., 2006). The treatment with dynasore did not influence $EPSC_1$ or
329 the early phase of facilitation, but it exacerbated depression during the later phase and
330 attenuated PTA (***Figure 4C***). This suggests that late synaptic transmission during the 40-Hz
331 train is limited by site clearance.
332 Given the involvement of actin polymerization in the multiple steps of vesicle docking and
333 recycling, we examined the effects of 20 μM latrunculin B (LatB), an inhibitor of actin
334 polymerization. The effects of LatB were complicated: (1) $EPSC_1$ was decreased, whereas PPR
335 was increased (***Figure 4Da***) and (2) facilitation and augmentation were reduced (***Figure 4Db***).
336 The latter effect may result from defects in vesicle replenishment rates, either through the
337 impaired physical movement of vesicles or disrupted site clearance (Sakaba and Neher, 2003;
338 Lee et al., 2012; Hallermann and Silver, 2013; Miki et al., 2016), as Ca^{2+}-dependent vesicle
339 refilling mediates facilitation and augmentation (***Figure 2 and Figure 3***). Meanwhile, the
340 former effect might be attributed to a shift in equilibrium in the priming states towards a

341 loosely docked state (LS) at rest. It is unknown whether actin affects vesicular fusogenicity in
342 neurons, although it facilitates multiple steps of vesicle fusion by enhancing membrane
343 tension in neuroendocrine cells (Wu and Chan, 2022).
344
345 *Short-term facilitation at both types of local excitatory synapses in L2/3 is abolished by Syt7*
346 *knockdown*

347 We examined whether Syt7 mediates facilitation at the PC-PC and PC-FSIN synapses in L2/3
348 of the mPFC. To this end, oChIEF and shRNA against Syt7 (shSyt7) were co-expressed in L2/3
349 PCs using IUE. Syt7 KD resulted in a complete loss of facilitation at all tested stimulation
350 frequencies (***Figure 5A-B***). Short-term depression and paired-pulse depression were more
351 pronounced as the stimulation frequency increased, and the frequency invariance of steady-
352 state EPSC was abolished, implying little contribution of Ca^{2+}-dependent vesicle recruitment
353 (***Figure 5C-F***). The lack of facilitation could not be attributed to the failure of APs in
354 presynaptic axons, as optical stimulation elicited reliable APs in Syt7 KD PCs during the 40-Hz
355 train (***Figure 1—figure supplement 2C***). Syt7 KD had little effect on the basal properties,
356 including the peak amplitude, rise/decay time, and time-to-peak, of single AP-induced EPSCs
357 (***Figure 1—figure supplement 3D-E***). Moreover, Syt7 KD did not significantly affect the
358 intrinsic properties of neurons (***Figure 5—figure supplement 1***).
359 To examine whether Syt7 KD had any nonautonomous effects on postsynaptic cells, we
360 measured the quantal size (q) from asynchronous events in the presence of Sr^{2+} and found
361 that the mean quantal size remained unaltered at Syt7 KD synapses (***Figure 3—figure***
362 ***supplement 1C***). This indicated that Syt7 did not influence postsynaptic parameters, including

363 the density and kinetic properties of AMPARs. To test the specificity of shSyt7, we examined
364 whether co-expression of the shRNA-resistant form of Syt7 restored synaptic facilitation in
365 both excitatory synapse types. Presynaptic overexpression of shRNA-resistant Syt7 under the
366 CAG promoter rescued the effect of shRNA at both PC-PC and PC-FSIN synapses (***Figure 5—***
367 ***figure supplement 2***). These results underscore the crucial role of presynaptic Syt7 in
368 mediating short-term facilitation at local excitatory synapses in L2/3 of the mPFC.
369
370 *Syt7 KD synapses exhibit complementary changes in the number of release sites and their*
371 *vesicle occupancy*

372 To estimate the quantal parameters in Syt7 KD synapses, we performed V-M analysis of EPSCs
373 evoked by train stimulation (***Figure 6A-B***). Fitting a parabola to the V-M plot revealed higher
374 baseline p_{occ} in Syt7 KD synapses than in wild-type (WT) synapses for both synapse types (KD,
375 0.68 and 0.42; WT, 0.32 and 0.31 for PC-PC and PC-FSIN synapses, respectively). The p_{occ}
376 monotonously decreased during a train stimulation, as expected from short-term depression.
377 Moreover, the number of release sites (N) was markedly lower in Syt7 KD synapses (N = 3.5)
378 than in WT synapses (N = 5.3; ***Figure 3A***). Next, we examined the PTA induced by the same
379 protocol as in ***Figure 3*** (two 40-Hz train stimulations separated by variable IBIs) at Syt7 KD
380 synapses (***Figure 6C***). The 40 Hz train-induced synaptic depression quickly recovered during
381 IBIs, but no PTA was observed at Syt7 KD synapses, indicating its crucial role in augmentation
382 (***Figure 6D-E***).
383

384 *TS vesicle recovery following depletion is accelerated by Syt7*

385 Syt7 is recognized not only for its crucial role in facilitation, but also for mediating Ca^{2+}-
386 dependent replenishment of releasable vesicles (Liu et al., 2014; Tawfik et al., 2021). Given
387 that facilitation at L2/3 excitatory synapses is driven by progressive overfilling (***Figure 2 and***
388 ***Figure 3***), the loss of facilitation at Syt7 KD synapses in the present study implies that Syt7
389 plays a key role in the Ca^{2+}-dependent acceleration of refilling and overfilling of the docking
390 sites with TS vesicles. To test this hypothesis, we examined the recovery kinetics of EPSCs
391 after TS vesicles were depleted by 3 pulses at 40 Hz in wild-type (WT) and Syt7 KD synapses
392 (***Figure 7***). Strong PPD was observed not only in the first burst, but also in the second burst.
393 This suggested that EPSCs in the second burst were mediated by TS vesicles, similar to those
394 in the first burst (***Figure 7A, D***). Therefore, $EPSC_1$ in the second burst can be interpreted as a
395 release from TS vesicles recovering during a given IBI. The recovery time course of $EPSC_1$ in
396 the second burst indicated full recovery of TS vesicles within 100 ms in both excitatory
397 synapse types in WT rats (***Figure 7C, F***). However, this recovery was remarkably slower in Syt7
398 KD synapses, requiring 5 s for full recovery, indicating that TS vesicle recovery was greatly
399 accelerated by Syt7. Overall, synaptic facilitation and augmentation at local excitatory
400 synapses in L2/3 can be ascribed to progressive activity-dependent increases in the occupancy
401 of TS vesicles (***Figure 2 and Figure 3***), in which Syt7 plays a key role.
402

403 *Behavioral consequences of Syt7 KD in L2/3 PCs of the mPFC*

404 We previously proposed that the disparity in STP between the PC-PC and PC-IN synapses
405 results in activity-dependent changes in the ratio of synaptic weights at the PC-PC and PC-IN
406 synapses (J_{ee}/J_{ie}). This may in turn have a profound influence on persistent activity in a
407 recurrent network by biasing the E-I balance (Yoon et al., 2020). Syt7 KD not only eliminated
408 facilitation, but also the activity-dependent increase in the J_{ee}/J_{ie} ratio in the L2/3 network
409 (***Figure 8—figure supplement 1***). This suggests the possibility that Syt7 contributes to the
410 persistent activity during working memory tasks in the L2/3 recurrent network. Trace fear
411 conditioning (tFC) requires associative learning between an auditory cue (conditioned
412 stimulus, CS) and temporally separate aversive events (unconditioned stimulus, US). The
413 formation of trace fear memory requires the prelimbic area of the mPFC and hippocampus.
414 mPFC activity during the trace interval is thought to temporarily hold CS information, enabling
415 the network to associate temporally discontinuous events (i.e., temporal associative learning)
416 (Gilmartin et al., 2013). Synaptic facilitation and augmentation are implicated in temporary
417 memory retention in recurrent networks (Mongillo et al., 2008; Mongillo et al., 2012).
418 Given that Syt7 is crucial for synaptic facilitation (***Figure 5***) and augmentation (***Figure 6***) in
419 prelimbic L2/3 recurrent excitatory synapses, we tested whether trace fear memory
420 formation was impaired in rats with depleted Syt7 transcripts, specifically in the L2/3 PCs of
421 the mPFC. Syt7-targeted shRNA (shSyt7) or scrambled shRNA (Scr) constructs were
422 transfected bilaterally into the L2/3 PCs of the mPFC using IUE (***Figure 8A***). Both the Scr-
423 transfected control and shSyt7-transfected rats underwent tFC training (***Figure 8B***).
424 Consistent with Gilmartin et al. (2013), in which prefrontal persistent firing was

425 optogenetically inhibited, WT and KD animals exhibited similar freezing behavior during this
426 acquisition phase. The following day, the rats were subjected to a tone test in which trace
427 fear memory formation was assessed from the freezing response to a tone alone in a distinct
428 context. Compared to the control group, the shSyt7-transfected group exhibited significantly
429 lower levels of freezing behavior in response to auditory cues during the first four trials,
430 suggesting an impairment in trace memory formation (***Figure 8C***).
431 The tone test was repeated the following day to test for extinction memory formation. Syt7
432 KD animals exhibited much less freezing to the CS (+1d) than did WT animals. Considering the
433 Rescorla-Wagner model (Yau and McNally, 2023), our results suggest that the strength of CS-
434 US associative memory was weaker in KD animals than in WT animals; thus, the acquisition
435 of trace fear memory was impaired by Syt7 KD in the L2/3 PCs of the mPFC.
436 Next, we assessed the locomotor activity and anxiety levels of rats using the open field test
437 (OFT) and elevated plus maze test (EPM). Scr controls and Syt7 KD rats showed similar
438 exploratory patterns, displaying comparable total distance moved and time spent in the
439 periphery of the OFT or the open/closed arms of the EPM (***Figure 8—figure supplement 2***).
440 Previous studies have suggested that the acquisition of trace fear memory requires elevated
441 trace activity in prelimbic PCs (Gilmartin et al., 2013). Given that c-Fos, an immediate early
442 gene, is upregulated following patterned activities in neurons, its expression is considered a
443 reliable indicator of recent neuronal activation (Fields et al., 1997; Guzowski et al., 2005). To
444 investigate the effect of Syt7 on neuronal activity *in vivo* during tFC, animals were sacrificed
445 at the time of peak c-Fos protein expression, approximately 90 min after completion of the
446 behavioral task (Arime and Akiyama, 2017).

447 To elucidate the activation patterns of specific neuronal populations, we performed
448 immunohistochemical co-labeling of c-Fos and GAD67 (a GABAergic interneuron marker)
449 upon tFC acquisition. The density of c-Fos(+) cells and the percentage of c-Fos(+) cells among
450 GAD67(+) cells in all prelimbic layers were significantly lower in Syt7 KD rats than in control
451 rats (***Figure 8F-G***). Additionally, analysis of the proportion of active cells among transfected
452 L2/3 PCs (c-Fos+/GFP+) revealed that Syt7 KD L2/3 PCs were significantly more active than
453 Scr-transfected PCs (***Figure 8H***). This unexpected hyperactivity of Syt7 KD PCs may be
454 attributed to the high reciprocal connectivity between PCs and FSINs in the neocortex
455 (Holmgren et al., 2003; Otsuka and Kawaguchi, 2009), as PCs with short-term depression
456 would transmit less input to FSINs, which in turn would exert less feedback inhibition on
457 reciprocally connected PCs. Collectively, these findings show that the slow activation of
458 vesicle refilling supports various forms of facilitation at excitatory synapses in PFC L2/3, which
459 is essential for neuronal activity during temporal associative learning.
460

461 ## Discussion

462 The present study characterized STP at local excitatory synapses in the L2/3 network of the
463 rat mPFC at physiological extracellular [Ca^{2+}]. These synapses displayed initial strong
464 depression and then delayed facilitation at 40 Hz, while monotonous slowly developing
465 facilitation was observed at lower frequencies. Our study suggests that such delayed
466 facilitation after a brief depression results from a high p_v (***Figure 2 and Figure 3 and Figure***
467 ***3—figure supplement 2***), along with Ca^{2+}-dependent delayed acceleration of vesicle refilling
468 and overfilling (***Figure 2 and Figure 3***). The two-fold increase in EPSC during 20 pulse train

469 stimulations under the condition of such a high p_v suggests a Ca^{2+}-dependent increase in the
470 vesicle replenishment rate during a train; this then leads to overfilling of release sites beyond
471 their incomplete basal occupancy (***Figure 3A-B***). The high p_v and strong PPD did not result
472 from artificial bouton stimulation, AMPAR desensitization, or any potential distortion caused
473 by optogenetic methods (***Figure 1—figure supplements 4 and 5***). Additionally, similar to
474 short-term facilitation, PTA could be explained by an increase in the TS vesicle occupancy
475 (***Figure 3C-E***), consistent with previous studies showing post-tetanic increases in the p_{occ}
476 and/or vesicle pool size at various types of synapses (Lee et al., 2010; Vandael et al., 2020;
477 Tran et al., 2023; Silva et al., 2024). These findings suggest that release sites are partially
478 occupied at rest and that a progressive overfilling of release sites with TS vesicles underlies
479 facilitation and augmentation at intracortical L2/3 excitatory synapses.
480

481 *Definition of vesicular release probability in the present and previous studies*

482 Previous studies for post-tetanic augmentation (Stevens and Wesseling, 1999; Garcia-Perez
483 and Wesseling, 2008) observed invariance of the RRP size after tetanic stimulation. In these
484 studies, the RRP size was estimated by hypertonic sucrose solution or as the sum of EPSCs
485 evoked 20 Hz/60 pulses train (denoted as 'RRP_{hyper}'). Because reluctant vesicles can be quickly
486 converted to TS vesicles (16/s) and are released during a train (Lee et al., 2012), it is likely that
487 the RRP size measured by these methods encompasses both LS and TS vesicles. In contrast,
488 we assert high p_v based on the observation of strong PPD, failure rates upon paired
489 stimulations at ISI of 20 ms (***Figure 2 and Figure 3—figure supplement 2***). Given that single

490 AP-induced vesicular release occurs from TS vesicles but not from LS vesicles, p_v in the present
491 study indicates the fusion probability of TS vesicles. From the same reasons, p_{occ} denotes the
492 occupancy of release sites by TS vesicles. Note that our study does not provide direct clue
493 whether release sites are occupied by LS vesicles that are not tapped by a single AP, although
494 an increase in the LS vesicle number may accelerate the recovery of TS vesicles. As suggested
495 in Neher (2024), even if the number of LS plus TS vesicles are kept constant, an increase in
496 p_{occ} (occupancy by TS vesicles) would be interpreted as an increase in 'vesicular release
497 probability' if it was measured based on RRP_{hyper}.
498 By the way, it should be noted that increases in both p_v and p_{occ} may contribute to PTA. Post-
499 tetanic potentiation (PTP) and phorbol esters had similar effects on baseline EPSCs
500 (Taschenberger et al., 2016), and PDBu increased both the TS vesicle pool size (1.9-fold) and
501 fusion probability of TS vesicles (1.3-fold) at the calyx synapses (Lin et al., 2025). Therefore, it
502 is possible that PTP may increase both p_{occ} and p_v of TS vesicles, although it has not been yet
503 directly tested. Moreover, acceleration of the latency in the hypertonicity-induced vesicle
504 release raises a possibility for an increase in fusion probability of TS vesicles (Stevens and
505 Wesseling, 1999; Garcia-Perez and Wesseling, 2008), although it is still controversial that the
506 acceleration of the release latency represents a reduction in the activation energy barrier for
507 vesicle fusion (Schotten et al., 2015).
508

509 *Progressive overfilling of docking sites*

510 Conventional models for facilitation assume an increase in p_v after an AP of RRP vesicles,
511 which had been in a low p_v or reluctant state at rest (Dittman et al., 2000; Turecek et al., 2016).
512 This facilitation model is unlikely to explain our results, because TS vesicles are predominantly
513 released not only at rest but also in the facilitated state during or after train stimulation, as
514 evidenced by the strong PPD (ISI, 25 ms) during a 5-Hz train (***Figure 2D-E***) and at different
515 intervals after a 40-Hz/30 pulse trains (***Figure 3D***). Moreover, the PPD (ISI, 25 ms) remained
516 pronounced during the early phase of facilitation (***Figure 7***), arguing against the possibility of
517 slowly increasing the p_v of reluctant vesicles. These results suggested that facilitation was
518 mediated by an activity-dependent progressive increase in occupancy of TS vesicles (TS
519 occupancy). Facilitation through an increase in TS occupancy has been termed frequency
520 facilitation, differentiating it from paired pulse facilitation that relies on a residual calcium-
521 dependent transient increase in the p_v of releasable vesicles (Neher, 2024).
522 Assuming an increase in TS occupancy, to attain a two-fold increase in EPSC (***Figure 1***), the
523 baseline occupancy of the docking sites by TS vesicles should be low, as estimated from the
524 V-M plot (c.a. 30% in ***Figure 3***). However, it is unclear whether the remaining docking sites
525 that are not occupied by TS vesicles are truly vacant or are occupied by reluctant LS vesicles
526 that are not released by the AP or AP trains. TS vesicles can be supplied by the transformation
527 of LS to TS vesicles, both of which reside at the same docking site (LS/TS model; reviewed in
528 Neher, 2024). Alternatively, TS vesicles can be supplied from a distinct replacement pool
529 (RS/DS model; reviewed in Silva et al., 2021). Further studies, including ultrastructural
530 analyses of active zones, are required to address this issue.

531 Given the crucial role of Syt7 in facilitation (***Figure 5***), the facilitation mechanisms are closely
532 related to how Syt7 mediates synaptic facilitation. Based on the slow kinetics of Syt7, previous
533 computational modeling studies have proposed that Syt7 may mediate short-term facilitation
534 through an activity-dependent increase in p_v (Turecek et al., 2016; Jackman and Regehr, 2017;
535 Turecek et al., 2017; Norman et al., 2023). The present study, however, found that both
536 facilitation and augmentation were mediated by overfilling of the release sites (***Figure 2 and***
537 ***Figure 3***). Moreover, Syt7-KD synapses displayed slower recovery of TS vesicles (***Figure 7***).
538 These results suggest that Syt7 is essential for activity-dependent overfilling of docking sites
539 with TS vesicles. Whereas the baseline EPSC was not altered (***Figure 1—figure supplement 3***),
540 and complementary changes in the number of docking sites and their baseline occupancy
541 were observed in Syt7 KD synapses (***Figure 6***). These results raise a possibility that Syt7 may
542 play a role in providing additional vacant docking sites that can be overfilled during facilitation.
543 It remains to be elucidated whether the decrease in the number of docking sites at Syt7 KD
544 synapses is related to the subcellular localization of Syt7 to the plasma membrane of the
545 active zone (Sugita et al., 2001; Vevea et al., 2021).
546

547 *STP model in light of known Ca^{2+} binding kinetics of Syt7*

548 Syt7 acts as an upstream Ca^{2+} sensor that facilitates activity-dependent replenishment (Liu et
549 al., 2014; Bacaj et al., 2015; Chen et al., 2017; Tawfik et al., 2021). The present study shows
550 that EPSCs are mediated by TS vesicles both at rest and during activity-dependent facilitation.
551 Releasable vesicles are homogeneous, and Syt7-dependent overfilling is responsible for the

552 activity-dependent enhancement of EPSCs. Thus, we tested whether slowly developing
553 facilitation could be replicated within the framework of a simple refilling model based on the
554 known Ca^{2+} binding kinetics of Syt7 (Brandt et al., 2012). To this end, we made following
555 assumptions: (1) a Ca^{2+}-dependent increase in the forward refilling rate (k_1) requires a full
556 Ca^{2+}-bound form of Syt7, to which multiple Ca^{2+} ions can bind (two or three Ca^{2+} ions on each
557 C2A and/or C2B domain) (Brandt et al., 2012; Chon et al., 2015; Voleti et al., 2017); (2)
558 sequential Ca^{2+} binding steps are synergistic through a cooperativity factor, *b*; (3) Syt7 senses
559 AP-induced local [Ca^{2+}] similar to that estimated at the calyx of Held (a Gaussian function with
560 a full width at half maximum value of 0.2 ms and a peak of 40 µM) (Wang et al., 2008); and
561 (4) residual [Ca^{2+}] follows a mono-exponential function with an amplitude of 1 µM and decay
562 time constant of 50 ms (Jackson and Redman, 2003).
563 Under these assumptions and the framework of the simple refilling model, slow and delayed
564 facilitation could be replicated by modeling k_1 as $k_1 = k_{1,b} + K_{1,max} \times$ [Syt7: n Ca^{2+}], where [Syt7:
565 n Ca^{2+}] represents the fraction of fully Ca^{2+}-bound Syt7 (***Figure 9A-C***). Syt7 was slowly
566 activated over the course of the stimulus train, suggesting that the cooperative binding of
567 multiple Ca^{2+} ions progressively increased the putative active form of Syt7 because of its slow
568 membrane binding/unbinding kinetics. Nevertheless, the late phase of facilitation at high
569 frequencies was predicted to be higher in the simulation than in the experiment. As expected,
570 based on the notion that sustained release during HFS is limited by site clearance in the active
571 zone (***Figure 4C***), the model/data discrepancy became more pronounced at higher
572 frequencies. Incorporating endocytic terms into the model may mitigate late-phase

573 discrepancies. Moreover, removal of the catalytic function of Syt7 in the model effectively
574 accounted for the complete loss of facilitation observed in Syt7 KD (***Figure 9D***).
575

576 *Conventional STF models involving cumulative increase in p_v of reluctant vesicles*

577 Conventional models for facilitation assume a post-AP residual Ca^{2+}-dependent step increase
578 in p_v of RRP (Dittman et al., 2000) or reluctant vesicles (Turecek et al., 2016). Given that p_v of
579 TS vesicles is close to one in the present study, an increase in p_v of TS vesicles cannot account
580 for facilitation. The possibility for activity-dependent increase in fusion probability of LS
581 vesicles (denoted as $p_{v,LS}$) should be considered in two ways depending on whether LS and TS
582 vesicles reside in distinct pools or in the same pool. Notably, strong PPD at short ISI implies
583 that $p_{v,LS}$ is near zero at the resting state. Whereas LS vesicles do not contribute to baseline
584 transmission, short-term facilitation (STF) may be mediated by cumulative increase in p_v of LS
585 vesicles that reside in a distinct pool. Because the increase in $p_{v,LS}$ during facilitation recruits
586 new release sites (increase in N), the variance of EPSCs should become larger as stimulation
587 frequency increases, resulting in upward deviation from a parabola in the V-M plane, as
588 shown in recent studies (Valera et al., 2012; Kobbersmed et al., 2020). This prediction is not
589 compatible with our results of V-M analysis (***Figure 3***), showing that EPSCs during STF fell on
590 the same parabola regardless of stimulation frequencies. Therefore, it is unlikely that an
591 increase in fusion probability of reluctant vesicles residing in a distinct release pool mediates
592 STF in the present study. For the latter case, in which LS and TS vesicles occupy in the same
593 release sites, it is hard to distinguish a step increase in fusion probability of LS vesicles from a

594 conversion of LS vesicles to TS. Nevertheless, our results do not support the possibility for a
595 gradual increase in $p_{v,LS}$ that occurs in parallel with STF. Strong PPD, indicative of high p_v, was
596 consistently found not only in the baseline (***Figure 2 and Figure 2—figure supplement 1***) but
597 also during post-tetanic augmentation phase (***Figure 3D***) and even during the early
598 development of facilitation (***Figure 2D-E and Figure 7***), arguing against the gradual increase
599 in $p_{v,LS}$. One may argue that STF may be mediated by a drastic step increase of $p_{v,LS}$ from zero
600 to one, but it is not distinguishable from conversion of LS to TS vesicles.
601

602 *Possible mechanisms underlying the depression-facilitation sequence*

603 Cooperative binding of multiple Ca^{2+} ions to Syt7 predicts delayed activation and a progressive
604 increase in vesicle refilling during train stimulations, resulting in delayed facilitation.
605 Combining this with a high p_v could replicate not only the slow facilitation at 5 Hz, but also
606 the unique depression-facilitation sequence observed at 40 Hz (***Figure 9***). As simulated by
607 Pulido and Marty (2018), delayed facilitation can be reproduced by a high p_v in combination
608 with a low baseline occupancy of replacement sites and subsequent Ca^{2+}-dependent refilling.
609 However, whether this model is also able to replicate slow facilitation at low frequencies
610 remains to be tested. An alternative, but not exclusive, possibility for delayed facilitation is
611 that the presynaptic global Ca^{2+} itself may undergo a slow buildup during a train due to the
612 saturation of endogenous Ca^{2+}-binding proteins.
613

614 *Facilitation requires not only Syt7, but also activation of PLC-DAG signaling*

615 To gain insight into the specific molecular mechanisms underlying the vesicle-loading
616 processes, we examined the effects of pharmacological inhibitors targeting PLC and DAG
617 (***Figure 4***). Both a PLC inhibitor and a DAG analog reduced facilitation and augmentation. This
618 suggests that the progressive overfilling of docking sites with TS vesicles is supported by the
619 PLC-DAG signaling pathway and probably downstream Munc13 (Rhee et al., 2002; Lou et al.,
620 2008). Phorbol esters have been recently shown to increase the TS fraction of docked vesicles
621 by enhancing vesicle priming without affecting Ca^{2+} transients or p_v (Taschenberger et al.,
622 2016; Aldahabi et al., 2022; Papantoniou et al., 2023; Aldahabi et al., 2024). In line with these
623 findings, signaling involving PLC and DAG may support Ca^{2+}-dependent overfilling of TS
624 vesicles by enhancing molecular priming processes.

625

626 *Roles of facilitation and augmentation in working memory and related tasks*

627 Previous theoretical studies have proposed that working memory in a recurrent network can
628 be maintained in the form of augmentation at synapses (Mongillo et al., 2008) and/or
629 persistent activity of neurons in a memory ensemble (Mongillo et al., 2012). The latter can be
630 supported by short-term facilitation of recurrent excitatory synapses or disparity in STP
631 between excitatory and inhibitory synapses (Mongillo et al., 2012; Yoon et al., 2020). However,
632 whether these short-term enhancements in synaptic efficacy contribute to working memory-
633 related behaviors has not been examined. tFC is a temporal associative learning task requiring
634 temporary retention of CS information during a trace period. The mPFC and hippocampus are

635 crucial for tFC, and tFC is interfered with by a load of working memory and attention
636 distraction, implying that the tFC shares the same neural substrate with other working
637 memory tasks (Raybuck and Lattal, 2014). In the present study, tFC in WT and Syt7 KD animals
638 revealed that Syt7 deficiency in the L2/3 PCs of the mPFC impaired the acquisition of trace
639 fear memory and reduced c-Fos expression associated with tFC training (***Figure 8***).
640 The acquisition of trace fear memory was impaired by inhibition of persistent activity in mPFC
641 during trace period (Gilmartin et al., 2013). The similar deficit observed in Syt7 KD animals is
642 consistent with the hypothesis that STF provides bi-stable ensemble activity in a recurrent
643 network (Mongillo et al., 2012). Nevertheless, alternative mechanisms may be responsible for
644 the behavioral deficit. Not only recurrent network but also long-range loop between the
645 mPFC and the mediodorsal (MD) thalamus play a critical role in maintaining persistent activity
646 within the mPFC especially for a delay period longer than 10 s (Bolkan et al., 2017). Prefrontal
647 L2/3 is heavily innervated by MD thalamus, and L2/3-PCs subsequently relay signals to L5
648 cortico-thalamic (CT) neurons (Collins et al., 2018). Given that L2/3 is an essential component
649 of the PFC-thalamic loop, loss of STF at recurrent synapses between L2/3 PCs may lead to
650 insufficient L2/3 inputs to L5 CT neurons and failure in the reverberant PFC-MD thalamic
651 feedback loop. Therefore, not only L2/3 recurrent network but also its output to downstream
652 network should be considered as a possible network mechanism underlying behavioral deficit
653 caused by Syt7 KD L2/3.
654 In summary, activity-dependent synaptic facilitation and augmentation at prelimbic L2/3
655 recurrent excitatory synapses can be attributed to overfilling of release sites with TS vesicles.
656 Given that the lack of short-term facilitation in L2/3 PCs impairs trace fear memory acquisition,

657 temporary maintenance of CS information during trace period may be supported by an
658 increase in the TS occupancy in presynaptic terminals of CS-representing neuronal ensembles.
659
660

661 ## Materials and Methods

662 *Animals/Subjects*

663 All experiments were carried out on Sprague-Dawley rats of either sex at p28-43. Rats were
664 maintained in temperature-controlled rooms on a constant 12 h light/12 h dark cycle and
665 were given access to water and food ad libitum. All animal procedures were approved and
666 performed in accordance with the Institutional Animal Care and Use Committee at Seoul
667 National University (SNU200522-1).

668

669 *In utero electroporation (IUE)*

670 Pregnant Sprague Dawley rats on embryonic day (E) 17.5 were deeply anesthetized with 5%
671 (v/v) isoflurane for the duration of surgery. The uterine horns were exposed by laparotomy
672 and wet with warm sterile phosphate-buffered saline (PBS). The plasmids (0.5-2 μg/μl)
673 together with the 0.01% fast green dye were injected into either the left or the right lateral
674 ventricle or bilateral ventricles of embryos through a thin glass capillary (Narishige PC-10).
675 The embryo's head was carefully held between the forceps-shaped electrodes of 10 mm
676 diameter (CUY65010; NEPAGENE, Japan). Electroporation pulses (50 V for 50 ms) were
677 delivered five times at intervals of 150 ms using a square-wave generator (ECM830; BEX,
678 United States). The uterine horns were placed back into the abdominal cavity and abdominal
679 muscles and skin were sutured. After the surgery, the animals were recovered under the
680 infrared lamp. For knockdown experiments in slice electrophysiology, the shRNA constructs
681 were co-electroporated with the pCAG-oChIEF-tdTomato, because of the consistent co-

682 expression of the two constructs in a significant proportion of the transfected cells in vivo
683 (Bony et al., 2013). Pups with robust fluorescence signals in the prefrontal cortex of both
684 hemispheres were used for behavior experiments.
685

686 *Preparation of vector constructs*

687 For an optic stimulation, pCAG-oChIEF-tdTomato was transfected by IUE. Short hairpin RNA
688 (shRNA) against Syt7 sequence (GATCTACCTGTCCTGGAAGAG) was expressed under the
689 control of an U6 promoter of pAAV-U6-GFP vector (Cell Biolabs, #VPK-413). This shRNA was
690 shown to effectively knockdown Syt7 in cell cultures and in vivo by previous reports from
691 more than one research group (Bacaj et al., 2013; Li et al., 2017). Scrambled control sequence
692 (TCGCATAGCGTATGCCGTT) was designed by shuffling the recognition region sequences and
693 a BLAST analysis confirmed that the control shRNA sequence had no target gene. For rescue
694 experiments, the cDNA for rat Syt7 (NM_021659) rendered resistant to the shRNA were
695 inserted in the KD vector and their expression was driven by the CAG promoter; the vector
696 also contained a self-cleaving P2A peptide sequence followed by EGFP, which ensures
697 visualization of transfected cells. All the constructs above were verified by DNA sequencing.
698 Plasmid DNA was purified and concentrated under endotoxin free conditions (Qiagen
699 EndoFree Maxi Kit).
700

701 *Slice preparation*

702 Acute brain slices were prepared by using a VT1200S Vibratome (Leica Microsystems) after
703 isoflurane anesthesia and decapitation. Coronal sections of the mPFC (300 µm in thickness)
704 were cut in ice-cold artificial cerebrospinal fluid (aCSF) composed of (in mM) 125 NaCl, 3.2
705 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 20 D-glucose, 2 NaPyr, 1 $MgCl_2$, and 1.3 $CaCl_2$ (Yoon et al.,
706 2020), bubbled with 95% O_2 and CO_2 (pH = 7.3; 310 mOsm). Slices were allowed to recover
707 for a minimum of 30 minutes at 36 °C, and subsequently maintained at room temperature
708 until recording. Composition of aCSF was identical throughout the preparation, incubation,
709 and recording periods unless otherwise stated. For the recordings, the mPFC slices were
710 continuously perfused with oxygenated aCSF at a flow rate of 1.5 ml/min, and the
711 temperature was maintained at 31–32 °C using an in-line temperature controller (Warner
712 Instruments).

713

714 *Electrophysiology*

715 Whole-cell patch-clamp recordings were performed from pyramidal cells and fast-spiking
716 interneurons in layer 2/3 of the prelimbic mPFC. Neuronal subtypes were initially selected
717 based on morphology under visual guidance by using an upright microscope equipped with
718 infrared differential interference contrast (IR-DIC) optics (BX51WI, Olympus), and were
719 further identified by their electrophysiological properties (see ***Figure 1—figure supplement***
720 ***1***; van Aerde and Feldmeyer, 2015; Tremblay et al., 2016). To analyze these intrinsic
721 properties in current-clamp experiments, we measured the following parameters: (1) resting

722 membrane potential (RMP), (2) input resistance (R_{in}), (3) sag ratio, and (4) the F-I curve, which
723 plots firing frequencies (F) against the amplitude of injected currents (I). Both cell types
724 exhibited a low sag ratio below 0.1, consistent with findings from other studies (van Aerde
725 and Feldmeyer, 2015; Yoon et al., 2020). PCs and FSINs were easily distinguished by their
726 unique firing frequency, Rin, spike adaptation, RMP and morphological characteristics
727 including the shape of soma and the thickness of apical dendrites. For experiments involving
728 oChIEF-expressing neurons in WT or Syt7 KD rats, whole-cell recordings were performed from
729 the fluorescently labeled pyramidal neurons in layer 2/3 of the prelimbic cortex using suitable
730 filter sets. Patch pipettes (tip resistance of 2–3 MΩ) were pulled from borosilicate glass and
731 filled with (in mM) 130 K-gluconate, 8 KCl, 10 HEPES, 0.2 EGTA, 20 Na_2Pcr, 0.3 Na_2GTP, 4
732 MgATP. Intracellular solutions were adjusted to pH 7.25 and 300–310 mOsm. Neurons were
733 voltage clamped at −78 mV and series resistance (Rs) were continuously monitored during
734 the EPSCs recordings. Experiments were discarded before analysis if the Rs changed by 20%
735 or larger deviation of baseline value. Measurements were not corrected for Rs compensation,
736 bridge balance, or liquid junction potential. Electrophysiological data were low-pass-filtered
737 at 1 kHz (Bessel) and acquired at 20 kHz using a Multiclamp 700B amplifier paired with
738 Digidata 1440A digitizer and Clampex 10.2 software (Molecular Devices).
739

740 *Synaptic stimulation*

741 IUE resulted in sparse expression of channelrhodopsin (10-20%) (***Figure 1A***). Exploiting the
742 sparse expression, we employed a minimal optical stimulation method to activate a single or
743 very few excitatory synapses in layer 2/3. The optic minimal stimulation was performed by

744 our published protocol with minor modifications (Yoon et al., 2020). This approach aimed to
745 minimize the risk of synaptic contamination, which could arise from engaging extra terminals
746 that were initially inactive (depolarized below threshold) but became active by temporal
747 summation during high-frequency trains. It also helped prevent the buildup of depolarization
748 at the synaptic terminals, which might influence the probability of release, as noted by
749 (Jackman et al., 2014). To achieve this, we employed a collimated digital micromirror device
750 (DMD)-coupled LED (Polygon400; Mightex Systems) to confine 470 nm blue light to a small
751 area with a radius of approximately 3–8 µm (typically 3–4 µm), as measured at the focal plane
752 of a 60× water immersion objective [numerical aperture (NA) = 1.0; LUMPlanFL, Olympus]
753 (***Figure 1—figure supplement 3A***). To find the minimal stimulation area, the radius of
754 illumination area was increased from 2 µm by 50% at each step (***Figure 1—figure supplement***
755 ***3A***), and selected the smallest illumination area that elicited EPSCs. Setting the duration of
756 illumination between 3 and 5 ms (typically 5 ms), photostimulation onto oChIEF-expressing
757 cells reliably induced action potentials (APs) during 600-pulse trains (see ***Figure 1—figure***
758 ***supplement 2***).
759 STP at various stimulus frequencies (5, 10, 20, and 40 Hz) was examined using 20-pulse trains.
760 Trials of train stimulation were repeated with a prolonged interval of 30 s to avoid change in
761 baseline properties. We observed no systematic changes in the amplitudes of the first EPSC
762 of each train, nor did we find significant differences in the paired-pulse ratio (PPR) across
763 stimulation trials in the same cell. For each stimulus frequency, approximately ten to twenty
764 consecutive traces were collected and then averaged across trials to yield a mean EPSC trace.
765 For recovery experiments, two consecutive trains of 3 or 30 stimuli at 40 Hz, with varying time

766 intervals (0.1-10 s), were applied every 10 s or 60 s, respectively, at both excitatory synapses.
767 For 600-stimulus trains at 10Hz, the trial was delivered only once for each cell to avoid
768 possible after-effects caused by prolonged synaptic stimulation (Hirsch and Crepel, 1990).
769 Prior to the stimulus trains, the EPSCs were normalized to the mean amplitude of the baseline.
770 For the baseline measurements, paired pulses with various ISIs (20-200 ms) were delivered
771 every 3 s (1/3 Hz) at least 20 times.
772 For all recordings, picrotoxin (PTX, 10 µM) was included in the recording solution to isolate
773 glutamatergic responses. mPFC PC terminals were entirely glutamatergic as the application
774 of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 µM), an AMPA receptor antagonist,
775 completely abolished the monosynaptic events evoked by photostimulation. A low
776 concentration of PTX was employed to minimize the relieving effects on spike suppression
777 caused by blockade of GABAa receptors. We confirmed that no synaptic response was elicited
778 by electrical stimulation in the presence of both 10 µM PTX and 20 µM CNQX.
779 In ***Figure 1—figure supplement 4A***, 1 µM TTX (1 mM stock solution) and 0.1 mM 4-AP (500
780 mM stock solution) were added to the bath solution to block action potentials and restore
781 presynaptic glutamate release, respectively (***Figure 1—figure supplement 4***). In ***Figure 1—***
782 ***figure supplement 5A***, 50 µM of cyclothiazide, a positive allosteric modulator of the AMPA
783 receptors, was included to minimize rapid desensitization of the postsynaptic receptors. In
784 ***Figure 1—figure supplement 5B***, 0.5 mM kynurenate was added to bath solution to prevent
785 possible saturation of AMPA receptors, especially during maximal synaptic facilitation. For
786 pharmacological manipulations of synaptic vesicle dynamics (***Figure 4***), drugs related to
787 vesicle supply including U73122, OAG, LatB, EGTA-AM, and dynasore at indicated

788 concentrations were added to the recording solution during baseline measurements or
789 PTA/recovery experiments (IBI = 0.5 s). Chemicals were from Sigma-Aldrich, Merck, or Tocris.
790

791 *Measurements of quantal size*

792 Experiments for measuring asynchronous release were performed as previously described
793 (Yoon et al., 2020). In brief, we replaced extracellular Ca^{2+} entirely with 2 mM Sr^{2+} to induce
794 asynchronous release. A single stimulus was delivered at 3-second intervals over 100
795 repetitions, and asynchronous release events were detected during a 100-ms window starting
796 25 ms after stimulus onset for each trial. The same procedure was also executed under sham
797 conditions (0% light intensity) to serve as a control, thus mitigating contamination from
798 nonspecific spontaneous events and potential errors associated with in the detection
799 algorithm. q was determined as the mean value from the first Gaussian function after fitting
800 the EPSC distribution with either a single or double Gaussian function (***Figure 3—figure***
801 ***supplement 1***).
802

803 *Estimation of Ca^{2+} kinetics at axonal boutons*

804 Cytosolic [Ca^{2+}] at single axonal boutons was estimated by employing the two-dye, dual-
805 excitation wavelength technique as described by (Oheim et al., 1998). This method involves
806 measuring the fluorescence ratio of a Ca^{2+}-sensitive dye to that of a Ca^{2+}-insensitive dye to
807 correct for dye concentrations. We used Fluo-5F at concentrations of 150, 250, or 500 µM
808 (dissociation constant, K_d = 2.3 µM) as the calcium indicator dye and Alexa Flour 555 (50 µM)

809 as the reference dye. The fluorophores were from Invitrogen. Both dyes were co-loaded into
810 presynaptic terminals of layer 2/3 pyramidal cells in mPFC through whole-cell patch clamp.
811 Before data acquisition, the dyes were allowed to diffuse to terminal boutons for at least 30
812 minutes after the patch break-in, a duration sufficient for the equilibrium of dye
813 concentrations between the soma and its axonal terminals.
814 Calcium imaging was performed by sequentially exciting Fluo-5F and AF555 at wavelengths
815 of 473 and 559 nm, respectively, using a confocal laser-scanning microscope (FV1200;
816 Olympus, Japan) equipped with a 60× water immersion objective (NA, 0.9; LUMPlanFl;
817 Olympus, Tokyo, Japan). Once a single axonal bouton was identified, lines for scanning were
818 drawn across boutons, oriented perpendicular to the axon (***Figure 2—figure supplement 2A-***
819 ***B***). After baseline fluorescence was measured, line scanning was proceeded while a single or
820 train APs were induced by applying current pulses. Upon stimulation, green fluorescence but
821 not red one increased along the vertical array of lines across the bouton (***Figure 2—figure***
822 ***supplement 2B-C***). Measurements were obtained under current-clamp conditions, with the
823 resting potential regularly monitored. The scanning speed was adjusted to 600-700 Hz by
824 controlling the line length. Line scan for measuring fluorescent transients in a bouton was
825 repeated at least 3 times, and then averaged to enhance the signal-to-noise ratio. To mitigate
826 photobleaching, minimal intensity of the laser power and maximal pinhole size were used.
827 Emitted light was filtered through 490/590 or 575/675 bandpass filters before detection by
828 photomultiplier tubes for green or red fluorescence, respectively.

829 Fluorescence measurements were converted to $[Ca^{2+}]_i$ using the ratio of background-
830 subtracted fluorescence ($F = F_0 - F_b$) from two dyes. The fluorescence ratio ($R = F_{green}/F_{red}$) in
831 data traces were converted to $[Ca^{2+}]_i$ using the following equation:

832 $$[Ca^{2+}]_i = K_d \times (R - R_{min}) / (R_{max} - R). \quad (1)$$

833 Calibration parameters were obtained using an in-cell calibration protocol (Helmchen, 2011),
834 wherein the minimum (R_{min}) and maximum ratio (R_{max}) values were established through the
835 use of intracellular solutions containing 10 mM EGTA or 10 mM $[Ca^{2+}]$, respectively.
836 When Ca^{2+} is extruded with a rate constant, γ, after an AP-induced short pulse of Ca^{2+} influx
837 to a single compartment, $[Ca^{2+}]_i$ initially rises to A_{Ca} and then undergoes a mono-exponential
838 decay with a time constant (τ_{Ca}). Under this framework, A_{Ca} and τ_{Ca} depend on Ca^{2+} binding
839 ratios of endo- and exogenous Ca^{2+} buffers (denoted as κ_S and κ_B, respectively) as following
840 equations (Neher, 1995; Kim et al., 2003):

841 $$1/A_{Ca} = (1 + \kappa_S + \kappa_B) / \Delta[Ca^{2+}]_T. \quad (2)$$

842 $$\tau_{Ca} = (1 + \kappa_S + \kappa_B) / \gamma. \quad (3)$$

843 , where $\Delta[Ca^{2+}]_T$ is an increment in total $[Ca^{2+}]$ (free plus bound) elicited by an AP. Ca^{2+} binding
844 ratio indicates the ratio of concentration changes in the Ca^{2+}-buffer complex relative to free
845 $[Ca^{2+}]$ change, and linearized κ_B is calculated from the dye concentration and the change from
846 $[Ca^{2+}]_1$ to $[Ca^{2+}]_2$ as follows:

847 $$\kappa_B = \Delta[CaB] / \Delta[Ca^{2+}] = [B]_t K_d / [([Ca^{2+}]_1 + K_d)([Ca^{2+}]_2 + K_d)]. \quad (4)$$

848 The total dye concentration, $[B]_t$, was taken as [Fluo-5F] in the patch pipette. With κ_B
849 determined from Equation 4 and τ_{Ca} from the decay of a Ca^{2+} transient, κ_S can be estimated

by plotting τ versus κ_B and applying a linear fit to the plot, which gives the x-intercept as -(1 + κ_S). Once determined, κ_S can be used to calculate $\Delta[Ca^{2+}]_T$.

Optimization of k_1 and p_v from failure rates

Let n_0 = the number of docked SVs before arrival of a first AP; n_1 = the number of docked SVs remaining after a first AP (i.e. n_0 - n_1 vesicles are released); n_2 = the number of docked SVs just before arrival of a 2nd AP (ISI = 20 ms; ***Figure 3—figure supplement 2***). If the number of release sites (N), forward and reverse rates for vesicle docking (k_1 and b_1, respectively), mean release site occupancy (p_{occ}) and vesicular fusion probability upon an AP (p_v) are given, we can predict $P(n_0, n_1, n_2)$, the probability for $[n_0, n_1, n_2]$, as following steps.

1) Probability for a synapse harbouring n_0 docked SVs just before arrival of a first AP is calculated as:

$$P(n_0) = C(N, n_1)\, p_{occ}^{n0} \times (1 - p_{occ})^{(N-n0)}.$$

2) Probability that n_1 vesicles remain after a first AP when the initial number of docked SVs is n_0 $[P(n_1|n_0)]$ is calculated as:

$$P(n_1|n_0) = C(n_0, n_1) \times (1-p_v)^{n1} \times p_v^{(n0-n1)}, \text{ if } n_0 \geq n_1 ,$$

$$P(n_1|n_0) = 0, \text{ if } n_0 < n_1.$$

3) When forward and backward rate constants are k_1 and b_1, respectively, probability that n_2 vesicles are docked just before arrival of a 2nd AP when n_1 SVs remain after a 1st AP (Δt = 20 ms) $[P(n_2 \mid n_1)]$ is predicted as follows: Assuming that docking and undocking follow a Poisson process,

$$P(n_2 \mid n_1) = C(N - n_1, n_2 - n_1)\,[1 - \exp(-k_1\,\Delta t)]^{(n2 - n1)}, \text{ if } n_2 > n_1 .$$

$$P(n_2 \mid n_1) = C(n_1, n_1 - n_2)\,[1 - \exp(-b_1\,\Delta t)]^{(n1 - n2)}, \text{ if } n_2 < n_1 .$$

$$P(n_2 \mid n_1) = \exp[-n_1\,k_1\,\Delta t]\,\exp[-n_1\,b_1\,\Delta t], \text{ if } n_2 = n_1 .$$

4) Combining probabilities calculated from above three steps, we can calculate $P(n_0, n_1, n_2)$:

$$P(n_0, n_1, n_2) = P(n_0) \times P(n_1|n_0) \times P(n_2|n_1)$$

5) Once $P(n_0, n_1, n_2)$ is given, we can predict probability for double failure (P_{00}) and probability for the 1st success and the 2nd failure (P_{10}) as:

$$P_{00} = \Sigma_{n0=0..N}\,\Sigma_{n1 = n0}\,\Sigma_{n2 = 0..N}\quad p(n_0, n_1, n_2)\,(1 - p_v)^{n2} .$$

$$P_{10} = \Sigma_{n0=0..N}\,\Sigma_{n1 \neq n0}\,\Sigma_{n2 = 0..N}\quad p(n_0, n_1, n_2)\,(1 - p_v)^{n2} .$$

6) Probability for the 1st failure and the 2nd success (P_{01}) and probability for double success (P_{11}) were calculated as:

$$P_{01} = P(F_1) - P(F_1, F_2)$$

$$P_{11} = P(S_1) - P(S_1, F_2)$$

, in which $P(F_1)$ and $P(S_1)$ are probability for the 1st failure and the 1st success, respectively, and these are calculated as:

$$P(F_1) = P(n_0)\,P(F_1 \mid n_0) = \Sigma_{n0=0..N}\,P(n_0)\,(1 - p_v)^{n0} ,$$

$$P(S_1) = 1 - P(F_1).$$

To optimize k_1 and p_v, we used the Matlab routine, *fmincon*, in which the sum of squared errors (= observed value minus predicted value) was set as a cost function to be minimized. Lower and upper bounds for k_1 and p_v were set as [4, 8] and [0.8, 1], respectively. Release probability (p_r) was calculated from the observed 1st failure rate, $P(F_1)$, as

$$p_r = 1 - [P(F_1)]^{(1/N)}$$

N was assumed to be six as shown in ***Figure 3***. Other parameters (b_1, and p_{occ}) were set according to following relationships:

$$p_{occ} = p_r / p_v .$$

$$b_1 = k_1 (1 - p_{occ})/p_{occ} .$$

Calculation of probability for double failure

Double failure probability, P_{00}, can be more easily predicted. By the Bayes rule,

$$P(F_1, F_2, n_1) = P(F_2 \mid n_1, F_1)\, P(F_1 \mid n_1)\, P(n_1).$$

, where F_1 and F_2 means 1st and 2nd failure. Moreover, and $n_0 = n_1$ because of the 1st failure. Thus, the probability for two consecutive failures can be calculated as:

$$P(F_1, F_2) = \Sigma_{n1=0..N}\, P(F_2 \mid n_1, F_1)\, P(F_1 \mid n_1)\, P(n_1).$$

$P(F_1)$ and N are observed values. For calculation in ***Figure 3—figure supplement 2***, k_1 and p_v were set free variables. Other parameters were set under the following constrains: $P(F_1) = (1 - p_r)^N$, $p_r = p_v\, p_{occ}$, and $p_{occ} = k_1 / (k_1 + b_1)$.

Behavioral tests

Trace fear conditioning: For trace fear conditioning (tFC) (Gilmartin et al., 2013), individual rats were placed in the training chamber (30.5 x 25.4 x 30.5 cm; Coulbourn Instruments) consisting of metal grid floor connected to an electrical stimulator (H10-11R-TC-SF, Coulbourn Instruments) for delivery of a footshock. After a 5 min habituation period, rats received five pairings of a 20 s tone conditioned stimulus (CS, 2.5 kHz, 75 dB), followed by empty 20s trace

914 period and a unconditioned stimulus (US; 1 s, 0.7 mA footshock) with a pseudorandom inter-
915 trial interval (ITI) of 240 ± 20 s. During this training session, rats learned to associate the
916 auditory CS with the shock US. The next day, the rats were tested for memory of CS-US trace
917 association (called tone test). The acquisition of fear learning, often indicated by the extent
918 of freezing to the CS, was assessed in a novel chamber (a white hexagonal enclosure) in a
919 dimly lit room. During this tone (or retrieval) test, rats were first allowed to explore the new
920 context for 5 min (habituation), followed by twelve 20 s CS presentations with a variable ITI
921 of 120 ± 20 s. This entire session was repeated on the next day for assessment of extinction
922 learning. Identities of the chambers were also determined with the presence of distinct odors;
923 the training and testing chambers were cleaned with 70% ethanol and 1% acetic acid
924 respectively before and after each session. For all experimental sessions, the activity of
925 animals in the chambers was recorded at 30 frames per second using the EthoVision XT
926 (Noldus Information Technology, Wageningen, Netherlands), and stored as a video file. The
927 freezing behavior, defined as behavioral immobility other than respiratory movement, was
928 assessed using an open-source video analysis pipeline ezTrack (Pennington et al., 2019). The
929 percentage of freezing was calculated as a total duration of freezing divided by the total
930 duration of observation.
931 **Elevated Plus Maze**: Elevated plus maze (EPM) test was performed using a plus-shaped
932 apparatus elevated 60 cm above the ground. The maze consisted of two open arms (50 x 10
933 x 0.5 cm (H)), two closed arms (50 x 10 x 40 cm (H)) and a center area (10 x 10 cm). Rats were
934 individually placed on the center area, facing an open arm, and the path of the animal was
935 recorded with a video camera for 5 min. We analyzed the total distance traveled for 5 min

936 and time spent in each arm. Animals were tracked offline using the open-source tracking
937 system ezTrack (Pennington et al., 2019).
938 **Open field test**: Open field exploration test (OFT) was performed in an open field apparatus
939 in a dimly lit room. The open field consisted of a white plastic board (1.20 m in diameter)
940 surrounded by white plastic walls (50 cm in height). Individual rats were placed in a center
941 zone (0.6 m in diameter), and allowed to freely explore the apparatus for 10 min. Time spent
942 in the center zone and total distance traveled were analyzed for initial 5 min. Animals were
943 tracked offline using the open-source tracking system ezTrack (Pennington et al., 2019).
944

945 *Histology and immunohistochemistry*

946 At the end of the behavioral experiments, rats were anesthetized using isoflurane inhalation
947 and transcardially perfused with PBS followed by 4% paraformaldehyde (PFA, T&I, Korea).
948 Brains were extracted and post-fixed in 4% PFA solution for 24 h. Perfused brains were sliced
949 at 100 μm for estimation of GFP expression or 50 μm for immunohistochemistry. For analysis
950 of c-Fos expression, rats were returned to their home cages immediately after the training
951 session and perfused approximately 90 minutes later (Arime and Akiyama, 2017). Brains were
952 then cryopreserved, frozen, and sectioned coronally. Immunofluorescence staining was
953 conducted on free-floating slices using a rabbit monoclonal anti c-fos (1:1000, Cell Signaling
954 Technology, cat# 2250s) and mouse monoclonal anti-GAD67 (1:500, Millipore, cat# mab5406).
955 Fluorescent secondary antibodies included Cy5-conjugated goat anti-rabbit IgG (1:1000,
956 Abcam, cat# ab97077) and Cy3-conjugated goat anti-mouse IgG (1:1000, Abcam, cat#
957 ab97035). Brain slices were permeabilized and blocked with PBS containing 0.5% Triton X-100

958 and 5% normal goat serum (NGS) for 1h at room temperature. Slices were incubated
959 overnight at 4 °C with primary antibodies, followed by 4 h incubation with secondary
960 antibodies at room temperature. Slices were then mounted with Antifading Mounting
961 medium (Abcam, cat# ab104139). Confocal images were acquired using a Leica TCS SP8
962 microscope set to the same laser intensity and acquisition parameters to compare the
963 immunosignals in sections from Scr and Syt7 KD groups. For cell counting and colocalization,
964 fluorescent protein-positive cells were automatically quantified with spot-detection
965 algorithms in Imaris 9.5 (Oxford instruments).
966

967 *Data analysis*

968 Data were analyzed and presented using ClampFit (Molecular Devices), Igor Pro
969 (Wavemetrics), MATLAB (Mathworks), and Prism (GraphPad). Data points and error bars
970 represent the mean ± standard error of the mean. All statistical analyses were conducted
971 using two-tailed comparisons. Sample sizes and statistical tests for each group are detailed in
972 the figure legends.
973

974 *Numerical integration of the simple refilling model*

975 This model posits reversible docking of vesicles at finite release sites (N_{max}) with forward (k_1)
976 and reverse rate constants (b_1) (Hosoi et al., 2007; Neher and Sakaba, 2008).

$$\underset{b_1}{\overset{k_1}{\rightleftarrows}} n \xrightarrow{p_v}$$

According to this scheme, $dn/dt = k_1\, u - b_1\, n - p_v\, n\, \delta(t - t_{AP})$, where u is the number of unoccupied sites (= $N_{max} - n$); t_{AP} is the timing of AP firing; and δ is the Dirac delta function. The p_v was assumed to be unity (***Figure 3—figure supplement 2***). The sum of basal k_1 (= $k_{1,b}$) and b_1 was be estimated as 23/s from the dependence PPR on ISIs (***Figure 3—figure supplement 2Ab***). From the baseline occupancy of 0.3 and $p_{occ} = k_{1,b} / (k_{1,b} + b_1)$, we could estimate baseline k_1 and b_1 as 6.9/s, and 16.1/s, respectively. Ca^{2+}- and Syt7-dependent increase of k_1 was modeled as $k_1 = k_{1,b} + K_{1,max}$ [Syt7: n Ca^{2+}], where [Syt7: n Ca^{2+}] represents the fraction of full Ca^{2+}-bound Syt7. For deterministic simulations, we time-integrated a set of differential equations for each model using Euler methods with a time step of 1 ms. All calculations were performed on the platform of Matlab (R2022b, Mathworks, USA).

Acknowledgements

We thank Dr. E. Neher and Dr. A. Marty for critical reading of this manuscript and invaluable comments.

Author contributions

All experiments were carried out at cell physiology lab under Y.K and S.H.L's supervision. Conception or design of the work: J.S. and S.H.L. Acquisition, analysis or interpretation of data

for the work: J.S, Y.K. and L.S.H. Drafting the work or revising it critically for important intellectual content: J.S., S.Y.L, Y.K. and L.S.H.

Funding

This study was supported by grants from the National Research Foundation of Korea (RS-2024-333669 to S-H. Lee; 2021R1I1A1A01059646 to Y. Kim), and Seoul National University Hospital (2024).

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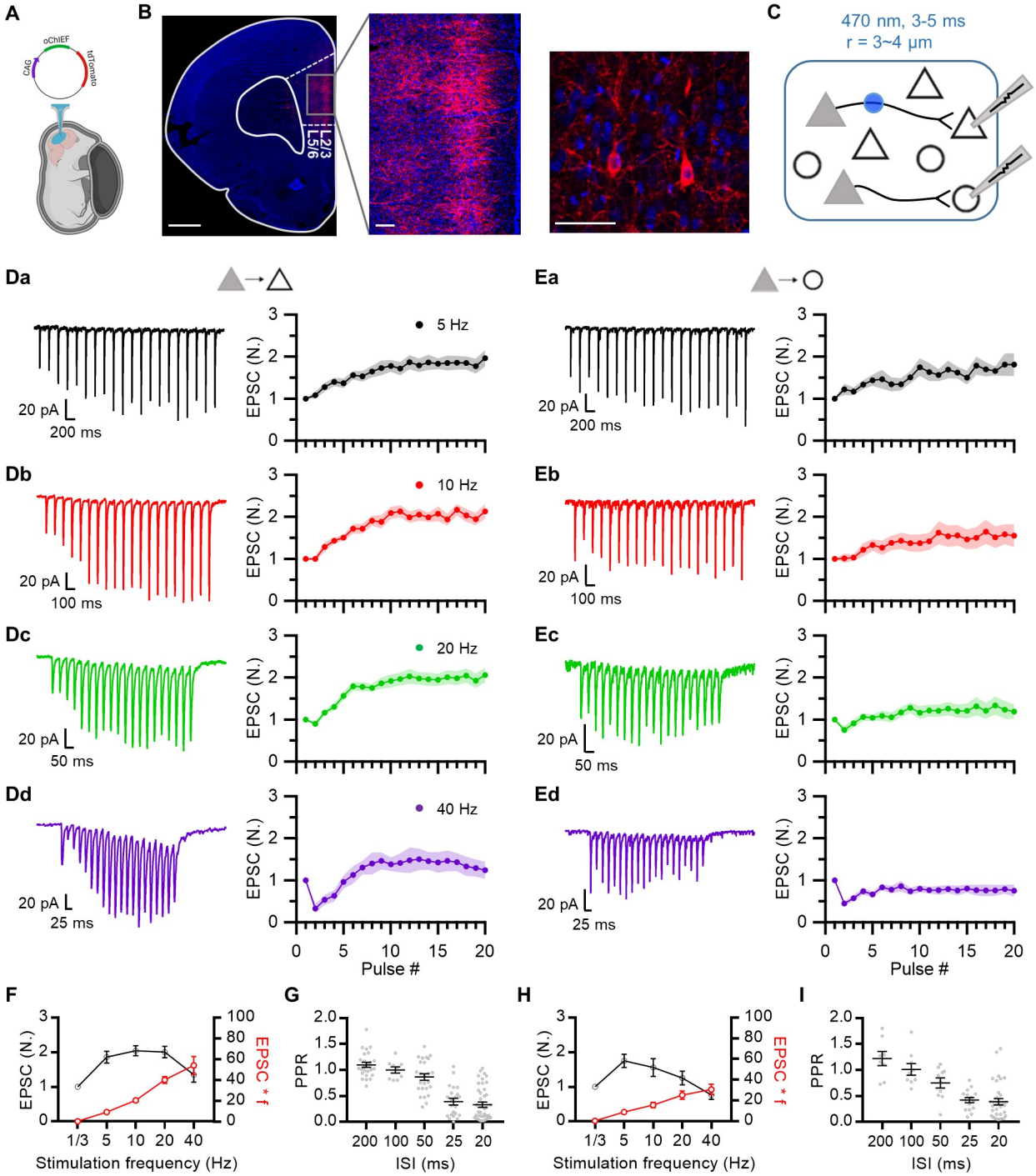

1 **Figure 1. Frequency-dependence of short-term synaptic plasticity at prelimbic L2/3**

2 **excitatory synapses.**

(**A**) In utero electroporation following injection of the plasmid (CAG-oChIEF-tdTomato) into the ventricle of an embryo (E17.5). (**B**) Representative images showing specific expression of oChIEF-tdTomato in L2/3 pyramidal cells after IUE. The red fluorescence of tdTomato clearly visualizes oChIEF-expressing cell bodies in L2/3 and axons in L5. Scale bar: 1 mm, 100 μm, 50 μm from left to right. (**C**) Recording schematic showing photostimulation of oChIEF-expressing axon fibers of transfected PCs (*filled triangles*) and a whole-cell recording from non-transfected PCs (*empty triangles*) or FSINs (*empty circles*). A collimated DMD-coupled LED was used to confine the area of excitation (typically 3–4 μm in diameter) to a small region (*blue circle*) near the soma. (**D, E**) Representative traces for EPSCs averaged over 10 trials at each frequency (*left*) and average amplitudes of baseline-normalized EPSCs (*right*) during 20-pulse trains at frequencies from 5 to 40 Hz at PC-PC (*D*; n = 12, 9, 21, 10 cells for 5, 10, 20, 40 Hz, respectively) and PC-FSIN (*E*; n = 8, 9, 10, 10) synapses. Each data point was normalized to the average of the first EPSC. (**F**) Baseline-normalized amplitudes of steady-state EPSCs ($EPSC_{ss}$; *black symbols*) and synaptic efficacy ($EPSC_{ss} \times f$; *red symbols*) as a function of stimulation frequency (*f*) at PC–PC synapses. $EPSC_{ss}$ was measured from the average of last 5 EPSCs from the 20-pulse trains (n = 12, 9, 21, 10). (**G**) Paired pulse ratio (PPR) as a function of inter-spike intervals (n = 25, 9, 25, 22, 43 for 200, 100, 50, 25, 20 ms ISI, respectively) at PC-PC synapses. (**H**) $EPSC_{ss}$ and synaptic efficacy at PC-FSIN (n = 8, 9, 10, 10). (**I**) PPR at PC-FSIN (n = 8, 9, 10, 15, 34). *Gray symbols*, individual data.

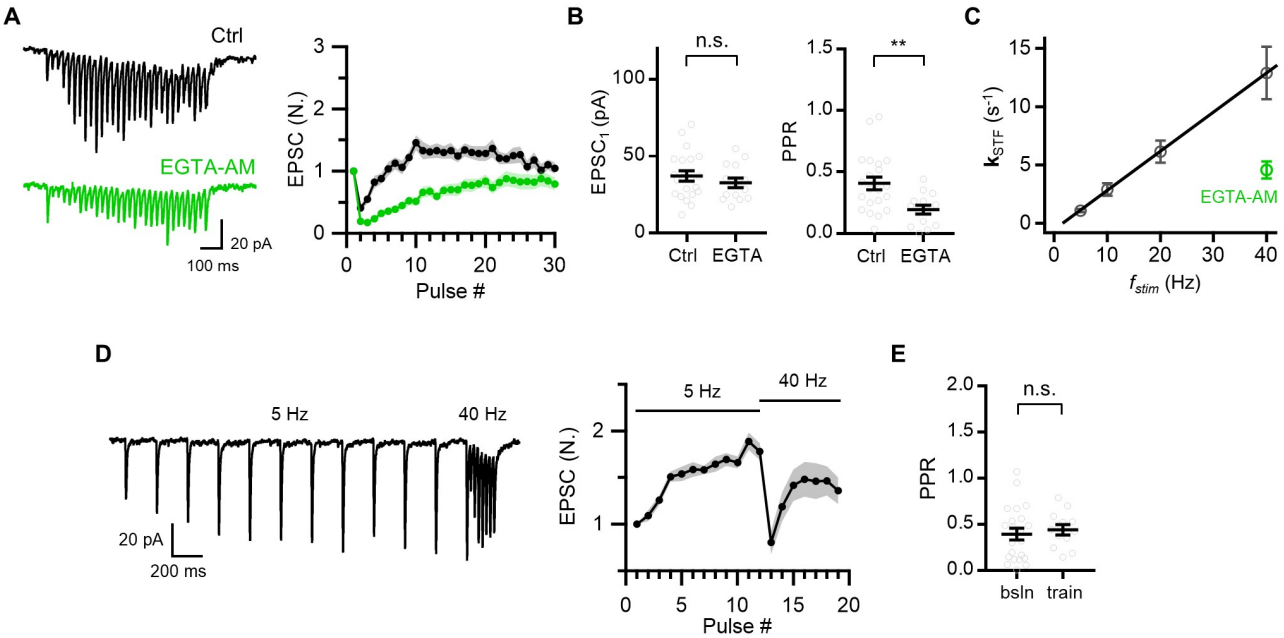

Figure 2. Delayed facilitation results from slow activation of Ca^{2+}-dependent vesicle replenishment at a constantly high vesicular fusion probability

(**A**) Representative traces (*left*) and mean baseline-normalized amplitudes (*right*) of EPSCs evoked by 30-pulse trains at 40 Hz in control (n = 21; *black*) and in the presence of 50 µM EGTA-AM (n = 14; *green*). (**B**) Mean values for the first EPSC amplitude ($EPSC_1$, *left*) and PPR (*right*) from the experiments displayed in (A). *Gray symbols*, individual data. (**C**) Plot of rate constants for short-term facilitation ($\mathbf{k}_{STF}$) as a function of stimulation frequency (f_{stim}), showing a linear relationship. The linear regression line (*black*) is shown fitted to $\mathbf{k}_{STF}$ values, estimated from ***Figure 1***. (**D**) Representative EPSC traces (*left*) and average of baseline-normalized EPSCs (*right*) evoked by 12-pulse stimulation at 5 Hz, followed by 40 Hz 7-pulse train (n = 12). Note that slowly developing facilitation was converted to rapid facilitation after strong PPD. (**E**) Mean values for PPR at 40 Hz. The baseline PPR was reproduced from ***Figure 1G*** and the PPR during 5 Hz train was calculated as (13th EPSC) / (12th EPSC). *Gray symbols*, individual data. All statistical data are represented as mean ± S.E.M.; n.s. = not significant; **, $P < 0.01$; unpaired t-test.

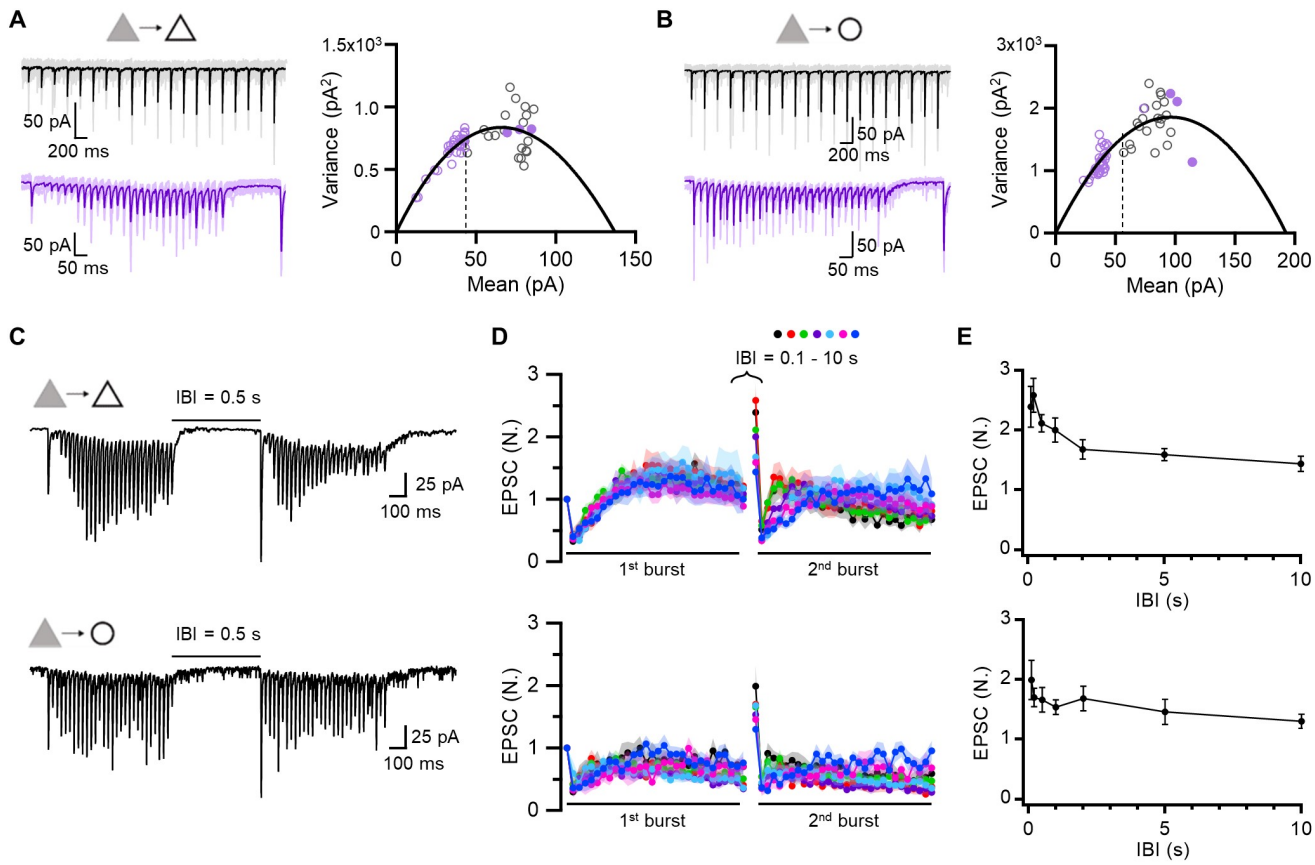

Figure 3. Low baseline occupancy of release sites and its increase during facilitation and post-tetanic augmentation

(**A, B**) *Left*, Representative EPSCs evoked by 5 and 40 Hz train stimulation (*black*, 5 Hz; *purple*, 40 Hz). *Right*, Variance-mean plots of EPSCs amplitude from averaged EPSCs recorded at PC-PC (A; n = 12, 17 for 5 and 40 Hz, respectively) and PC-FSIN (B; n = 8, 10) synapses. The data were fitted using multiple-probability fluctuation analysis (MPFA). Error bars are omitted for clarity. The 1st EPSC of 5 Hz train (*broken line*) was used to estimate the resting level of p_{occ}. *Filled circles* were measured from post-tetanic augmented EPSCs (*A*, n = 12, 12, 9; *B*, n = 7, 7, 7 for 0.1, 0.2, 0.5 s IBIs, respectively). (**C-E**) Post-tetanic augmentation (PTA) experiments at PC-PC (*top*) and PC-FSIN (*bottom*) synapses. (**C**) Representative traces for EPSCs evoked by double 40 Hz train stimulations separated by 0.5 s. (**D**) Mean baseline-normalized amplitudes of EPSCs evoked by double 40 Hz trains at different inter-burst intervals (IBIs, 0.1, 0.2, 0.5, 1, 2, 5, 10 s). *Upper*, PC-PC synapse (n = 12, 12, 21, 11, 11, 16, 11 from short to long IBIs,

respectively). *Lower*, PC-FSIN synapse (n = 10, 9, 8, 9, 9, 7, 9). (**E**) PTA time course. The baseline-normalized amplitudes of 1st EPSC from the 2nd train were plotted as a function of IBIs.

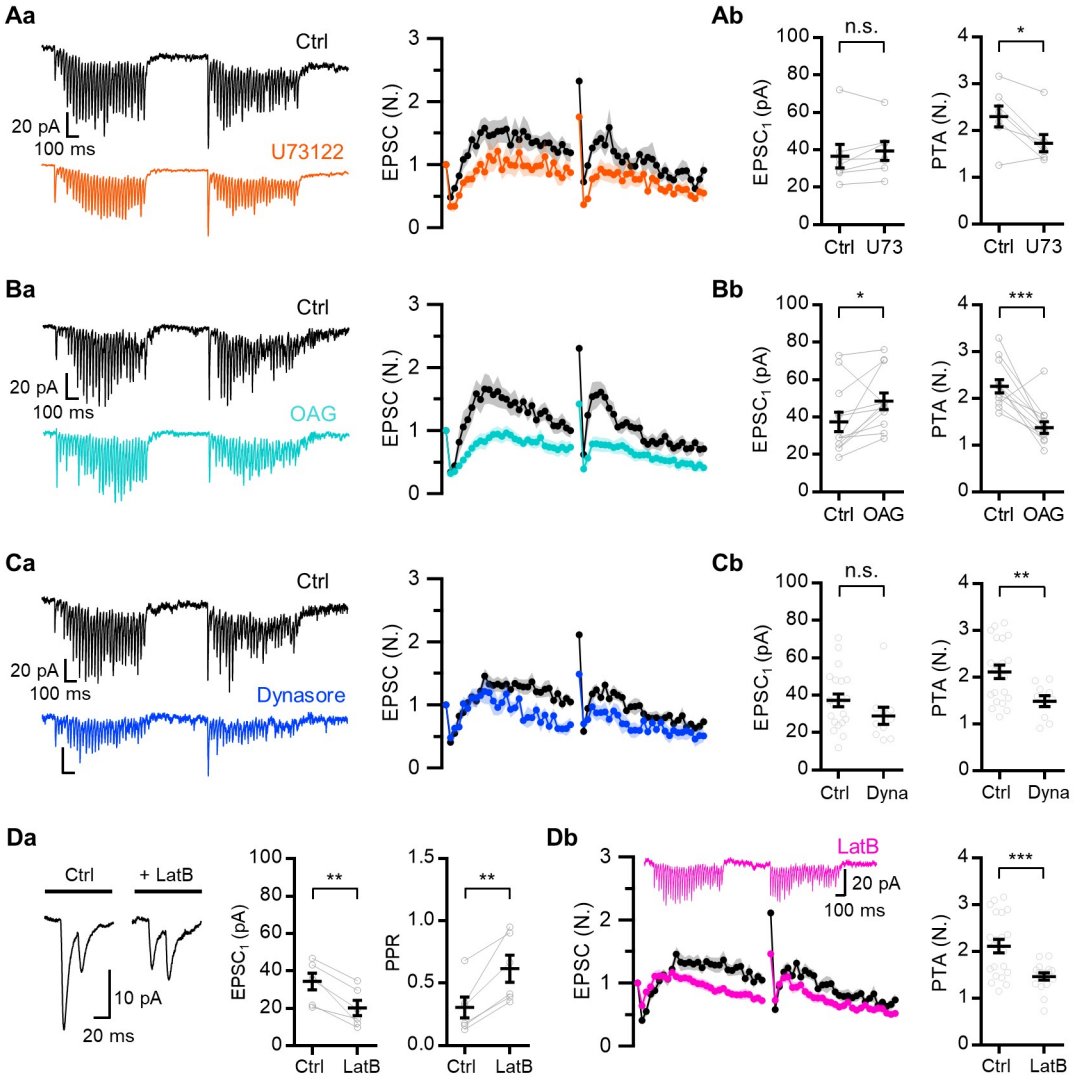

Figure 4. Pharmacological experiments reveal specific molecular mechanisms underlying vesicle loading processes

(**A-C**) (**a**) Representative EPSC traces (*left*) and mean baseline-normalized EPSCs (*right*) evoked by double 40 Hz train stimulations separated by 0.5 s inter-burst interval (IBI) in control and in the presence of 5 µM U73122 (*Aa*, n = 7, *orange*), 20 µM OAG (*Ba*, n = 12, *cyan*) or 100 µM dynasore (*Ca*, n = 10, *blue*). (**b**) Mean values for baseline EPSCs ($EPSC_1$, *left*) and augmentation (*right*) from the experiments shown in corresponding *a* panel. (**Da**) Representative EPSC traces evoked by paired pulses (*left*) and mean values for baseline EPSC amplitude (*middle*) and PPR (*right*) before and after applying 20 µM LatB (n = 6). (**Db**) Representative EPSC traces

66 (*left, upper*) and average of normalized EPSCs (*left, lower*) evoked by double 40 Hz train
67 stimulation separated by 0.5 s in control (n = 21) and in 20 µM LatB conditions (n = 16; *pink*).
68 *Right*, Mean values for augmentation in control and LatB conditions. *Gray symbols*, individual
69 data. All statistical data are represented as mean ± S.E.M., *, $P < 0.05$; **, $P < 0.01$; ***, $P <$
70 0.001; unpaired or paired t-test; n.s. = not significant.
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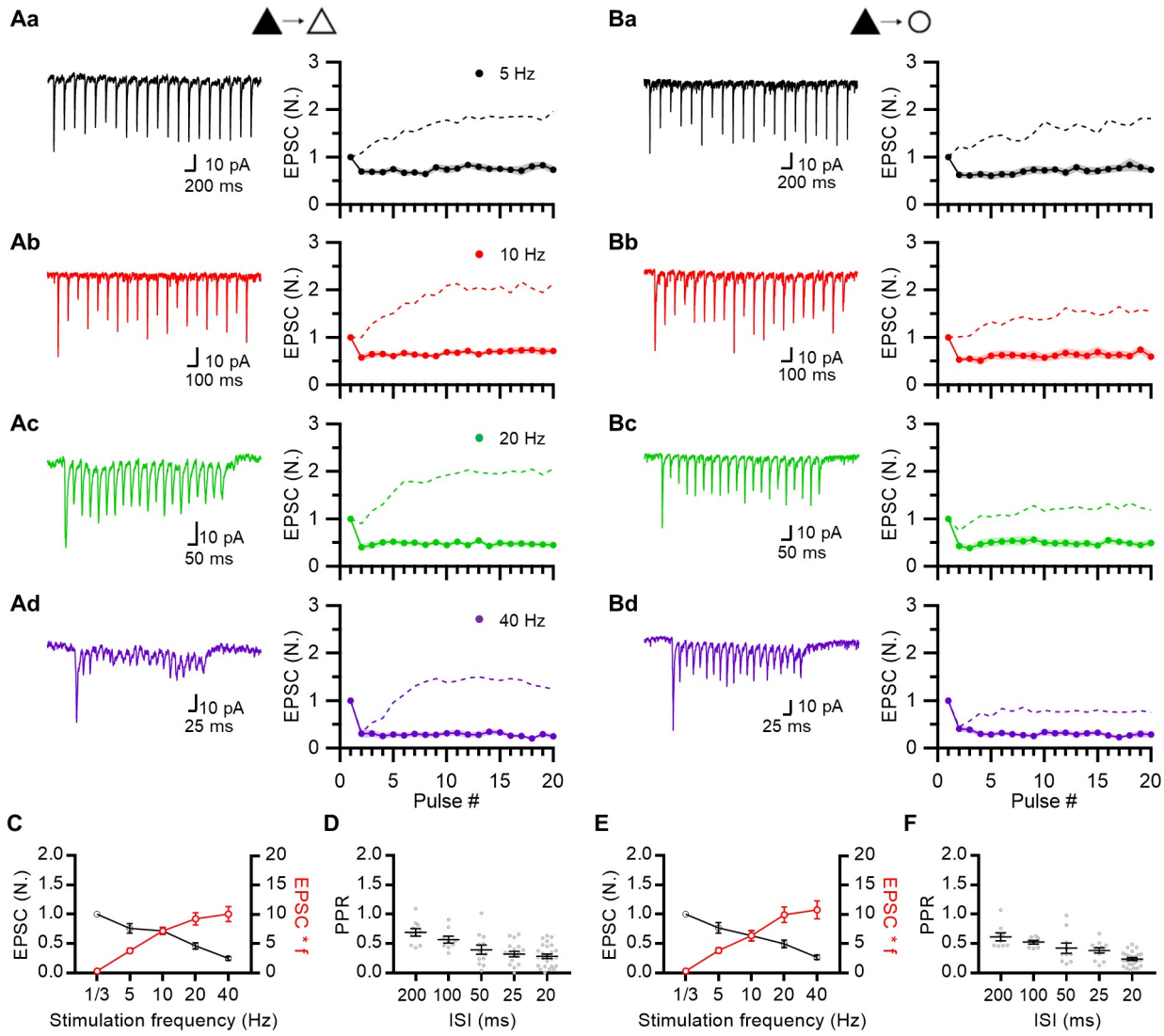

73 **Figure 5. STF at both types of local excitatory synapses is abolished by Syt7 KD**

74 (**A, B**) Representative EPSC traces (*left*) and mean baseline-normalized amplitudes of EPSCs
75 (*right*) evoked by 20-pulse trains at 5 to 40 Hz. STP was measured at PC-PC (*A*; n = 10, 9, 12,
76 9) and PC-FSIN (*B*; n = 9, 8, 10, 9) synapses, in which presynaptic Syt7 transcripts were
77 depleted (Syt7 KD). Syt7 KD pyramidal cells are indicated as *black triangles* on the top. For
78 comparison, STP in WT synapses is reproduced from ***Figure 1*** (*dotted lines*). Same frequency
79 color codes were used as in ***Figure 1***. (**C**) Baseline-normalized amplitudes of steady-state EPSC
80 ($EPSC_{ss}$; *black symbols*) and synaptic efficacy ($EPSC_{ss} \times f$; *red symbols*) as a function of

81 stimulation frequency (*f*) at PC-PC synapses. $EPSC_{ss}$ was defined as the average of last 5 EPSC
82 amplitudes from 20-pulse trains. (**D**) PPR as a function of inter-spike intervals (n = 10, 9, 12,
83 18, 26). (**E**) $EPSC_{ss}$ and synaptic efficacy at PC-FSIN synapses. (**F**) PPR at PC-FSIN synapses (n =
84 9, 8, 10, 12, 24). *Gray symbols*, individual data.
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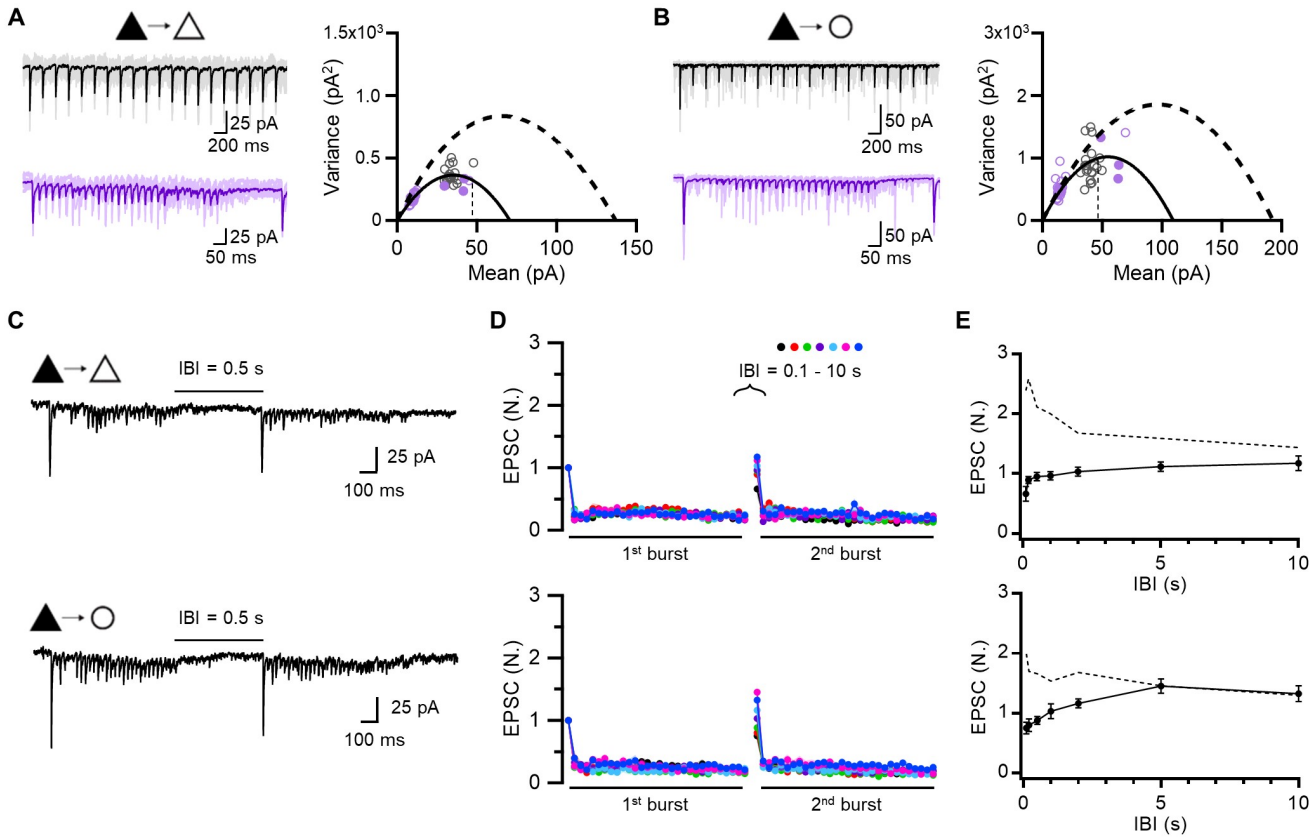

Figure 6. Syt7 KD synapses exhibit complementary changes in the number of release sites and their vesicle occupancy

(**A, B**) *Left*, Representative traces of EPSCs evoked by 5 and 40 Hz train stimulations (*black*, 5 Hz; *purple*, 40 Hz). *Right*, Variance-mean plots of EPSCs amplitude from averaged EPSCs recorded at PC-PC (*A*; n = 9, 15, 12, 10, 9) and PC-FSIN (*B*; n = 4, 14, 10, 9, 8) synapses in which presynaptic Syt7 has been knocked down. The data were fitted using MPFA and error bars are omitted for clarity. The mean 1st EPSC amplitude of 5 Hz train (*vertical broken line*) was used for estimation of baseline p_{occ}. Dashed parabolas indicate MPFA fits to variance-mean plot of WT synapses reproduced from *Figure 3*. (**C-E**) Recovery experiments at PC-PC (*top*) and PC-FSIN (*bottom*) synapses in which presynaptic Syt7 was knocked-down. (**C**) Representative EPSCs evoked by double 40 Hz train stimulations separated by 0.5 s. (**D**) Mean baseline-normalized amplitudes of EPSCs evoked by double 40 Hz trains at PC-PC (n = 12, 10, 9, 9, 10, 9, 8) and PC-FSIN (n = 10, 9, 10, 11, 8, 9, 10) synapses at different interburst intervals (IBIs).

100 (**E**) Recovery time course. Baseline-normalized amplitudes of 1st EPSC from the 2nd burst were
101 plotted as a function of various IBIs. *Dotted lines* indicate augmented EPSCs in the WT
102 reproduced from ***Figure 3***.
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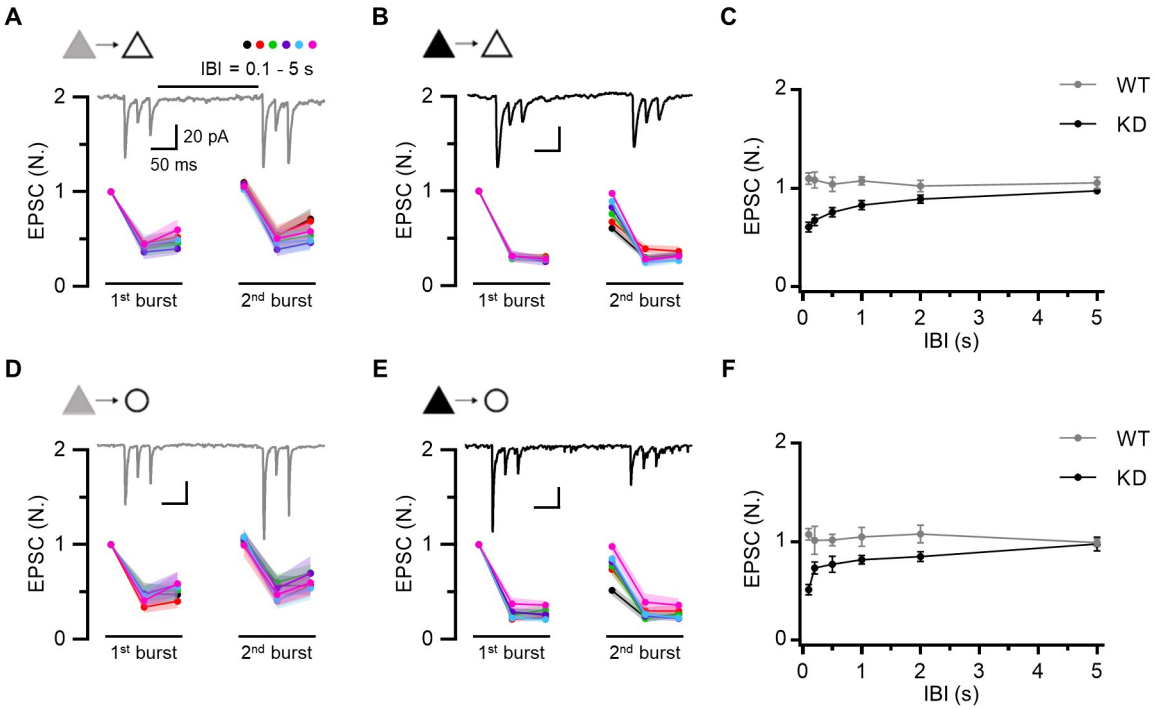

105 **Figure 7. Recovery of TS vesicles following depletion is accelerated by Syt7**

106 (**A-C**). Recovery experiments at PC-PC synapses in WT (*A*) and Syt7 KD (*B*). Mean baseline-
107 normalized amplitudes of EPSCs evoked by two consecutive 3-pulse 40 Hz trains in WT (n =
108 16, 13, 11, 11, 11, 9 from short to long IBIs, respectively) and KD (n = 13, 11, 12, 8, 10, 11)
109 synapses at different IBIs (0.1, 0.2, 0.5, 1, 2, 5 s). *Inset*, representative traces of EPSCs evoked
110 by two consecutive 3-pulse 40 Hz train stimulations separated by 0.2 s. (**D-F**). Recovery
111 experiments at PC-FSIN synapses in WT (*D*) and Syt7 KD (*E*). Mean baseline-normalized
112 amplitudes of EPSCs evoked by two consecutive 3-pulse 40 Hz trains in WT (n = 9, 11, 11, 9,
113 10, 13) and KD (n = 9, 12, 9, 7, 10, 9) synapses at different IBIs. *Inset*, representative traces of
114 EPSCs evoked by two consecutive 3-pulse 40 Hz train stimulations separated by 0.2 s. (**C, F**)
115 Recovery time course. Baseline-normalized amplitudes of 1st EPSC from the 2nd burst were
116 plotted as a function of various IBIs.

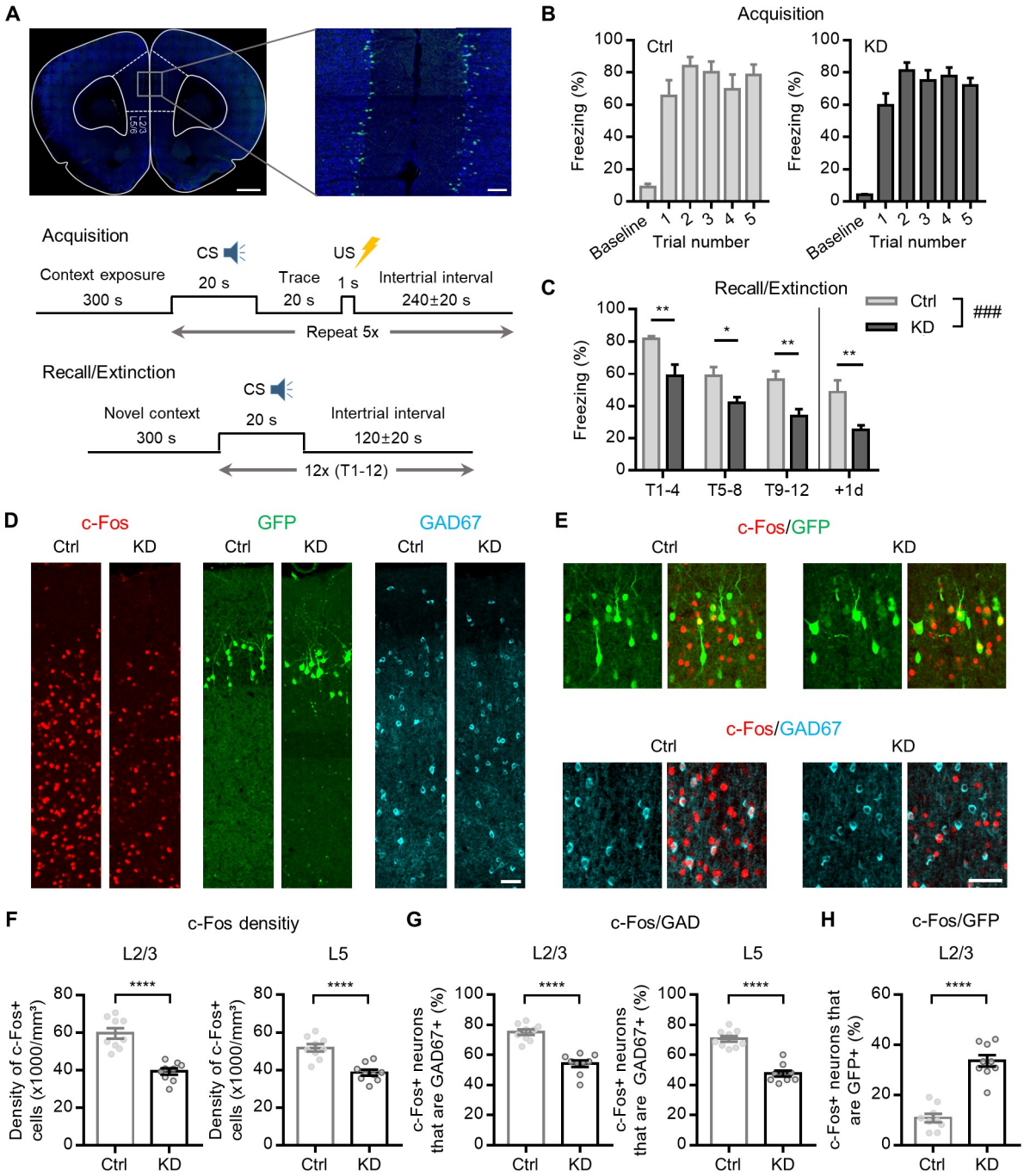

Figure 8. Behavioral effects of Syt7 deficiency in L2/3 PCs of the mPFC

(**A**) *Top*, Representative images showing bilateral expression of U6-GFP in L2/3 of PCs after IUE at E17.5. Scale bar: 1 mm, 100 µm. *Bottom*, Schematic of trace fear conditioning and extinction (tone test) protocol. (**B**) Freezing behavior of control (expressing scrambled shRNA, *Scr*) and Syt7 KD rats during acquisition of tFC. (**C**) Freezing ratio during tone tests on following

122 days. Data are shown as average freezing during T1–T4, T5–T8, or T9–T12 (T, trials). The
123 freezing on T1-T4 was significantly lower in KD rats suggesting that formation of trace memory
124 was impaired in Syt7 KD rats (n = 10, 11 for Ctrl and KD, respectively; P = 0.0007, F(1, 19) =
125 16.43, two-way repeated measures ANOVA; P = 0.0064, 0.0211, 0.0064, 0.0064; Holm-Sidak
126 test). (**D**) Representative images of c-Fos immunoreactivity in the prelimbic cortex of control
127 or Syt7 KD rats 90 min after tFC acquisition. c-Fos (*left*, *red*, Cy5) and GAD67 (*right*, *cyan*, Cy3)
128 were immunostained in the same brain slice expressing U6-GFP (*middle*; *green*). Scale bar, 50
129 µm. (**E**) Exemplar images of c-Fos positive neurons expressing GFP (*top*) or GAD67 (*bottom*)
130 in control (*left*) or Syt7 KD rats (*right*). Scale bar, 50 µm. (**F-H**) Effects of Syt7 KD on c-Fos
131 density (*F*) and percentage of c-Fos positive neurons co-labeled with GAD67 (*G*) or GFP (*H*) in
132 L2/3 or L5 of prelimbic cortex (n = 9, 9 for Ctrl and KD, respectively). *Open symbols*, individual
133 data. All statistical data are represented as mean ± S.E.M.; ****, P＜0.0001; unpaired t-test.
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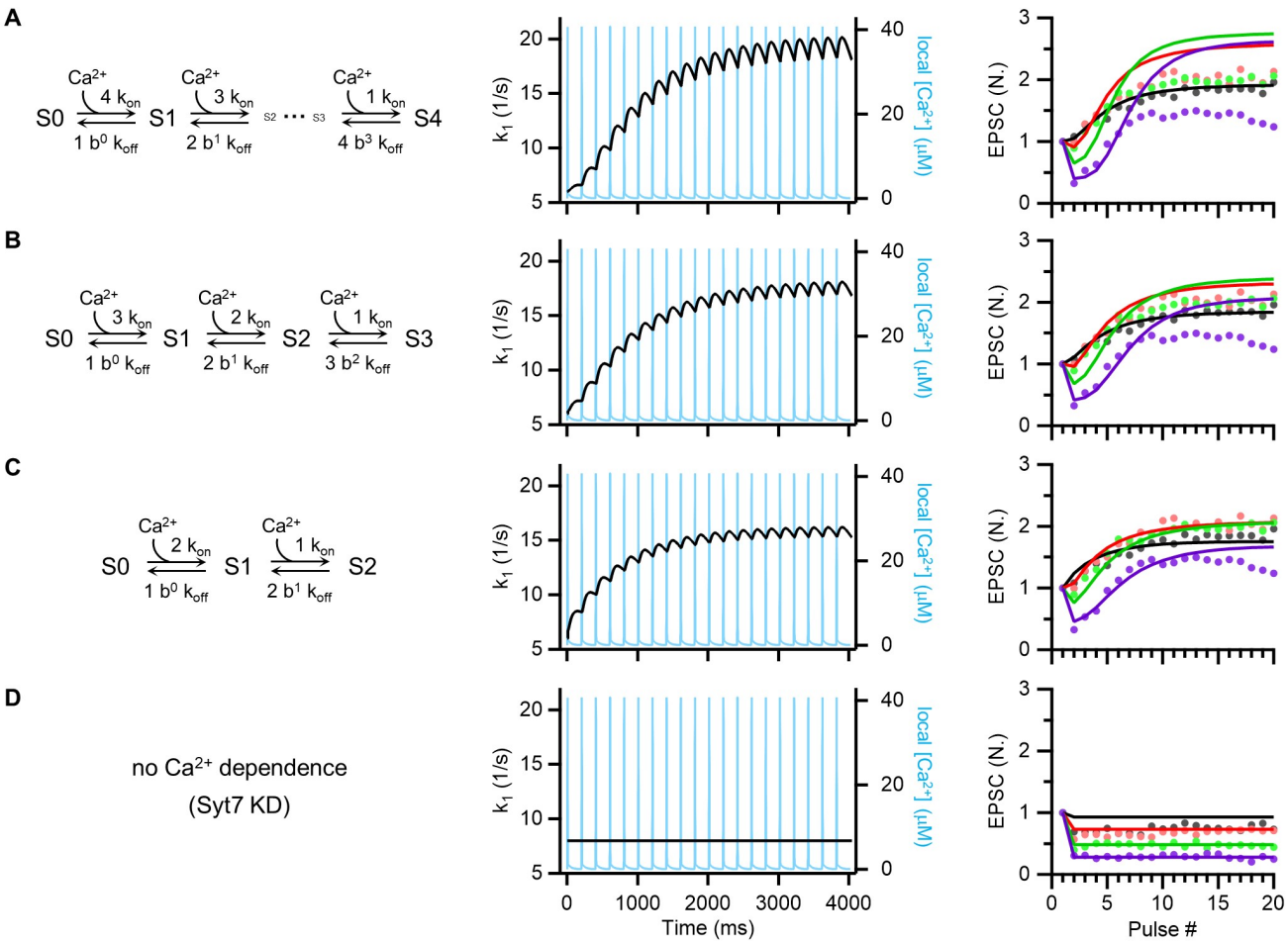

136 **Figure 9. STP model in light of known Ca^{2+} binding kinetics of Syt7**

137 (**A-D**) *Left*, Schematic of allosteric calcium binding to Syt7. The number of Ca^{2+} bound to Syt7
138 was denoted as # in 'S#' in the reaction scheme. k_{on} = 7/μM/s, k_{off} = 10/s. *Middle*, Simulated
139 changes of k_1 (*black*) in response to 5 Hz train of Ca^{2+} transients (*light blue traces*). The priming
140 step of the simple refilling model was assumed to be catalyzed by full Ca^{2+}-bound form of Syt7.
141 Accordingly, Ca^{2+}-dependent increase in k_1 was calculated as $K_{1,max}$ multiplied by a fraction of
142 full Ca^{2+}-bound form of Syt7. We assumed that local $[Ca^{2+}]_i(t)$ follows a Gaussian function: $(1/\sigma$
143 $\sqrt{2\pi})\exp[-(t-t_p)^2/2\sigma^2]$, in which t_p = 0.25 ms and σ = 0.085 ms. *Right*, Fits of the Syt7 model to
144 the STP data. To fit this model to the STP data, $K_{1,max}$ was set to 300/s (*A*), 220/s (*B*), and 180/s
145 (*C*). Cooperativity factor (*b*) was set to 0.35 (*A*), 0.2 (*B*), and 0.05 (*C*). k_1 is set to be constant
146 for Syt7 KD (*D*). Same frequency color codes were used as in ***Figure 1***.

Supplementary Figures

Progressive overfilling of readily releasable pool underlies short-term facilitation at recurrent excitatory synapses in layer 2/3 of the rat prefrontal cortex

Jiwoo Shin[1,2,3], Seung Yeon Lee[2], Yujin Kim[1,2,3*], Suk-Ho Lee[1,2,3*]

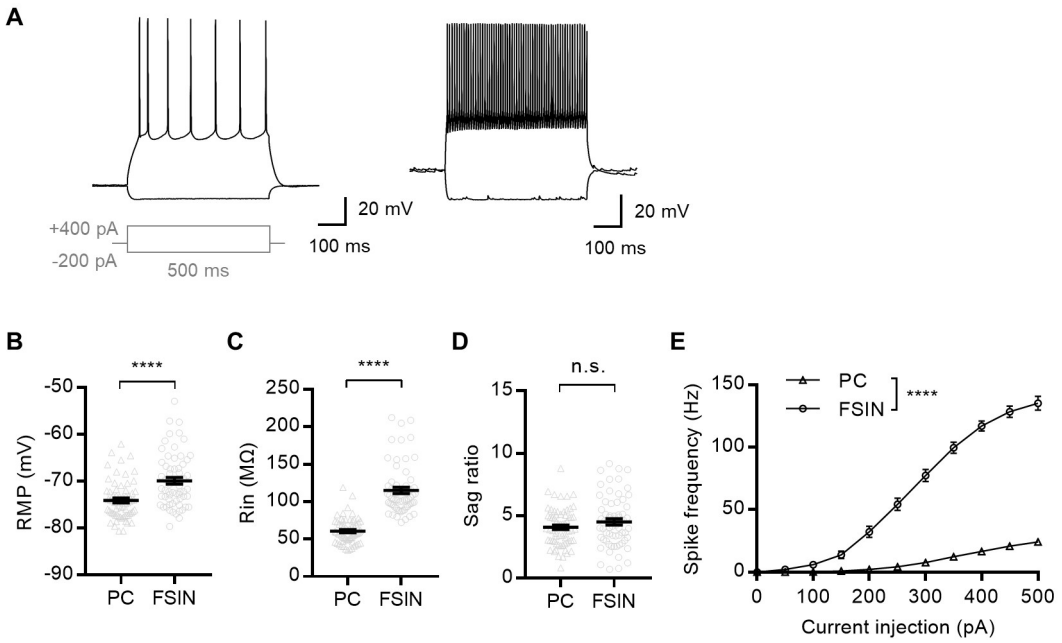

Figure 1—figure supplement 1. Intrinsic membrane properties of mPFC neurons.

(**A**) Neuronal subtypes in mPFC layer 2/3. Cells were characterized based on their electrophysiological properties (n = 66, 64 for PC and FSIN, respectively). Representative voltage responses to current injection (-200 or +400 pA) of pyramidal cells (*left*) and fast-spiking interneurons (*right*). (**B-D**) Mean resting membrane potential (*B*), input resistance (*C*), and Sag ratio (*D*) measured in layer 2/3 PCs and FSINs. *Gray symbols*, individual data. (**E**) Current-spike relationship in each cell type. All statistical data are represented as mean ± S.E.M.; n.s. = not significant; ****, $P < 0.0001$; unpaired t-test or two-way ANOVA.

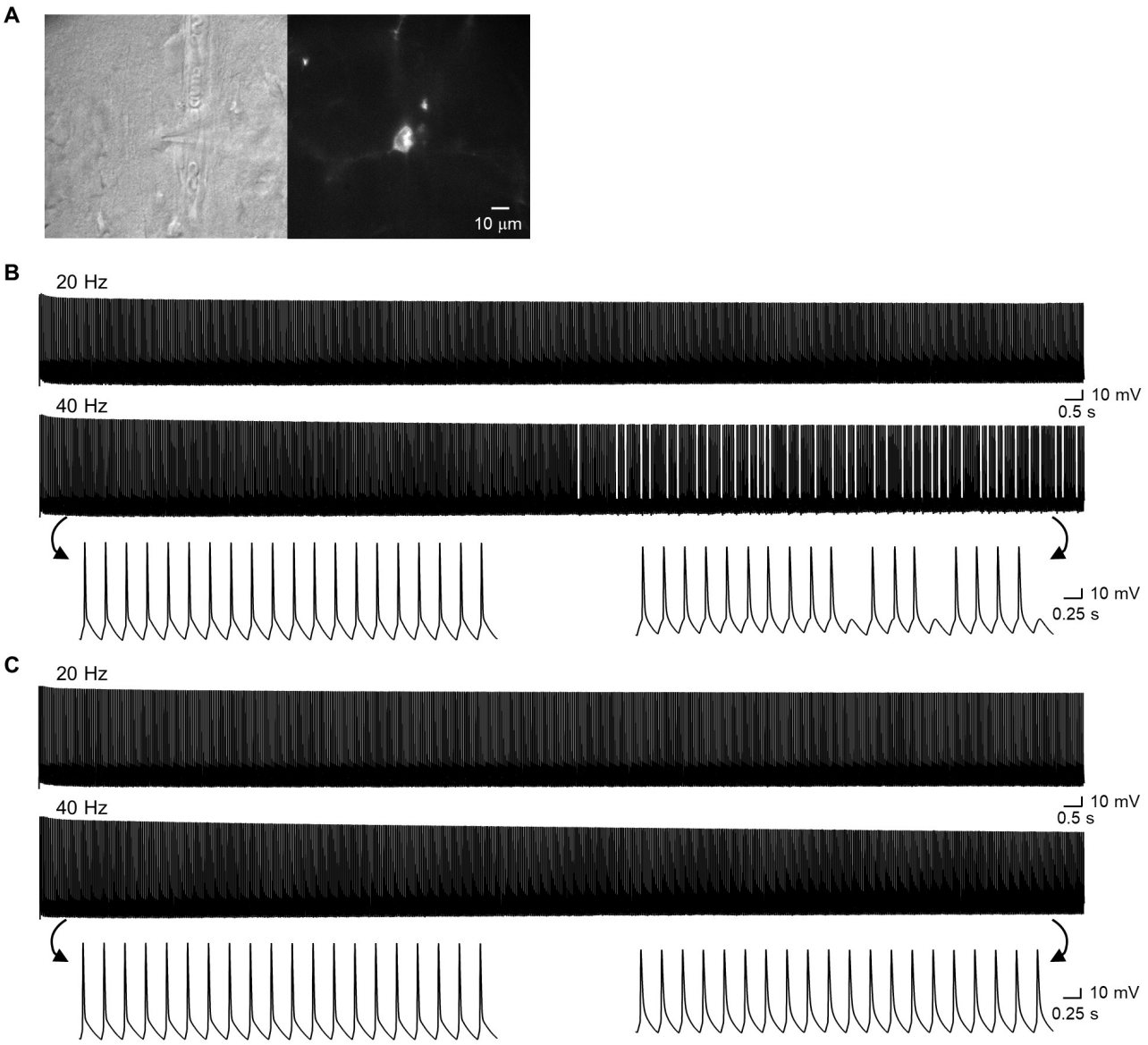

Figure 1—figure supplement 2. Test for consistency of light stimulation in oChIEF-expressing L2/3 pyramidal cells.

(**A**) Representative images of an oChIEF-expressing neuron. Whole-cell recordings were performed from the fluorescently labeled neurons in layer 2/3 of the prelimbic cortex. *Left*, IR-DIC image (60×) of the pyramidal cell under whole-cell patch clamp. *Right*, fluorescence image of td-Tomato in the same cell excited at 530 nm wavelength. (**B**) Current-clamp recordings of action potentials evoked by 600 light pulse trains from an oChIEF-expressing

26 L2/3 cell. For somatic stimulation, we used the typical light stimulation conditions used in STP
27 experiments (5 ms in duration and 4 µm in radius; see *Methods*). Uniform action potentials
28 were produced with high fidelity, albeit not without compromise towards the end of the train
29 in the case of 40 Hz stimulation. Note that these results are similar to previously reported
30 values from neocortical neurons (Lin et al., 2009; Yoon et al., 2020). (**C**) Same experiments as
31 (*B*) but in Syt7 KD pyramidal cells that express oChIEF in L2/3 of the mPFC.
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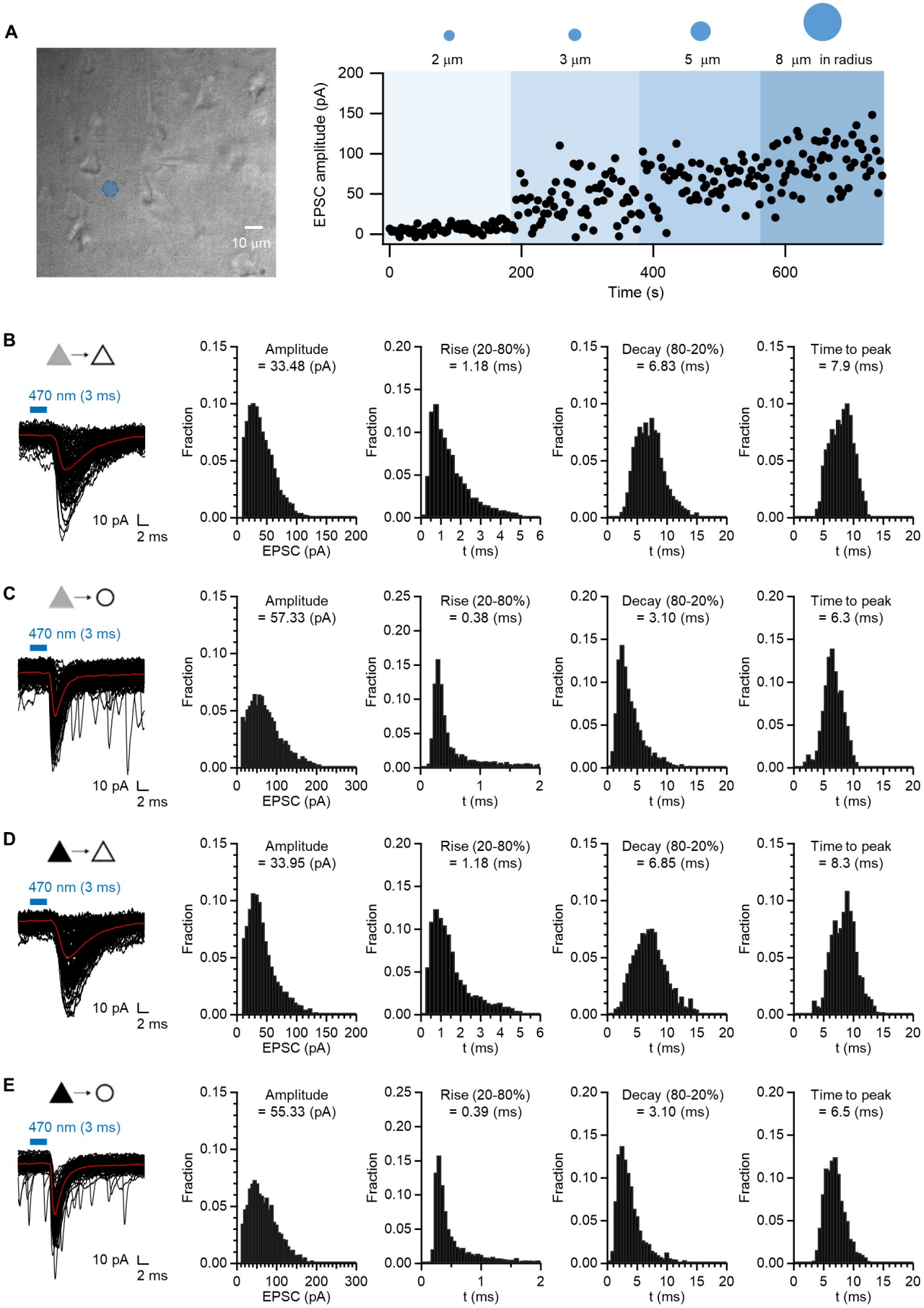

A
10 µm
2 µm
3 µm
5 µm
8 µm in radius
EPSC amplitude (pA)
Time (s)
B
470 nm (3 ms)
10 pA
2 ms
Amplitude = 33.48 (pA)
Rise (20-80%) = 1.18 (ms)
Decay (80-20%) = 6.83 (ms)
Time to peak = 7.9 (ms)
C
Amplitude = 57.33 (pA)
Rise (20-80%) = 0.38 (ms)
Decay (80-20%) = 3.10 (ms)
Time to peak = 6.3 (ms)
D
Amplitude = 33.95 (pA)
Rise (20-80%) = 1.18 (ms)
Decay (80-20%) = 6.85 (ms)
Time to peak = 8.3 (ms)
E
Amplitude = 55.33 (pA)
Rise (20-80%) = 0.39 (ms)
Decay (80-20%) = 3.10 (ms)
Time to peak = 6.5 (ms)
Fraction
EPSC (pA)
t (ms)

35 **Figure 1—figure supplement 3. Properties of EPSCs evoked by minimal stimulation.**

36 (**A**) Optical minimal stimulation. A collimated DMD-coupled LED was used to confine the area
37 of excitation to a small region near the soma. To stimulate a minimal number of excitatory
38 axon fibers, the radius of illumination area was increased from 2 µm by 50% at each step, and
39 selected the smallest illumination area that elicits EPSCs (Yoon et al., 2020) before starting
40 each recording session. The illumination area was in the range of 3 to 8 µm in radius, when
41 measured at the focal plane (typically 3–4 µm) of a 60× water immersion objective (*blue circle*
42 in the photograph). (**B-E**) Properties of minimally stimulated baseline EPSCs (failure-excluded).
43 Medians of frequency distributions are presented. Decay times below 1 ms were excluded
44 from analysis. From left to right, representative traces (100 pulses), EPSC amplitude, rise time
45 (20-80%), decay time (80-20%), and time to peak (from stimulus onset) from WT PC-PC (*B*, n
46 = 81), WT PC-FSIN (*C*, n = 77), Syt7 KD PC-PC (*D*, n = 72), and Syt7 KD PC-FSIN (*E*, n = 78)
47 synapses.
48
49

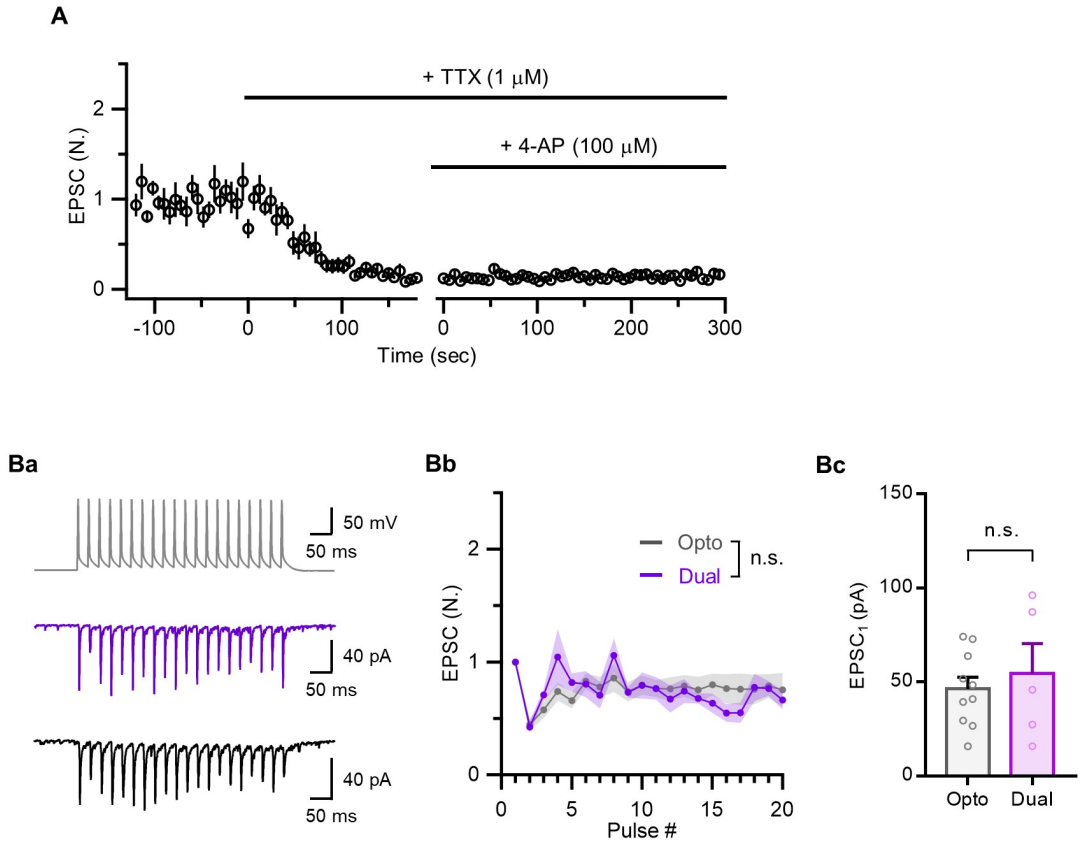


Figure 1—figure supplement 4. Validation of STP of optically evoked EPSCs

(**A**) The high p_v at PC-PC synapses may result from direct photo-stimulation of axon boutons. To test if optically evoked EPSCs depend on APs, the effect of 1 µM tetrodotoxin (TTX) was tested. Applying 1 µM tetrodotoxin (TTX) and additionally 0.1 mM 4-aminopyridine, a D-type K channel blocker abolished optically evoked EPSCs (n = 8). Even when repolarization was hindered by subsequent addition of 100 µM 4-aminopyridine (4-AP), a potassium channel blocker, EPSCs were not rescued. These findings suggest that the light-evoked responses are dependent on APs of presynaptic axon fibers rather than photo-induced depolarization of presynaptic boutons (Little and Carter, 2013). (**B**) Comparison of STP evoked by optical stimulation with that by dual patch techniques. To test validity of STP of photostimulation-evoked EPSCs, we compared STP of unitary EPSCs evoked by dual whole-cell patch at PC-FSIN synapses. Both of EPSC1 and STP at 40 Hz recorded by using paired whole-cell recordings were not significantly different from those evoked by photo-stimulation, supporting the validity of

our optogenetic methods. (**Ba**) Representative EPSC traces evoked by 20-pulse 40 Hz trains stimulation at pyramidal cell to fast spiking interneuron (PC-FSIN) synapses. A train of APs in a presynaptic PC (*top*), evoked EPSCs in a FSIN by dual whole-cell patch clamp (*middle*) or by optical stimulation (*bottom*) under same conditions. (**Bb**) Mean values for baseline-normalized amplitudes of EPSCs evoked by 40 Hz train at PC-FSIN synapses. *Opto*, optical stimulation (n = 10); *Dual*, dual patch techniques (n = 5). STP evoked by the two techniques were not significantly different (two-way repeated measures ANOVA, $F(1, 13) < 0.01$, $P = 0.955$). (**Bc**) Mean values for the first EPSC in different stimulation methods ($P = 0.5724$, unpaired t-test). *Open circles*, individual data. All statistical data are represented as mean ± S.E.M.

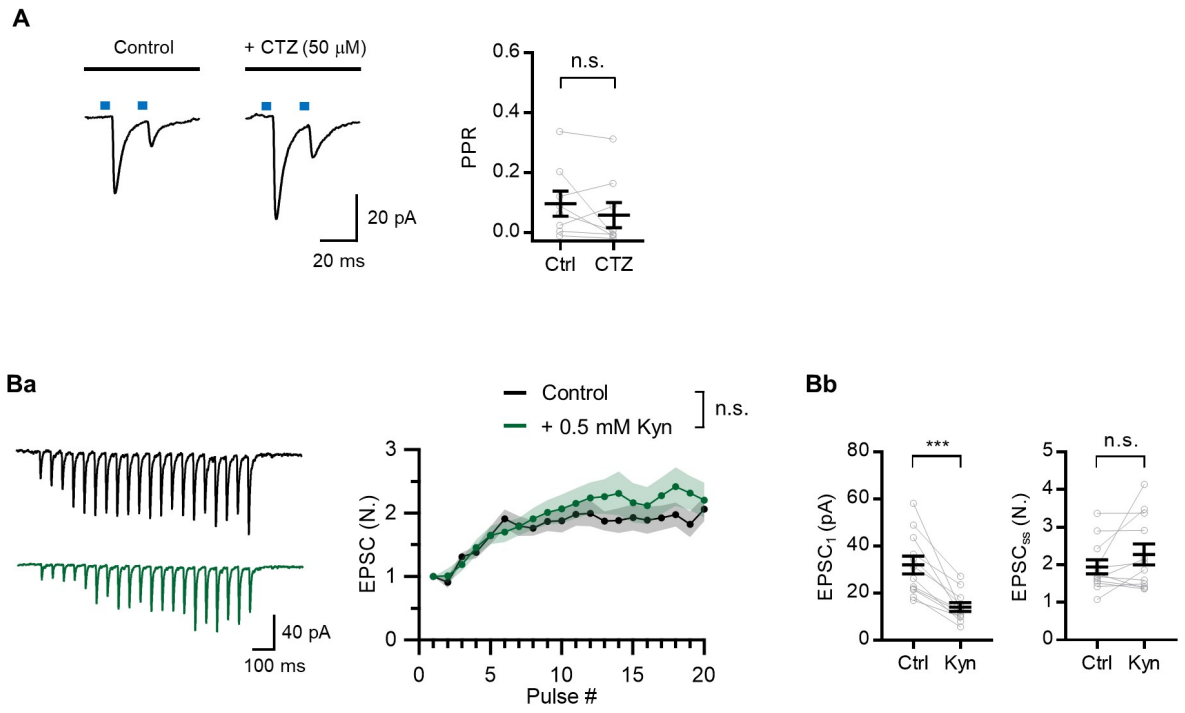
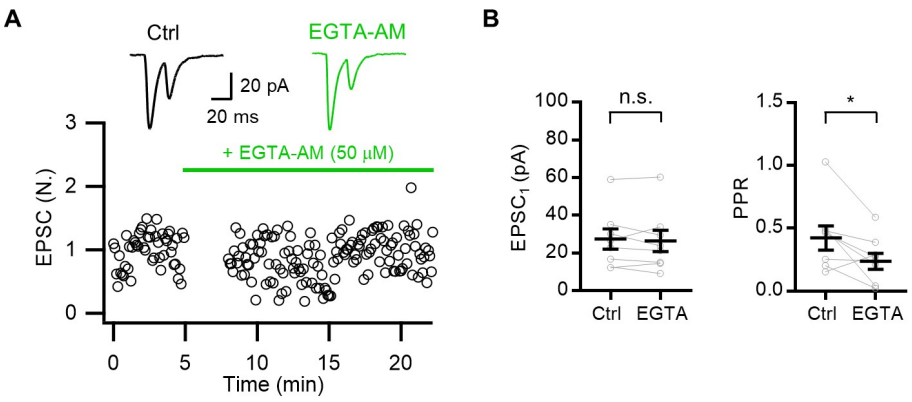


Figure 1—figure supplement 5. Test for AMPAR desensitization and saturation

(**A**) Representative traces of EPSCs evoked by paired-pulse photostmulation at 20 ms ISI (*left*) and mean values for paired pulse ratio (PPR, *right*) before and after applying 50 μM cyclothiazide (CTZ, n = 8), an AMPA receptor (AMPAR) desensitization inhibitor. CTZ induced no significant change in PPR arguing against the possibility that strong PPD is caused by AMPAR desensitization. *Gray symbols*, individual data. (**B**) It has been shown that multi-vesicular release occurs at recurrent excitatory synapses in neocortical L2/3 (Holler et al., 2021). Whereas a single vesicle release is insufficient to saturate postsynaptic AMPARs, an increase in the number of vesicular release during facilitation may saturate AMPAR resulting in underestimation of facilitation. To test this possibility, facilitation at 20 Hz was measured in the presence of 0.5 mM kynurenic acid (Kyn), a fast competitive antagonist (Diamond and Jahr, 1997; Wadiche and Jahr, 2001). Fast blockade of AMPA receptors did not have significant effects on short-term facilitation at excitatory synapses in the mPFC L2/3, suggesting that AMPAR saturation may not be significant during facilitation. (**Ba**) Representative EPSC traces (*left*) and average of baseline-normalized EPSCs (*right*) evoked by 40 Hz 20-pulse trains in

control and in the presence of 0.5 mM kynurenic acid (Kyn). Although facilitation was slightly increased by Kyn, it was not statistically significant (n = 12; two-way repeated measures ANOVA, F (1, 22) = 0.575, P = 0.456). The slightly higher facilitation may result from reduced blocking efficiency of Kyn upon increased glutamate concentration in the synaptic cleft (Tong and Jahr, 1994; Meyer et al., 2001). (**Bb**) Mean values for the baseline EPSC (*left*) and the steady-state EPSC (*right*) before and after applying 0.5 mM kynurenate as shown in *Ba*. The baseline EPSC was reduced by Kyn to 43.5% of control. *Gray symbols*, individual data. All statistical data are represented as mean ± S.E.M.; n.s. = not significant; ***, $P < 0.001$; paired t-test.



101 **Figure 2—figure supplement 1. Effects of EGTA-AM on synaptic transmission**

102 (**A**) Representative traces (*top*) and time courses of normalized amplitudes of the first EPSCs
103 (*bottom*) during paired-pulse photostimulation at 20 ms ISI in control and in the presence of
104 50 μM EGTA-AM (n = 8). The green horizontal bar indicates application of 50 μM EGTA-AM.
105 (**B**) Mean values for the first EPSC (*left*) and PPR (*right*) before and after EGTA-AM application.
106 *Gray symbols*, individual data. All statistical data are represented as mean ± S.E.M.; n.s. = not
107 significant; *, $P < 0.05$; paired t-test.

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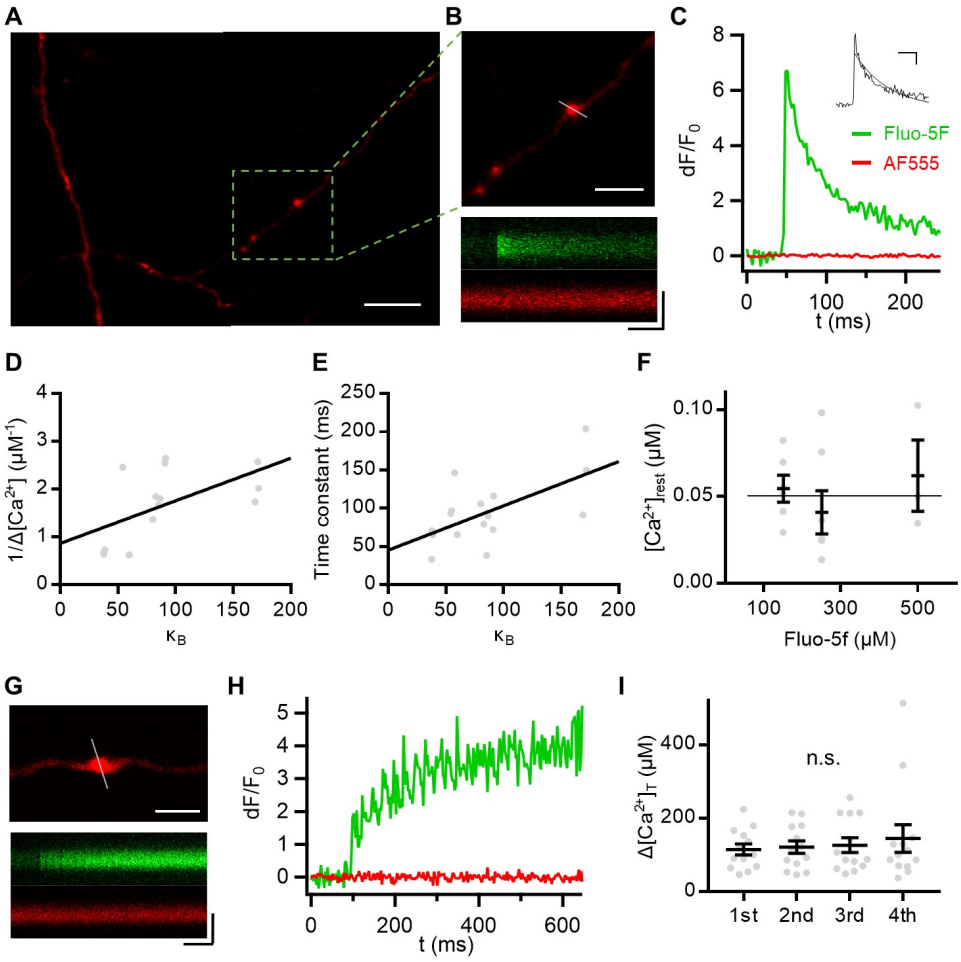

110 **Figure 2—figure supplement 2. Presynaptic calcium kinetics at boutons of layer 2/3**
111 **pyramidal cells**

112 (**A-C**) A mixture of Fluo-5F (150, 250 or 500 μM) and Alexa Flour 555 was loaded on their
113 axonal boutons of L2/3-PCs through whole-cell patch techniques for dual excitation
114 ratiometric measurements of the cytosolic [Ca^{2+}] at axonal boutons. (**A**) The long-branched
115 stretch of axon from L2/3 PCs visualized through maximum fluorescence intensity projection
116 using confocal laser scanning of AF555 perfused via a patch clamp pipette. Scale bar, 10 μm.
117 (**B**) *Top*, the selected bouton for recordings, among the three, is displayed with the
118 corresponding scanning line. Scale bar, 5 μm. *Bottom*, Line-scan images for fluorescence of
119 Fluo-5F and AF555. Scale bars, 50 ms and 2 μm. (**C**) Baseline-normalized fluorescence changes
120 evoked by an AP. Fluorescence intensities were spatially averaged over the bouton shown in

121 (*B*), background-subtracted and normalized to the baseline value (dF/F_0). *Inset*, Ca^{2+} transient
122 estimated from dF/F_0. Scale bar, 50 ms and 100 nM. (**D, E**) The kinetic parameters of an AP-
123 CaT were determined from a plot for the inverse of AP-evoked [Ca^{2+}] increment ($1/\Delta[Ca^{2+}]$) *vs*.
124 calcium binding ratio (κ_B; Eq. 4 in *Material and Methods*). (**D**) Reciprocal plot of the AP-induced
125 [Ca^{2+}] changes ($1/\Delta[Ca^{2+}]$) vs. corresponding calcium binding constants (κ_B) according to Eq. 2
126 (n = 14). A linear fit to the plot gave the amplitude of AP-CaT (A_{Ca} = 1.16 μM) from the y-
127 intercept, representing the amplitude of free [Ca^{2+}] increase in the absence exogenous Ca^{2+}
128 buffer. The calcium binding ratio of endogenous static Ca^{2+} buffers (κ_S) was estimated as 96
129 from the x-intercept of the linear regression based on Eq. 2 in *Material and Methods*. Linear
130 regression line, P = 0.035. (**E**) Plot of [Ca^{2+}] decay time constant versus κ_B (n = 16). The time
131 constant for [Ca^{2+}] decay from mono-exponential fit was plotted vs. κ_B, and a linear regression
132 based on Eq. 3 yielded the κ_S value of 77 from the x-intercept. The y-intercept was 43 ms, an
133 estimate for a dye-independent value for Ca^{2+} decay time constant (τ_c) (Eq. 3 in *Material and*
134 *Methods*). Linear regression line, P = 0.011. (**F**) Resting [Ca^{2+}] measured from basal
135 fluorescence ratio using Eq. 1 in *Material and Methods* as a function of [Fluo-5F] in patch
136 pipettes. The horizontal line indicates 50 nM. The mean $[Ca^{2+}]_{rest}$ at three different
137 concentrations of Ca^{2+} indicator dye were not different and measured as 50 nM, suggesting
138 that the dye did not influence $[Ca^{2+}]_{rest}$. (**G-I**) Strong PPD and the subsequent STF may be
139 caused by Ca^{2+} channel inactivation and Ca^{2+}-dependent facilitation (CDF) of calcium influx
140 into presynaptic boutons, respectively. To test these possibilities, we measured presynaptic
141 Ca^{2+} increments evoked by each of successive APs applied in a train at 40 Hz. (**G**) Fluorescence
142 signals from a selected bouton with a scanning line. *Scale bars*, 3 μm (*top*), 100 ms and 2 μm
143 (*bottom*). (**H**) Normalized fluorescence (dF/F_0) responses to a train of APs at 40 Hz. (**I**) Total
144 Ca^{2+} increments ($\Delta[Ca^{2+}]_T$; free plus bound form) are plotted against the number of APs in the
145 train (n = 13). $\Delta[Ca^{2+}]_T$ were calculated based on Eq. 2 with κ_S set to 90. No significant change
146 was observed for the estimates of $\Delta[Ca^{2+}]_T$ at each of first 4 APs during 40 Hz train, indicating
147 that contributions of Ca^{2+} channel inactivation and CDF to STP are unlikely. Mean ± S.E.M.,
148 One-way ANOVA; n.s. = not significant.

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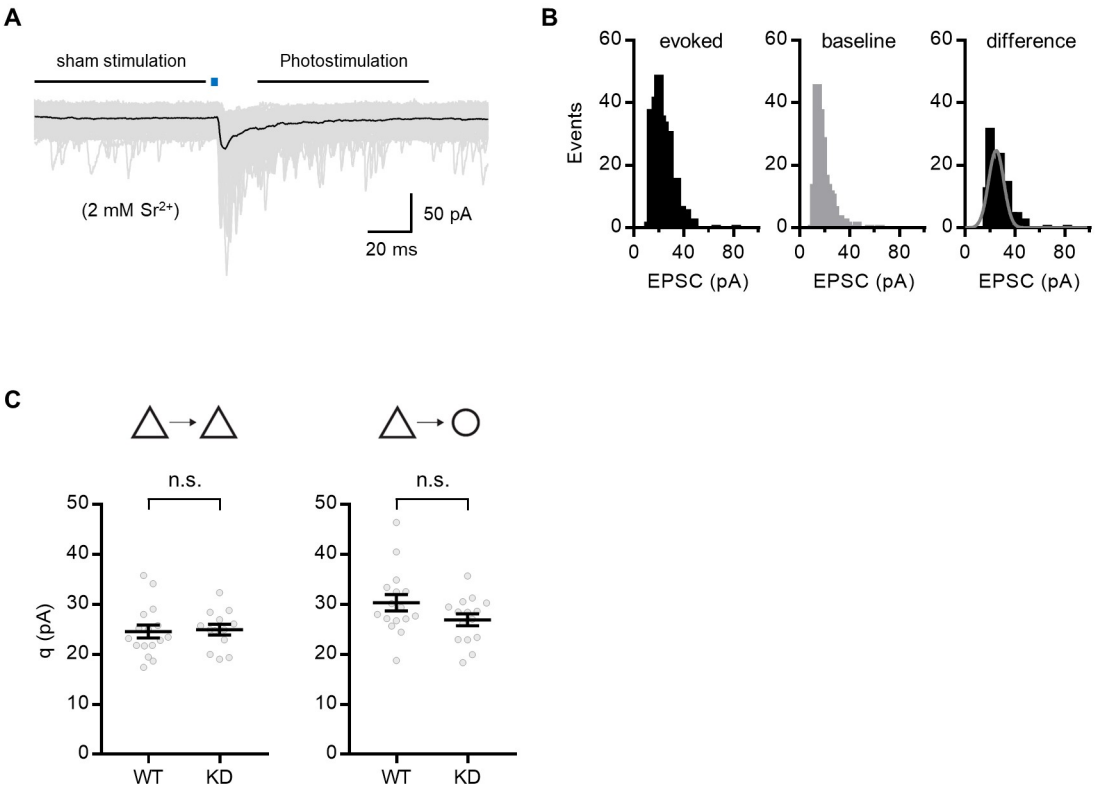

150 **Figure 3—figure supplement 1. Quantal size estimated from Sr^{2+}-induced asynchronous**
151 **release.**

152 (**A**) Representative traces (100 pulses) from PC-PC synapses. Black bars indicate the detection
153 window (100 ms) for release events in sham stimulation (*left bar*) and photostimulation (*right*
154 *bar*). (**B**) Representative distribution of asynchronous EPSCs at PC-PC synapses. *Left*, after
155 photostimulation. *Middle*, before stimulation. *Right*, difference between left and middle
156 panels. The difference distribution was fitted with a Gaussian function (m = 24.93, σ = 6.09).
157 (**C**) Comparisons of estimates for quantal size (q) between WT and Syt7 KD synapses. From
158 left to right: PC-PC (n = 16, 13 for WT and KD, respectively), PC-FSIN (n = 16, 15). *Gray symbols*,
159 individual data. All data are represented as mean ± S.E.M., unpaired t-test; n.s. = not
160 significant.
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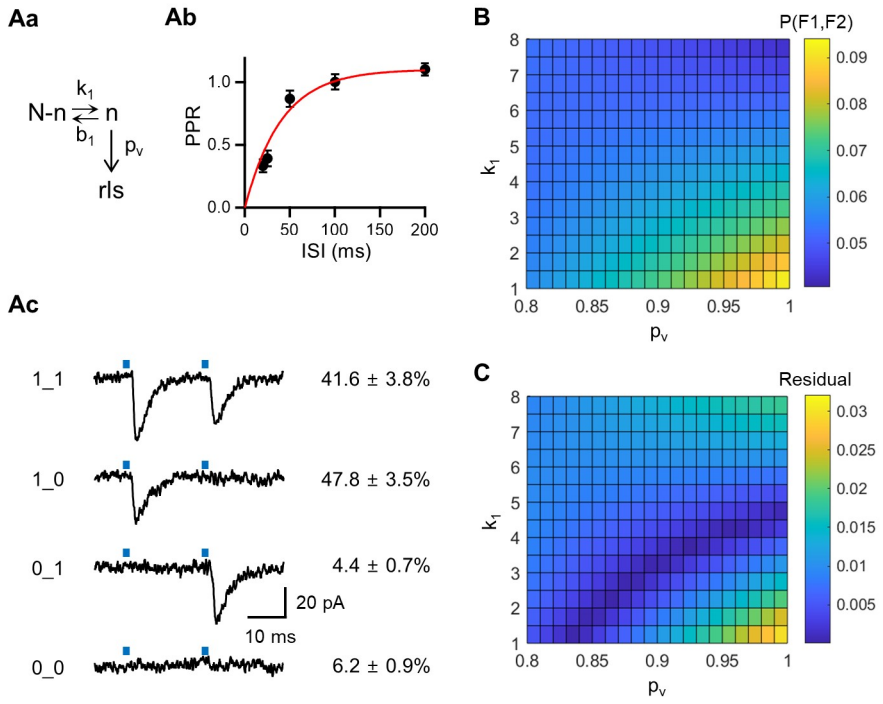
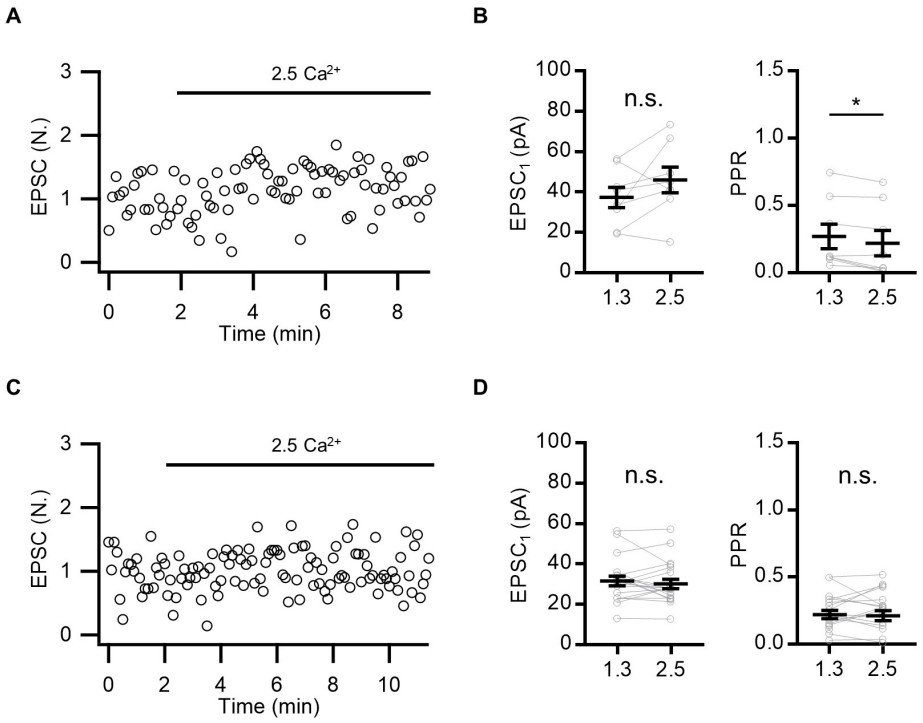


Figure 3—figure supplement 2. Analysis of double failure rate supports high p_v at excitatory synapses

(**Aa**) Schematic of simple refilling model. (**Ab**) An exponential fit to the PPR plot as a function of ISIs reproduced from *Figure 1G*. *Fitted function*, PPR = 1.1 [1 − exp(− t/43.5 ms)]. (**Ac**) Representative traces for four combinations of success and failure of 1st and 2nd EPSCs evoked by paired pulses with ISI of 20 ms at PC-PC synapses, showing successes in both pulses (1_1), failures in both pulses (0_0), and mixed responses (1_0; 0_1). On the right side of each trace, corresponding observation probability is shown in percentages (mean ± S.E.M. n = 57). (**B-C**) Plots of the calculated P(F1, F2) values (*B*) and their differences (*C*) from the observed value (6.2%) under the simple refilling model are displayed on the plane of k_1 versus p_v.



174 **Figure 3—figure supplement 3. Minimal EPSC increase upon elevated $[Ca^{2+}]_o$ indicates**

175 **saturated p_v at baseline**

176 (**A**) Representative time courses of normalized amplitudes of the first EPSCs during paired-
177 pulse photo-stimulation at 20 ms ISI under control conditions (1.3 mM $[Ca^{2+}]_o$) and elevated
178 extracellular $[Ca^{2+}]$ (2.5 mM; n = 8). (**B**) Mean values for the first EPSC (*left*) and PPR (*right*)
179 under the two conditions. (**C-D**) Experiments of (A-B) were repeated in the slices that were
180 pre-incubated in aCSF containing 50 µM EGTA-AM (n = 20). Note that the modest extracellular
181 $[Ca^{2+}]$-dependence of the first EPSC and PPR observed in (A-B) was abolished by 50 µM EGTA-
182 AM pre-incubation. *Gray symbols*, individual data. All statistical data are represented as mean
183 ± S.E.M.; n.s. = not significant; *, $P < 0.05$; paired t-test.

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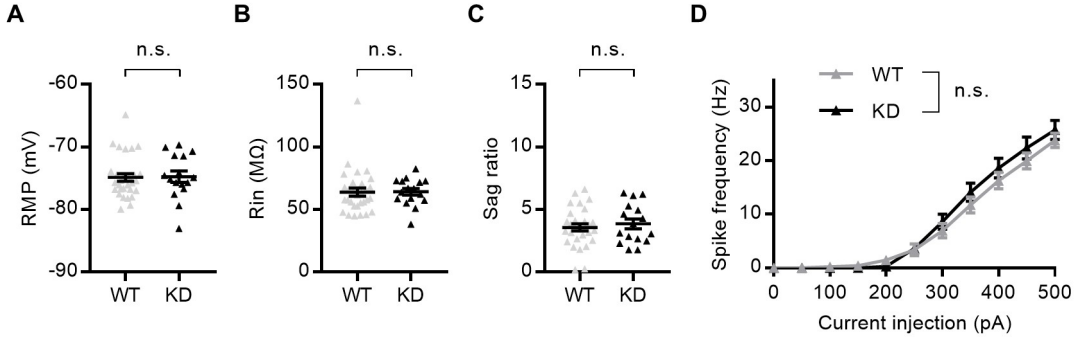

186 **Figure 5—figure supplement 1. Little effects of Syt7 KD on intrinsic properties of neurons**

187 Whole-cell recordings were performed from the fluorescently labeled pyramidal neurons in
188 the prelimbic L2/3 of WT (n = 30) or Syt7 KD (n = 16) rats. (**A**) Resting membrane potential. (**B**)
189 Input resistance. (**C**) Sag ratio. *Filled triangles*, individual data. (**D**) Current-spike frequency
190 relationship in WT and KD cells. All statistical data are represented as mean ± S.E.M., unpaired
191 t-test or two-way ANOVA; n.s. = not significant.

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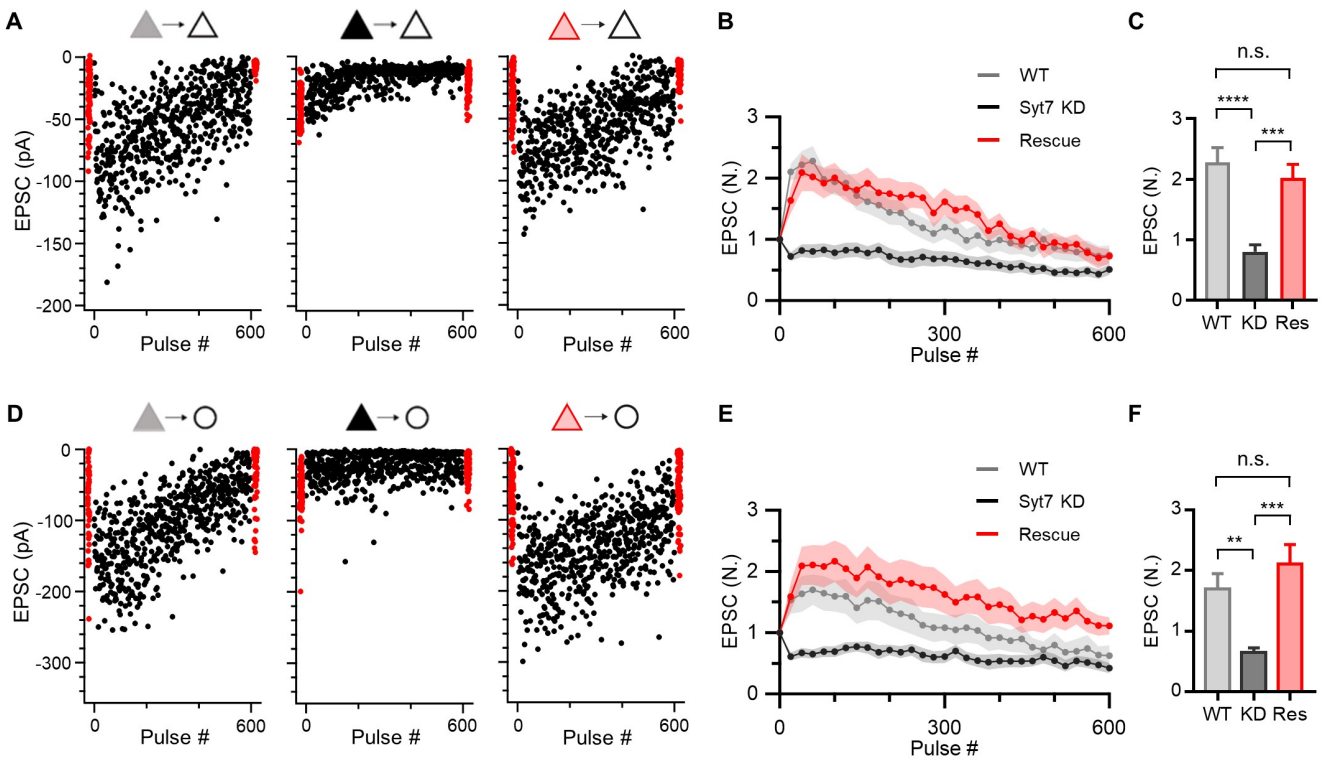
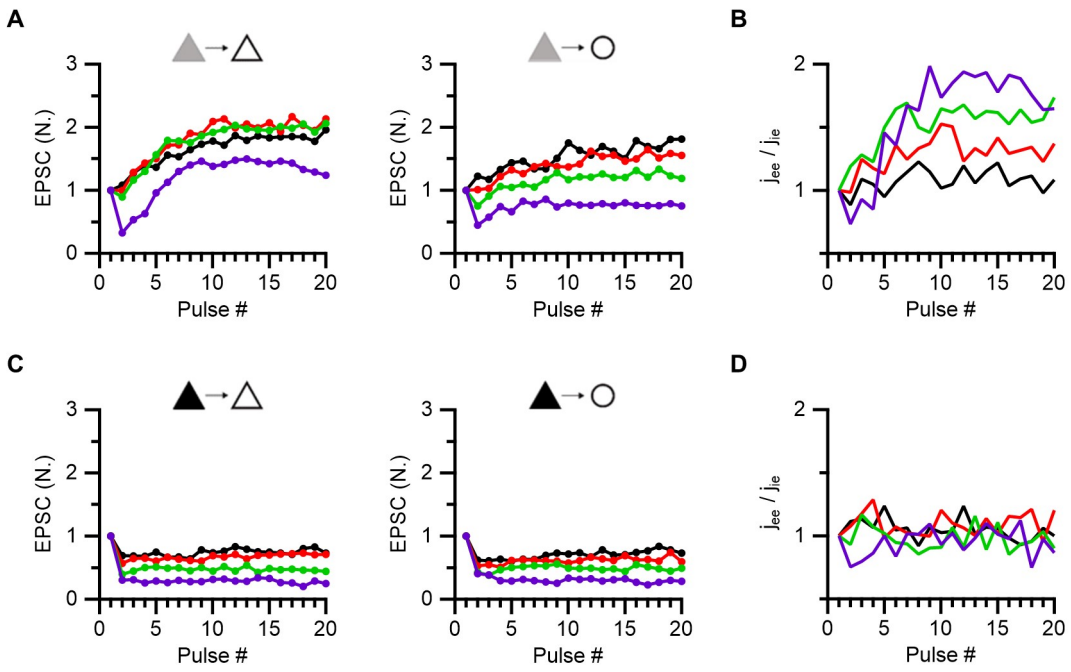


194 **Figure 5—figure supplement 2. Presynaptic expression of Syt7 rescues facilitation at L2/3**
195 **excitatory synapses**

196 (**A, D**) Representative amplitudes of EPSC trains evoked by 600-pulse 10 Hz trains (flanked by
197 2 baseline periods comprised of 40 pulses each) at PC-PC (*A*) and PC-FSIN (*D*) synapses, in
198 which presynaptic WT (*left, gray triangles*) pyramidal cells and those transfected with plasmid
199 encoding shRNA against Syt7 (sh-Syt7, KD, *middle, black triangles*) or sh-Syt7 plus Syt7
200 insensitive to shRNA (rescue, *right, red triangles*). (**B, E**) STP of EPSCs evoked by 600-pulse
201 trains at 10 Hz at L2/3 PC-PC (*B*; n = 9, 10, 7; WT, Syt7 KD, Rescue, respectively) and PC-FSIN
202 (*E*; n = 12, 10, 8) synapses. Each data point represents an average from a moving window of
203 20 pulses, normalized to the baseline EPSC. (**C, F**) Mean values of 41-60th pulses from *B* and *E*.
204 Mean ± S.E.M.; n.s. = not significant; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; one-way
205 ANOVA with Tukey's post-hoc test.



206 **Figure 8—figure supplement 1. Activity-dependent increase in the synaptic weight ratio at**

207 **PC-PC to at PC-FSIN connections (J_{ee}/J_{ie}) is abolished by Syt7 KD**

208 (**A, C**) Average amplitudes of baseline-normalized EPSCs during 20-pulse trains at frequencies
209 from 5 to 40 Hz at PC-PC (*left*) and PC-FSIN (*right*) synapses in WT (*A*) and Syt7 KD (*C*). Data
210 are reproduced from ***Figure 1*** (*A*) and ***Figure 5*** (*C*). (**B, D**) Relative synaptic weights of
211 excitatory input to PCs over that onto FSINs (*Jee/Jie*) in WT (*B*) and Syt7 KD (*D*). Note that
212 excitatory influence (*Jee/Jie*) to the recurrent network increases as the firing number and
213 frequency increase in WT but not in Syt7 KD.

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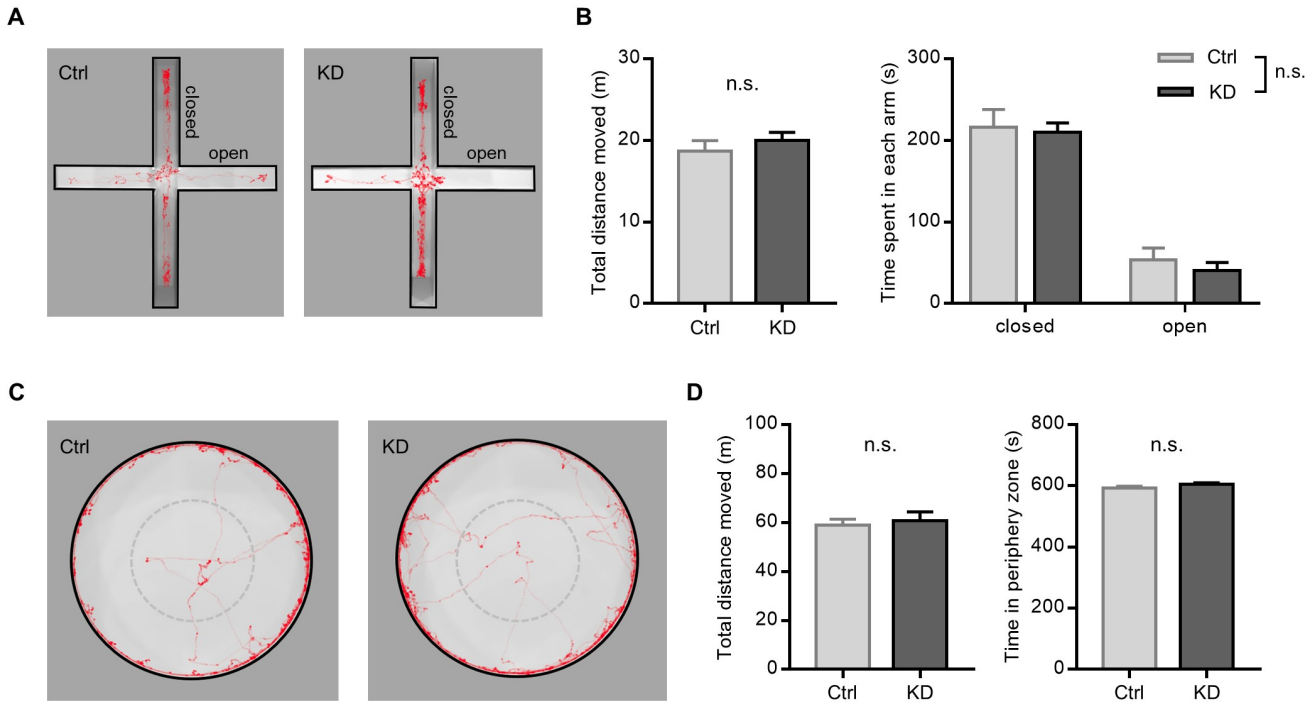

Figure 8—figure supplement 2. Locomotor activity and anxiety levels in Syt7 KD rats using OFT and EPM

(**A, C**) Representative tracking trace of the elevated plus maze (*A*) and open field test (*C*). (**B, D**) Mean values for total distance moved (*left*) and time spent (*right*) in each arm (*B*) or in periphery zone (*D*) in control (n = 13) or Syt7 KD rats (n = 9). Mean ± S.E.M.; Unpaired t-test or two-way ANOVA; n.s. = not significant.

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