Unique dynamics and exocytosis of single inhibitory synaptic vesicles

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42

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45   tracking; real-time imaging

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47   **Author Contributions**

48   C.P. and H.P. designed the experiments. C.P., X.C., and C.-L.T., performed the

49   experiments. C.P, C.-L.T., G.N.P., N.C., and HL analyzed data. C.P., C.-L.T., X.Y.Y.,

50   S.J., G.B., R.W.T., and H.P. discussed and interpreted results, C.P., X.C., C.L.T., S.J.,

51   R.W.T., and H.P. wrote the manuscript. S.J., G.B., and H.P. supervised students.

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54   **This PDF file includes:**

55   Main Text

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57

58 **Abstract**

59 The balance between excitation and inhibition is essential for maintaining proper

60 brain function in the central nervous system. Inhibitory synaptic transmission plays an

61 important role in maintaining this balance. Inhibitory synaptic transmission faces

62 greater kinetic demands than excitatory synaptic transmission, yet remains less well

63 understood. In particular, the dynamics and exocytosis of single inhibitory vesicles

64 have not been investigated due to both technical and practical limitations. Using

65 quantum dots (QDs)-conjugated antibodies against the luminal domain of the

66 vesicular GABA transporter (VGAT) to selectively label single GABAergic inhibitory

67 vesicles and dual-focus imaging optics, we tracked single inhibitory vesicles up to the

68 moment of exocytosis (i.e., fusion) in three dimensions in real time. Using three-

69 dimensional trajectories, we found that the total travel length before fusion of

70 inhibitory synaptic vesicles was smaller than that of synaptotagmin-1 (Syt1)-labeled

71 vesicles. Fusion times of inhibitory vesicles were shorter compared with those of

72 Syt1-labeled vesicles. We also found a close relationship between release probability

73 to the proximity to fusion sites and total travel length of inhibitory synaptic vesicles.

74 Furthermore, inhibitory synaptic vesicles exhibited a higher prevalence of kiss-and-

75 run fusion than Syt1-labeled vesicles. Thus, our results showed that inhibitory

76 synaptic vesicles have the unique dynamics and fusion properties that facilitate their

77 ability to support fast synaptic inhibition.

78

79 **Significance**

80 Despite of its importance, the dynamics of single inhibitory synaptic vesicles before

81 exocytosis has not been investigated. Here, we tracked single inhibitory vesicles up to

82 the moment of exocytosis in three dimensions in real time using QDs-conjugated

83 antibodies against the luminal domain of the vesicular GABA transporter (VGAT). We

84 found that inhibitory synaptic vesicles had smaller total travel length before fusion,

85 shorter fusion time and a higher prevalence of kiss-and-run than synaptotagmin-1-

86 lableled vesicles. For the first time, we showed the unique dynamics and exocytosis of

87 single inhibitory synaptic vesicles before exocytosis, which supports fast inhibitory

88 synaptic transmission. Thus, our results suggest that unique dynamics and exocytosis

89 of inhibitory synaptic vesicles is an important factor in inhibitory synaptic transmission.

90

Introduction

Neurons communicate with other neurons and different types of cells by releasing neurotransmitters in presynaptic terminals through exocytosis, and subsequently activating receptors in postsynaptic terminals (1-4). The central synapses can be broadly classified into excitatory or inhibitory synapses, depending on the types and effects of neurotransmitters they release. While the excitatory synapses act to generate, propagate and potentiate neuronal responses for information processing (5), the inhibitory synapses are essential for feedback or feedforward inhibition to control neural excitability (6). Inhibitory synaptic transmission, which is mediated primarily through the release of GABA in the central nervous system, coordinates the pattern of excitation and synchronization of the neuronal network to regulate neuronal excitability (7, 8). Thus, the tight regulation of both excitatory and inhibitory neurotransmission is essential for the proper functions of the brain.

The systematic analysis of the components and molecular events of vesicle fusion and neurotransmitter release has generated general models for the organization and functional operation of the pre- and postsynaptic components of synapses (9-11). The high local Ca^{2+} concentration due to activity-induced activation of Ca^{2+} channels triggers the local buckling of the plasma membrane through the direct interaction between the C2B domain of synaptotagmin-1 (Syt1) and the lipid on the membrane (12-14). This leads to synchronous fusion of the membrane and the release of its content into the synaptic cleft (15). Once released, the excitatory and inhibitory

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112   neurotransmitters exert their respective effects through glutamate or GABA receptors

113   on the postsynaptic membrane.

114   The generalized understanding of vesicular release built on excitatory

115   neurotransmission is not able to explain distinct inhibitory synaptic transmission. The

116   size of the readily releasable pool (RRP) of synaptic vesicles in the striatal inhibitory

117   GABAergic neurons, probed by the use of hypertonic sucrose solution, is three times

118   higher than that of excitatory hippocampal glutamatergic neurons (16). Furthermore,

119   the average vesicular release probability and number of release sites of the inhibitory

120   neurons are higher than those of excitatory neurons (16-18). These results suggest at

121   least quantitative differences in properties of vesicle release and recycling in the

122   GABAergic synapses. In addition, the disproportionate effects of the knockout of the

123   individual or all synapsin genes between the two types of synapses, provide a molecular

124   perspective on the potential differences between excitatory and inhibitory synapses (20-

125   23).

126   Despite the crucial roles of inhibitory synaptic vesicles in information processing

127   in the neuronal network, the dynamics of the inhibitory synaptic vesicles in presynaptic

128   terminals is poorly understood. In particular, the real-time dynamics of single inhibitory

129   synaptic vesicles in hippocampal neurons upon stimulation have not been investigated

130   due to technical and practical limitations, including the lack of specific single inhibitory

131   vesicular markers, the structural complexity of synapses in three dimensions, and the

132   extreme small size of synaptic vesicles (diameter of 40–50 nm, which is well below the

133   resolution of conventional light microscopy)(2).

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134   Here, we report the real-time three-dimensional tracking of single inhibitory

135   synaptic vesicles in dissociated hippocampal neurons using dual-focus optics and QD-

136   conjugated antibodies against the luminal domain of the vesicular GABA transporter

137   (VGAT) to selectively label single GABAergic vesicles. We found that fusion times of

138   inhibitory vesicles were significantly shorter than those of Syt1-labeled vesicles and the

139   total travel length before fusion of inhibitory vesicles was smaller compared with the

140   Syt1-labeled vesicles. The release probability of inhibitory synaptic vesicles was

141   closely correlated to the proximity to fusion sites and the total travel length before

142   fusion. Inhibitory synaptic vesicles exhibited a higher prevalence of kiss-and-run fusion

143   compared with Syt1-labeled vesicles. Thus, our results indicate that inhibitory synaptic

144   vesicles have unique dynamics and fusion properties.

145

146   **Results**

147   **Real-time three-dimensional tracking of single inhibitory synaptic vesicles in**

148   **hippocampal neurons**

149   In order to study the dynamics of single inhibitory synaptic vesicles in mature

150   synapses, we tracked single inhibitory synaptic vesicles in three dimensions in real time

151   up to the moment of exocytosis in mature dissociated hippocampal neurons (i.e., days

152   in vitro (DIV) 14 – 21) similar to the previous method used in the tracking of single

153   synaptic vesicles (24) of undetermined identity. We previously reported the real-time

154   three-dimensional tracking of single synaptic vesicles loaded with a streptavidin-

155   conjugated quantum dots (QDs)-conjugated to biotinylated antibodies against the

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156   luminal domain of endogenous synaptotagmin-1 (Syt1) (or Syt1-labeled synaptic

157   vesicles) with an accuracy of 20 to 30 nanometers (less than the synaptic vesicle

158   diameter) in neurons (24). In order to specifically label inhibitory synaptic vesicles with

159   QDs in living hippocampal neurons, we conjugated streptavidin-coated QDs with a

160   commercially available biotinylated antibody which is specific to the luminal domain

161   of the endogenous vesicular GABA transporter (VGAT, also termed vesicular inhibitory

162   amino acid transporter (VIAAT))(Fig. 1A). The QDs-conjugated antibodies bind to the

163   luminal domain of VGAT, exposed after exocytosis during electrical stimulation.

164   CypHer5E-labeled antibodies against the luminal domain of VGAT (VGAT-CypHer5E

165   - a fluorescent marker that selectively labels inhibitory synaptic vesicles (25)) were

166   used to label spontaneously released inhibitory synaptic vesicles during 2 hours of

167   incubation in order to locate the inhibitory presynaptic terminals. The colocalization

168   between fluorescence signals of QDs and VGAT-CypHer5E shown in Fig. 1B indicates

169   that QDs-conjugated antibodies against VGAT successfully labeled inhibitory synaptic

170   vesicles in the inhibitory presynaptic terminals marked by VGAT-CypHer5E. Only

171   colocalized QD-labeled synaptic vesicles were used for data analysis. Exocytosis of

172   single QD-loaded vesicles was reflected by a sudden irreversible drop in the fluorescent

173   signal caused by the quenching of exposed QD by extracellular trypan blue

174   (extracellular quencher) (24). The positions of single synaptic vesicles loaded with QDs

175   in the x-y plane were localized with an accuracy of tens of nanometers using a technique

176   called fluorescence imaging with one nanometer accuracy (FIONA)(24, 26, 27). The z-

177   positions of single vesicles loaded with QDs were localized with an accuracy of tens of

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178 nanometers using dual-focusing imaging optics (24, 28). Using these techniques, we

179 successfully localized three-dimensional positions of single inhibitory synaptic vesicles

180 loaded with QD-conjugated antibodies against the luminal domain of VGAT. Figure 1C

181 shows the real-time three-dimensional trace of one single QD-loaded inhibitory

182 synaptic vesicle in living neurons with the x-y plane projection overlaid on the

183 fluorescence image of an inhibitory presynaptic terminal with VGAT-CypHer5E-

184 labeled vesicles during 1200 stimuli (10 Hz for 120 s). The trace in Fig. 1C indicates

185 that the single inhibitory synaptic vesicle remained at the edge of a presynaptic terminal,

186 before undergoing exocytosis at 32.0 s. Fluorescence images of the QD-loaded

187 inhibitory synaptic inside the vesicles before and after exocytosis (Fig. 1D) clearly

188 show almost complete quenching of the QD. We further analyzed average fluorescence

189 intensity within the region-of-interest (ROI) and the radial distance from the momentary

190 position to the fusion site ($R = \sqrt{\Delta X^2 + \Delta Y^2 + \Delta Z^2}$) of the single QD-loaded inhibitory

191 synaptic vesicle before exocytosis (Fig. 1E). The fluorescence trace in Fig. 1E included

192 photoblinking events at ~8, ~13 and ~15 s, indicating the presence of a single QD inside

193 a single vesicle. At 32.0 s, the fluorescence signal displayed a sharp drop in brightness

194 caused by the exposure of the vesicle lumen to the quencher-containing external

195 solution, indicating the full-collapse fusion of the single inhibitory synaptic vesicle

196 loaded with a single QD. Thus, the example shown in Fig. 1 demonstrates that a single

197 inhibitory synaptic vesicle was successfully labeled with a QD-conjugated antibodies

198 against the luminal domain of VGAT by external stimuli and this single QD-labeled

199 inhibitory synaptic vesicle was tracked in three dimensions up to the moment of

200   exocytosis during electrical stimulation.

201   In order to check whether QD-conjugated antibodies against the luminal domain of

202   Syt1 also label inhibitory synaptic vesicles, we measured the colocalization between

203   VGAT-CypHer5E-labeled inhibitory presynaptic terminals and synaptic vesicles

204   loaded with QD-conjugated antibodies against Syt1. The colocalization percentage

205   between VGAT-CypHer5E and Syt1-labeled vesicles was 12 ± 1.4 % (N = 13

206   images)(*SI Appendix,* Fig. S1), implying that our previous observed dynamics of these

207   Syt1-labeled synaptic vesicles (24) mainly represented the dynamics of excitatory

208   synaptic vesicles in the dissociated hippocampal neurons.

209   In order to find relative proportion of inhibitory neurons in dissociated

210   hippocampal cultures, we co-immunostained for MAP2 (a neuron-specific marker) and

211   glutamic acid decarboxylase 67 (GAD67 - a marker for inhibitory neurons) in our

212   hippocampal cultures (Fig. 2A). The colocalization percentage between fluorescence

213   puncta of MAP2 and GAD67 was 6 ± 0.8 % (N = 23 images)(Fig. 2B), which indicates

214   that our dissociated hippocampal cultures are predominantly excitatory neurons. To

215   further find the identity of inhibitory neurons in our hippocampal cultures, we co-

216   immunostained GAD67 and parvalbumin (PV) in fixed primary hippocampal neurons.

217   The colocalization percentage between fluorescence puncta of GAD67and PV was 82

218   ± 7.7 % (N = 19 images)(Fig. 2C), indicating that PV-expressing fast-spiking

219   GABAergic interneurons were dominant in our inhibitory neurons in our dissociated

220   hippocampal cultures. Therefore, our VGAT-labeled synaptic vesicles likely represent

11

221   the dynamics of inhibitory synaptic vesicles in fast-spiking PV-expressing GABAergic

222   interneuron.

223

224   **Unique spatiotemporal dynamics of single inhibitory synaptic vesicles**

225   In order to understand the dynamics of exocytosed inhibitory synaptic vesicles, we

226   investigated the net displacements between the initial positions and their fusion sites of

227   single releasing inhibitory synaptic vesicles during electrical stimulation. Three-

228   dimensional net displacement was calculated as Pythagorean displacements using our

229   real-time 3D traces of single QD-loaded inhibitory synaptic vesicles. Figure 3A shows

230   the net displacements of inhibitory synaptic vesicles compared with our previously

231   reported net displacements of Syt1-labeled synaptic vesicles (24). Net displacements

232   between the initial and fusion sites of inhibitory synaptic vesicles were not significantly

233   different from that of Syt1-labeled synaptic vesicles ($p>0.7$, Kolmogorov–Smirnov (K-

234   S) test) (Fig. 3A).

235   To further understand the motion of inhibitory vesicles, we calculated the three-

236   dimensional total travel length before fusion of inhibitory synaptic vesicles. The total

237   travel length before fusion of inhibitory synaptic vesicles undergoing exocytosis

238   (average, 10.8 ± 0.94 (standard error of the mean) μm (n = 80)) was significantly

239   smaller than that of Syt1-labeled vesicles (18.7 ± 2.32 μm (n = 49), $p<0.05$, K-S test;

240   Fig. 3B). These results indicated that inhibitory synaptic vesicles traveled less to reach

241   fusion sites than excitatory synaptic vesicles since Syt1-labeled vesicles are likely to

242   represent excitatory synaptic vesicles in our cultures. The smaller travel length before

12

243 fusion and not significantly different net displacement of inhibitory synaptic vesicles

244 suggest more exocytosis at earlier times compared with excitatory synaptic vesicles.

245   In order to test that hypothesis, we measured the fusion time between initial

246 stimulation and fusion of inhibitory synaptic vesicles. Figure 3C shows the cumulative

247 fusion time of inhibitory synaptic vesicles compared with that of Syt1-labeled synaptic

248 vesicles. The fusion time of inhibitory synaptic vesicle during stimulation (26.2 ± 2.24

249 s), was significantly shorter than Syt1-labeled vesicles (44.2 ± 4.89 s)(*p<0.01*, K-S test).

250 These results indicate that inhibitory synaptic vesicles undergo faster exocytosis than

251 the excitatory synaptic vesicles. We further investigated exocytosis kinetics by

252 calculating the number of fusion events every 20 s. A higher percentage of inhibitory

253 synaptic vesicles underwent exocytosis in the first two 20 s ($31/80 = 38.8\%$ in t < 20 s,

254 $35/80 = 43.8\%$ in $20 \leq t < 40$ s) compared with Syt1 labeled vesicles ($16/49 = 32.7\%$ in

255 t < 20 s, $10/49 = 20.4\%$ in $20 \leq t < 40$ s), which is consistent with the higher release

256 probability of inhibitory synaptic vesicles compared with excitatory synaptic vesicles

257 (16, 17).

258

259 **Relation of release probability to position and travel length of inhibitory synaptic**

260 **vesicles**

261   Whether vesicles' proximity to fusion sites determines their release probability ($P_{r/v}$)

262 is a crucial question in neuroscience. Recent real-time tracking of Syt1-labeled vesicles

263 indicates that vesicles' proximity to fusion sites is a key factor in determining the

264 release probability of Syt1-labeled synaptic vesicles (24). However, the relationship

13

265 between the proximity to their fusion sites and the release probability ($P_{r/v}$) of inhibitory

266 synaptic vesicles is unknown. Thus, we answered this question by investigating the

267 relationship between the fusion time and their net displacements. Figure 4A shows that

268 the net displacement of inhibitory synaptic vesicles is related to their fusion time

269 (Pearson's r = 0.77), indicating a correlation between the proximity to fusion sites and

270 release probability of inhibitory synaptic vesicles. The slope of inhibitory synaptic

271 vesicles in Fig. 4A (0.079 ± 0.00745 (± SEM)) was smaller than that of Syt1-labeled

272 vesicles (0.11346 ± 0.01632)(*SI Appendix,* Fig. S3), indicating the faster exocytosis of

273 inhibitory synaptic vesicles located in the same net displacement. Using net

274 displacement, we calculated net velocity of releasing inhibitory synaptic vesicles.

275 Average net velocity of releasing inhibitory synaptic vesicles was 5.4 ± 0.54 nm/s,

276 which was higher than that of releasing Syt1-labeled vesicles (4.7 ± 0.74 nm/s). The

277 difference was not significant (*p>0.2, K-S test*). The coefficient of variation (CV) in net

278 velocity of inhibitory synaptic vesicles was 89%, which is smaller than that of Syt1-

279 labeled synaptic vesicles (110%). The smaller CV indicates that net speed of inhibitory

280 synaptic vesicles is less variable to reach their fusion sites.

281   We further investigated the relationship between the total travel length before

282 fusion and fusion time of inhibitory synaptic vesicles. The total travel length before

283 fusion of inhibitory synaptic vesicles is highly related to their fusion time (Pearson's r

284 = 0.94)(Fig. 4C), indicating a close correlation between the travel length and release

285 probability of inhibitory synaptic vesicles. This higher value of Pearson

286 correlation coefficient compared with net displacement indicates that fusion time is

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287   more closely related to total travel length before fusion than net displacement of

288   synaptic vesicles. The slope of inhibitory synaptic vesicles (1.43 ± 0.059 ($\pm$ SEM)) was

289   smaller than that of Syt1-labeled vesicles (1.58 ± 0.095), indicating the faster exocytosis

290   of inhibitory synaptic vesicles. Using total travel length before fusion, we calculated

291   speed of releasing inhibitory synaptic vesicles. Speed of releasing inhibitory synaptic

292   vesicles (405 ± 13.9 nm/s) was significantly lower than that of releasing Syt1-labeled

293   vesicles (448 ± 28.1 nm/s). The CV in speed of inhibitory synaptic vesicles (31%) was

294   smaller than that of Syt1-labeled synaptic vesicles (44%), indicating that inhibitory

295   synaptic vesicles undergo more tightly regulated movement. The higher velocity and

296   lower speed before fusion of inhibitory synaptic vesicles imply that inhibitory synaptic

297   vesicles moved to fusion sites more straightly than Syt1-labeled vesicles.

298

299   **Exocytotic fusion mode of single inhibitory synaptic vesicles**

300   Finally, we investigated the exocytotic fusion mode of single inhibitory

301   synaptic vesicles. Since fusion pore opening is brief in kiss-and-run fusion, trypan blue

302   (quencher) in external solution equilibrate only partially, which give partial quenching

303   compared with full-collapse fusion (24). Figure 5A shows the fluorescence images of a

304   QD inside an inhibitory synaptic vesicle undergoing kiss-and-run fusion, taken before

305   any fluorescence drop (70.2 s), after partial loss of fluorescence (71.2 and 105 s), and

306   after subsequent loss of the remaining fluorescence (105.9 s). A fluorescence trace,

307   obtained by analysis of the region-of-interest (ROI) containing the QD (Fig. 5B),

308   displays a sudden and irreversible decrease in fluorescence (red arrow) at 70.5 s. At

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309   105.2 s, the fluorescence signal dropped further (blue arrow) and settled at a lower level

310   reflecting near-complete quenching, representing full-collapse fusion. The average

311   fluorescence of the QD for the period from 70.5 s to 105.2 s was reduced to 0.35

312   compared with an initial level, significantly larger than that expected for QDs steadily

313   exposed to 2 μM TB (0.12). To further confirm that the partial quenching can be used

314   as indicator of kiss-and-run fusion, we divided all fluorescence traces depending on the

315   quenching pattern. We observed three patterns: almost complete quenching

316   (presumably full-collapse fusion), partial quenching followed by almost complete

317   quenching (presumably the first kiss-and-run fusion followed by full-collapse fusion or

318   reuse after the first kiss-and-run fusion), and partial quenching not followed by

319   complete quenching (the first kiss-and-run not followed by full-collapse fusion or no

320   immediate reuse after the first kiss-and-run fusion). Figure 5C shows the average

321   normalized fluorescence intensities with SEM in time aligned with the first fusion

322   depending on the quenching pattern. The average remaining normalized fluorescence

323   right after the full-collapse fusion (Fig. 5C1) was smaller 0.12 whereas the kiss-and-

324   run fusion (Fig. 5C2 and 5C3) was higher than 0.12. This clear difference in the

325   remaining normalized fluorescence after the first fusion depending on fusion mode

326   confirms that the partial quenching is a good indicator of kiss-and-run fusion in our

327   experiments.

328   Based on the partial quenching as the reporter of kiss-and-run fusion, we also

329   calculated the prevalence of kiss-and-run fusion. We observed ~2.5 fold greater

330   prevalence of kiss-and-run fusion in inhibitory synaptic vesicles compared with Syt1-

331   labeled synaptic vesicles (Fig. 5D), indicating that inhibitory synaptic vesicles undergo

332   kiss-and-run fusion more frequently than excitatory synaptic vesicles. The high

333   prevalence of kiss-and-run fusion of inhibitory synaptic vesicles may make inhibitory

334   synaptic vesicles available for continuous release of neurotransmitter during prolonged

335   stimulation in inhibitory synaptic transmission.

336   We further investigated the dynamics of inhibitory synaptic vesicles based on

337   fusion modes and compared them with Syt1-labeled vesicles. The total travel length of

338   inhibitory synaptic vesicles undergoing full-collapse fusion (16.9 ± 1.66 (n = 28) μm)

339   was larger than that of Syt1-labeled vesicles (30.6 ± 3.09 (n = 36) μm, $p<0.05$, K-S

340   test)(Fig. 5E). Total travel length of inhibitory synaptic vesicles undergoing kiss-and-

341   run fusion (20.4 ± 1.48 (n = 52) μm) was similar to that of Syt1-labeled vesicles (21.0

342   ± 3.09 s (n = 13) μm, $p>0.7$, K-S test)(Fig. 5F). The fusion time of inhibitory synaptic

343   vesicles undergoing full-collapse fusion was significantly shorter than that of Syt1-

344   labeled vesicles undergoing full-collapse fusion (20.3 ± 3.03 s (n = 28) vs. 44.7 ± 5.84

345   s (n = 36), respectively; ($p<0.01$, K-S test)) (Fig. 5G). Fusion times of inhibitory

346   synaptic vesicles undergoing kiss-and-run fusion were not significantly different from

347   those of Syt1-labeled synaptic vesicles undergoing kiss-and-run fusion ($p>0.4$, K-S test)

348   (Fig. 5H). These results indicate that the observed unique dynamics of inhibitory

349   synaptic vesicles is mainly caused by the difference in the dynamics of inhibitory

350   synaptic vesicles undergoing full-collapse fusion (significantly shorter travel length and

351   faster fusion time).

352

17

Discussion

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354   The structures and functions of GABAergic inhibitory synapses are different from

355   those of glutamatergic excitatory synapses. GABAergic inhibitory neurons are crucial

356   in controlling the number and activity level of glutamatergic excitatory neurons and

357   firing frequency via fast feedforward and feedback inhibition in neuronal networks (17,

358   30, 31). To allow for fast synaptic transmission without synaptic failure, inhibitory

359   neurons adopt a distinct set of morphology, multiple release sites, biogenesis and

360   trafficking processes for synaptic vesicles in presynaptic terminals (20, 32-34). In

361   particular, Ca^{2+} channels are tightly coupled to the GABA release molecular machinery,

362   to increase the speed and efficacy of GABA transmission, which in turn provides a

363   relatively higher energy efficiency of vesicle release in the presynaptic terminals of

364   inhibitory neurons (35). Although the higher release probability and reliability of action

365   potential-evoked Ca^{2+} signal play an important role in the GABA release mechanisms

366   to maintain timely synaptic transmission, the actual exocytosis and dynamics of single

367   synaptic vesicles throughout the processes of vesicle release and recycling in

368   GABAergic inhibitory presynaptic terminals has been unknown.

369   In this study, we loaded single inhibitory synaptic vesicles with QDs-conjugated

370   antibodies against the luminal domain of VGAT in hippocampal neurons and tracked

371   single inhibitory synaptic vesicles in three dimensions up to the moment of exocytosis.

372   To compare dynamics of inhibitory vesicles with that of glutamatergic excitatory

373   synaptic vesicles, we used synaptic vesicles labeled with QD-conjugated antibodies

374   against the luminal domain of Syt1 for excitatory synaptic vesicles in cultured

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375   hippocampal neurons because the great majority of neurons in our hippocampal cultures

376   are excitatory (Fig. 2) and Syt1-labeled synaptic vesicles mainly label excitatory

377   synaptic vesicles (SI Appendix Fig. 1). Although around 10 % of Syt1-labeled synaptic

378   vesicles were colocalized with VGAT-CypHer5E-labeled presynaptic terminals and

379   Syt1 plays a key role as a Ca^{2+} sensor in both glutamatergic and GABAergic presynaptic

380   terminals (36), our previous results obtained from Syt1-labelled synaptic vesicles in

381   cultured hippocampal neurons mainly represent the properties of excitatory synaptic

382   vesicles (24). Interestingly, the fast synchronous vesicle release was abolished in

383   glutamatergic presynaptic terminals (granule cell-basket cell synapse) but not

384   GABAergic presynaptic terminals (basket cells-granule cells synapse) in the

385   hippocampus of Syt1 KO mice (37). In addition, a subset of PV-expressing

386   hippocampal interneurons mainly use Syt2 as the main Ca^{2+} sensor (38) and

387   GABAergic inhibitory neurons have an alternative high-affinity Ca^{2+} sensor, DOC2β,

388   to control spontaneous release (36, 38). Even though more than 21 types of interneurons

389   are localized in the hippocampus, our immunostaining results showed that less than 10%

390   of the cultured hippocampal neurons are inhibitory neurons and ~80% of those

391   inhibitory neurons were PV)-expressing interneurons. Given these pieces of evidence,

392   our previous results obtained from Syt1-labelled synaptic vesicles in cultured

393   hippocampal neurons likely represent the properties of excitatory synaptic vesicles (24).

394   Here, we found that the total travel length before exocytosis of inhibitory synaptic

395   vesicles were smaller than those of Syt1-labelled synaptic vesicles. Intriguingly,

396   inhibitory synaptic vesicles underwent exocytosis earlier than those of Syt1-labeled

397   vesicles and a larger portion of the inhibitory synaptic vesicles was released within 40

398   s of the onset of stimulation. Release probability of inhibitory synaptic vesicles was

399   closely related to the proximity to fusion sites and total travel length. These results

400   indicate that inhibitory synaptic vesicles move to their fusion sites more directly than

401   Syt1-labeled synaptic vesicles. Inhibitory synaptic vesicles also exhibited a higher

402   prevalence of kiss-and-run fusion. The smaller   travel length before exocytosis,

403   shorter fusion time, and a higher prevalence of kiss-and-run fusion of inhibitory

404   synaptic vesicles represent the unique dynamics and exocytosis of inhibitory synaptic

405   vesicles, which is consistent with previously reported electrophysiological and

406   ultrastructural features of inhibitory synaptic transmission–a larger RRP size, fast short-

407   term depression and higher release probability in inhibitory synaptic vesicles (16, 17).

408   Furthermore, these observed dynamics suggests efficient and fast neurotransmitter

409   release of inhibitory neurons in our primary hippocampal neurons - mainly PV-

410   expressing interneurons. This fast and efficient release of inhibitory synaptic vesicles

411   enables PV-expressing interneurons to signal rapidly, which makes possible higher

412   microcircuit functions in the brain including feedforward and feedback inhibition and

413   high-frequency network oscillations on fast timescales (40). The differences in vesicle

414   dynamics between inhibitory and excitatory synaptic vesicles suggest that the distinct

415   dynamics of synaptic vesicles affect the synaptic functions, despite the overlapping

416   molecular machinery of synaptic release. In line with the importance of localization and

417   movement of synaptic vesicles, the altered relationship between the release probability

418   and position of synaptic vesicles can affect the release of neurotransmitters in

419   neurodegenerative diseases, such as Huntington's disease (41, 42).

420   The unique dynamics of single inhibitory synaptic vesicles can contribute to fast

421   inhibitory synaptic transmission and fast short-term depression. The shorter travel

422   length before exocytosis and proximity of inhibitory synaptic vesicles with high release

423   probability ($P_{r/v}$) to fusion sites enable inhibitory synaptic vesicles to undergo

424   exocytosis earlier than excitatory synaptic vesicles, observed as significantly shorter

425   fusion time of inhibitory synaptic vesicles (Fig. 3). The shorter fusion time will

426   contribute to the faster inhibitory synaptic transmission upon the stimulation. In

427   addition to unique dynamics of inhibitory synaptic vesicles, other mechanisms can also

428   contribute to fast inhibitory synaptic transmission.

429   The specific mechanism driving the unique dynamics of single inhibitory synaptic

430   vesicles compared to excitatory synaptic vesicles is not known. However, the

431   underlying principles can be deduced from the distinct molecular and structural profiles

432   of the inhibitory presynaptic terminals, which influence the encoding of signals via fast

433   and efficient synaptic transmission. The distinct types of Ca^{2+} channels, larger RRP size,

434   higher release probability and tight coupling between the Ca^{2+} channels and Ca^{2+} sensor

435   have been thought to support fast inhibitory synaptic transmission and prevent synaptic

436   fatigue in the inhibitory presynaptic terminals.

437   In summary, we showed that inhibitory synaptic vesicles travel less to reach their

438   fusion sites before exocytosis. We also found that inhibitory synaptic vesicles undergo

439   faster exocytosis and exhibit a higher prevalence of kiss-and-run fusion. This unique

440   dynamics and exocytosis of inhibitory synaptic vesicles can be a novel process to

21

441 mediate the precise conversion of input signals into output signals in hippocampal

442 inhibitory neurons. Thus, our data suggest that unique dynamics and exocytosis of

443 inhibitory synaptic vesicles is one of important factors in inhibitory synaptic

444 transmission.

445 Materials and methods

446 Primary hippocampal neuron culture.

447 Rat (Sprague Dawley) pups at the postnatal 0-day (P0) were sacrificed to harvest CA1

448 and CA3 of the hippocampal regions of the brain tissue as previously described[24, 43].

449 All procedures were performed according to the animal protocol approved by the

450 Department of Health, Government of Hong Kong. Around 2×10^4 neurons were plated

451 on each Poly D-Lysine (P7886, Sigma-Aldrich, USA)-coated cover glass with a

452 diameter of 12 mm (Glaswarenfabrik Karl Hecht GmbH & Co KG, Germany) in 24-

453 well plates. Two days after plating the neurons, 10 µM uridine (U3003, Sigma-Aldrich)

454 and 10 µM 5-fluorodeoxyuridine (F0503, Sigma-Aldrich) were added to inhibit the

455 proliferation of glial cells in cultured neurons[44]. The neurons were cultured in the

456 Neurobasal^{TM}-A Medium (10888022, Thermo Fisher Scientific, USA) supplemented

457 with 2.5 % FBS, 1 % Penicillin-Streptomycin (15140122, Thermo Fisher Scientific),

458 500 µM GlutaMAX^{TM}-I Supplement (A1286001, Thermo Fisher Scientific), and 2 %

459 B-27^{TM} Supplement (17504001, Thermo Fisher Scientific). Cultured neurons were

460 maintained in a 5% CO_2 incubator at 37°C before experiments were performed.

461

462 Microscopy setup.

22

463 A real-time three-dimensional tracking microscope with a custom-built dual-focus

464 imaging optics was built as previously described (24). The dual-focus imaging optics

465 was made up of an aperture, a beam splitter, lenses, mirrors and a right angle mirror. In

466 the dual-focus imaging optics, the beam splitter divided fluorescent signals into two

467 beam pathways, the focus plane of each beam pathway was determined by its lens, and

468 two fluorescence signals from each beam pathway were imaged side-by-side in an

469 electron multiplying (EM) CCD camera. An Olympus IX-73 inverted fluorescence

470 microscope (Olympus, Japan) equipped with an EMCCD camera (iXon Ultra, Andor,

471 UK) was used to acquire fluorescence signals as previously described (45). An oil

472 immersion 100x objective with NA = 1.40 (UPlanSApo, Olympus) was used for

473 imaging Streptavidin-coated quantum dots (QD 625, A10196, Life Technologies)-

474 conjugated antibodies against VGAT and CypHer5E-conjugated antibodies against

475 VGAT. In order to locate two-dimensional centroids and to calculate the fluorescent

476 peak intensity at different focal planes (I_1 and I_2), we used custom-written IDL coded

477 programs (L3Harris Geospatial, USA) to fit a two-dimensional Gaussian function to

478 the fluorescence image. An objective scanner (P-725.2CD, Physik Instrumente (PI),

479 Germany) was used to generate a calibration curve based on the z-position dependence

480 of the normalized intensity difference ($(I_1-I_2)/(I_1+I_2)$). Custom-made programs written

481 in LabVIEW (National Instruments, USA) were used to control an objective scanner

482 and acquire data on the z-position. The z-positions of quantum dots (QDs) were

483 obtained from the relative peak intensity differences using the empirically determined

484 calibration curve, as previously described (24, 28). A 405 nm laser (OBIS, Coherent

23

485   Inc., USA) and a 640 nm laser (OBIS, Coherent Inc.) were used to illuminate

486   streptavidin coated QDs and CypHer5E, respectively. A 15× beam expander (Edmund

487   Optics) and a focus lens (Newport Corporation, USA) were used to illuminate the

488   sample uniformly.

489

490   **Real-time imaging of single inhibitory synaptic vesicles.**

491   Imaging experiments were performed using 14 - 21 days in vitro (DIV) rat primary

492   hippocampal neurons at room temperature. Neurons were pre-incubated with 3 nM of

493   CypHer5E-tagged anti-VGAT antibodies (131 103CpH, Synaptic Systems, Germany)

494   in the culture medium and incubated in the 5% CO_2 incubator for 2 h at 37°C to

495   specifically label inhibitory synaptic vesicles in presynaptic terminals. Then, single

496   inhibitory synaptic vesicles were labeled by QDs conjugated with antibodies against

497   the luminal domain of VGAT. To conjugate QDs to antibodies, Streptavidin-coated

498   quantum dots 625 (A10196, Thermo Fisher Scientific) were incubated with biotinylated

499   antibodies against the luminal domain of VGAT (131 103BT, Synaptic Systems) in a

500   modified Tyrode's solution (4 mM KCl, 150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10

501   mM D-glucose, 10 mM HEPES, 310-315 mOsm/kg, and pH 7.3-7.4) with casein

502   (C7078, Sigma-Aldrich) for 50 min at room temperature. After the incubation, the

503   conjugated solution was added to a sample chamber containing a cover glass with

504   neurons dipped in the modified Tyrode's solution. The labeling of single inhibitory

505   synaptic vesicles with QDs was conducted by triggering evoked release of synaptic

506   vesicles using 10 Hz electric field stimulation for 120 s, which was induced by parallel

24

507   platinum electrodes connected with the SD9 Grass stimulator (Grass Instruments, USA).

508   Following 2 min incubation after stimulation, the Tyrode's solution was superfused for

509   15 min at 1.0 ~ 2.0 ml/min. Subsequently, 2 µM of trypan blue (15250061, Thermo

510   Fisher Scientific) were superfused before imaging. During the imaging, trypan blue

511   (quencher) were kept in the chamber to quench the fluorescence of QDs exposed to

512   external solution during exocytosis. Fluorescence images of CypHer5E-labeled and

513   QD-labeled synaptic vesicles were acquired using an Olympus IX-73 inverted

514   microscope. A ZT640rdc-UF1 (Chroma Technology, USA) and an ET690/50M

515   (Chroma Technology) were used to acquire fluorescence images of CypHer5E-labeled

516   presynaptic boutons. A ZT405rdc-UF1 (Chroma Technology) and an ET605/70M

517   (Chroma Technology) were used to acquire fluorescence images of QD-labeled

518   inhibitory synaptic vesicles. 2000 frame images were obtained using a frame-transfer

519   mode at 10 Hz for 200 s using an EMCCD camera. The EMCCD camera and the

520   stimulator were synchronized with Axon Digidata 1550 (Molecular Devices, USA) to

521   trigger an electric field stimulation at 10 Hz for 120 s while recording. Clampex

522   (program, Molecular Devices) was used to generate stimulation protocols and control

523   stimulation based on the protocol.

524

525   **Analysis of the real-time single vesicle-tracking images.**

526   Custom-written IDL coded programs were used to localize the centroids of raw

527   fluorescence images. Two-dimensional centroids and peak intensities of synaptic

528   vesicles loaded with QDs at two different focal planes were calculated by fitting a

529   Gaussian function to fluorescence images using custom-written programs using IDL.

530   The position along the z-axis was calculated based on a calibration curve while the

531   average intensity of the fluorescence of a QD within a region of interest (ROI) was

532   calculated using MetaMorph (Molecular Devices). Fusion positions and times of single

533   synaptic vesicles were determined as the first quenching positions and times of

534   fluorescence intensity by trypan blue during electrical stimulation similar to the

535   previous report (24). Two-sample Kolmogorov-Smirnov tests (K-S test) were used for

536   statistical analysis, using OriginPro 9.1 (OriginLab, USA). Data are presented as the

537   mean ± standard error of the mean (SEM). Differences with $p<0.05$ was considered

538   significant.

539

540   **Immunofluorescence.**

541   For immunocytochemistry, neurons at DIV14 were washed with PBS once, then fixed

542   with ice-cold 100% methanol for 10 minutes at -20°C. After three washes in PBS for

543   30 min at room temperature, cells were incubated with Chicken antibody against MAP2

544   (1:1000, ab5392, Abcam), Rabbit antibody against Parvalbumin (1:500, PA1-933,

545   Thermo Fischer Scientific) and Mouse antibody against GAD67 (1:500, MAB5406,

546   Sigma-Aldrich) in staining buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton X-100, 30 mM

547   phosphate buffer, pH 7.4) overnight at 4°C. Neurons were then washed three times in

548   PBS for 30 min at room temperature and incubated with Alexa Fluor conjugated

549   secondary antibodies (Goat anti-Chicken-Alexa 488, A11039, Life Technologies; Goat

550   anti-Rabbit-Alexa 568, A11011, and Donkey anti-Mouse-Alexa 647, A31571, Life

551   Technologies) with 1:1000 dilution in staining buffer for 2 hours at room temperature

26

552   and stained with DAPI (300 nM, D1306, Thermo Fisher Scientific) for 10 minutes at

553   room temperature. After washed three times in PBS for 30 min, Cover glasses were

554   mounted onto microscope slides with HydroMount medium (National Diagnostics).

555   Confocal images were acquired using a SP8 confocal microscope (Leica) with a 40x

556   oil objective.

557

558   **Quantification of colocalization.**

559   Biotinylated mouse monoclonal antibody against the luminal domain of vesicular

560   protein Syt1 (105 311BT, Synaptic System) were conjugated with QD 625 streptavidin-

561   conjugates (A10196, Thermo Fisher Scientific) in order to label single SVs. The

562   conjugation and labeling methods are the same as the previously described methods for

563   VGAT-QDs. VGAT-CypHer5E (131 103CpH, Synaptic System) were used to label

564   inhibitory presynaptic terminals by the incubation for 3 hours after experiments. Syt1-

565   labeled SVs localized in inhibitory presynaptic boutons were counted, based on the

566   colocalization of the fluorescence signals of QDs and CypHer5E.

567

568   **Supporting Information**

569   This article contains supporting information online at

570

571   **Acknowledgements**

579

580   **Declaration of interests**

581   The authors declare no conflict of interest.

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688

689   **FIGURE LEGENDS**

690

691   **Fig. 1. Real-time three-dimensional tracking of a single inhibitory synaptic**

692   **vesicle. (A)** Schematic diagram of a streptavidin-conjugated quantum dot (QD)

693   conjugated with biotinylated antibodies against the luminal domain of VGAT by the

694   streptavidin-biotin interaction. **(B)** Colocalization of single inhibitory synaptic

695   vesicles loaded with QDs (VGAT-QD; green) and CypHer5E-labeled inhibitory

696   presynaptic boutons (VGAT-CypHer5E; red). Scale bar: 1 μm. **(C)** Three-dimensional

697   trajectory of an inhibitory synaptic vesicle loaded with a QD laid on the x-y plane of a

698   CypHer5E-labeled presynaptic bouton. A color bar represents elapsed time. This

699   inhibitory synaptic vesicle underwent exocytosis at 32.0 s. **(D)** Fluorescence images

700   of a VGAT-QD shown in Fig 1C taken before exocytosis (20 s and 31.9 s) and after

701   exocytosis (32.3 s). Scale bar: 0.5 μm. **(E)** Three-dimensional trajectory, radial

702   distance from the momentary position to the fusion site ($R = \sqrt{\Delta X^2 + \Delta Y^2 + \Delta Z^2}$), and

703   fluorescence intensity trace of the synaptic vesicle with a QD up to the moment of

704   exocytosis shown in Fig. 1C. Photoblinking events at 8 s, 13 s and 15 s, indicate the

705   presence of a QD inside a synaptic vesicle. Quenching (irreversible loss of

706   fluorescence) at 32.0 s indicates exocytosis of the synaptic vesicle loaded with a QD.

707   Stimulation (10 Hz, 120 s) started at 20 s.

708

709   **Fig. 2.** Inhibitory neurons in primary hippocampal cultures. (A) Representative

710   confocal images of co-immnuonstained cultured hippocampal neurons with MAP2 (a

711   neuron-specific marker; green), glutamic acid decarboxylase 67 (GAD67; an

712   inhibitory neuronal marker; red) and parvalbumin (PV; a marker for PV-expressing

713   interneuron; yellow). Nuclei were counterstained with DAPI (blue). Scale bar: 1 µm.

714   (B) Percentage of GAD67 expressing neurons in MAP2-expressing hippocampal

715   neurons. (C) The percentage of PV-expressing interneurons in GAD67 positive

716   hippocampal neurons.

717

718   **Fig. 3. Distinct spatiotemporal dynamics of single inhibitory synaptic vesicles**

719   **before exocytosis. (A)** Net displacement between initial and fusion sites of VGAT-

720   labeled synaptic vesicles (VGAT SVs, n = 80) and Syt1-labeled synaptic vesicles (Syt1

721   SVs, n = 49). **(B)** Total travel length before exocytosis of VGAT-labeled synaptic

722   vesicles. Total travel length of VGAT-labeled synaptic vesicles was significantly

723   smaller than that of Syt1-labeled synaptic vesicles. **(C)** Fusion time of VGAT-labeled

724   synaptic vesicles and Syt1-labeled synaptic vesicles. Fusion time of VGAT-labeled

725   synaptic vesicles was significantly shorter than that of Syt1-labeled synaptic vesicles.

726   ***p<0.01, *p<0.05* and NS, not significant (K-S test).

727

728   **Fig. 4. Relation of fusion time to net displacement and travel length of inhibitory**

729   **synaptic vesicles. (A)** Relationship between fusion time and net displacement of

730   inhibitory synaptic vesicles. Each dot represents inhibitory synaptic vesicles, and a

731   solid red line represents the linear regression line (Pearson's r = 0.77), indicating close

732   relationship between release probability and position of inhibitory synaptic vesicles. **(B)**

733   Cumulative fraction of net velocity. Net velocity of inhibitory synaptic vesicle was not

734 significantly different from Syt1-labeled synaptic vesicles. **(C)** Relationship between

735 fusion time and total travel length for inhibitory synaptic vesicles. A larger

736 value of Pearson's correlation (Pearson's r = 0.94), indicating strong linear association

737 between release probability and total travel length of inhibitory synaptic vesicles. **(D)**

738 Cumulative fraction of speed. Speed of inhibitory synaptic vesicle was significantly

739 different from Syt1-labeled synaptic vesicles. ***p<0.01* and NS, not significant (K-S

740 test).

741

742 **Fig. 5. Exocytotic fusion mode of inhibitory synaptic vesicles. (A)** Images before any

743 fluorescence drop (70.2 s), after partial loss of fluorescence (71.2 s), before (105 s), and

744 after subsequent loss of the remaining fluorescence (105.9 s). Scale bar, 0.8 μm. **(B)** 3D

745 position and fluorescence of a QD-loaded vesicle that undergoes kiss-and-run fusion.

746 The unquenched fraction with partial quenching (0.35) was larger than expected

747 unquenched value (0.12). This partial quenching indicates kiss-and-run fusion. A red

748 arrow marks the time of kiss-and-run fusion, and a blue arrow marks the time of full-

749 collapse fusion. Green bar, 10 Hz stimulation for 120 s. **(C)** Average normalized

750 fluorescence intensities with SEM in time aligned with the first fusion depending on

751 three quenching patterns - almost complete quenching (C1), partial quenching followed

752 by almost complete quenching (C2), and partial quenching not followed by complete

753 quenching (C3). The horizontal dot line represents the normalized remaining

754 fluorescence intensity of 0.12 (the expected normalized fluorescence right after full-

755 collapse fusion). **(D)** Prevalence of kiss-and-run fusion. The prevalence of kiss-and-run

34

756   fusion in inhibitory synaptic vesicles (65%) was higher than that in Syt1-labeled

757   synaptic vesicles (27%). **(D)** Total travel length of inhibitory synaptic vesicles

758   undergoing full-collapse fusion (VGAT FCF) was significantly different from that of

759   Syt1-labeled synaptic vesicles (Syt1 FCF). **(E)** Total travel length of inhibitory synaptic

760   vesicles undergoing kiss-and-run fusion (VGAT K&R) was not significantly different

761   from that of Syt1-labeled synaptic vesicles (Syt1 K&R). **(F)** Fusion time of inhibitory

762   synaptic vesicles undergoing full-collapse fusion (VGAT FCF) was not significantly

763   different that of Syt1-labeled synaptic vesicles (Syt1 FCF) **(G)** Fusion time of inhibitory

764   synaptic vesicles undergoing kiss-and-run fusion (VGAT K&R) was not significantly

765   different that of Syt1-labeled synaptic vesicles (Syt1 K&R). *$p<0.01$, **$p<0.01$, and*

766   NS, not significant (K-S test).

767

768

35

A Streptavidin-coated quantum dot (QD) | Biotin-labeled IgG against VGAT

B VGAT-CypHer5E | VGAT-QD | Merge

C Z (nm) vs X (nm) vs Y (nm), Time (s) color scale 0.1–32.0

D 20.0 s | 31.9 s | 32.3 s

E Stim. at 10 Hz

Pos. (nm): X, Y, Z

R (nm)

F (a.u.)

Time (s)
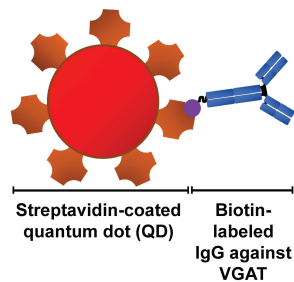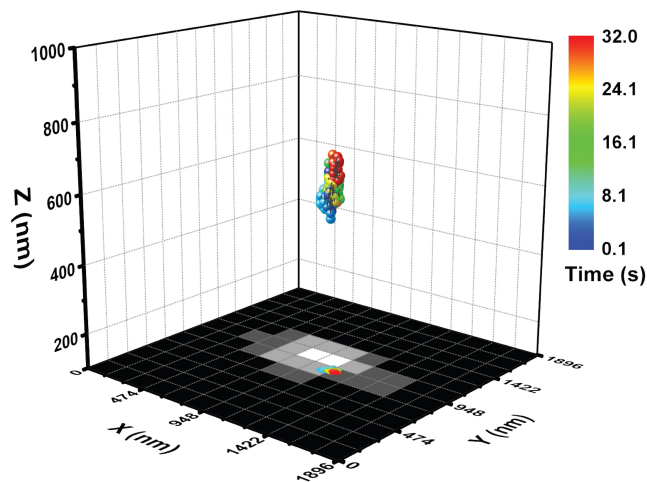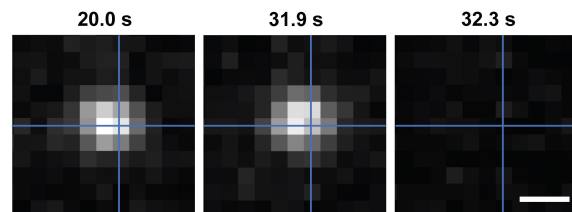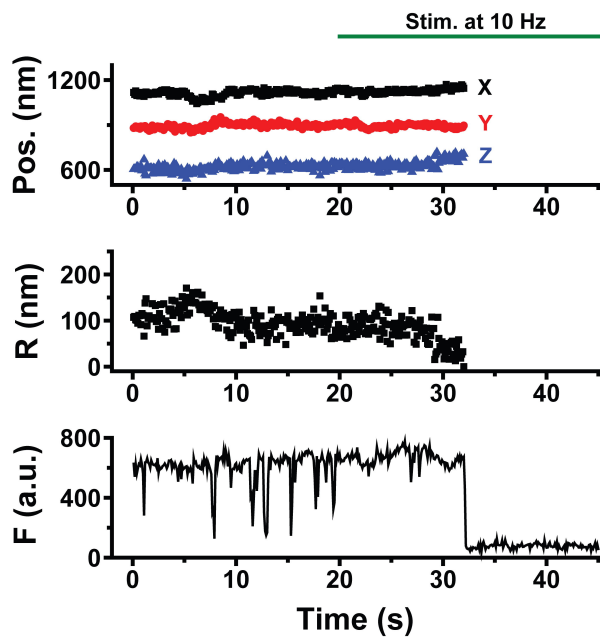
A

| DAPI | MAP2 | GAD67 | Parvalbumin | Merge |

B

C

A

Fusion time (s) vs Net displacement between initial and fusion sites (nm)

B

Cum. Fraction vs Net velocity (nm/s)

NS

VGAT
Syt1

C

Fusion time (s) vs Total travel length before fusion (μm)

D

Cum. Fraction vs Speed (nm/s)

**

VGAT
Syt1