

Supplemental Material

Supplemental Methods

Electrical stimulation of CX-G3-labeled hippocampal neurons

Following 5 min incubation in 0.5 μ M CX-G3 and washes, 18-20 DIV neurons were imaged in normal Tyrodes solution in a custom-built imaging chamber as described previously. Images were acquired every 5 seconds over a 5 min timecourse, under baseline conditions or in the presence of electrical stimulation (10Hz, 60 seconds; 10 mA current, 1 msec pulse width), administered following the first 3 images (15 sec into timecourse). To calculate the change in CX-G3 fluorescence intensity over time, we used the Time Series Analyzer V 2.0 plugin (Balaji) in Image J. Fluorescence intensity for each punctum at any given time 't' (F_t) was expressed as a fraction of its starting fluorescence (F_0) using the equation: $(F_t - F_0)/F_0$. These values were averaged for all puncta in a field of view and normalized to the peak fluorescence intensity, making '1' the highest value.

Triton X-100 extraction

For 18-20 DIV hippocampal neurons labeled with CX-G3 and imaged in Tyrodes solution, images were acquired before and after application of 0.01% Triton X-100.

CX-G3 labeling in astrocyte cultures

Astrocytes were prepared as previously described¹, plated into 96-well plates coated with poly-L-lysine, and imaged after 1 week in culture. Prior to imaging, cells were incubated with 2 μ M CX-G3 and 5 μ M Calcein AM (Thermofisher) for visualization.

Tables**Table S1.** Colocalization of GFP-Syn and mCh-Rab3 with synaptic and non-synaptic markers.

Marker protein	Fraction colocalization with GFP-Syn (avg +/- STD)	Fraction colocalization with mCh-Rab3 (avg +/- STD)
MAP2 (cell body/dendrite)	0.158 +/- 0.03	0.194 +/- 0.01
LAMP1 (lysosome)	0.119 +/- 0.06	0.126 +/- 0.02
SV2 (synaptic)	0.595 +/- 0.12	--
VGLUT1 (synaptic)	0.607 +/- 0.17	--
Synaptophysin (synaptic)	--	0.649 +/- 0.08
Synapsin (synaptic)	--	0.834 +/- 0.07
GFP (control for GFP-Syn)	1.05 +/- 0.11	--
mCherry (control for mCh-Rab3)	--	0.876 +/- 0.12

Table S2. Organization of fluorescent dyes into groups, based on excitation/emission wavelength, for high-throughput screening

Filter combination	Number of compounds	Number of 96wells plates	Synaptic marker used
CFP:YFP	503	6	mCherry-Rab3
DAPI:Cy3	80	1	mCherry-Rab3
DAPI:FITC	961	12	mCherry-Rab3
FITC:Cy3	669	8	mCherry-Rab3
FITC:FITC	633	8	mCherry-Rab3
FITC:YFP	831	10	mCherry-Rab3
YFP:YFP	38	1	mCherry-Rab3
CFP:Cy3	324	4	GFP-Synapsin
Cy3:Cy3	311	4	GFP-Synapsin
Cy5:Cy3	31	1	GFP-Synapsin
DAPI:CFP	401	5	GFP-Synapsin
DAPI:DAPI	401	5	GFP-Synapsin
dsRed:dsRed	968	12	GFP-Synapsin
FITC:TexasRed	82	1	GFP-Synapsin
TexasRed:Cy5	94	2	GFP-Synapsin
TexasRed:TexasRed	665	9	GFP-Synapsin

Table S3. List of hit compounds from each group, with group average mean colocalization and hit threshold (** = groups that exhibited significant bleedthrough into synaptic marker channel)

Filter combination	Mean colocalization with synaptic marker	Hit threshold (StdDev >2.5)	Number of hits (StdDev >2.5)
FITC: Cy3**	33.8 %	86.8 %	4
Cy3: Cy3**	29 %	73.0 %	2
dsRed: dsRed	24.9 %	67.4 %	-
CFP: Cy3**	14.5 %	63.5 %	4
TexasRed: TexasRed	24.7 %	62.2 %	1
FITC: YFP **	18.6 %	59.6 %	3
Cy5: Cy3	25.5 %	58.7 %	-
FITC: FITC	15.3 %	56.2 %	-
TexasRed: Cy5	27.4 %	56.2 %	-
YFP: YFP	16.2 %	52.6 %	-
DAPI: FITC	12.4 %	49.8 %	1
CFP: YFP **	13.9 %	47.9 %	3
DAPI: Cy3	13.3 %	44.6 %	3
DAPI: CFP	8.6 %	27.8 %	8
FITC: TexasRed	3.1 %	25.8 %	2
DAPI: DAPI	4.1 %	25.1 %	22

Table S4. Colocalization of CX compounds with GFP-Synapsin, arranged from highest to lowest average fraction colocalization, for 3 experiments

Compound Name	Expt. 1	Expt. 2	Expt. 3	Average colocalization	STDEV
CX-G3	0.400091624	0.35887369	0.330652323	0.363205879	0.03492177
CX-D10	0.332167577	0.37816616	0.371329948	0.360554562	0.024820337
CX-D5	0.344172452	0.368942561	0.348666409	0.353927141	0.013196441
CX-A6	0.347260702	0.336724169	0.36113565	0.348373507	0.012243727
CX-E3	0.383308985	0.372455505	0.273921309	0.3432286	0.060266698
CX-E6	0.362289168	0.332792115	0.305939387	0.333673557	0.028185229
CX-A4	0.340068341	0.337636462	0.292401054	0.323368619	0.026846249
CX-A8	0.295907985	0.336455464	0.334761601	0.322375017	0.022936763

CX-B9	0.329788244	0.325802873	0.3001188	0.318569972	0.016102954
CX-B11	0.308280392	0.321880349	0.262925852	0.297695531	0.030869688
CX-F8	0.262217893	0.316582542	0.305546188	0.294782208	0.028736322
CX-D6	0.269480519	0.329993605	0.274290557	0.291254894	0.033634802
CX-D11	0.293083573	0.306454272	0.260283092	0.286606979	0.023757192
CX-D8	0.301872752	0.316968915	0.240726589	0.286522752	0.040372515
CX-E5	0.305103931	0.311484305	0.24026863	0.285618955	0.039403887
CX-C4	0.313543381	0.287095336	0.228452656	0.276363791	0.043548621
CX-E2	0.286845579	0.298420294	0.238445724	0.274570532	0.031815795
CX-G6	0.30675619	0.271348448	0.235994699	0.271366446	0.035380749
CX-C7	0.20177268	0.372809228	0.237732815	0.270771574	0.090177852
CX-A2	0.227790815	0.290228977	0.287364352	0.268461381	0.035250855
CX-A7	0.272355541	0.268108062	0.26480874	0.268424114	0.003783314
CX-G4	0.261982186	0.284447917	0.254360214	0.266930106	0.015642215
CX-E4	0.276600955	0.248416548	0.256436267	0.26048459	0.014521773
CX-D2	0.249287536	0.267239046	0.25713439	0.257886991	0.008999388
CX-A11	0.169431759	0.271175391	0.30620577	0.24893764	0.071046961
CX-C11	0.273662551	0.238315238	0.207543925	0.239840571	0.033085694
CX-B10	0.223882839	0.229948854	0.201726488	0.218519394	0.014855989
CX-A5	0.211844031	0.213865977	0.226887948	0.217532652	0.008164756
CX-C3	0.244762514	0.228802685	0.156312577	0.209959259	0.047139727
CX-D4	0.211501597	0.201562449	0.212182162	0.208415403	0.005944579
CX-B7	0.209573514	0.214468881	0.196198235	0.206746877	0.009457618
CX-E9	0.168491891	0.257078524	0.187798049	0.204456155	0.046583444
CX-H3	0.222857143	0.26364841	0.125556955	0.204020836	0.070946581
CX-B4	0.219330105	0.224126635	0.150895296	0.198117345	0.040965756
CX-B5	0.197003478	0.204168032	0.175157252	0.192109587	0.015111882
CX-C6	0.197344322	0.194346674	0.172707889	0.188132962	0.013442326
CX-B8	0.191304348	0.206979475	0.147796589	0.182026804	0.030662813
CX-H2	0.235697607	0.240964139	0.032258065	0.169639937	0.119005329
CX-B2	0.159160789	0.187548905	0.122838106	0.156515933	0.032436373
CX-H6	0.1697385	0.191867759	0.107667043	0.156424434	0.043650755
CX-C5	0.137692839	0.199177768	0.125581321	0.154150643	0.039462054
CX-C2	0.138428918	0.174863828	0.128513383	0.14726871	0.024406912
CX-A10	0.19108663	0.13199922	0.112741396	0.145275749	0.040825159
CX-B6	0.148930154	0.177660302	0.10101998	0.142536812	0.038718095
CX-A3	0.108695652	0.141789647	0.131479897	0.127321732	0.016934311
CX-H11	0.132135704	0.130083251	0.094606049	0.118941668	0.021100235
CX-F5	0.126860088	0.119274363	0.107762467	0.117965639	0.009615839
CX-G11	0.120705475	0.13121043	0.084410093	0.112108666	0.024555992
CX-H10	0.122125095	0.120981777	0.083070518	0.108725797	0.022225476
CX-C9	0.087229331	0.108337436	0.10485418	0.100140316	0.011316069
CX-C8	0.091809267	0.110867474	0.088861468	0.097179403	0.011945495

CX-F4	0.096732673	0.09429083	0.095372796	0.095465433	0.001223554
CX-B3	0.119477588	0.089790455	0.053502544	0.087590196	0.03304251
CX-G9	0.088948606	0.103500863	0.03922309	0.077224186	0.033704666
CX-D7	0.069134292	0.091011942	0.058659218	0.072935151	0.016507864
CX-G10	0.074237856	0.070176239	0.022324578	0.055579558	0.02887117

Figure S1: CX compound lipophilicity vs. number of puncta

A) Heat map of Q4 plate, showing side chain structures of CX xanthone compounds. Darker color corresponds to higher number of puncta that colocalize with GFP-Syn . B) Plot of xanthone compound side chain calculated lipophilicity (LogP) vs. number of puncta that colocalize with GFP-Syn. CX-G3 and other compounds with high colocalization are highlighted in red. Note that the side chain of CX-G3 has a LogP value of 3.27. C) Graph of LysoTracker colocalization with GFP-Synapsin (fraction of LysoTracker puncta containing GFP signal). Colocalization of LysoTracker with GFP-Synapsin in randomized images (depicted with *) are included for comparison (n=12 images acquired from two independent batches of neurons).

Figure S2: Properties of CX-G3 labeling in neurons

A) Images of CX-G3 in hippocampal neurons at t=5 sec (1st image) and t=5 min (60th image), from a 5-minute timecourse in which images were acquired every 5 sec and neurons were not subject to electrical stimulation (control condition), in contrast to panels C and D. Size bar is 10 μ m. B) Quantification of normalized CX-G3 fluorescence intensity over time in this image series (see Supplemental Methods for details of analysis). Red arrows indicate t=5 sec and t=5 min. No photobleaching is observed; CX-G3 fluorescence actually increases slightly over the timecourse as indicated on the graph. Similar results were obtained for 4 fields of view, each from a different coverslip. C-D) Similar to A and B, but for a timecourse in which neurons were electrically stimulated (10Hz, 1 min; red bar in D) to induce synaptic vesicle recycling. Very little change in fluorescence intensity was observed during or after stimulation. Similar results were obtained from 4 fields of view, each from a different coverslip. Size bar is 10 μ m. E) Images showing CX-G3 labeling in hippocampal neurons before and after mild detergent extraction with 0.01% Triton X-100. Note that Triton removes virtually all of the CX-G3 signal. Size bar is 10 μ m. F)

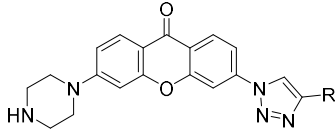
Fluorescence excitation spectrum of CX-G3 obtained on the two-photon microscope. G) Images of CX-G3 labeling in glia. Cultured astrocytes were labeled with calcein dye (green) together with CX-G3.

Note that CX-G3 exhibits a perinuclear labeling pattern in astrocytes. Size bar is 10 μ m.

1. Cortese, G. P.; Zhu, M.; Williams, D.; Heath, S.; Waites, C. L., Parkin Deficiency Reduces Hippocampal Glutamatergic Neurotransmission by Impairing AMPA Receptor Endocytosis. *J Neurosci* **2016**, *36* (48), 12243-12258.

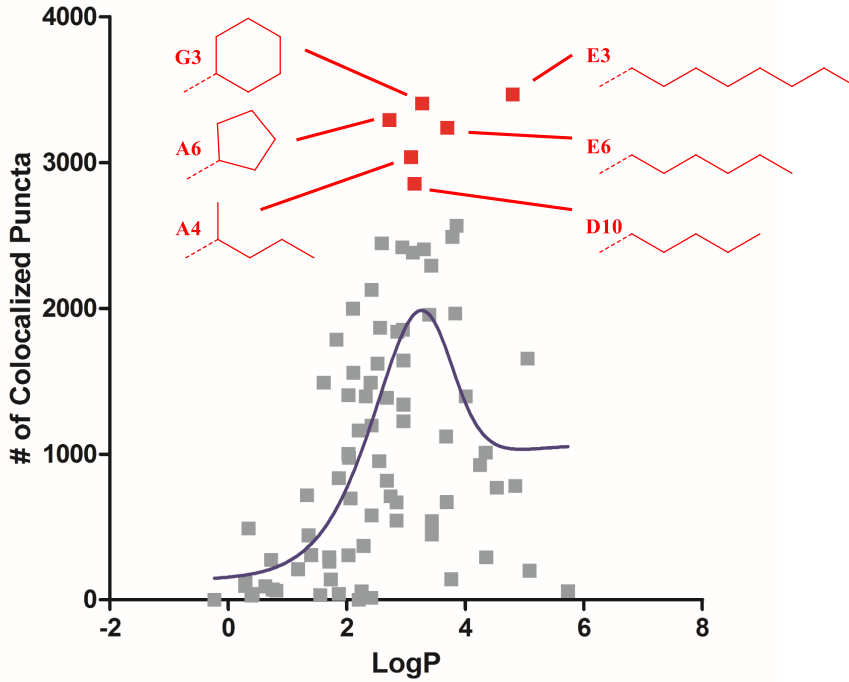
a

General Structure



Side Chains

	2	3	4	5	6	7	8	9	10	11
A	 149194	 49154	 303747	 149195	 329131	 240544	 256760	 162022	 133974	 101040
B	 76948	 71235	 139540	 112074	 66883	 116227	 69734	 241923	 139650	 249154
C	 67113	 165550	 195682	 54527	 119740	 122517	 20015	 29178	 81831	 196376
D	 184073	 58109	 138682	 238217	 185461	 14205	 212620	 95309	 285579	 244692
E	 186674	 346874	 164243	 229452	 323974	 97656	 78143	 92577	 6184	 5907
F	 6293	 4161	 31003	 44247	 1488	 11857	 155908	 27381	 71895	 37181
G	 70	 340690	 199970	 3362	 178517	 9768	 14091	 29293	 21104	 26292
H	 140492	 100230	 7219	 4250	 83690	 3072	 9474	 30665	 44850	 54080

b**c**

Lysotracker colocalization with GFP-Synapsin

