Methods

Cell culture and transfection
Dissociated hippocampal cultures were obtained from newborn rats as described previously [1]. In brief, hippocampi were dissected in HBBS (Invitrogen) supplemented with 7 mM HEPES and digested with 0.25% trypsin (Invitrogen) at 37°C for 20 min. After trituration with a fire-polished glass pipette, cells were plated at a density of 60,000 cells/well on top of a pregrown rat glia monolayer on 25-mm coverslips. Cultures were grown in Neurobasal supplemented with B27, 18 mM Hepes, 0.5 mM GlutaMAX, and penicillin/streptomycin (all obtained from Invitrogen), and half the medium was replaced once every week. 5 µM arabinofuranosyl cytidine was added at DIV 2 to prevent microglia proliferation. Autaptic island cultures were cultured similarly as previously described in [2]. Islands of collagen/poly-d-lysine substrate were applied to glass coverslips using a stamp with evenly spaced squares. Astrocytes were cultured on these islands for 4–7 d before the addition of neurons. Mouse neurons were obtained from E18 pups, obtained by caesarian section of pregnant females from previously documented mouse lines (Syt1-KO [3], Doc2ab DKO [4, 5], MEN1-lox [6]). Animals were housed and bred according to institutional guidelines and Dutch law.

Calcium transfection
For live-imaging experiments, neurons were transfected with 3 µg/well SypHy (gift from A. Jeromin, Allen Institute for Brain Science, Seattle, WA; [7]) and 0.5 µg/well eCFP at DIV 6–8 using Ca2+ phosphate [8]. The eCFP signal was used to identify the transfected cell. The transfection protocol was optimized to yield only a few transfected cells per coverslip.

Viral transduction
Lenti viral particles encoding for eGFP or Cre-eGFP were produced as described previously [9, 10]. Viral transduction was performed at DIV1 for mouse hippocampal neurons, or DIV9 for rat hippocampal neurons. Semliki forest viral constructs encoding for synaptotagmin1(wt)-IRES-eGFP, synaptotagmin1(T112A)-IRES-eGFP were a generous gift from Jakob Sørenson (Kopenhagen University, Denmark, [11]). Viral particles were produced as described in reference [12], and viral transduction was performed 16 hours before start of the experiment.

Electrophysiology
Whole-cell recordings were performed at DIV 12–16 with borosilicate glass pipettes (2–4 mOhm) containing 125 mM K+-gluconic acid, 10 mM NaCl, 4.6 mM MgCl$_2$, 4 mM K$_2$-ATP, 15 mM creatine phosphate, 1 mM EGTA, and 20 U/ml phosphocreatine kinase, pH 7.3. Intracellular solution for the postsynaptic cell contained 80 µm of fixable Alexa Fluor 468–dextran (Invitrogen). In chapter 2 & 3, samples were constantly superfused with external solution containing 2 mM CaCl$_2$, 2.5 mM KCl, 119 mM NaCl, 2 mM MgCl$_2$, 30 mM glucose, and 25 mM Hepes, pH 7.4. Experiments in chapter 4 & 5 were performed with extracellular solution containing 140 mM NaCl, 2.4 mM KCl, 4 mM CaCl$_2$, 4 mM MgCl$_2$, 10 mM HEPES and 10 mM Glucose, pH 7.3. Cells were kept in a voltage clamp (membrane potential = −70 mV) using Axopatch 200B amplifiers (Axon Instruments). In chapter 4, series resistance was 90% compensated (20 µs lag time), and recordings were discarded if series resistance exceed 10 mOhm, holding current was lower than -300 pA or the
evoked EPSC amplitude did not exceed 500 pA. APs were induced by 0.5–2 ms steps to 30 mV, which was controlled by a stimulator (Master-8; A.M.P.I.). Signals were recorded with Digidata 1440A and pCLAMP 10 software (both obtained from Axon Instruments). All experiments were performed at RT (20–23°C). Glutamate application was performed using pipettes identical to recording pipettes, placed near the soma of interest. Pipette was filled with extracellular solution plus 100 µM glutamate, which was applied for 20 ms with 20 psi. Acquired signals were analyzed in ClampFit 10 (Axon Instruments), MiniAnalysis 6 (Synaptosoft) and custom written programs in Matlab (the Matworks).

Live imaging
SypHy imaging was performed at RT, using an inverted microscope (IX71; Olympus) equipped with an 40x oil objective, NA 1.41, a xenon lamp (DG-4; Sutter Instrument), and 488/6- and 520/35-nm filter sets (Semrock). Images were acquired with an intensified charge-coupled device camera (XR/TURBO-120EX; Stanford Photonics) at 60 Hz (unless otherwise stated) controlled by Piper v1.51 (Stanford Photonics). Regions of interest of 2.2 × 2.2 µm were placed based on the pHluorin response to 900 APs at 20 Hz with subsequent application of 50 mM NH4+ and were omitted from further analyses when they did not respond to this AP train (response of <2× SD of baseline). Raw traces were extracted from the images using ImageJ (National Institutes of Health) and analyzed with custom-written programs in Matlab (MathWorks). A running mean of 10 frames was applied to the raw traces to reduce noise. Cell pairs were rejected if the imaging results showed substantial activity asynchronous to the applied AP pattern, as it suggests that the SypHy+ synapses were formed by more than one transfected cell. At the end of the experiment, samples were fixed in 4% formaldehyde. The recorded cells were relocated on a confocal microscope (LSM510; Carl Zeiss) to identify the SypHy+ synapses that were formed on the postsynaptic cell.

Immunocytochemistry and confocal microscopy
Cultures were fixed at DIV 14 (unless otherwise stated) with 4% formaldehyde and permeabilized with 0.5% Triton X-100. Neurons were then stained with primary antibodies for 1 h at RT, washed with PBS, and stained for 1 h at RT with secondary antibodies with conjugated Alexa Fluor (1:1,000; Invitrogen). The primary antibodies and dilution used were chicken anti-MAP (1:10,000; Abcam), mouse anti-VAMP2 (1:1,000; Synaptic Systems), rabbit anti-Synapsin I (1:1,000; P610; a gift from T.C. Sudhof, Stanford University School of Medicine, Stanford, CA), mouse anti-Bassoon (1:500; Stressgene), mouse anti–Munc18-1 (1:500; Synaptic Systems), mouse anti–Rab3-interacting molecule (1:200; BD), rabbit anti-Syntaxin (1:1,000; Synaptic Systems), rabbit anti-vGAT (1:500; Synaptic Systems), rabbit anti-Prox1 (1:5,000; Covance), rabbit anti-vGlut1 (1:500; Synaptic Systems), guinea pig anti-vGlut1 (used in combination with Prox1; 1:5,000; Millipore), and rabbit anti–synaptotagmin-1 (1:2,000; W855; a gift from T.C. Sudhof). Coverslips were mounted with 1,4-diazobicyclo-[2.2.2]-octane–Mowiol (Invitrogen) and examined on a confocal microscope (LSM510). Images were acquired with a 40x oil objective, NA 1.3, and 0.7x mechanical zoom. In chapter 3, all images were z stacks with a 1-µm interval and were collapsed to maximal projection before analysis. All images were analyzed in Matlab with SynD [13] and custom-written programs. Synapses were detected based on the staining for VAMP, Synapsin, vGAT, or vGlut, depending on antibody compatibility. Detected
regions were subsequently used to measure the synaptic intensity of additional proteins. Arc distance from the soma was measured along the dendritic marker MAP or GFP. For distance-binned cumulative histograms, synapses were grouped based on distance from postsynaptic soma in 20 µm bins. Fluorescence intensity was normalized to the brightest synapse formed on each neuron.

Statistics
Statistical significance between experimental groups was tested using a Kruskal-Wallis analysis of variance. Statistics for Sholl analysis was only performed at bin with largest difference in average number of branches, because observations in different bins are interdependent. To test the distance dependency of the measured parameters per cell, the ratio of proximal synapses (<50 µm from the soma) over distal synapses (last 50 µm) was calculated per cell (P/D ratio). Significance of this ratio was tested with a one-sample Wilcoxon rank test, assuming that P/D = 1 if the measured parameter is independent of distance. All tests were performed in Matlab. N indicates the number of independent experiments. Differences were considered significant if P < 0.05. All bar graphs represent means ± SEM.

Chemicals
TTX and AP5 were obtained from Ascent, and DNQX was purchased from Enzo Life Sciences. All other chemicals were obtained from Sigma-Aldrich.

References