On-Line Only Methods

Plasmids

VAMP-mCherry was designed by replacing the pHluorin of VAMP-pHluorin[31] with mCherry. Vesicular Glutamate-mOrange2 (vGmOr2) was designed by replacing the pHluorin of vGlut1-pHluorin[32] with mOrange2[33]. GFP-$\alpha1_A$ was kindly provided by Gerald Obermair[12], E1656K mutagenesis of was outsourced to Mutagenex Inc. (Piscataway, NJ). GCaMP3[19] cDNA was kindly provided by Loren Looger. The auxiliary subunits used were rat $\alpha_2\delta$-1 (Genbank accession number AF_286488), mouse $\alpha_2\delta$-2 (AF_247139), rat $\alpha_2\delta$-3 (NM_175595) and rat β4 (NM_001105733). The α2δ-1 and α2δ-2 MIDAS^{AAA} constructs were made by standard molecular biological techniques and verified by DNA sequencing. MIDAS mutations were generated by mutating 3 MIDAS motif amino acids to Ala. D^{259}VSGS in α2δ-1 and D^{300}VSGS in α2δ-2 became AVAGA. All auxillary subunits were cloned into pcDNA3.0 vectors. For knockdown of endogenous $\alpha1_A$ or α2δ–1 subunits, mRNA target sequences (GCATTCTCCTCTGGACTTTCG) and (ACTCAACTGGACAAGTGCCTTAGATGAAG) respectively, were cloned into an shRNA vector[30]

Immunofluorescence and Quantification

To quantify $\alpha1_A$ in presynaptic boutons , following live cell imaging, neurons were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 5% BSA and 1% goat serum for 1 hour, and subsequently incubated overnight at 4°C with [anti-GFP (Invitrogen, CA) 1:3000, anti-$\alpha1_A$ (Synaptic Systems, Germany)]. Alexa-488- or Alexa-546-conjugated secondary antibodies (1:1000) were applied post primary antibody incubation. Expression level ratios of $\alpha1_A$ were measured as follows: 2μm diameter circular ROIs were centered on transfected synaptic marker to indentify the axonal boutons of transfected neurons and then compared to an equal number of adjacent ROIs that were centered on any small punctate spots of $\alpha1_A$ fluorescence within 2-10 μm of measured transfected boutons with local background correction. For measurements of α2δ-1, cells were fixed and permeabilized with 10% MES buffer pH6.9 and 90% MeOH at -20°C for 5 minutes then blocked for 2 hours at RT. Primary antibodies for α2δ-1 (1:100 dilution; C14882 LSBio) were incubated at 37°C for 4 hours prior to wash and detection with secondary antibodies**.**

Live Cell Imaging

Action potentials were evoked by passing 1-ms current pulses, yielding fields of ~10 V/cm via platinum-iridium electrodes. Live-cell imaging experiments were performed at 30.0 ± 0.2°C. Cells were continuously perfused at 0.2–1.0 ml/min. in a saline solution containing (in mM) 119 NaCl, 2.5 KCl, 4 $CaCl_2$, 25 HEPES, buffered to pH 7.4, 30 glucose, 10 μM 6-cyano-7- nitroquinoxaline-2,3-dione (CNQX), and 50 μM D,L-2-amino-5-phosphonovaleric acid (AP5). NH_4Cl applications were done with 50 mM NH_4Cl in substitution of 50 mM of NaCl (buffered to pH 7.4). All chemicals were obtained from Sigma except for Ca^{2+} channel toxins (Alomone Labs) and Ca^{2+}dyes (Invitrogen). During experiments, cells were allowed to rest for ~30 s between 1 AP trials and at least 5 min between 100 Hz AP bursts. All RRP measurements were the average of 8 trials of 20 AP at 100 Hz, details of measurements previously described[17]. 1 Khz imaging used 0.972 ms in frame transfer mode with an imaging field of 5 pixel width (2 μm) and 512 pixel height. Toxins to block P/Q- and N-type channels were applied for ~2 min prior to stimulation with washout at the following concentrations: agatoxin IVA (400 nM) and

conotoxin GVIA (40 μM). Fluo5F measurements were obtained by diluting a DMSO stock 1μg/μl 1:150 and loading or 10 minutes at 30°C before washing for 30 minutes prior to imaging. For experiments with vGlut-pHluorin involving EGTA-AM, 200 μM was loaded for 90 s, followed by a 10 minute wash prior to beginning experiments.

GCaMP3 measurements

We found that GCaMP3 had a highly non-linear response to changes in [Ca^{2+}] as previously described in vitro[19]. We compared the change in fluorescence relative to the response of single AP in cells transfected with GCaMP compared to similar cells loaded with Magnesium Green Dye (MgG AM-ester dye)[17]. Cytosolic GCaMP3 used to measure presynaptic bouton intracellular Ca^{2+} in hippocampal neurons stimulated by field potential-generated action potentials. GCaMP3 peak fluorescence (ΔF) for each stimulation was found by averaging the 5 highest points post stimulation and subtracting the average of 10 points prior to stimulation. All changes in fluorescence were normalized to GCaMP intensity to Ca^{2+} saturation (F_{MAX}). FMAX was calculated by applying a solution of Tyrodes buffer (pH 6.9) at the end of experiments containing ionomycin (1 mM. Ionomycin exposure caused a 6.1-6.6 fold increase in GCaMP fluorescence in good agreement with results found previously under these pH conditions (personal communication – Loren Looger, Janelia Farms, HHMI). These values were then compared to MgG values under similar conditions. We found that these changes in fluorescence could be well fit with a Hill equation: Vmax=1, k=9.077, n=2.46; adjusted R^2=0.997. The fit to the Hill equation was in good agreement with published expectations[19]. To linearize GCaMP 3 signals we inverted the Hill equation to obtain an expression of the signal relative to that obtained with MgG following 1 AP stimulation using the following equation: Linearized GCaMP = $(((\Delta F/F)/F_{MAX})*k^n)/(1-((\Delta F/F)/F_{MAX}))^{(1/n)}$.

Electrophysiology

Calcium channel expression in tsA-201 cells was investigated by whole cell patch clamp recording. The patch pipette solution contained in mM: Cs-aspartate, 140; EGTA, 5; $MgCl_2$, 2; $CaCl_2$, 0.1; ATP, 2; HEPES, 20; pH 7.2, 310 mOsm with sucrose. The external solution for recording Ba^{2+} currents contained in mM: tetraethylammonium (TEA) Br, 160; KCl, 3; $NaHCO_3$, 1.0; $MgCl_2$, 1.0; HEPES, 10; glucose, 4; $BaCl_2$, 1, pH 7.4, 320 mosM with sucrose. Measurements and analysis were performed as previously described[28].

Action potential recordings were performed in a bath solution containing (mM): NaCl 145, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1, HEPES 10, Glucose 10, pH 7.4. The internal solution contained (mM): KCl 130, EGTA 10, HEPES 10, NaCl 8, Mg-ATP 4, $MgCl_2$ 1, $CaCl_2$ 1, Na_2-GTP 0.4, pH 7.25 adjusted with 1M KOH, 318 mOsm. Data were analysed with Clampfit 9 (Molecular Devices), recorded traces were post-processed with 1 kHz 8-pole Bessel digital filter, the AP initiated at rheobase was used for measurement of the peak of AP overshoot, the peak of after-hyperpolarization (AHP) and the duration of AP. Measured parameters between two groups were compared using t-Student's test. Dorsal root ganglion neurons (DRGs) were prepared from P10 Sprague Dawley rats and transfected as previously described[34].

31. Miesenbock, G., De Angelis, D. A. & Rothman, J. E. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature* **394**, 192-5 (1998).
32. Voglmaier, S. M. et al. Distinct endocytic pathways control the rate and extent of synaptic vesicle protein recycling. *Neuron* **51**, 71-84 (2006).
33. Shaner, N. C. et al. Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nat Methods* **5**, 545-51 (2008).
34. Viard, P. et al. PI3K promotes voltage-dependent calcium channel trafficking to the plasma membrane. *Nat Neurosci* **7**, 939-46 (2004).