

Optogenetic Inhibition of Synaptic Release with Chromophore-Assisted Light Inactivation (CALI)

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SUMMARY

Optogenetic techniques provide effective ways of manipulating the functions of selected neurons with light. In the current study, we engineered an optogenetic technique that directly inhibits neurotransmitter release. We used a genetically encoded singlet oxygen generator, miniSOG, to conduct chromophore assisted light inactivation (CALI) of synaptic proteins. Fusions of miniSOG to VAMP2 and synaptophysin enabled disruption of presynaptic vesicular release upon illumination with blue light. In cultured neurons and hippocampal organotypic slices, synaptic release was reduced up to 100%. Such inhibition lasted >1 hr and had minimal effects on membrane electrical properties. When miniSOG-VAMP2 was expressed panneuronally in *Caenorhabditis elegans*, movement of the worms was reduced after illumination, and paralysis was often observed. The movement of the worms recovered overnight. We name this technique *Inhibition of Synapses with CALI (InSynC)*. InSynC is a powerful way to silence genetically specified synapses with light in a spatially and temporally precise manner.

INTRODUCTION

Optogenetic approaches allow experimenters to control neurophysiological functions of a genetically defined neuronal population through expression of light-responsive activity-modulating proteins. For example, microbial opsin pumps hyperpolarize membrane potentials of expressing neurons during light illumination, reducing the probability of the neurons to achieve suprathreshold depolarization with excitatory inputs (Han and Boyden, 2007). Chemical-biological optogenetic approaches can also be used to hyperpolarize membrane potential (Levitin et al., 2013; Janovjak et al., 2010). The microbial opsin channels, channelrhodopsins, can be used to achieve suprathreshold depolarization with light pulses in the expressing neurons (Boyden et al., 2005; Lin et al., 2009). When channelrhodopsins are expressed

at high levels at the membrane of presynaptic terminals, light can induce direct release of neurotransmitters without triggering action potentials, so that focused illumination can be used to map the synaptic inputs to a neuron (Petreanu et al., 2009). Currently there is no technique that allows direct inhibition of synaptic release with light. Optogenetic inhibition of synaptic transmission would be very valuable to dissect the contribution of individual synapses or defined populations to the behavior of defined circuits and whole animals. Synaptic transmission could be blocked by interference with either presynaptic release or postsynaptic receptors. We chose to target presynaptic release because it occurs by a relatively well-conserved mechanism, in contrast to the enormous diversity of postsynaptic receptors.

Vesicular synaptic release is mediated by the SNARE protein complex located at the presynaptic terminal of neurons. Proteins in the SNARE complex have been well characterized and studied, however, there are also multiple associated proteins with unknown functions (Sudhof, 2004). The SNARE proteins, synaptobrevin 2/VAMP2, SNAP-25, and syntaxin, are believed to be the essential proteins for synaptic release in the central nervous system. During synchronized release, calcium influx from voltage-gated calcium channels triggers the binding of VAMP2 and synaptotagmin on the vesicular membrane to the SNAP-25 and syntaxin on the plasma membrane, allowing for the fusion of the vesicular membrane to plasma membrane and the release of vesicular contents. With asynchronous release, the fusion of vesicles is thought to occur without the involvement of voltage-gated calcium channels and synaptotagmin (Smith et al., 2012). Engineering a method to inhibit synaptic release in neurons with light would require the disruption of the endogenous SNARE complex to inhibit their normal function.

Chromophore-assisted light inactivation (CALI) is a powerful technique that can be used to selectively inactivate proteins during excitation of chromophores placed in the proximity of a protein (Jay, 1988; Marek and Davis, 2002; Tour et al., 2003). The reactive oxygen species generated by the chromophore during illumination oxidize nearby susceptible residues (tryptophan, tyrosine, histidine, cysteine and methionine), interfering with protein function. Synthetic chromophores such as malachite green (Jay, 1988), fluorescein (Beck et al., 2002), FIAsh (Marek and Davis, 2002), ReAsH (Tour et al., 2003), and eosin (Takemoto et al., 2011) have been shown to be effective CALI agents. CALI has

also been demonstrated with genetically encoded chromophores such as eGFP (Rajfur et al., 2002) and KillerRed (Bulina et al., 2006), although these fluorescent protein-based techniques are much less efficient (Takemoto et al., 2011). A recently engineered flavoprotein, miniSOG, has been shown to be an effective chromophore for the photo-oxidation of diaminobenzidine to introduce contrast in electron microscopy of fixed tissue (Shu et al., 2011). Singlet oxygen is generated when the flavin mononucleotide within miniSOG is illuminated by light with wavelength <500 nm. Flavin mononucleotide is sufficiently ubiquitous within cells to avoid any need to administer exogenous cofactor molecules. Judicious fusion of miniSOG to a mitochondrial transporter enables photoablation of genetically targeted neurons in *Caenorhabditis elegans* (Qi et al., 2012). Due to the high quantum efficiency for singlet oxygen photogeneration by miniSOG, it should be a more effective genetically encoded CALI chromophore than eGFP or KillerRed.

In the current study, we fuse miniSOG to the SNARE proteins VAMP2 and synaptophysin (SYP1) to inactivate the SNARE complex with light. We were able to achieve the reduction of synaptic release with 480 nm light with both constructs in hippocampal neurons, with the SYP1-based system achieving greater reduction than the VAMP2-based system. When mammalian VAMP2 fused to miniSOG was expressed pan-neuronally in *C. elegans*, we were able to induce long-lasting paralysis (>1 hr) with 480 nm light. Animals recovered movement when re-tested 24 hr later. We name this technique *Inhibition of Synapses with CALI* (InSynC). We believe InSynC is a powerful optogenetic technique for inhibiting neurotransmitter release with light and interrogating neurocircuitry in a spatially precise manner.

RESULTS

Design and Assaying of the CALI-Based Synaptic Inhibition System

To design a CALI-based synapse inhibition system, we chose candidate fusion proteins based on two criteria: (1) the protein is essential for vesicular synaptic release in synapses of the central nervous system; and (2) the engineered protein can achieve inhibition in a dominant-negative manner, without the need to eliminate endogenous protein expression.

The SNARE protein synaptobrevin 2/VAMP2 is the core protein in the vesicular SNARE complex, with a cytosolic N-terminal α -helix capable of binding to the α helices of SNAP-25 and syntaxin during vesicle fusion. The C-terminal of VAMP2 consists of a transmembrane domain that is anchored to the vesicular membrane. Both N and C termini of VAMP2 have previously been fused to fluorescent proteins without disrupting function (Deák et al., 2006). The second protein candidate that we chose was synaptophysin (SYP1), which is closely associated with the VAMP2 protein (Arthur and Stowell, 2007), although its role in vesicular release is still unclear. SYP1 has 4 proposed transmembrane domain helices transversing the vesicular membrane, with both N and C termini facing the cytosol. The C terminus of SYP1 has been previously tagged with fluorescent proteins without affecting its function (Dreosti et al., 2009). We genetically fused miniSOG to the N terminus and C terminus of VAMP2 and SYP1, respectively (Figure 1A). To visualize expressing cells,

mCherry was placed after the coding sequence of miniSOG-VAMP2 and SYP1-miniSOG, connected by a cotranslationally self-cleaving *Thomosa asigna* virus 2A-like sequence (T2A) (Osborn et al., 2005). The expression of the tagged synaptic proteins and the cytosolic red fluorescent protein were tightly linked genetically, even though the proteins were not fused to each other.

To assay the effects of miniSOG fused to VAMP2 and SYP1 on synaptic release, cultured hippocampal neurons were plated on microislands to induce autaptic synapse formation. The self-stimulated excitatory postsynaptic potential (EPSP) was typically observed as a prolonged depolarization after an action potential in current-clamp recording in response to a depolarizing current injection pulse (Wyart et al., 2005; Figure 1D). In voltage-clamp recording, a depolarizing voltage step can evoke a self-stimulated excitatory postsynaptic current (EPSC; Figure 1B). After establishing a stable baseline with repetitive stimulation, the recorded cell was illuminated for 2.5 min with 9.8 mW/mm² of 480 nm light. In the nonexpressing cells, a gradual rundown of EPSCs was often observed with repetitive voltage steps independent of light illumination (6.5% \pm 7.0% reduction in amplitudes, $n = 7$). In the miniSOG-VAMP2 expressing cells, a decrease of 29.4% \pm 3.3% ($n = 4$, $p = 0.006$) in EPSC amplitude was observed after light illumination (Figures 1B and 1C). In the SYP1-miniSOG expressing cells, the reduction of EPSC amplitude was significantly greater at 82.6% \pm 8.5% ($n = 6$, $p = 0.0002$). In two of the cells that exhibited the reduction of EPSC with SYP1-miniSOG, a stable recording was maintained for 45 min to 1 hr and no recovery in EPSC amplitude was observed. The reduction of EPSC amplitudes was associated with insignificant changes of electrophysiological properties such as membrane resistance (105.6 \pm 35.6 M Ω to 73.6 \pm 23.2 M Ω , $n = 6$, $p = 0.20$) or capacitance (62.5 \pm 13.9 pF to 56.9 \pm 6.7 pF, $n = 6$, $p = 0.55$). The reduction of EPSC amplitude did not alter the ability of the cell to fire action potentials in response to depolarizing current injections (Figure 1D; $n = 5$). Active and continual synaptic release during illumination was not essential for the inhibition of synaptic release, as the inhibition was still observed in cells where the self-stimulation was discontinued during light illumination (63.7% and 45.7% inhibition in two cells tested with SYP1-miniSOG).

In order to examine whether we can selectively inhibit specific synapses with high spatial resolution, we performed an imaging-based assay with the membrane dye FM4-64 in combination with SYP1-miniSOG. In this assay, SYP1-miniSOG fused to eGFP or Citrine was expressed in cultured cortical neurons. A quadrant of the field of view (159 μ m \times 159 μ m) was scanned with 488 nm laser to conduct CALI of the presynaptic terminals. Vesicular release was induced with 40 mM KCl solution containing 10 μ M FM4-64. The high potassium solution and dye solution was subsequently washed out to repolarize the membrane and remove membrane bound FM4-64 (Cousin, 2008). We then measured and averaged the FM4-64 fluorescence of the puncta positive for both FM4-64 and SYP1-miniSOG-eGFP/Citrine inside and outside the CALI region (Figure 1E). The SYP1-miniSOG-eGFP/Citrine positive puncta within the CALI region had significant less FM4-64 dye uptake compared to the puncta outside the CALI region (5911.2 \pm 687.5 and 8118.3 \pm 763.2

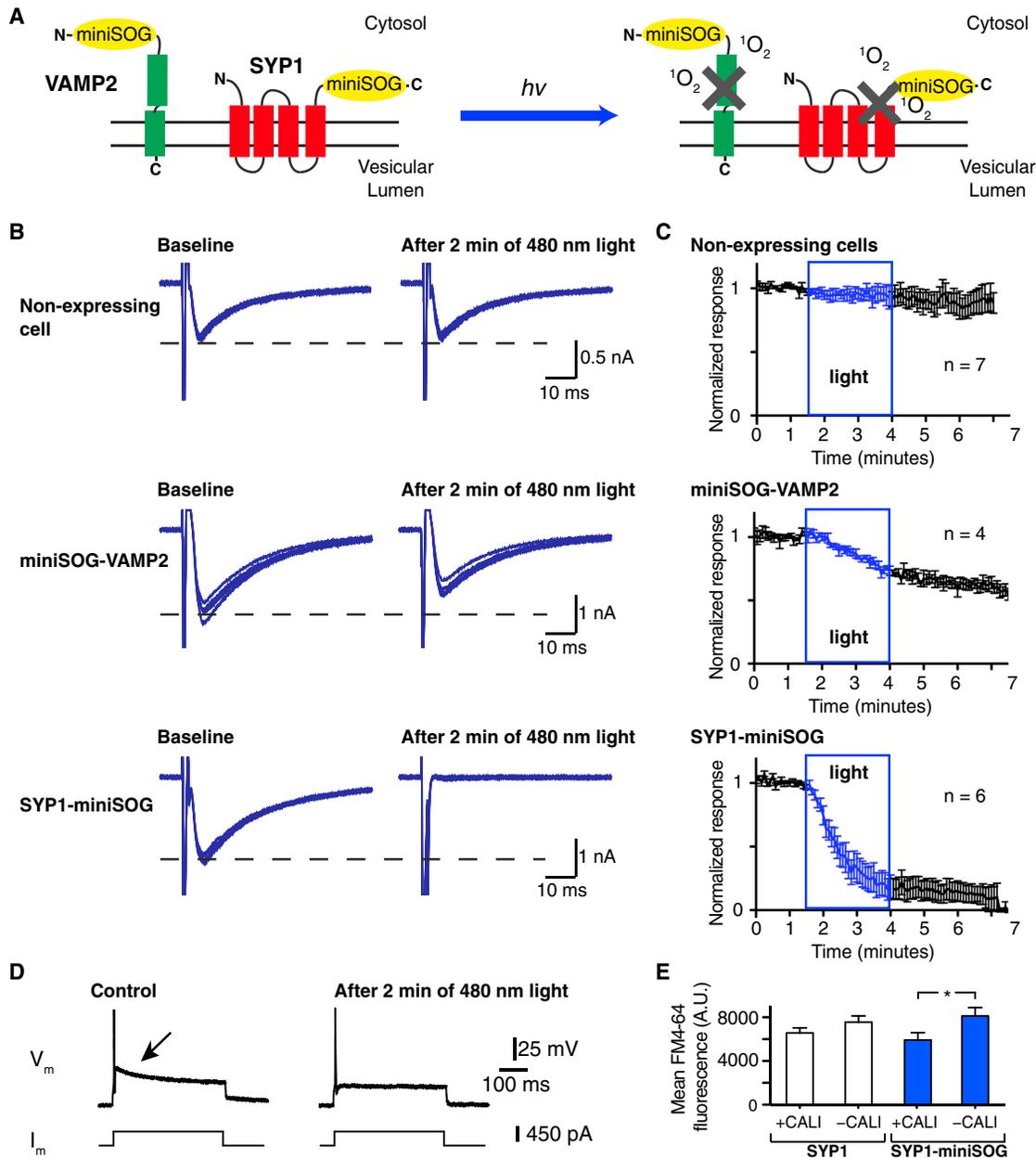


Figure 1. The Designs and Testing of the CALI-Based Synaptic Inactivation System

(A) The two designs of CALI-based synaptic inactivation system with miniSOG fused to Vesicular Associated Membrane Protein 2 (VAMP2) or Synaptophysin (SYP1). In the design with VAMP2, miniSOG is fused to the N terminus of VAMP2 facing the cytosolic space. With the SYP1 design, miniSOG is fused to the C terminus of the SYP1 also facing the cytosol. After light illumination, singlet oxygen (1O_2) is generated by miniSOG leading to the inactivation of fusion protein. (B) Representative examples of the effects of light on self-stimulated EPSCs recorded from nonexpressing control neurons (top), miniSOG-VAMP2-expressing neurons (middle), and SYP1-miniSOG-expressing neurons (bottom) before and after 2 min of 480 nm light illumination (9.8 mW/mm²). Each panel shows the overlap of 6 events (30 s). The synaptic release is induced with a 2 ms voltage step (-60 to 0 mV) at 0.2 Hz.

(C) Summaries of the inhibition of the self-stimulated EPSC amplitudes of the non-expressing control (top), miniSOG-VAMP2-expressing (middle), and SYP1-miniSOG-expressing (bottom) with blue light of 2.5 min duration.

(D) Current-clamp recordings from a SYP1-miniSOG expressing neuron in response to current injection. Action potential and self-stimulated excitatory postsynaptic potential (arrowed) can be evoked by the current step (left). After 2 min of 480 nm light illumination, the self-stimulated excitatory postsynaptic potential is abolished but the action potential remains (right).

(E) Mean FM4-64 fluorescence of SYP1-eGFP (SYP1) and SYP1-miniSOG-eGFP/Citrine (SYP1-miniSOG) puncta inside and outside the regions of CALI with 488 nm laser scan (25 mW and cumulative pixel illumination time of 13.86 ms).

See Figure S1 for example of FM4-64 images used for quantification. * indicates difference of $p < 0.05$. The error bars indicate SEM.

arbitrary units, $n = 78$ and $n = 95$, respectively; $p = 0.037$). However, this measurement is likely an underestimate of the true level of synaptic inhibition, as we only quantified puncta that are positive for both eGFP/Citrine and FM4-64 and omitted eGFP/Citrine positive puncta that did not have FM4-64 fluorescence. This selection was necessary as not all fluorescent protein-positive puncta were functional presynaptic boutons capable of dye uptake even in the absence of light illumination (see Figure S1 available online). No significant difference was detected between the puncta inside and outside the CALI region in SYP1-eGFP positive puncta (6562.6 ± 466.9 and 7551.1 ± 560.6 arbitrary units, $n = 114$ and $n = 157$, respectively; $p = 0.20$). These results demonstrated that we were able to inhibit presynaptic terminals with high spatial specificity.

Overall, the fusion of miniSOG to the synaptic proteins VAMP2 and SYP1 functionally inhibited synaptic release, with SYP1-miniSOG demonstrating greater effects under the same expression system in the cultured hippocampal neurons. We named this approach *Inhibition of Synapses with CALI* (InSynC).

Validation of InSynC in Synaptic Connections of Hippocampal Organotypic Slices

To test whether InSynC can depress synaptic connections in a nonautaptic system and whether illumination of presynaptic terminals is sufficient to inhibit vesicular release, we infected the CA3 region of hippocampal organotypic slices with recombinant adenoassociated virus (rAAV) containing *SYP1-miniSOG* under the human synapsin promoter and assayed the synaptic inputs in the CA1 region with field potential recordings and electrical stimulation. We fused the yellow fluorescent protein variant Citrine (Griesbeck et al., 2001) at the C terminus of SYP1-miniSOG, which enabled us to directly visualize the expression of InSynC at the CA3 presynaptic terminals projecting to CA1 (Figures 2A and 2B). When CA1 neurons were independently infected with Sindbis virus expressing the red fluorescent protein tdTomato, SYP1-miniSOG-Citrine punctate fluorescence signals were detected in the proximity of the tdTomato-expressing dendritic shaft (Figures 2A and 2B). Illumination of the local dendritic recording site in CA1 with 480 nm light led to 86.64% \pm 8.55% depression in field excitatory postsynaptic potential ($n = 6$, $p < 0.0001$) while the amplitude of the fiber volley remained unchanged after light illumination (100.04% \pm 10.38%), indicating minimal effects on the action potentials at presynaptic terminals (Figures 2C, 2D, and 2F). No significant reduction in field excitatory postsynaptic potential was detected in slices infected with rAAV expressing SYP1 directly fused to Citrine (96.30% \pm 10.85%, $n = 10$; Figure 2E). We also expressed SYP1-miniSOG fused with T2A-mCherry sequence, and we observed 82.06% \pm 1.99% reduction in electrically evoked EPSC amplitudes in whole-cell recordings of CA1 cells after 5 min illumination of 480 nm light ($n = 8$; $p < 0.0001$) (Figure 2G), whereas the slices expressing cytosolic mCherry alone (mCherry; 0.60% \pm 6.45% increase, $n = 9$), cytosolic miniSOG and mCherry (miniSOG-T2A-mCherry; 11.49% \pm 10.72%, $n = 8$) did not have significant decreases in EPSC amplitude (Figures 2G and 2H). Interestingly, in slices expressing miniSOG fused to membrane anchored mCherry (miniSOG-mCherry-CAAX), light caused a nonsignificant increase in electrically evoked EPSC amplitude

(32.48% \pm 10.61%, $n = 12$) (Figure 2H and S2). As synaptophysin overexpression had previously been reported to change release probability at presynaptic terminals (Alder et al., 1995), we measured the pair-pulse facilitation from cells in SYP1-miniSOG expressing slices, and compared to the cells from slices expressing mCherry, miniSOG-T2A-mCherry, and miniSOG-mCherry-CAAX (Figure S2 and Table 1). Despite the slight increase in pair-pulse facilitation in SYP1-miniSOG-expressing slices, the values between the five conditions were not significantly different. Light illumination did not change pair-pulse facilitation in any of the conditions. We also observed a slight increase in mEPSC frequency but not the mEPSC amplitude in SYP1-miniSOG-expressing slice (Figures 2J, S2, and Table 1). Both the frequency and the amplitude were nonsignificantly different from values in nonexpressing, mCherry, miniSOG-T2A-mCherry, and miniSOG-mCherry-CAAX-expressing slices prior to light illumination (Figures 2J, S2, and Table 1). Light illumination greatly increased the mEPSC frequencies in slices expressing miniSOG-mCherry-CAAX, ($p < 0.0001$), SYP1-miniSOG ($p = 0.012$), and miniSOG-T2A-mCherry ($p = 0.047$), whereas the mEPSC frequencies were not affected by light in mCherry-expressing slices (Figures 2I, 2J, and S2; Table 1). We were unable to accurately measure the mEPSC amplitudes after light illumination as the increased frequency of mEPSC leads to the superimposition of many events. In the miniSOG-mCherry-CAAX and SYP1-miniSOG recordings, the membrane resistance of the postsynaptic cells were not altered by light illumination (post-light/before light ratio of 0.95 ± 0.06 and 1.01 ± 0.04 , respectively). To investigate the effects of membrane targeted miniSOG and its effects on EPSC, we expressed miniSOG-mCherry-CAAX in cultured cortical neurons and conducted whole-cell patch-clamp recordings. In two cells expressing miniSOG-mCherry-CAAX at high level, blue light illumination leads to the appearance of an inward current (129.0 and 57.4 pA) that is associated with a decrease in membrane resistance (14.3% and 55.4% decrease), indicative of increased permeability of the plasma membrane (Figure S2). This effect was not seen in non-expressing cells with light illumination.

Validation of InSynC In Vivo in *C. elegans*

To test whether we can utilize InSynC in behaving animals in vivo, we expressed InSynC in the nematode *Caenorhabditis elegans*. Mammalian VAMP2 shares 62.9% overall homology with *C. elegans* synaptobrevin and 86.4% homology in the N-terminal α helices that interact with the SNAP-25 and syntaxin. VAMP2 was chosen over SYP1, because mammalian SYP1 has no homologs in *C. elegans*. We expressed mammalian VAMP2 fused to miniSOG pan-neuronally in *C. elegans*. To confirm expression and trafficking of mammalian VAMP2 in *C. elegans*, Citrine was fused to the luminal C-terminal of miniSOG-VAMP2. Pan-neural-miniSOG-VAMP2-Citrine showed punctate expression in the nerve cords, corresponding to presynaptic terminals (Figure 4A). When miniSOG-VAMP2-Citrine was expressed in the synaptobrevin (*snb-1*) mutant worm strain *md247* (Nonet et al., 1998), the movement phenotype of this strain was rescued (9.31 ± 3.14 bends/min in *md247*, $n = 7$ to 26.70 ± 5.19 bends/min in *md247* + miniSOG-VAMP2, $n = 6$, $p = 0.013$) (Figure 3A). This functional rescue of the movement

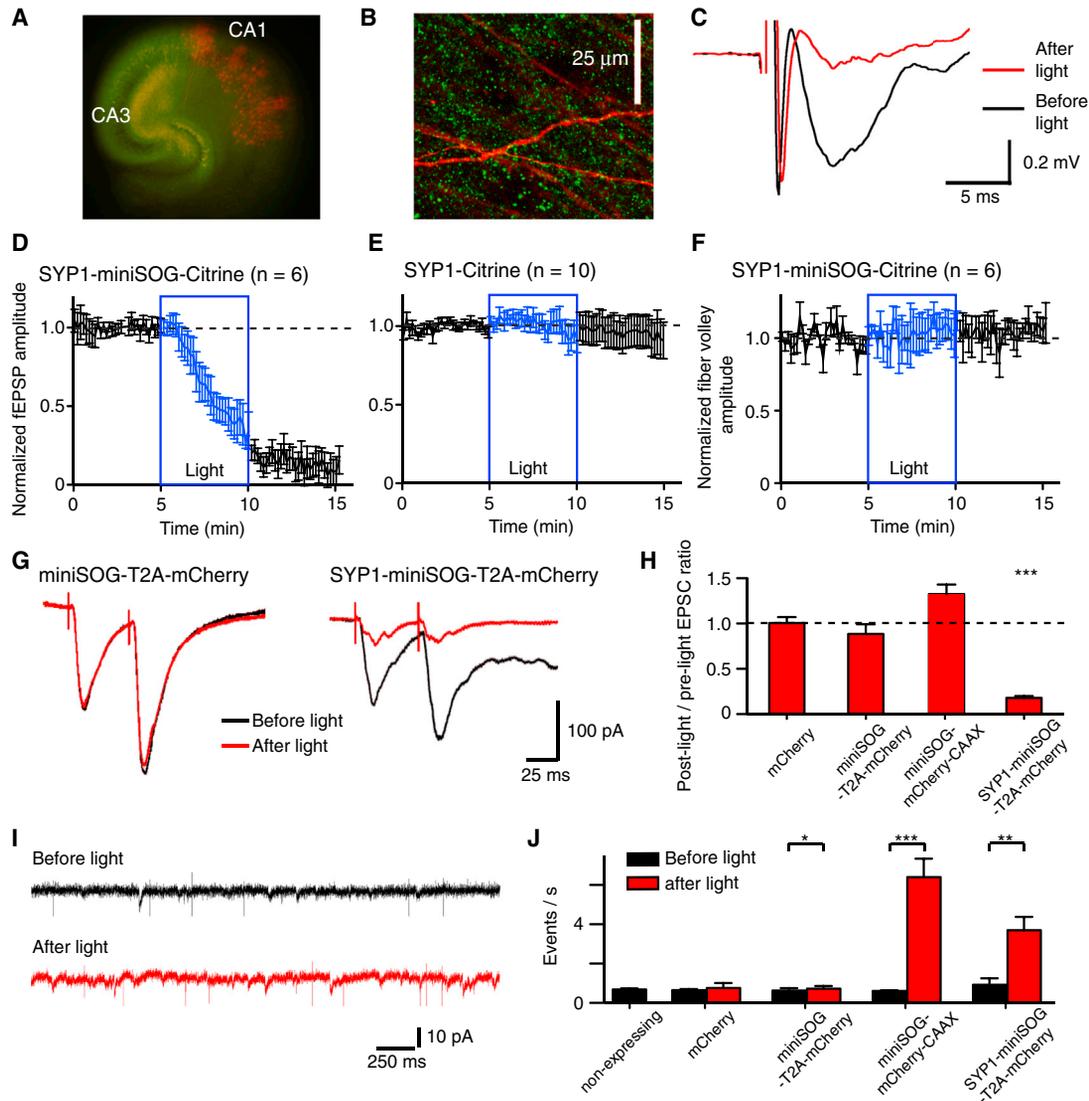


Figure 2. Validation of InSynC in Hippocampal Synapses

(A) Organotypic hippocampal slice culture coexpressing SYP1-miniSOG-Citrine (green) in presynaptic CA3 neurons and tdTomato (red) in sparsely labeled postsynaptic CA1 neurons. Recombinant AAV expressing SYP1-miniSOG-Citrine was injected into the CA3 region and yellow-green fluorescence can be detected in the CA1 region along the Schaffer collateral pathway. Sindbis virus expressing tdTomato was injected into CA1 to indicate region of field EPSP recording. (B) High-magnification image of the stratum radiatum of CA1, punctate fluorescence of Citrine signal (green) can be detected throughout the field of view in close proximity of the tdTomato labeled apical dendrites (red).

(C) Example of field excitatory postsynaptic potential (EPSP) recording of the stratum radiatum of CA1 from SYP1-miniSOG-Citrine expressing slice before (black) and after (red) 480 nm light illumination showing the inhibition of field EPSP by light.

(D) Quantification of the light-induced inhibition of field EPSP recorded from SYP1-miniSOG-Citrine-expressing slices.

(E) Quantification of the light-induced inhibition of field EPSP recorded from SYP1-Citrine-expressing slices. Light-induced inhibition of field EPSP was observed in SYP1-miniSOG-Citrine-expressing slices but not in SYP1-Citrine-expressing slices.

(F) Quantification of the fiber volley amplitude in SYP1-miniSOG-Citrine-expressing slices before and after light illumination showing light has no effect on the presynaptic action potential as measured with fiber volley amplitude.

(G) Examples of electrically-evoked EPSCs recorded in CA1 neurons from slices expressing cytosolic miniSOG and mCherry (miniSOG-T2A-mCherry; left) and SYP1-miniSOG-T2A-mCherry (right) before (black) and after (red) light illumination.

(H) Summary graph of the EPSC amplitude ratio (post-light/pre-light) in slices expressing mCherry, cytosolic miniSOG/mCherry (miniSOG-T2A-mCherry), membrane tethered miniSOG and mCherry (miniSOG-mCherry-CAAX) and SYP1-miniSOG/cytosolic mCherry (SYP1-miniSOG-T2A-mCherry).

(I) Example traces of miniature EPSC recordings of CA1 neurons from SYP1-miniSOG-T2A-mCherry-expressing slice before (black) and after (red) light illumination. The fast events that lacked the characteristic profiles of EPSCs were electrical noises.

(J) Summary graph of miniature EPSC frequencies from slices expressing mCherry, miniSOG-T2A-mCherry, miniSOG-mCherry-CAAX, and SYP1-miniSOG-T2A-mCherry.

*, **, and *** indicate differences of $p < 0.05$, 0.01 , and 0.001 , respectively. The error bars indicate SEM. See Figure S2 for additional examples and analysis.

Table 1. Summary of Pair-Pulse Facilitation Ratio and Miniature EPSC (mEPSC) Properties of Nonexpressing Organotypic Slices and Organotypic Slices Expressing mCherry or miniSOG Fusion Proteins

	Pair-Pulse Facilitation Ratio	mEPSC Amplitude before Light (pA)	mEPSC Frequency before Light (Hz)	mEPSC Frequency after Light (Hz)
Nonexpressing	1.58 ± 0.10 n = 7	12.67 ± 0.46 pA n = 7	0.68 ± 0.06 Hz n = 7	Not tested
mCherry	1.64 ± 0.13 n = 9	9.78 ± 1.36 pA n = 6	0.65 ± 0.05 Hz n = 6	0.76 ± 0.25 Hz
miniSOG-T2A-mCherry	1.55 ± 0.15 n = 8	10.61 ± 0.63 pA n = 6	0.63 ± 0.12 Hz n = 6	0.72 ± 0.14 Hz
miniSOG-mCherry-CAAX	1.61 ± 0.09 n = 12	11.25 ± 0.74 pA n = 7	0.61 ± 0.03 Hz n = 7	6.4 ± 0.94 Hz
SYP1-miniSOG-T2A-mCherry	1.93 ± 0.17 n = 8	12.70 ± 1.50 pA n = 8	0.92 ± 0.33 Hz n = 8	3.7 ± 0.68 Hz n = 8

phenotype of *snb-1(md247)* mutant strain confirms the incorporation of mammalian VAMP2 into the *C. elegans* SNARE complex. Illumination of single worms carrying the *miniSOG-VAMP2-Citrine* transgene with 480 nm light (5.4 mW/mm²) for 3 and 5 min reduced the movements by 68.2% ± 4.2% and 89.9% ± 3.7%, respectively (7.87 ± 1.07 bends/min, *p* = 0.008 and 3.07 ± 1.19 bends/min, *p* = 0.003 for 3 and 5 min illumination, respectively) (Figure 3A). Complete paralysis was observed in three of the six worms illuminated with light. The animals were returned to agar plates containing bacteria for recovery, and after 2–3 hr some gradual recovery of movements was noticeable. When the animals were re-tested in identical condition 24 hr later, some recovery of the movements was observed (10.34 ± 3.48 bends/min, *n* = 4, *p* = 0.08 compared to before illumination).

A more challenging test of CALI was to determine whether illumination of miniSOG-VAMP2-Citrine could inhibit synaptic release in the presence of normal endogenous VAMP2. When miniSOG-VAMP2-Citrine was expressed in wild-type (N2) worms, the animals showed normal movement under standard culture condition. When single miniSOG-VAMP2-Citrine expressing worms were illuminated for 5 min with 480 nm light (5.4 mW/mm²), we were able to achieve 80.6% ± 7.3% reduction of movements (24.06 ± 3.28 bends/min before light and 4.89 ± 1.76 bends/min after light; *n* = 9, *p* = 0.0004) (Figure 3C and Movie S1). Four of the nine worms tested were paralyzed after illumination. Partial recovery of movements was observed in some of the worms re-tested after 6 hr (16.69 ± 5.74 bends/min, 52.7% ± 32.4% of movements before illumination, *n* = 3). Full recovery of the movement was observed after 24 hr when the same worms were retested (27.38 ± 4.84 bends/min, 140.0% ± 4.0% of movements before illumination; *n* = 3) (Figure 3C). Control worms expressing miniSOG fused to Citrine without VAMP2 showed a smaller 19.8% ± 4.0% reduction in movements after illumination (25.55 ± 2.40 bends/min and 20.61 ± 2.52 bends/min before and after light illumination, respectively, *p* = 0.01; *n* = 5) (Figure 3B). As expected, the fluorescence of miniSOG-Citrine was located at soma and not presynaptic terminals (Figure S3).

We also tested whether we can achieve the same effect with weaker illumination intensity for longer duration (480 nm light for 25 min at 0.7 mW/mm²) in a population of expressing worms. In this experiment, multiple worms were moved to a bacteria-free

agar plate and the entire plate was illuminated. The movements of different worms in multiple regions on the plates were imaged and quantified separately. Illumination of the agar plate significantly reduced the movements of the miniSOG-VAMP2-Citrine-expressing worms from 29.04 ± 4.66 body bends/min before light, (*n* = 11) to 10.49 ± 4.18 bends/min after light, (*n* = 12), a 63.9% reduction (*p* = 0.007) (Figures 3D and 3E). In 5 of 12 worms (42%), movement was eliminated by the illumination. We did not observe significant changes in the movement of control worms expressing miniSOG-Citrine after light illumination (34.01 ± 5.91 bends/min before light, *n* = 11, and 31.15 ± 7.61 bends/min after light, *n* = 6, 8.4% reduction, *p* = 0.77) (Figure 3E). The recovery of movement in miniSOG-VAMP2-expressing worms was observed after 20–22 hr in miniSOG-VAMP2 expressing worms (21.76 ± 1.79 bends/min, *p* = 0.44) on bacteria containing agar plates (Figure 3E). In multiple animals on the recovery dish, the worms aggregated in groups on the bacterial lawn and the movements were not quantified. However, tracks from the animals could be seen on the dish, indicative of movements prior to aggregation. In some animals, the movements were interrupted when they encountered other animal and these were not quantified.

We then performed patch-clamp recording of the *C. elegans* muscles to confirm the reduction of synaptic inputs onto muscles after illumination with 480 nm light (15 or 30 mW/mm²). The recordings were done on miniSOG-VAMP2-Citrine worms of wild-type background. The spontaneous EPSC frequency was reduced from 47.67 ± 7.00 to 5.22 ± 1.98 events/s after 3 min of light illumination (89.1% reduction, *n* = 7; *p* < 0.0001) (Figures 4B and 4C). The inhibition of spontaneous EPSCs occurred largely within 1 min of illumination. There was also a significant reduction in the mean amplitude in electrically evoked EPSCs after 2–3 min of light (0.247 ± 0.12 nA, *n* = 8) compared to the mean amplitudes without light illumination (2.88 ± 0.41 nA, *n* = 4; *p* < 0.0001) (Figures 4D and 4E). In 4 of 8 animals, the electrically evoked EPSCs were abolished by illumination. No effects of light were observed in the nonexpressing progeny from the same parent. To test whether overexpression of miniSOG-VAMP2-Citrine altered vesicular fusion mechanisms, we compared the amplitudes, frequency and the kinetics of spontaneous release in non-expressing and miniSOG-VAMP2-Citrine-expressing worms. None of the parameters measured were

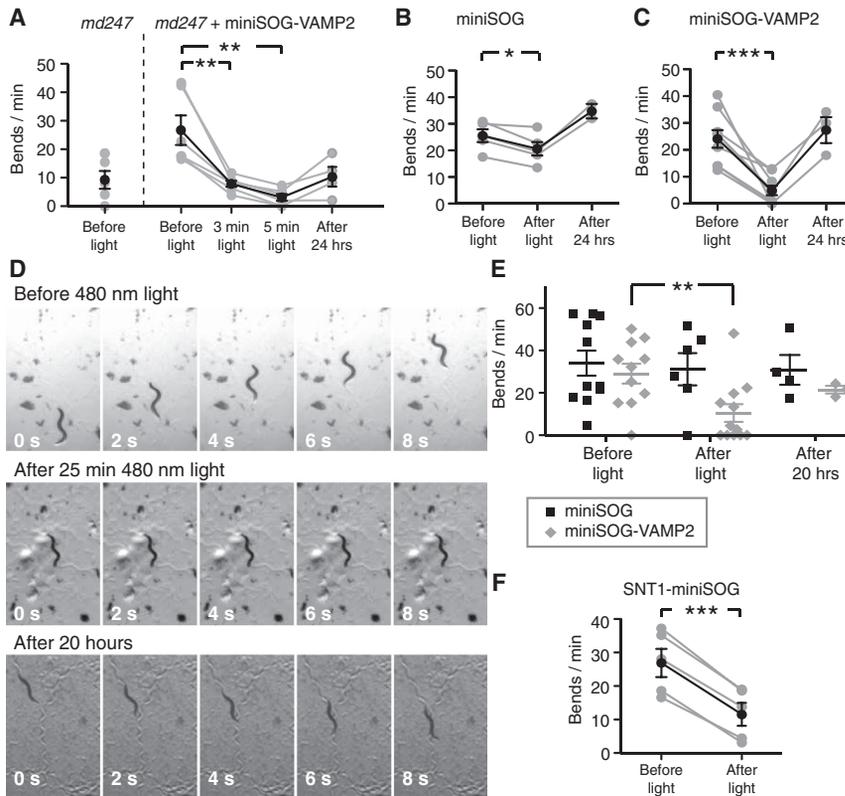


Figure 3. The Effects of InSynC on Locomotion of *C. elegans*

(A) Expression of miniSOG-VAMP2-Citrine (miniSOG-VAMP2) in synaptobrevin mutant worm strain *md247* functionally rescued the movement phenotype of *md247* as quantified by body bends/min. Illumination of miniSOG-VAMP2-Citrine expressing *md247* worms with 480 nm light (5.4 mW/mm², 3 and 5 min) reduced the movements of the worms. Some recovery was observed when the same worms were re-tested after 24 hr. (B) The effects of 480 nm light illumination (5.4 mW/mm², 5 min) on the movements of miniSOG-Citrine (miniSOG)-expressing worms of the wild-type background.

(C) The effects of 480 nm light illumination (5.4 mW/mm², 5 min) on miniSOG-VAMP2-Citrine (miniSOG-VAMP2; B2)-expressing worms of the wild-type background. Light illumination strongly reduced the movements of the miniSOG-VAMP2-Citrine-expressing worms, with full recovery of movements 24 hr later. See also [Movie S1](#).

(D) Examples of images extracted from movies of miniSOG-VAMP2-Citrine expressing *C. elegans* before (top) and after (middle) illumination with 480 nm light (0.7 mW/mm², 25 min), and after 20 hr of recovery in the dark (bottom). Worms successfully transferred after the testing were re-tested for the recovery of movements 20 hr later on agar plate containing bacteria after initial testing.

(E) Quantification of the *C. elegans* movements on the agar dish before and after light illumination (0.7 mW/mm², 25 min) and after recovery period.

(F) The effects of 480 nm light illumination (5.4 mW/mm², 5 min) on the movements of SNT1-miniSOG-Citrine-expressing worms (SNT1-miniSOG).

In (A), (B), and (C), the same worms were tested before and after light illumination, and allowed to recover for 24 hr on agar dishes seeded with bacteria before retesting on bacteria-free agar. The gray lines and symbols represent results from individual worms and the black lines and symbols represent the mean. In (D), multiple worms on the same plate were simultaneously tested. *, **, and *** indicate differences at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively. The strain numbers for miniSOG-VAMP2-Citrine expressing animal in (A) is CZ13749 and CZ13748 for (C) and (D). The strain number for *snt-1*-miniSOG-Citrine-expressing animal in (E) is CZ13750 and the miniSOG-Citrine animal in (B) and (D) is CZ14344. The error bars indicate SEM.

significantly different between the two groups without blue light illumination (Figure S4 and Table S1).

Specificity of the CALI Approach

To test the specificity of the InSynC approach, we made additional worms expressing miniSOG-Citrine fused to the C terminus of *C. elegans* synaptotagmin (SNT-1) (Figure S3). Whereas the synaptobrevin deletion mutation in *C. elegans* is lethal (Nonet et al., 1998), the *snt-1(md290)* deletion mutant is viable and retains the ability to move, although at reduced capacity (Nonet et al., 1993). When SNT-1-miniSOG was expressed on wild-type background illumination (5.4 mW/mm², 5 min) reduced movement by only 60.7% \pm 7.4% (27.13 \pm 4.2 bends/min and 11.78 \pm 3.4 bends/min before and after illumination, respectively, $n = 5$; $p = 0.0001$), and complete paralysis was not observed in any of the five worms tested (Figure 3F). The milder effect of CALI on synaptotagmin versus synaptobrevin is consistent with the reduced penetrance of the respective genetic deletions, as if CALI were preferentially inactivating the protein species to which the miniSOG was directly fused, although this could also be explained by other factors such as the expression level of the fusion protein and the susceptibility of the fusion protein to oxidation.

To further test the extent of singlet oxygen mediated CALI in living cells, we expressed singlet-oxygen sensitive fluorescent protein IFP1.4 (Shu et al., 2011) in cultured neurons fused directly to SYP1, SYP1-miniSOG, rat synaptotagmin-1 (SYT1) or expressed as a plasma membrane tethered form (pm-IFP) (Figure 5). In cells expressing SYT1-IFP and pm-IFP, SYP1-Citrine or SYP1-miniSOG-Citrine were coexpressed to test the bleaching of the IFP by differentially-located miniSOG. Exogenously expressed SYT1 with fluorescent protein at the C-terminal has previously been shown to localize to synaptic vesicles but not incorporated in the SNARE complex (Han et al., 2005). IFP fused to SYP1-miniSOG had significant greater bleaching after 93 s of cumulative 495 nm light illumination compared to SYP1-IFP (49.7% \pm 1.5% versus 28.0% \pm 1.0% bleaching, $n = 85$ and $n = 85$, respectively; $p < 0.0001$). The bleaching of SYT1-IFP in the presence of miniSOG fused to SYP1 (34.6% \pm 1.5%, $n = 81$) was greater than SYP1 control (14.4% \pm 1.4%, $n = 56$; $p < 0.0001$). The bleaching of pm-IFP in the presence of miniSOG fused to SYP1 (21.5% \pm 1.0%, $n = 144$) was also significant greater than SYP1 control (15.6% \pm 1.1%, $n = 102$; $p < 0.0001$). However, the difference of pm-IFP bleaching between the SYP1 control and SYP1-miniSOG (5.9%; 95% confidence interval of 3.0% to 8.8%) was smaller than the difference of

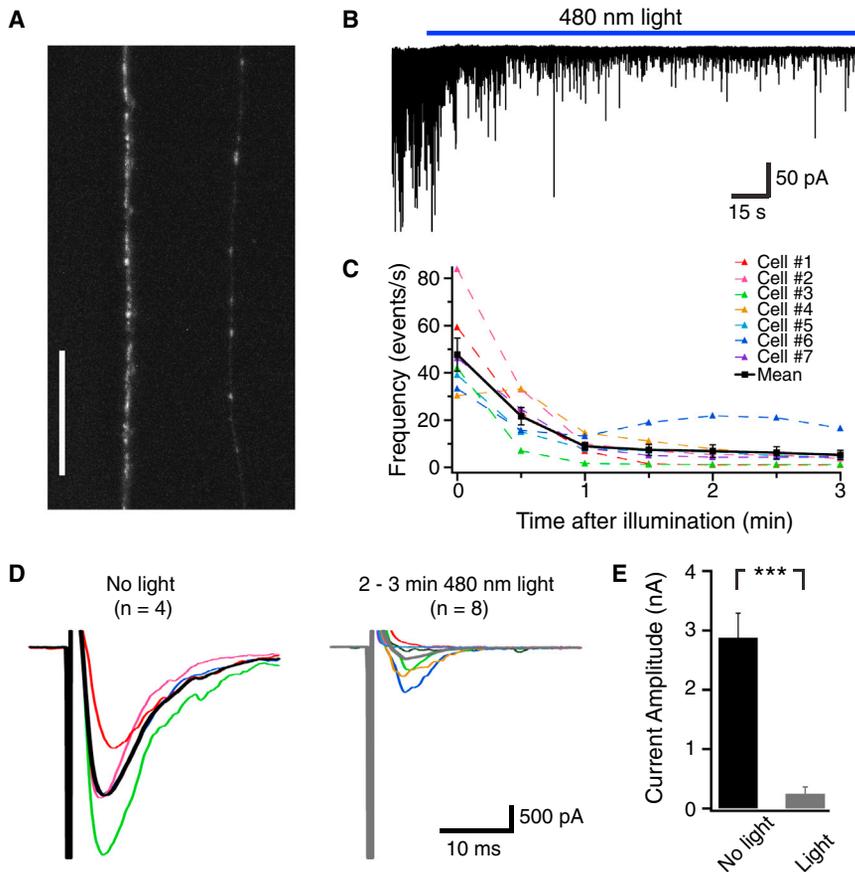


Figure 4. Validation of InSynC on Postsynaptic Currents at Neuromuscular Junctions of *C. elegans*

Expression of miniSOG-VAMP2-Citrine fusion protein in *C. elegans* neurons as visualized by Citrine fluorescence on a confocal microscopy (N2 wild-type background, strain number CZ13748). Punctate fluorescence at synaptic sites of the ventral and dorsal cords of the nematodes can be observed. See Figure S3 for miniSOG-Citrine expression.

(B) An example electrophysiological recording of the muscle of miniSOG-VAMP2 expressing *C. elegans* (CZ13748) and the effects of 480 nm light (30 mW/mm²) on spontaneous synaptic events.

(C) Quantification of the spontaneous event frequency with the illumination of light.

(D) Example traces of the electrically-evoked postsynaptic current before and after light illumination.

(E) Quantification of the peak amplitudes of electrically-evoked postsynaptic current before and after light illumination.

In (C) and (D), responses in individual cells are shown in different colors, and the mean responses before and after illumination are in black and gray, respectively. The scale in (A) is 25 μ m. *** indicates difference at $p < 0.001$. The error bars indicate SEM.

shown to be sensitive to CALI (Tour et al., 2003). Gratifyingly, we were able to inhibit synaptic release efficiently by illuminating miniSOG fusion proteins

SYT1-IFP bleaching between the SYP1 control and SYP1-miniSOG (20.2%; 95% confident interval of 16.0% to 24.5%) or the difference of bleaching between SYP1-IFP and SYP1-miniSOG-IFP (21.7%; 95% confidence interval of 18.1% to 25.4%). These results demonstrated singlet oxygen generated by SYP1-miniSOG can oxidize other synaptic proteins on the vesicles, and to a smaller extent, the proteins located on the plasma membrane, although this could potentially due to the plasma membrane localization of exogenously-expressed SYP1 (Li and Tsien, 2012) or the vesicular uptake of some pm-IFP.

DISCUSSION

In the current study, we developed an optogenetic technique, InSynC, to inhibit synaptic release with light using chromophore-assisted light inactivation. InSynC with synaptophysin (SYP1) is much more efficient than the corresponding VAMP2 version in the mammalian system. The exact function of synaptophysin in synaptic release is unclear, although it is known to be closely associated with VAMP2 (Washbourne et al., 1995). Both exogenously expressed VAMP2 and synaptophysin tagged with fluorescent proteins are known to incorporate into endogenous v-SNARE (Deák et al., 2006; Dreosti et al., 2009). It has been proposed that synaptophysins are assembled with a connexon-like structure (Arthur and Stowell, 2007), and membrane channels such as connexins or calcium channels have previously been

without replacing the endogenous proteins, either due to the dominant-negative effect of inactivated miniSOG-fusion proteins within the SNARE complex, or the extension of the CALI effect beyond the fusion protein. In the current study, we cannot conclusively distinguish between one mechanism over the other, and it is possible that both mechanisms play a role in inactivating the synaptic release. Our IFP bleaching results demonstrated that the effects of singlet oxygen can extend beyond the fusion protein. However, the concentration of singlet oxygen decreases exponentially from the site of generation, and its effect should be strongest on the fusion protein. The different efficiency with SYP1-miniSOG and miniSOG-VAMP2 in hippocampal culture, and miniSOG-VAMP2 and SNT-1-miniSOG in *C. elegans*, supported this hypothesis, although this could also be potentially explained by the difference in expression level or the residues susceptible to oxidation on the proteins. The estimated exponential space constant for singlet oxygen diffusion in the cytosol is 70 nm (Hatz et al., 2007), which is greater than the diameter of an average synaptic vesicle (~50 nm) (Kim et al., 2000). The inhibition of synaptic response is only observed when miniSOG is tethered to synaptophysin or VAMP2 and not with membrane-tethered miniSOG, suggesting the inhibition of synaptic release with InSynC requires the specific inhibition of vesicular proteins. It is interesting to note that mEPSC frequency is increased by light in both membrane-tethered miniSOG and SYP1-miniSOG after light illumination, possibly due to the localization of some

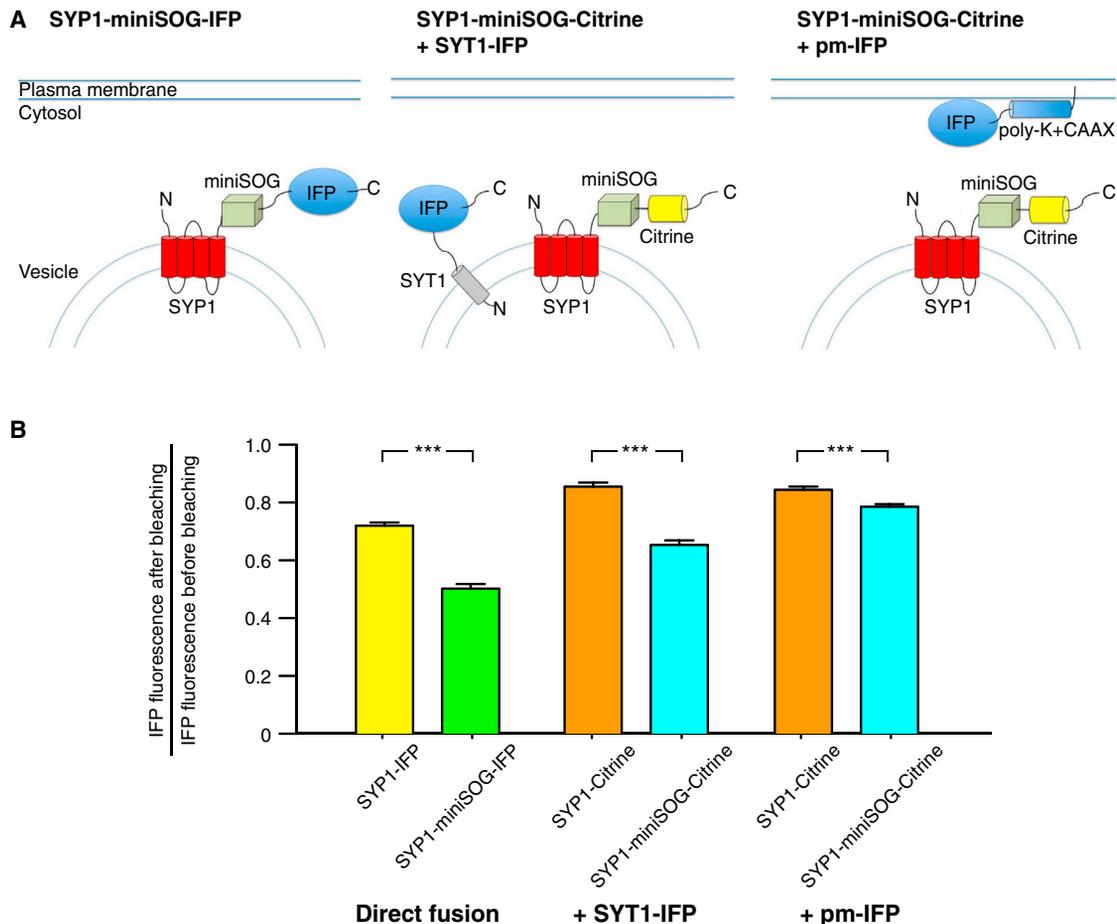


Figure 5. The Extent of Singlet Oxygen-Mediated Photo-oxidation as Measured with IFP Photobleaching

(A) Schematic drawing showing the three conditions tested in the IFP bleaching experiments. IFP was expressed in neurons either fused to the C terminus of the SYP1 or SYP1-miniSOG (left panel). In the other conditions, IFP was coexpressed as synaptotagmin-1 (SYT1) fusion (middle panel) or tethered to the plasma membrane (pm-IFP) in neurons expressing SYP1-miniSOG-Citrine or SYP1-Citrine.

(B) Summary graph showing the IFP bleaching in the six conditions tested. Significant greater IFP bleaching were observed in all three conditions. A smaller difference was observed when IFP was expressed on the plasma membrane and SYP1-miniSOG or SYP1 were expressed on the vesicles. MiniSOG was excited by 93 s of 495 nm light at 20 mW/mm² and IFP was imaged with 665 nm excitation light.

*** indicates difference at $p < 0.001$. The error bars indicate SEM.

SYP1-miniSOG onto the plasma membrane (Li and Tsien, 2012) or the oxidation of membrane protein by singlet oxygen diffused to the membrane (see IFP bleaching). The enhanced mEPSC and electrically evoked EPSC by membrane targeted miniSOG after illumination is likely to result from the inward current and the potential depolarization associated with illumination. The mechanism responsible for this inward current is unknown and requires further investigation. The physiological functions of spontaneous release in neuronal signaling are not known, although it has been suggested that spontaneous release stabilizes synapse (McKinney et al., 1999) and tune the sensitivity of the postsynaptic membrane to neurotransmitters (Sutton et al., 2006). The users of the InSynC technology need to be aware of these possible effects when interpreting the results, especially in long-term behavior experiments. Our results also indicated that synaptophysin may have distinct roles in synchronous and asynchronous release at presynaptic terminals as has been suggested

with other SNARE proteins (Deitcher et al., 1998; Schulze et al., 1995).

Due to the cuticle, *C. elegans* resists the introduction of many chemicals. However, genetic modification of *C. elegans* is relatively easy. *C. elegans* is an ideal model for the use of InSynC. Mammalian VAMP2 shares a high degree of homology to *C. elegans* synaptobrevin, and the miniSOG-VAMP2 protein can rescue the behavioral abnormality of the synaptobrevin mutant strain *md247*, suggesting that mammalian VAMP2 can efficiently incorporate into the *C. elegans* SNARE complex. The stronger inhibitory effects of mSOG-VAMP2 in *C. elegans* compared to the mammalian system is likely to be associated with the stronger expression of miniSOG-VAMP2 in *C. elegans* than in primary hippocampal cultures with human synapsin promoters. We were also able to reduce the movements of worms with synaptotagmin (SNT-1)-miniSOG but its effect was weaker than miniSOG-VAMP2. Therefore, the best InSynC system to

utilize will depend on the organism and the phenotype the experimenters wish to achieve.

The replacement of inactivated proteins with newly synthesized proteins is likely the mechanism of recovery. Presynaptic proteins are believed to be synthesized in the soma and transported down the axon, with minimal local protein translation at the presynaptic terminal (Hannah et al., 1999). In our experiments with primary cultured hippocampal neurons and in *C. elegans*, we illuminated the whole neuron or the whole worm, potentially destroying the newly synthesized protein at the soma and the protein en route to the presynaptic terminal, in addition to the proteins already present in the presynaptic vesicles. It is likely the recovery of the synaptic function can be quicker if illumination is focused on the presynaptic terminal only. In the organotypic slices, only the presynaptic terminals were illuminated, and this is sufficient to inhibit presynaptic vesicular release efficiently. The time required for recovery may also depend on the axon length if the whole neuron is illuminated. The long duration of the effect can be advantageous in experiments where the behavior tested is complex and long lasting.

Compared to current techniques of inhibiting neuronal activities with microbial opsin pumps, InSynC has the following differences: (1) InSynC inhibits synaptic release and not the firing of action potentials and therefore can be used to inhibit a single, spatially distinct axonal innervation without inhibiting other axonal projections made by the same cell. (2) InSynC takes more time to build up but has a long-lasting effect (>1 hr) that persists after the termination of the light pulse. The slower kinetics of InSynC will prevent some biophysical applications requiring precision timing but should facilitate experiments in which synapses are sequentially inactivated to titrate effects on circuit dynamics. (3) Effective light illumination for InSynC is on the presynaptic site and not the soma, potentially reducing light-mediated toxicity to the cell. (4) The effects of InSynC can be graded and not all-or-none. As with all other optogenetic techniques, the efficiency of such techniques depends heavily on the expression level and the properties of the cells targeted. Given the widespread distribution of VAMP2 and synaptophysin in the mammalian nervous system (Marquèze-Pouey et al., 1991; Trimble et al., 1990), it is likely that InSynC will be applicable to the majority of neurons targeted.

In conclusion, we have demonstrated that it is possible to use a genetically-encoded singlet oxygen generator to conduct CALI experiments in vitro and in vivo, and that CALI can be used to engineer new optogenetic techniques by inhibiting the function of specific proteins. Our optogenetic technique, InSynC, is a powerful method for inhibiting synaptic release with light, and is currently the only optogenetic approach that can efficiently inhibit a specific axonal projection in vivo and in vitro. This approach complements the existing optogenetic tools and can be used to study the function of specific projections.

EXPERIMENTAL PROCEDURES

Constructs and Molecular Cloning

Complementary DNA (cDNA) encoding Vesicle-associated membrane protein (VAMP2), *C. elegans* synaptotagmin 1 (SNT-1) and synaptophysin (SYP1) were fused to *miniSOG* by polymerase chain reaction with Phusion (New England

Biolabs). *VAMP2* and *SYP1* fused with *miniSOG* were inserted into a lentiviral vector (gift from Ed Boyden, MIT) with the hSynapsin promoter and Woodchuck Posttranscription Regulatory Element (WPRE). A *Thoesa asigna* virus 2A (T2A) sequence was fused in frame with *mCherry* in the lentiviral vector at 3' end of the transgene. The AAV2 vector (gift from Dr. Lin Tian, University of California, Davis) contained the hSynapsin promoter and WPRE flanking the *SYP1-miniSOG* or *SYP1*. The sequence coding for Citrine was inserted in frame at the 3' end. *VAMP2* cDNA was provided by Dr. S. Andrew Hires (Janelia Farm Research Campus) and *SYP1* cDNA was amplified by RT-PCR from rat brain RNA (Clontech). The *C. elegans* synaptotagmin 1 (*snt-1*) cDNA was provided by Dr. Erik Jorgensen (University of Utah).

For the worm constructs, *miniSOG-VAMP2*, *miniSOG*, and *snt-1-miniSOG* were fused to the *Citrine* cDNA at the 3' end and inserted into the Gateway entry vector (Life Technologies). LR reaction (Life Technologies) was used to introduce this insertion into the Prgef-1 destination vector PCZGY66 vector for injection into *C. elegans*.

The annotated DNA and protein sequences of InSynC are provided in Supplemental Information.

Recombinant Adenoassociated Virus Production

Recombinant adenoassociated virus with serotype 8 containing the *SYP1-miniSOG-Citrine*, *SYP1-Citrine*, *SYP1-miniSOG-T2A-mCherry*, *mCherry*, *miniSOG-T2A-mCherry*, and *miniSOG-mCherry-CAAX* were produced according to the protocols at <http://vectorcore.salk.edu/protocols.php> with minor modifications. In brief, AAV2 plasmid and helper plasmids XX6-80 and XR8 (National Vector Biorepository) were transfected into 293A cells (Life Technology) with calcium phosphate precipitation (Clontech). Recombinant AAV2/8 were released from the cells by freeze-thawing and purified with iodixanol gradient purification. The virus was further concentrated using 50 kDa Amicon Ultra centrifugal filters (Millipore). The rAAV-*SYP1-miniSOG-Citrine* titer was measured by quantitative PCR to be 6.6×10^{13} genome copy (GC)/ml (Salk Vector Core). The rAAV-*SYP1-miniSOG-T2A-mCherry* titer was estimated to be 2.3×10^{13} GC/ml with Quant-IT picogreen dsDNA dye (Life Technologies).

Sindbis Virus Production

Sindbis virus containing the *tdTomato* transgene is produced as described previously (Malinow et al., 2010). In brief, BHK cells were electroporated with RNAs transcribed from pSinRep5-*tdTomato* and DH(26S) plasmids. The media was collected 40 hr later and centrifuged to obtain the concentrated virus.

Hippocampal and Cortical Neuronal Culture and Electrophysiological Recording

Hippocampal microisland cultures were made by a protocol modified from Bekkers (2005). In brief, a collagen (0.5mg/ml, Affymetrix)/poly-D-lysine (0.1mg/ml) mixture was sprayed onto the glass surface of glass bottom dishes (MatTek) with an atomizer. Hippocampal and cortical neurons were extracted from P2 Sprague-Dawley rat pups with papain digestion and mechanical trituration. Hippocampal neurons were transfected by electroporation (Lonza) and plated at $1.5-3 \times 10^4$ cells per dish. Cortical neurons were plated on poly-D-lysine coated dish and infected with rAAV three days after plating. The procedures of extracting cultured neurons and organotypic slices (below) from rat pups were approved by the UCSD Institutional Animal Care and Use Committee.

Cultured hippocampal neurons were placed on an Olympus IX71 microscope with 20× air phase contrast objective for the recording (Olympus). Illumination (9.8 mW/mm^2) from a xenon arc lamp (Opti-quip) was filtered through a 480/40 nm filter and reflected to the specimen with a full-reflective mirror (Chroma). Illumination was controlled with a mechanical shutter (Sutter Instrument). Recordings were performed with an Axopatch 200B patch amplifier, Digidata 1332A digitizer, and pCLAMP 9.2 software (Molecular Devices). EPSCs were evoked with a 2 ms voltage step from -60 mV to 0 mV at 0.2 Hz. Illumination was initiated after 1.5 min of stable baseline (changes <10%) of EPSC amplitude. One hundred percent response for each cell was the mean EPSC amplitude of the 1 min prior to light illumination and the amplitudes of each EPSC were normalized to this 100% response. Reduction of

EPSC amplitudes was measured as the mean amplitudes of 6 EPSCs (25 s) after light illumination. Only cells with series resistance $<10\text{ M}\Omega$ and changes of series resistance $<20\%$ after light illumination were analyzed. The external solution contained 118 mM NaCl, 3 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, and 20 mM glucose (pH 7.35, 315 mOsm). The intracellular pipette solution contained 110 mM K-gluconate, 30 mM KCl, 5 mM NaCl, 2 mM MgCl_2 , 0.1 mM CaCl_2 , 2 mM MgATP, 0.3 mM TrisGTP, and 10 mM HEPES (pH 7.25, 285 mOsm). Cortical neurons were recorded with intracellular solution containing 110 mM Cs methanesulfonate, 30 mM tetraethylammonium chloride, 10 mM EGTA, 10 mM HEPES, 1 mM CaCl_2 , 1 mM MgCl_2 , 2 mM Mg-ATP, 0.15 mM $\text{Na}_3\text{-GTP}$ (pH 7.25, 285 mOsm). With cortical neuron recording, the extracellular solution contained 1 μM TTX. Light intensity was measured with a calibrated photometer with an integrating sphere detector (International Light Technologies) placed on the objective. A glass slide with a semispherical lens was used to direct the light into the integrating sphere. The area of illumination was measured with a stage micrometer for illumination intensity.

Hippocampal Organotypic Slice Culture and Electrophysiological Recording

Organotypic hippocampal slices were prepared from 2 days old rat pups as described previously (Shi et al., 1999). The various constructs were expressed using rAAV virus in CA3 neurons in 2 DIV slice cultures. Cells were allowed to express for 14–17 days before being used for recordings. CA3 region was surgically removed to prevent stimulus induced bursting. Recordings were done in ACSF containing 119 mM NaCl, 2.5 mM KCl, 26 mM NaHCO_3 , 1 mM NaH_2PO_4 , 11 mM glucose, 4 mM MgCl_2 , and 4 mM CaCl_2 , 100 μM picrotoxin and 4 μM 2-chloroadenosine (pH 7.4) at 24°C – 28°C .

Organotypic hippocampal slices were placed in a recording chamber on an Olympus BX50WI microscope with $60\times$ water immersion objective (Olympus). Extracellular field potential was recorded in stratum radiatum with glass electrodes (1–2 $\text{M}\Omega$) filled with the perfusion solution. Synaptic responses were evoked by stimulating two independent pathways using bipolar stimulating electrodes (Frederick Haer) placed 150–250 μm down the apical dendrites, 100 μm apart, and 150–200 μm laterally in opposite directions. Field EPSP was measured by averaging the response over a 5 ms fixed window covering the peak amplitude. Results from two pathways were averaged and analyzed as $n = 1$. Whole-cell patch-clamp recordings were done with intracellular solution containing 115 mM Cs-Methanesulfonate, 20 mM CsCl, 10 mM HEPES, 2.5 mM MgCl_2 , 4 mM Na_2ATP , 0.4 mM Na_3GTP , 10 mM Na-phosphocreatine, 0.6 mM EGTA (pH 7.25). Electrically evoked EPSCs and miniature EPSCs were recorded under voltage clamping ($V_{\text{hold}} = -60\text{ mV}$; junctional potential not corrected). Recording and analysis were done with IGOR Pro (WaveMetrics). For the analysis of mEPSC frequency, EPSCs that had the characteristic excitatory EPSC profile (Bekkers et al., 1990) was manually identified over 1 min period of recording. Light illumination was provided from a 100 W mercury arc lamp filtered through a eGFP filter set with 480/40 nm excitation filter (Olympus). The illumination area was 360 μm in diameter.

Confocal and Fluorescence Imaging

Hippocampal organotypic slices infected with rAAV and Sindbis virus were imaged under low magnification with a MVX10 Macroview microscope (Olympus). Citrine fluorescence was imaged with the eGFP filter set and tdTomato was imaged with a Texas Red filter set. For high magnification, the slices were fixed with 4% paraformaldehyde and imaged with a Zeiss LSM 780 confocal microscope (Zeiss).

C. elegans Expression and Behavior

Germline transmission of Prgef-1-*miniSOG-VAMP2-Citrine*, Prgef-1-*snt-1-miniSOG-Citrine*, and Prgef-1-*miniSOG-Citrine* was achieved following standard procedures (Mello and Fire, 1995). The plasmids were injected at 5–15 ng/ μl into N2 or NM467 SNB-1 (*md247*) (Nonet et al., 1998) using *Pmyo2-mCherry* or *Ptx3mCherry* as coinjection markers. For behavioral experiments, a single worm (L4 stage) was transferred onto glass bottom culture dishes (MatTek) coated with a thin layer of Nematode Growth Medium (NGM) agar. The worms were illuminated (480/40 filter) with a $20\times$ air objective on an Olympus IX71 inverted microscope (Olympus). The illumination is reflected

onto the specimen plane with a 518 nm dichroic to allow visualization of the worms for tracking during the illumination period (5 min). The light intensity was measured to be 5.4 mW/mm^2 with the procedure described under the Hippocampal Neuronal Culture and Electrophysiological Recording section. The worms were manually tracked during illumination for continuous illumination. After testing, the worms were transferred back to the NGM agar dish seeded with bacteria for recovery. The animals successfully transferred between the dishes were used for testing recovery the next day. For experiment with weaker light intensity and longer illumination duration, multiple L4 worms were placed on a 35 mm dish coated with agar were illuminated under a Zeiss Discovery V12 dissecting microscope with an eGFP filter set (480/40 nm excitation) at $9.3\times$ magnification for 25 min. The light intensity was measured to be 0.7 mW/mm^2 with a planar light detector connected to a power meter (Newport). Movement was recorded using a Nikon DS-QiMc camera and NIS elements F3.0 software (Tokyo, Japan). In some of the regions, multiple worms aggregate in regions with bacteria carried over with the animal transfer, the movements of these worms were not used for quantification. The quantification of movement was done according to Sawin et al. (2000). The strain numbers for N2 worms with pan-neuronal expression of *miniSOG-VAMP2-Citrine*, *snt-1-miniSOG-Citrine*, and *miniSOG-Citrine* are CZ13748, CZ13750, and CZ14344, respectively. The strain number for *miniSOG-VAMP2-Citrine* expression in *md247* background is CZ13749.

C. elegans Electrophysiology

Dissection methods were described as previously (Stawicki et al., 2011). Adult worms were immobilized on Sylgard-coated coverslips with cyanoacrylate glue. A dorsolateral incision was made with a sharp glass pipette and the cuticle flap was glued down to expose the ventral medial body wall muscles. The preparation was then treated by collagenase type IV (0.4 mg/ml; Sigma-Aldrich) for $\sim 30\text{ s}$.

The bath solution containing (in mM): 127 NaCl, 5 KCl, 26 NaHCO_3 , 1.25 NaH_2PO_4 , 2 CaCl_2 , 4 MgCl_2 , 10 glucose, and sucrose to 340 mOsm, bubbled with 5% CO_2 , 95% O_2 at 20°C . The pipette solution containing (in mM): 120 Cs-methanesulfonate, 4 CsCl, 15 CsF, 4 MgCl_2 , 5 EGTA, 0.25 CaCl_2 , 10 HEPES, and 4 Na_2ATP , adjusted to pH 7.2 with CsOH. Whole-cell recordings ($V_{\text{hold}} = -60\text{ mV}$) from muscle cells were performed at 20°C with 2–3 $\text{M}\Omega$ pipettes. An EPC-10 patch-clamp amplifier was used with the Patchmaster software package (HEKA Electronics). An electrode positioned close to the ventral nerve cord was used to stimulate evoked release by applying a 0.5 ms 85 μA square pulse with a stimulus current generator (WPI). Further analysis was done with IGOR Pro (WaveMetrics). Illumination was provided with a Sutter Instrument Lambda LS with a filter wheel for shuttering. The excitation light was filtered with 480 nm excitation filter (N41012; Chroma) and focused on the specimen with a $63\times$ water immersion objective (Olympus). Light intensity measurement was done similarly to that described for cell culture recordings.

FM4-64 Dye Imaging and Analysis

Cultured cortical neurons (15 DIV) plated on a glass-bottom culture dish were imaged on a Zeiss Live 5 Confocal Microscope with a $20\times$ air objective. The neurons were electroporated with the indicated plasmids prior to plating. Prior to imaging, the cells were treated briefly with balanced saline solution containing 40 mM KCl before placed in the same external solution used in cell culture recordings. Five to seven regions ($318\text{ }\mu\text{m} \times 318\text{ }\mu\text{m}$) on each dish were tested per experiment. After acquiring the initial image of eGFP/Citrine fluorescence of each region, a quadrant of the field of view ($159\text{ }\mu\text{m} \times 159\text{ }\mu\text{m}$) was scanned with 488 nm laser (25% of 100 mW) with pixel dwelling time of 154 μs for 90 frames, given each pixel the cumulative illumination time of 13.86 ms. The sample was then perfused for 2.5 min ($\sim 3.5\text{ ml}$) of 40 mM KCl containing-external solution containing 10 μM FM4-64FX (Life Technologies) before washout with standard external solution with no FM4-64 for 7.5 min ($\sim 10\text{ ml}$). The same regions were then re-imaged for FM4-64 fluorescence. The eGFP/citrine fluorescence was excited with 488 nm laser (5% intensity of 100 mW) and imaged with a 505 LP emission filter and pixel dwell time of 154 μs . The FM4-64 fluorescence was excited with 532 nm laser (10% of intensity of 75 mW) and imaged with a 650 LP emission filter (pixel dwell time of 131 μs). For both imaging and CALL, 5 optical slices with 0.84 μm spacing were acquired in the z series.

For the analysis of the results, the CALI regions were identified on the images and the FM4-64 fluorescence of the puncta inside and outside the CALI region were quantified separately. As not all eGFP or Citrine positive puncta are pre-synaptic boutons capable of vesicular release (Figure S1), only puncta positive for both eGFP/Citrine and FM4-64 were used for quantification to avoid false negative. However, this criterion also underestimates the inhibition of vesicular release with InSynC, as presynaptic boutons that were strongly inhibited and failed to take up FM4-64 were not quantified. The mean fluorescence value from a region with no fluorescent structures in the FM4-64 image was chosen to provide the background value to be subtracted. The fluorescence values were measured on ImageJ. For statistical comparison, unpaired Student's *t* tests were performed on the mean FM4-64 fluorescence values inside and outside the CALI region.

IFP Bleaching

Cultured cortical or hippocampal neurons plated on glass-bottom culture dish (7 DIV) were imaged on an inverted microscope with a 40× oil objective (Zeiss). IFP (Shu et al., 2009) was imaged with a 665/45 nm excitation filter, 725/50 nm emission filter, and 695 nm dichroic mirror. Citrine and miniSOG were excited with 495/10 nm excitation filter, 515 nm dichroic mirror, and imaged with 535/25 nm emission filter. The light intensity of 495 nm excitation was 20 mW/mm². The field of view and focus were adjusted with IFP fluorescence. The images of IFP and citrine were acquired at 512 × 512 pixel resolution (150 ms exposure time) with a Cascade 1024 EMCCD camera (Photometric). IFP bleaching and imaging were mediated by alternating 495 nm (3 s) and 665 nm excitation (150 ms) for 31 frames given accumulative 495 nm excitation of 93 s and 665 nm excitation of 4.65 s. For the analysis of the bleaching, puncta positive for both IFP and citrine were selected and the mean fluorescence was measured. The IFP fluorescence was normalized to the initial fluorescence intensity. Image acquisition and analysis were performed on the Slidebook 5.0 software (Intelligent Imaging Innovations, Inc.). The fluorescence intensities of the corresponding treatment pairs were compared with unpaired Student's *t* tests.

Plasma membrane targeting of IFP (pm-IFP) was achieved with a C terminus CaaX motif preceded by a lysine-rich sequence (KKKKKKSKTK). For C-terminal fusion of IFP to SYP1 and SYT1, flexible linkers (>25 amino acids) were used to ensure expression and trafficking. Both SYT1-IFP and pm-IFP were expressed under the control of the truncated hSynapsin promoter and a WPRE sequence was inserted after the stop codon. Expression in the neurons was achieved with electroporation prior to plating. For IFP imaging, the cells were incubated with 5 μM billiverdin in neurobasal media for 15 min prior to imaging.

Statistical Analysis

All values are expressed as mean ± SEM. Two-tailed paired student's *t* tests were used for the comparison of the same sample before and after light illumination. Two-tailed unpaired Student's *t* tests were used to compare two unmatched samples. For multiple comparisons, one-way ANOVA was used followed by Tukey's multiple comparison tests between all pairs. The exact *p* values reported were not adjusted for multiple comparisons. Statistical tests were done with Graphpad Prism 5.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, one movie, and Supplemental Text and can be found with this article online at <http://dx.doi.org/10.1016/j.neuron.2013.05.022>.

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represent the official views of the National Institute of Neurological Disorders and Stroke or the National Institutes of Health. J.Y.L. and S.B.S. designed the InSynC constructs. J.Y.L. conducted and analyzed the hippocampal microisland recordings. J.Y.L. and S.B.S. conducted and analyzed the worm movement and imaging experiments. K.Z. conducted and analyzed the electrophysiological recordings from *C. elegans* muscle cells. S.N. conducted and analyzed the organotypic slice experiments. R.Y.T., C.D.P., R.M., and Y.J. contributed to the design and analysis of the experiments. All authors contributed to the writing and discussion of the manuscript.

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Supplemental Information

Optogenetic Inhibition of Synaptic Release

with Chromophore-Assisted Light Inactivation (CALI)

John Y. Lin, Sharon B. Sann, Keming Zhou, Sadegh Nabavi, Christophe D. Proulx, Roberto Malinow, Yishi Jin, and Roger Y. Tsien

Inventory of Supplemental Materials

Figure S1. Representative images of SYP1-eGFP fluorescence and FM4-64 dye uptake in cultured cortical neurons.

This supplemental figure shows the representative images of the data used in Figure 1E.

Figure S2. Additional examples and summaries of whole-cell patch-clamp recording of electrically-evoked EPSC and miniature EPSC in organotypic hippocampal slices and primary cultured neurons.

This supplemental figure provides additional representative electrophysiological recordings and summary graphs not shown Figure 2.

Figure S3. Expression of miniSOG-Citrine and SNT-1-miniSOG-Citrine in *C. elegans*.

This supplemental figure provides the representative fluorescent images of miniSOG-Citrine and SNT1-miniSOG-Citrine worms that are acquired similarly to Figure 4A.

Figure S4. Analysis of spontaneous EPSCs recorded from miniSOG-VAMP2-Citrine (miniSOG-VAMP2) expressing worms and non-expressing (Wt) N2 worms.

This supplemental figure provides the properties of the spontaneous EPSC recorded from non-expressing (Wt) worms and miniSOG-VAMP2-Citrine expressing worms prior to light illumination to demonstrate the over-expression of miniSOG-VAMP2-Citrine in Wt

worms does not significantly changed the vesicular release. This supplemental figure complements the data shown in Figure 4B.

Table S1. Analysis of spontaneous EPSCs recorded from miniSOG-VAMP2-Citrine (miniSOG-VAMP2) expressing worms and non-expressing (Wt) N2 worms.

This supplemental table provides values shown in **Figure S4**.

Movie S1. An example of the inhibition of *C. elegans* movements with light illumination.

This movie shows the typical movies used for the quantification shown in **Figure 3A-C**.

Additional Supplemental Material

Protein and DNA sequences of InSynC

- miniSOG-VAMP2-T2A-mCherry
- miniSOG-VAMP2-Citrine
- SYP1-miniSOG-T2A-mCherry
- SYP1-miniSOG-Citrine
- SNT-1-miniSOG-Citrine

Supplemental Material

Optogenetic inhibition of synaptic release with chromophore-assisted light inactivation (CALI)

John Y. Lin *et al.*

Figure S1

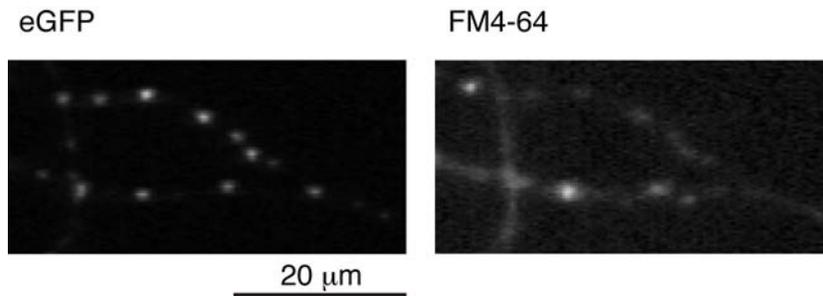


Figure S1. Representative images of SYP1-eGFP fluorescence and FM4-64 dye uptake in cultured cortical neurons.

Fluorescence images of eGFP puncta (left) and the corresponding FM4-64 dye uptake (right) after stimulation with 40 mM KCl. Not all eGFP positive puncta had detectable dye uptake, and FM4-64 on the plasma membrane was not removed completely after washout. Fluorescence was quantified to produce the graph shown in Figure 1E.

Figure S2

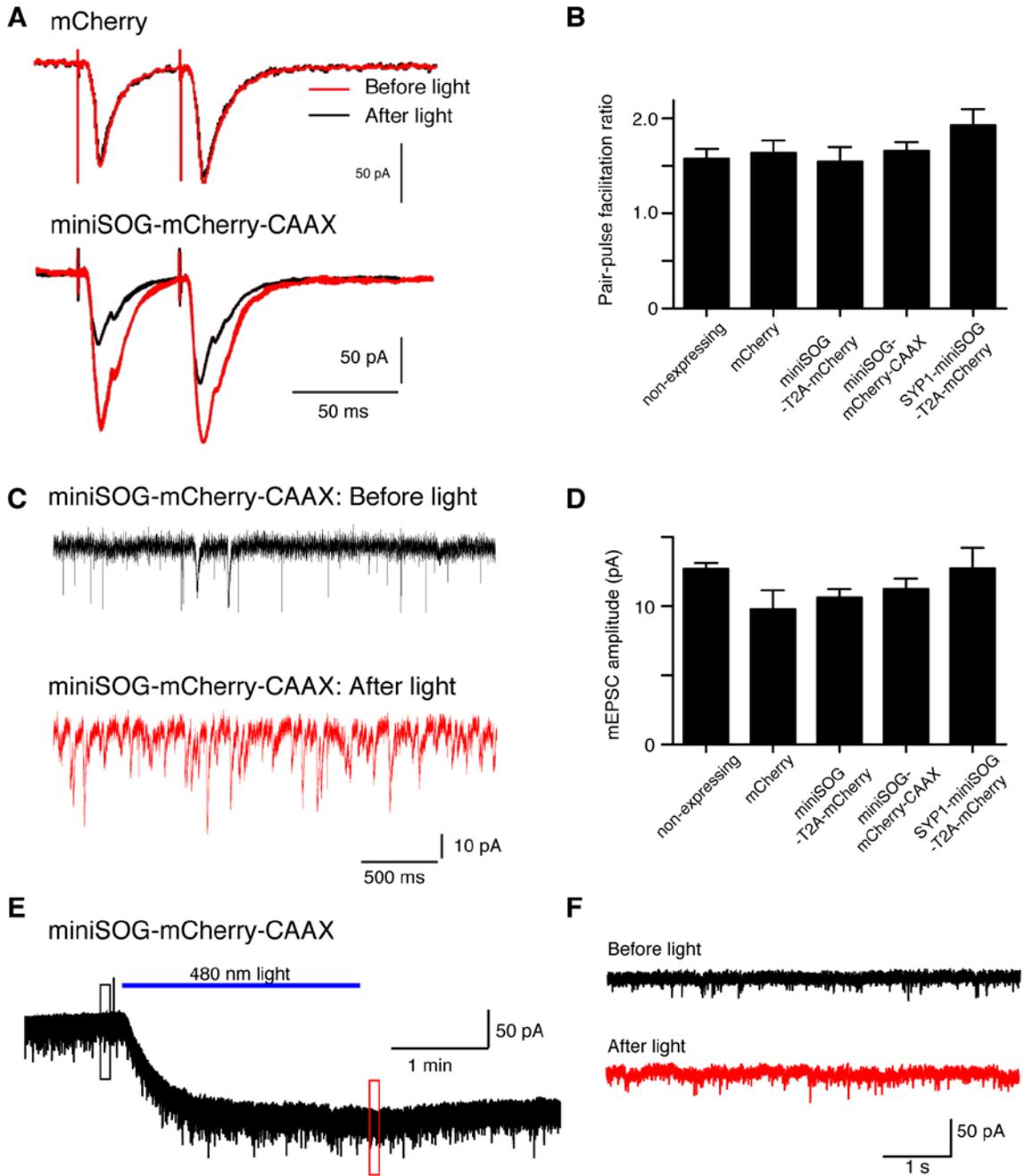


Figure S2. Additional examples and summaries of whole-cell patch-clamp recording of electrically-evoked EPSC and miniature EPSC in organotypic hippocampal slices and primary cultured neurons.

(A) In the organotypic hippocampal slices infected with rAAV expressing mCherry alone, the electrically-evoked EPSC was not inhibited after 480 nm light illumination. In the organotypic hippocampal slices infected with rAAV expressing membrane tethered miniSOG and mCherry (miniSOG-mCherry-CAAX), the electrically-evoked EPSC was sometimes increased after 480 nm light illumination. (B) Expression of mCherry, miniSOG-T2A-mCherry, miniSOG-mCherry-CAAX and SYP1-miniSOG-T2A-mCherry did not significantly change the pair-pulse facilitation ratio. (C) In the organotypic hippocampal slices infected with rAAV expressing miniSOG-mCherry-CAAX, light illumination increased the frequency mEPSC. (D) The mean mEPSC amplitudes in the slices expressing the different constructs prior to light illumination were not significantly different. (E) Voltage-clamp recording of miniSOG-mCherry-CAAX-expressing cortical neurons before and after light illumination (illumination indicated by blue horizontal bar). A long-lasting inward current is observed after light illumination. (F) Higher magnification of the boxed area in (E). Both graphs are shown as mean \pm S.E.M.. This supplemental figure provides additional representative electrophysiological recordings and summary graphs not shown Figure 2.

Figure S3

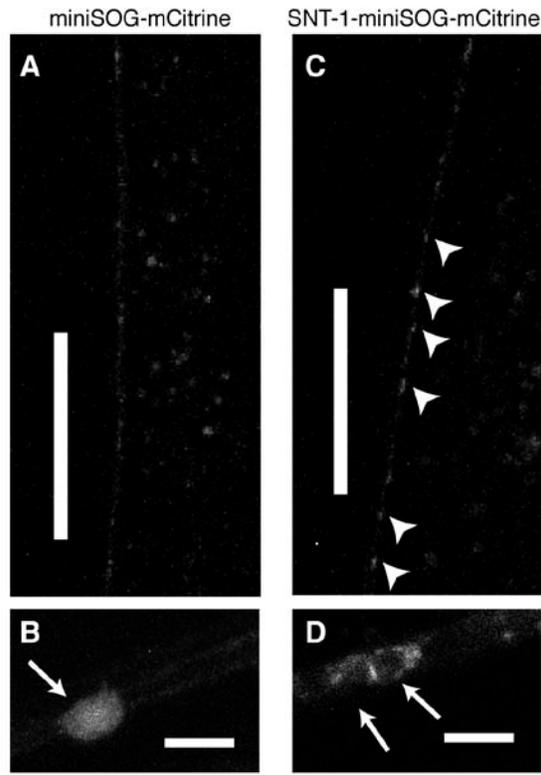


Figure S3. Expression of miniSOG-Citrine and SNT-1-miniSOG-Citrine in *C. elegans*.

Confocal images of Citrine fluorescence in miniSOG-mCitrine- (A and B) and SNT-1-miniSOG-Citrine- (C and D)-expressing worms. (A) In miniSOG-Citrine-expressing animals, punctate fluorescence signals corresponding to presynaptic terminals were not observed at the dorsal cord (A) although fluorescence is observed in the motor neuron somas located ventrally (B; arrowed). (C) In SNT-1-miniSOG-Citrine-expressing worms, punctate fluorescence signals at the dorsal cord (indicated by arrowheads) were consistent with synaptic localization along axonal projections (C). Somatic fluorescence signals (arrowed) were also observed in SNT-1-miniSOG-Citrine-expressing worms, presumably from newly synthesized protein prior to axonal trafficking (D). The animals expressing miniSOG-VAMP2-Citrine show similar expression pattern to SNT-1-miniSOG-Citrine-expressing animals. Scale bar: 25 μm for (A) and (C) and 5 μm for (B) and (D). These images were acquired similarly to Figure 4A

Figure S4

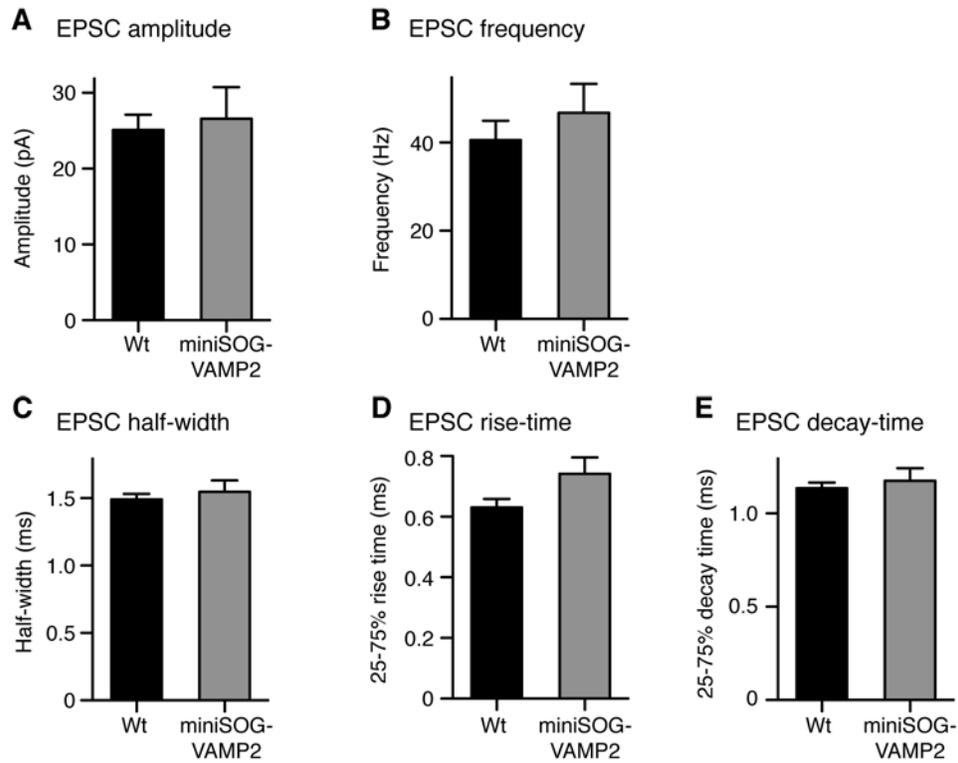


Figure S4. Analysis of spontaneous EPSCs recorded from miniSOG-VAMP2-Citrine (miniSOG-VAMP2) expressing worms and non-expressing (Wt) N2 worms prior to light illumination.

Analysis of the mean spontaneous EPSC amplitudes (A), frequency (B), half-width (C), rise time (D) and decay time (E) of whole-cell patch-clamp recordings from the muscle of miniSOG-VAMP2 (n = 11) and wild-type (n = 15) worms. None of the parameter compared were significantly different between the two groups. All graphs are shown as mean \pm S.E.M..

Table S1. Analysis of spontaneous EPSCs recorded from miniSOG-VAMP2-Citrine (miniSOG-VAMP2) expressing worms and non-expressing (Wt) N2 worms.

	Amplitude (pA)	Frequency (Hz)	Half-width (ms)	Rise-time (ms)	Decay-time (ms)
Wt (N2)	25.10 ± 2.01	40.57 ± 4.39	1.492 ± 0.041	0.063 ± 0.028	1.137 ± 0.029
miniSOG-VAMP2	26.57 ± 4.18	46.73 ± 6.55	1.548 ± 0.083	0.742 ± 0.054	1.175 ± 0.068

Movie S1. An example of the inhibition of *C. elegans* movements with light illumination.

This composite movie shows the inhibition and the recovery of the movements of a miniSOG-VAMP2-mCitrine expressing worm (N2 wild-type background) on a bacteria-free agar surface after the illumination of light. The movie is shown in real time.

Protein and DNA sequences of InSynC

miniSOG-VAMP2-T2A-mCherry

miniSOG
Linker
VAMP2
T2A sequence
mCherry

gctagc NheI restriction site
cgtacg BsiWI restriction site

```
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21 S D G F L E L T E Y S R E E I L G R N G 40
1128 TCCGATggCTTCTCCTGGAGCTGACCGAGTATTCCAGAGAGGAGATCCTGGGCCGCAATGgC 1187

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101 G V Q L D D G S G G G S G G G M S A T A A 120
1368 GGAGTGCAGCTGGATGGATCAGGAGGAGGCTCAGGAGGAGGAATGTGGCTACCGCTGCC 1427

121 T V P P A A P A G E G G P P A P P P N I 140
1428 ACCGTCCCgCCTGCCGCCCGGGCCGGCGAGGGTGGCCCCCTGCACCTCCTCAAACCTT 1487

141 T S N R R L Q Q T Q A Q V D E V V D I M 160
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181 A D A L Q A G A S Q F E T S A A K L K R 200
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1668 AAATACTGGTGGAAAAACCTCAAGATGATGATCATCTTGGGAGTGATCTGCGCCATCATC 1727

221 L I I I I V Y F S T A S G G G S G G G 240
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```

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421 **L K G E I K Q R L K L K D G G H Y D A E** 440
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441 **V K T T Y K A K K P V Q L P G A Y N V N** 460
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461 **I K L D I T S H N E D Y T I V E Q Y E R** 480
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481 **A E G R H S T G G M D E L Y K** * 496
2508 gccgagggccgcccactccaccggcggcatggacgagctgtacaagtaa 2555

miniSOG-VAMP2-Citrine

miniSOG
Linker
VAMP2
Citrine

ACCGGT AgeI restriction site
ATCGAT ClaI restriction site

1 **M E K S F V I T D P R L P D N P I I F A** 20
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 4765 gatgccacctacggcaagctgaccctgaagttcatctgcaccaccggcaagctgcccgtg 4824
 301 P W P T L V T T F G Y G L M C F A R Y P 320
 4825 ccctggcccaccctcgtgaccacctcggctacggcctgatgtgcttcgcccgtacccc 4884
 321 D H M K Q H D F F K S A M P E G Y V Q E 340
 4885 gaccacatgaagcagcagcacttcttcaagtcggccatgcccgaaggctacgtccaggag 4944
 341 R T I F F K D D G N Y K T R A E V K F E 360
 4945 cgcaccatcttcttcaaggacgacggcaactacaagaccgcgccgaggtgaagttcgag 5004
 361 G D T L V N R I E L K G I D F K E D G N 380
 5005 ggcgacaccctggtgaaccgcatcgagctgaaggcatcgacttcaaggaggacggcaac 5064
 381 I L G H K L E Y N Y N S H N V Y I M A D 400
 5065 atcctggggcacaagctggagtacaactacaacagccacaacgtctatatcatggccgac 5124
 401 K Q K N G I K V N F K I R H N I E D G S 420
 5125 aagcagaagaacggcatcaaggtgaacttcaagatccgccacaacatcgaggacggcagc 5184
 421 V Q L A D H Y Q Q N T P I G D G P V L L 440
 5185 gtgcagctcggcgaccactaccagcagaacacccccatcggcgacggccccgtgctgctg 5244
 441 P D N H Y L S Y Q S A L S K D P N E K R 460
 5245 cccgacaaccactacctgagctaccagtccgcccctgagcaaagaccccaacgagaagcgc 5304
 461 D H M V L L E F V T A A G I T L G M D E 480
 5305 gatcacatggctcctgctggagttcgtgaccgcccgggatcactctcggcatggacgag 5364
 481 L Y K * 484
 5365 ctgtacaagTAG 5376

SYP1-miniSOG-T2A-mCherry

Synaptophysin (SYP1)

Linker

miniSOG

T2A sequence

mCherry

accggt AgeI restriction site

gctagc NheI restriction site

cgtacg BsiWI restriction site

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1  M D V V N Q L V A G G Q F R V V K E P L 20
1083 atggacgtggtgaatcagctggtggtcgggggtcagttccgggtggtcaaggagccctt 1142

21  G F V K V L Q W V F A I F A F A T C G S 40
1143 ggcttctgaaggtgctgcagtggtcctttgccatcttcgcctttgtactcgtgtggcagc 1202

41  Y T G E L R L S V E C A N K T E S A L N 60
1203 tacaccggggagcttcggctgagcgtggagtgtgccacaagacggagagtgcctcaac 1262

61  I E V E F E Y P F R L H Q V Y F D A P S 80
1263 atcgaagttgaattcgagtacccttcaggctgcaccaagtgtactttgatgcacctcc 1322

81  C V K G G T T K I F L V G D Y S S S A E 100
1323 tgcgtcaaagggggcactaccaagatcttctcgttggggactactcctcgtcggctgaa 1382

101 F F V T V A V F A F L Y S M G A L A T Y 120
1383 ttctttgtcaccgtggctgtgtttgccttctactccatgggggcctggccacctac 1442

121 I F L Q N K Y R E N N K G P M M D F L A 140
1443 atcttctgcagaacaagtaccgagagaacaacaaagggcctatgatggactttctggct 1502

141 T A V F A F M W L V S S S A W A K G L S 160
1503 acagccgtgttcgcttcatgtggctagttagttcatcagcctgggcaaaggcctgtcc 1562

161 D V K M A T D P E N I I K E M P M C R Q 180
1563 gatgtgaagatggccacggaccagagaacattatcaaggagatgcccatgtgcccag 1622

181 T G N T C K E L R D P V T S G L N T S V 200
1623 acaggaacacatgcaaggaactgaggaccctgtgacttcaggactcaacacctcagtg 1682

201 V F G F L N L V L W V G N L W F V F K E 220
1683 gtgtttggcttctgaacctggtgctctgggttggaacttatggttcgtgttcaaggag 1742

221 T G W A A P F M R A P P G A P E K Q P A 240
1743 acaggctgggcagccccattcatgcgcgcacctccaggcggccccgaaaagcaaccagca 1802

241 P G D A Y G D A G Y G Q G P G G Y G P Q 260
1803 cctggcgatgcctacggcgatgcgggtacgggcagggccccggaggctatgggccccag 1862

261 D S Y G P Q G G Y Q P D Y G Q P A S G G 280
1863 gactcctacgggcctcagggtggttatcaaccgatctacgggcagccagccagcgggtggc 1922

281 G G Y G P Q G D Y G Q Q G Y G Q Q G A P 300
1923 ggtggtacgggcctcagggcgactatgggcagcaaggctatggccaacaggggtgcgccc 1982

301 T S F S N Q M K T G G G G S G G G S M E 320
1983 acctccttccaatcagatgaaaaccggtgggtggcggcagtggtggcggcagcatggag 2042

321 K S F V I T D P R L P D N P I I F A S D 340
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2043 aaaagtttcgtgataactgatccacggctgccagacaatcccatcatcttcgcatccgat 2102

341 G F L E L T E Y S R E E I L G R N G R F 360
2103 ggcttctcggagctgaccgagatattccagagaggagatcctgggcccgaatggcgccttt 2162

361 L Q G P E T D Q A T V Q K I R D A I R D 380
2163 ctgcaggaccagagacagaccaggccacagtgcagaagattcgcgatgccattagagat 2222

381 Q R E I T V Q L I N Y T K S G K K F W N 400
2223 cagcgcgagattaccgtgcagctgataaactacacaaaaagcgggaagaaattctggaac 2282

401 L L H L Q P M R D Q K G E L Q Y F I G V 420
2283 ctcctgcacctccagcccatgagggaccagaagggtgagctccagtatttcatcggagtg 2342

421 Q L D G A S G G G S G G G E G R G S L I 440
2343 cagctggatggagctagcggaggtggatctgggtggcggtgagggcagaggaagtcttcta 2402

441 T C G D V E E N P G P R T M V S K G E E 460
2403 acatgcggtgacgtggaggagaatcccggccctcgtaccatgggtgagcaagggcagggag 2462

461 D N M A I I K E F M R F K V H M E G S V 480
2463 gataacatggccatcatcaaggagttcatgcgcttcaagggtgcacatggagggctccgtg 2522

481 N G H E F E I E G E G E G R P Y E G T Q 500
2523 aacggccacgagttcagatcagagggcgagggcgagggcccccctacgagggcaccag 2582

501 T A K L K V T K G G P L P F A W D I L S 520
2583 accgccaagctgaaggtgaccaagggtggccccctgccttgcctgggacatcctgtcc 2642

521 P Q F M Y G S K A Y V K H P A D I P D Y 540
2643 cctcagttcatgtacggctccaaggcctacgtgaagcaccgccgacatccccgactac 2702

541 L K L S F P E G F K W E R V M N F E D G 560
2703 ttgaagctgtccttccccgagggcttcaagtgggagcgcgtgatgaacttcgaggacggc 2762

561 G V V T V T Q D S S L Q D G E F I Y K V 580
2763 ggcgtggtgaccgtgaccaggactcctcctgcaggacggcgagttcatctacaaggtg 2822

581 K L R G T N F P S D G P V M Q K K T M G 600
2823 aagctgcgcggccaccaacttcccctccgacggccccgtaatgcagaagaagaccatgggc 2882

601 W E A S S E R M Y P E D G A L K G E I K 620
2883 tgggaggcctcctccgagcggatgtaccccgaggacggcgcctgaagggcgagatcaag 2942

621 Q R L K L K D G G H Y D A E V K T T Y K 640
2943 cagaggctgaagctgaaggacggcggcactacgacgtgaggtcaagaccacctacaag 3002

641 A K K P V Q L P G A Y N V N I K L D I T 660
3003 gccaaagaagcccgtgcagctgcccggcgcctacaacgtcaacatcaagttggacatcacc 3062

661 S H N E D Y T I V E Q Y E R A E G R H S 680
3063 tcccacaacgaggactacaccatcgtggaacagtagaacgcgcccagggccgcccactcc 3122

681 T G G M D E L Y K * 690
3123 accggcggcatggacgagctgtacaagtaa 3152

SYPI-miniSOG-Citrine

SYPI
miniSOG
Linker

Citrine

ACCGGT AgeI restriction site

ATCGAT ClaI restriction site

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1 M D V V N Q L V A G G Q F R V V K E P I 20
1065 ATGGACGTGGTGAATCAGCTGGTGGCTGGGGGTCAAGGAGCCCTT 1124

21 G F V K V L Q W V F A I F A F A T C G S 40
1125 GGCTTCGTGAAGGTGCTGCAGTGGGTCTTTGCCATCTTCGCCTTTGCTACGTGTGGCAGC 1184

41 Y T G E L R L S V E C A N K T E S A L N 60
1185 TACACCGGGGAGCTTCGCTGAGCGTGGAGTGTGCCACAAGACGGAGAGTGCCCTCAAC 1244

61 I E V E F E Y P F R L H Q V Y F D A P S 80
1245 ATCGAAGTTGAATTTCGAGTACCCCTTCAGGCTGCACCAAGTGTACTTTGATGCACCTCC 1304

81 C V K G G T T K I F L V G D Y S S S A E 100
1305 TGCGTCAAAGGGGGCACTACCAAGATCTTCTGGTTGGGGACTACTCCTCGTCGGCTGAA 1364

101 F F V T V A V F A F L Y S M G A L A T Y 120
1365 TTCTTTGTCACCGTGGCTGTGTTTGCCTTCTCTACTCCATGGGGCCCTGGCCACCTAC 1424

121 I F L Q N K Y R E N N K G P M M D F L A 140
1425 ATCTTCTGCAGAACAAGTACCGAGAGAACAACAAGGGCCTATGATGGACTTTCTGGCT 1484

141 T A V F A F M W L V S S S A W A K G L S 160
1485 ACAGCCGTGTTTCGCTTTCATGTGGCTAGTTAGTTCATCAGCCTGGGCCAAAGGCCTGTCC 1544

161 D V K M A T D P E N I I K E M P M C R Q 180
1545 GATGTGAAGATGGCCACGGACCCAGAGAACATTATCAAGGAGATGCCATGTGCCGCCAG 1604

181 T G N T C K E L R D P V T S G L N T S V 200
1605 ACAGGGAACACATGCAAGGAAGTGGGACCCCTGTGACTTCAGGACTCAACACCTCAGTG 1664

201 V F G F L N L V L W V G N L W F V F K E 220
1665 GTGTTTGGCTTCTGAACCTGGTGTCTTGGGTTGGCAACTTATGGTTCGTGTTCAAGGAG 1724

221 T G W A A P F M R A P P G A P E K Q P A 240
1725 ACAGGCTGGGCAGCCCCATTCATGCGCGACCTCCAGGCGCCCCGAAAAGCAACCAGCA 1784

241 P G D A Y G D A G Y G Q G P G G Y G P Q 260
1785 CCTGGCGATGCCTACGGCGATGCGGGCTACGGGCAGGGCCCCGAGGCTATGGGCCCAg 1844

261 D S Y G P Q G G Y Q P D Y G Q P A S G G 280
1845 GACTCCTACGGGCCTCAGGGTGGTTATCAACCCGATTACGGGCAGCCAGCCAGCGGTGGC 1904

281 G G Y G P Q G D Y G Q Q G Y G Q Q G A P 300
1905 GGTGGCTACGGGCCTCAGGGCGACTATGGGCAGCAAGGCTATGGCCAACAGGGTGCGCC 1964

301 T S F S N Q M K T G G G G S G G S M E 320
1965 ACCTCCTTCTCCAATCAGATGaaaaccgggTGGTGGCGCAGTGGTGGCGGCAGCATgGAG 2024

321 K S F V I T D P R L P D N P I I F A S D 340
2025 AAAAGTTTCGTGATAAcTGATCCACGGCTGCCAGACAATCCCATCATCTTCGCATCCGAT 2084

341 G F L E L T E Y S R E E I L G R N G R F 360
2085 ggCTTCTGAGCTGACCGAGTATTCCAGAGAGGAGATCCTGGGCCGCAATGgCCGCTTT 2144

361 L Q G P E T D Q A T V Q K I R D A I R D 380
2145 CTGCAGGACCAGAGACAGACCAGGCCACAGTGCAGAAGATTTCGCGATGCCATTAGAGAT 2204
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381 Q R E I T V Q L I N Y T K S G K K F W N 400
 2205 CAGCGCGAGATTACCGTGCAGCTGATAAACTACACAAAAGCGGGAAGAAATTCTGGAAC 2264

 401 L L H L Q P M R D Q K G E L Q Y F I G V 420
 2265 CTCCtGcACCTCCAGCCCATGAGGGACCAGAAGGGTGAGCTCCAGTATTTTCATCGGAGTG 2324

 421 Q L D G S G G G S G G G G A G I D M V S 440
 2325 CAGCTGGATGGATCAGGAGGAGGCTCAGGAGGAGGAGGCGCGggAATCGATatggtgagc 2384

 441 K G E E L F T G V V P I L V E L D G D V 460
 2385 aagggcgaggagctgttcaccgggggtggtgcccatcctggtcgagctggacggcgacgta 2444

 461 N G H K F S V S G E G E G D A T Y G K L 480
 2445 aacggccacaagttcagcgtgtccggcgagggcgagggcgatgccacctacggcaagctg 2504

 481 T L K F I C T T G K L P V P W P T L V T 500
 2505 accctgaagttcatctgcaccaccggcaagctgcccgtgccctggcccaccctcgtgacc 2564

 501 T F G Y G L M C F A R Y P D H M K Q H D 520
 2565 accttcggctacggcctgatgtgcttcgcccgtaccccgaccacatgaagcagcacgac 2624

 521 F F K S A M P E G Y V Q E R T I F F K D 540
 2625 ttcttcaagtccgccatgcccaaggtacgtccaggagcgcaccatcttcttcaaggac 2684

 541 D G N Y K T R A E V K F E G D T L V N R 560
 2685 gacggcaactacaagaccgcgcccaggtgaagttcgagggcgacaccctggtgaaccgc 2744

 561 I E L K G I D F K E D G N I L G H K L E 580
 2745 atcgagctgaagggcatcgacttcaaggaggacggcaacatcctggggcacaagctggag 2804

 581 Y N Y N S H N V Y I M A D K Q K N G I K 600
 2805 tacaactacaacagccacaacgtctatatcatggccgacaagcagaagaacggcatcaag 2864

 601 V N F K I R H N I E D G S V Q L A D H Y 620
 2865 gtgaacttcaagatccgccacaacatcgaggacggcagcgtgcagctcgccgaccactac 2924

 621 Q Q N T P I G D G P V L L P D N H Y L S 640
 2925 cagcagaacacccccatcggcgacggccccgtgctgctgcccgacaaccactacctgagc 2984

 641 Y Q S A L S K D P N E K R D H M V L L E 660
 2985 taccagtcgcccctgagcaaagacccaacgagaagcgcgatcacatggtcctgctggag 3044

 661 F V T A A G I T L G M D E L Y K * 677
 3045 ttcgtgaccgcccgggatcactctcggcatggacgagctgtacaagTAG 3095

SNT-1-miniSOG-Citrine

miniSOG
 Linker
 SNT-1
 Citrine

accggt AgeI restriction site
 ATCGAT ClaI restriction site

1 M V K L D F S S Q D E E N D E D L T K E 20
 692 ATGGTGAAATTAGACTTTTCGTGCGCAAGACGAAGAGAACGACGAAGACTTGACAAAAGAG 751

 21 F V R D E A P M E E T T S E A V K Q I A 40

752 TTTGTAAGGGATGAAGCACCAATGGAAGAAACAACATCGGAAGCAGTAAAGCAAATAGCA 811

41 T T T K E T L K D V V V N K V I D V K D 60
812 ACAACGACAAAGGAGACGCTGAAAGATGTGGTTGTAATAAAGTGATTGATGTGAAAGAC 871

61 V V K E K V M Q Q T G M P E W A F V F L 80
872 GTTGTGAAAAGAAAAGTTATGCAACAAACTGGGATGCCTGAATGGGCGTTCGTATTTCTT 931

81 G F V F I L L V L A C A F C L I R K L F 100
932 GGATTCGTATTTATTCTGCTGGTTCCTCGCGTGTGCATTCTGTCTCATTTCGGAAGTTATTT 991

101 G K K R H G E K N K K G G L K G F F G K 120
992 GGAAAAAAGCGGCATGGTGAGAAGAACAAAAAGGGTGATTGAAAGGATTCTTTGGTAAA 1051

121 G Q D V V D G K N I Q G M A Q D L E E L 140
1052 GGACAGGATGTCGTTGATGGAAAAAATATTCAAGGGATGGCTCAAGACTTGAAGAAGACTT 1111

141 G D A M E Q N E K E Q A E E K E E V K L 160
1112 GGTGATGCGATGGAACAAAATGAAAAAGAACAAGCTGAAGAAAAAGAAGAAGTGAAGACTT 1171

161 G R I Q Y K L D Y D F Q Q G Q L T V T V 180
1172 GGAAGGATACAATATAAACTTGATTATGATTTCCAACAAGGTCAACTAACTGTAAGTACTGTA 1231

181 I Q A E D L P G M D M S G T S D P Y V K 200
1232 ATTCAAGCAGAAGATTTACCAGGAATGGACATGTTCAGGAACATCAGATCCATATGTAAAA 1291

201 L Y L L P E K K K K V E T K V H R K T L 220
1292 TTGTATTTGTTACCGGAGAAAAAGAAGAGTTGAGACGAAAGTACATCGAAAAACTCTT 1351

221 N P V F N E T F I F K V A F N E I T A K 240
1352 AATCCAGTATTCAATGAGACATTCATTTTAAAGTCGCTTTCAACGAAATTACGGCAAAA 1411

241 T L V F A I Y D F D R F S K H D Q I G Q 260
1412 ACTCTTGTCTTTGCAATTTATGATTTTCGATCGGTTTCAGTAAGCACGATCAAATCGGACAA 1471

261 V L I P L G K I D L G A V I E E W K D I 280
1472 GTTCTCATTCCGCTTGAAAAATTGATTTGGGAGCTGTATCGAAGAATGGAAGGATATT 1531

281 A P P P D D K E A E K S L G D I C F S I 300
1532 GCACCACCACAGATGACAAAGAAGCTGAGAAGAGTCTTGGTGACATTTGCTTCTCACTT 1591

301 R Y V P T A G K L T V V I L E A K N L K 320
1592 CGGTACGTCCCAACTGCTGGTAAATTGACAGTGGTCATTCTGGAAGCAAAAAATCTTAAG 1651

321 K M D V G G L S D P Y V K I V L M Q G G 340
1652 AAAATGGACGTCGGTGGTTTATCAGATCCTTATGTGAAGATTGTGTTGATGCAAGGTGGA 1711

341 K R L K K K K T S I K K C T L N P Y Y N 360
1712 AAACGACTGAAAAAGAAGAAGACATCAATCAAAAAGGTACACTTAACCCATATTATAAC 1771

361 E S F S F E V P F E Q I Q K V S L M I T 380
1772 GAATCGTTCAGCTTTGAAGTGCCTTTGAAACAAATTCAGAAAGTTTCCCTTATGATCACT 1831

381 V M D Y D K L G S N D A I G R C L L G C 400
1832 GTGATGGATTATGATAAACTTGGATCCAATGACGCTATTGGAAGGTGTCTATTGGGATGT 1891

401 N G T G A E L R H W M D M L A S P R R P 420
1892 AATGGAACCGGTGCCGAGCTGAGGCATTGGATGGATATGTTGGCTTACCACGTCGTCCA 1951

421 I A Q W H T L G P V E E E G D K K D D K 440
1952 ATTGCTCAATGGCATACTTGGACCAGTTGAAGAAGAAGGTGATAAGAAAGATGATAAG 2011

441 K T G G G G S G G G S M E K S F V I T D 460

2012 AAAaccggtGGTGGCGGCAGTGGTGGCGGCAGCATgGAGAAAAGTTTCGTGATAAcTGAT 2071

461 P R L P D N P I I F A S D G F L E L T E 480
2072 CCACGGCTGCCAGACAATCCCATCATCTTCGCATCCGATggCTTCCTGGAGCTGACCGAG 2131

481 Y S R E E I L G R N G R F L Q G P E T D 500
2132 TATTCCAGAGAGGAGATCCTGGGCCGAATGgCCGCTTCTGCAGGGACCAGAGACAGAC 2191

501 Q A T V Q K I R D A I R D Q R E I T V Q 520
2192 CAGGCCACAGTGCAGAAGATTTCGCATGCCATTAGAGATCAGCGCGAGATTACCGTGCAG 2251

521 L I N Y T K S G K K F W N L L H L Q P M 540
2252 CTGATAAACTACACAAAAAGCGGGAAGAAATTCTGGAACCTCtGcACCTCCAGCCCATG 2311

541 R D Q K G E L Q Y F I G V Q L D G S G G 560
2312 AGGGACCAGAAGGGTgAGCTCCAGTATTTTCATCGGAGTGCAGCTGGATGGATCAGGAGGA 2371

561 G S G G G G A G I D M V S K G E E L F T 580
2372 GGCTCAGGAGGAGGAGGCGGGGAATCGATatggtgagcaagggcgaggagctgttcacc 2431

581 G V V P I L V E L D G D V N G H K F S V 600
2432 ggggtggtgcccatcctggtcgagctggacggcgacgtaaacggccacaagttcagcgtg 2491

601 S G E G E G D A T Y G K L T L K F I C T 620
2492 tccggcgagggcgagggcgatgccacctacggcaagctgaccctgaagttcatctgcacc 2551

621 T G K L P V P W P T L V T T F G Y G L M 640
2552 accggcaagctgcccgtgccctggcccaccctcgtgaccaccttcggctacggcctgatg 2611

641 C F A R Y P D H M K Q H D F F K S A M P 660
2612 tgcttcgcccgtacccccgaccacatgaagcagcagcacttcttcaagtccgccatgcc 2671

661 E G Y V Q E R T I F F K D D G N Y K T R 680
2672 gaaggctacgtccaggagcgcaccatcttcttcaaggacgacggcaactacaagaccgc 2731

681 A E V K F E G D T L V N R I E L K G I D 700
2732 gccgaggtgaagttcgagggcgacaccctggtgaaccgcatcgagctgaagggcatcgac 2791

701 F K E D G N I L G H K L E Y N Y N S H N 720
2792 ttcaaggaggacggcaacatcctggggcacaagctggagtacaactacaacagccacaac 2851

721 V Y I M A D K Q K N G I K V N F K I R H 740
2852 gtctatatcatggccgacaagcagaagaacggcatcaaggtgaacttcaagatccgccac 2911

741 N I E D G S V Q L A D H Y Q Q N T P I G 760
2912 aacatcgaggacggcagcgtgcagctcggcaccactaccagcagaacacccccatcggc 2971

761 D G P V L L P D N H Y L S Y Q S A L S K 780
2972 gacggccccgtgctgctgcccgacaaccactacctgagctaccagtcgcgcctgagcaaa 3031

781 D P N E K R D H M V L L E F V T A A G I 800
3032 gaccccaacgagaagcgcgatcacatggtcctgctggagttcgtgaccgcccgggatc 3091

801 T L G M D E L Y K * 810
3092 actctcggcatggacgagctgtacaagTAG 3121