A fully genetically encoded protein architecture for optical control of peptide ligand concentration

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Ion channels are among the most important proteins in biology, regulating the activity of excitable cells and changing in diseases. Ideally it would be possible to actuate endogenous ion channels, in a temporally precise and reversible manner, and without requiring chemical cofactors. Here we present a modular protein architecture for fully genetically encoded, light-modulated control of ligands that modulate ion channels of a targeted cell. Our reagent, which we call a lumitoxin, combines a photoswitch and an ion channel-blocking peptide toxin. Illumination causes the photoswitch to unfold, lowering the toxin’s local concentration near the cell surface, and enabling the ion channel to function. We explore lumitoxin modularity by showing operation with peptide toxins that target different voltage-dependent K⁺ channels. The lumitoxin architecture may represent a new kind of modular protein-engineering strategy for designing light-activated proteins, and thus may enable development of novel tools for modulating cellular physiology.
on channels govern cellular signaling and computation, in neurons and neural compartments as well as other excitable cell classes, and are significant drug targets for a variety of disorders. Ideally, one could genetically target ion channels for perturbation to assess their causal contribution to complex systems. Earlier studies have approached this problem by several kinds of innovation. For example, one line of inquiry has resulted in genetically encoded membrane-targeted peptide toxins that can be expressed in cell types of interest. Peptide toxins comprise a broad class of genetically encoded ion channel modulators from venomous animals that are capable of recognizing targets from every major ion channel family, with extraordinary specificity. These reagents function without requiring exogenously supplied chemicals, and are inducible and reversible over timescales of hours to days, and have been shown to function in mammalian brain in vivo. A second line of inquiry has utilized light-responsive chemical modulators that are anchored to specific sites engineered into ion channels (for example, SPARK, LiGluR and HyLighter), and thus are capable of fast induction and reversal with light. They incorporate exogenously supplied engineered chemicals, which bind to engineered sites on targeted ion channels. The first technology is fully genetically encoded but not light-modulatable; the second technology is rapidly light-modulatable but not genetically encoded.

Here we devise a novel protein architecture capable of modulating endogenous ion channels, but which is both fully genetically encoded, and actutable by light. We accordingly here adapt three different technology building blocks in a novel protein architecture to yield a fully genetically encoded reagent that can actuate endogenous voltage-gated K⁺ (Kv) channels with light. We create a fusion protein containing a peptide toxin as the ion channel ligand and a light-oxygen-voltage (LOV) domain photoswitch tethered to the cell membrane. We hypothesize that upon illumination, conformational changes in the LOV domain lower the local concentration of the peptide toxin near the cell surface, resulting in a decreased apparent association rate constant (k⁺) for binding to the channel (Ch) and then blocking it (Ch × LL or Ch × LD). As approximations, we assume that the lumitoxin and channel are expressed to similar levels, and further assume that the dynamics of light-dependent conformational changes in the LOV-Jz photoreceptor are independent of the peptide toxin/channel binding equilibrium.

We found that when using published values for the binding of α-DTX to Kv24,25 and the light-dependent transitions of an isolated LOV2-Jz domain from Avena sativa26,27 (see Methods for details), the system equilibrates with most ion channels (>90%) being occupied by a peptide toxin—that is, most Kv channels are blocked by the tethered α-DTX in the dark state (Fig. 1d, compare cyan (blocked channel) and orange (unblocked channel) lines). Illumination leads to a rapid increase in unblocked ion channels, followed by a somewhat slower re-blocking of those channels when illumination ceases. On the basis of our simulations we predict that a synthetic fusion protein, as we described above, will react to illumination with reasonably fast response times (s) and will allow fully reversible repeated activation/deactivation cycles.

**Lumitoxins mediate optical modulation of cellular K⁺ current.** We synthesized a gene coding for a lumitoxin that contained Dendroaspis angusticeps α-DTX, which specifically binds to Kv1.1 and Kv1.2 channels, connected to the A. sativa LOV2-Jz domain (AsLOV2) via a 26-residue flexible linker. This fusion protein was targeted for the secretory pathway using a cleavable signal peptide and was anchored to the extracellular side of the cell membrane by a single-pass transmembrane domain derived from the human platelet-derived growth factor receptor (PDGF-R). We expressed α-DTX-lumitoxins in cultured PC12 cells co-transfected with Kv1.2, and saw healthy expression (Fig. 2a), as might be expected, given that both AsLOV2-containing proteins and peptide toxins had previously been shown separately to express in mammalian cells. Whole-cell patch clamp recordings showed characteristic baseline voltage-dependent K⁺ currents in a cell expressing α-DTX–lumitoxins (Fig. 2b, left panel). Illumination of the same cell with modest levels (500 μW mm⁻²) of blue (455 nm) light increased the whole-cell K⁺ current approximately twofold within seconds (Fig. 2c, orange circles and Fig. 2b, middle panel). After cessation of illumination, the whole-cell K⁺ current recovered to pre-illumination levels within 2 min (Fig. 2b, right panel).

As predicted by our model (Fig. 1), the majority of ion channels were blocked in the dark state, as judged by the baseline behaviour to gain insight into the characteristic parameters. Our simulation predicts that the number of peptide collisions with the cell surface is decreased as the tether length is increased, the computational analogue to the unfolding of the Jz helix. Specifically, by inspecting the probability distribution in the x-z plane (that is, looking at the lumitoxin from the side as in Fig. 1a) we get an appreciation of the changes in the mean peptide localization. Shown are the simulation results for a six-membered and a 27-membered membrane linker (Fig. 1b, upper left and lower left panels, respectively). The distribution difference demonstrates that after unfolding of the Jz helix, it is more likely to find the peptide 6 nm away from the membrane plane; the local concentration close to the peptide toxin’s binding site on a membrane-bound ion channel would accordingly be greatly reduced (Fig. 1b, right panel).
K⁺ currents recorded in cells co-expressing both αDTX–lumitoxin and Kv1.2 versus cells expressing Kv1.2 alone (mean current at +50 mV: 40 ± 12 pA/pF versus 206 ± 24 pA/pF, P < 0.0001 two-tailed Student’s t-test, n = 7–20). Furthermore, as predicted by our model, the whole-cell K⁺ current rose within seconds and then, post illumination, attenuated back to baseline somewhat more slowly, but completely (Fig. 2c, orange circles).

An important aspect of the utility of peptide toxins in physiology and neuroscience is their excellent specificity; they can differentiate between subfamilies of closely related ion channels and receptors. To test whether the observed light-dependent current increase was ion channel-specific, we co-expressed the Shaker channel and αDTX–lumitoxin in the same cell. Kv1.2 and Shaker are similar in many aspects, but differ in affinity, while its affinity for Shaker is very low (micromolar)²⁵. As expected, illumination did not alter whole-cell K⁺ current in cells that co-expressed Shaker and αDTX–lumitoxin, demonstrating that peptide toxins embedded within lumitoxins maintain their binding specificity (Fig. 2c, black circles). Neither were the properties of the AsLOV2 domain overtly perturbed by embedding within a lumitoxin, as current modulation was dependent on blue light, and was not affected by green light (Fig. 2d). We measured the time constant for light-driven lumitoxin effect—that is, the time constant constant by which the Jα helix unfolds and presumably lowers the local αDTX concentration near the cell surface—leading to an increase in available Kv1.2 channels. We found the time constant to be a function of irradiance, consistent with Jα helix unfolding being dependent on photon absorption. The apparent half-maximum time constant is achieved at 80 μW mm⁻² (Fig. 2e, left panel). Thus, note that K⁺ current could be modulated by non-damaging, relatively low light intensities. On the other hand, the post-illumination recovery of K⁺ current to pre-illumination baseline, associated with an increase in αDTX concentration close to the membrane-bound ion channel and increased block of Kv1.2, solely depends on the spontaneous dark-state refolding of LOV2–Jα and is thus expected to be independent of irradiance, which we confirmed experimentally (Fig. 2e, right panel). Repeated stimulation with blue light demonstrates that αDTX–lumitoxins are able to carry out consecutive K⁺ current modulations and are not undergoing destructive conformational changes (Fig. 2f).

The lumitoxin architecture enables rational protein design. We assessed the tunability of the lumitoxin architecture by utilizing a family of mutated αDTX variants, which are known to possess altered affinity towards Kv1.2. High-affinity binding of αDTX depends crucially on the peptide residues K5 and L9, and changing them to alanine decreases the binding affinity by three orders of magnitude²⁴. We thus expect two things when introducing the
corresponding mutations K5A and L9A into αDTX-lumitoxins. First, we expect the initial K\(^+\) current to increase, as our model predicts that decreasing the peptide affinity results in fewer channels being blocked in the dark (off) state. Second, we expect the activation ratio \((I_{\text{off}}/I_{\text{dark}})\) to decrease as fewer channels are now primed to become unmasked by illumination. We experimentally confirmed these expectations, and, after investigation of a suite of mutated αDTX–lumitoxins (Fig. 3a), we found that both baseline current and activation ratio depended monotonically on peptide toxin affinity, as would be expected given previous reports on free, that is, non-tethered, toxin\(^2^4\), and consistent with our predictions. For example, the mutations R3A and R4A, which decrease the binding affinity 10-fold for free αDTX, decreased the activation ratio by 20%. The mutations K5A and L9A, which decrease the binding affinity >1,000-fold, completely abolished the light-dependent increase in K\(^+\) current (Fig. 3b).

We do not know whether mutations in αDTX affect the lumitoxin affinity through altering peptide toxin/channel association or dissociation. However, by using the model developed above, we can simulate the theoretical activation ratios of αDTX–lumitoxin variants using either assumption and found that the effects of all mutations except H10A on the experimentally determined activation ratios were consistent with a decrease in the association rate constant (Fig. 3b, solid lines).

**Lumitoxins are modular and channel specificity is adjustable.** In the ideal case, it would be possible to tether multiple light-switchable protein ligands to the cell membrane without any optimization of the non-ligand protein parts. Consequently, we explored whether the lumitoxin architecture fulfills this requirement by creating lumitoxin genes containing different peptide toxins that target distinct Kv channel types. Whereas αDTX blocks both Kv1.1 and Kv1.2 channels\(^2^8\), *Dendroaspis polyplepis* DTX-K, which shares only 63% sequence identity with αDTX, blocks mainly Kv1.1 channels\(^2^9\). Conkunitzin-S1 (CONK1), isolated from the marine cone snail *Conus striatus*, specifically blocks the Drosophila Shaker Kv channel\(^3^0\). We expressed the resulting lumitoxins in mammalian cell culture co-transfected with the Kv channels Kv1.1, Kv1.2 and Shaker. We used a variant of the Shaker Kv containing a mutation (K427D) shown to increase CONK1 affinity\(^3^0\). Whole-cell patch clamp electrophysiology reveals a clear specificity in the ability of lumitoxins to modulate K\(^+\) currents in response to blue-light illumination (500 \(\mu\)W mm\(^{-2}\)). Whereas αDTX-containing

![Figure 2](image-url)
lumitoxins affect both Kv1.1 and Kv1.2, but not Shaker (K427D) (Fig. 3c upper row, Fig. 3d). DTX-K-containing lumitoxins are specific for Kv1.1 (Fig. 3c middle row, Fig. 3d). Similarly, CONK1-containing lumitoxins only affect Shaker Kv channels, but not the mammalian homologs Kv1.1 or Kv1.2 (Fig. 3c bottom row, Fig. 3d). These results imply that lumitoxin specificity can be altered by swapping the genetically encoded protein ligand without the necessity of optimizing non-ligand protein domains, resulting in multiple genetically encoded reagents for the actuation of specific subclasses of endogenous Kv channels.

**Discussion**

We here present the architecture of a fully genetically encoded tool for modulating the concentration of a protein ligand near the surface of a specified cell or set of cells by light. The resultant ‘lumitoxins’ are fusion proteins of two fundamentally useful functional elements, peptide neurotoxins and the photoreceptor LOV2-Jα, which serves as the tether between the protein ligand and the membrane. We demonstrated the functionality of our technology by creating several genetically encoded peptide toxins, targeting different classes of Kv channels, whose concentration could be modulated by light, in a fully reversible manner. Our architecture was capable of yielding lumitoxins that modulated K⁺ currents with subfamily precision (for example, favouring Kv1.1 over Kv1.2 with perfect discrimination), demonstrating that peptide toxins, when part of a light-activated membrane-tether fusion protein, retain their activity and specificity. This specificity might be further facilitated by the addition of subcellular protein-trafficking motifs so that, for example, just the subset of Kv channels located on the axons or dendrites might be modulated. Exploring whether other classes of ion channels, and other membrane proteins such as neurotransmitter receptors, GPCRs, receptor tyrosine kinases and so forth, can be actuated by lumitoxins may represent a natural field of exploration in the future.

In earlier studies, the LOV domain had been customized in several ways to embed various different peptides and enzymes within or next to the LOV domain. Our new architecture is modular in the sense that the LOV domain does not require customization for each target peptide and thus might present a more general solution to make peptides or proteins available...
upon illumination. Another set of earlier studies fused protein ligands to membrane anchors, enabling fully genetically encoded blockade of endogenous ion channels9–11. Our architecture builds on these pioneering studies by enabling ion channel activity to be dynamically controlled over rapid timescales. Finally, a third line of past inquiry utilizes light-responsive, externally added chemical ligands anchored to specific sites engineered into ion channels13–19, enabling fast induction and reversal, by modulating the concentration of the ligand relative to the ion channel surface. Our current methodology extends these ideas by being fully genetically encoded, eliminating the need for exogenous chemical application.

| Table 1 | Lumitoxin construct generation. |
|---------------------------------|---------------------------------|
| **Fusion protein component**    | **Amino acid sequence**         | **DNA sequence** |
| Secretion signal/FLAG tag       | MSALL ILALV GAAVA               | atg aqc gcc ctg ctg atc ctg ggc ctg atc ctg ggc gcc |
|                                 | DYKDDD DDKL                    | gcc ggc ggc gac aag gag gcag gac aag ctg |
| 2DTX (wt)                       | QPRRK LCLIL RNPGGR             | cag ccc aga aga aag ctg tgc atc ctg cag aca aac |
|                                 | CYDKF PAPFY NQKKK              | ccc ggc aga tgc tac gac aag acc gcc ttc ttc tac |
|                                 | QCERF DWSGC GNNSN              | taa cacc cag aag cag tgc gag aga ttc gag |
|                                 | RFKFR EECRR TCIG                | tgg aqc ggc tgc ggc gac aac agc aag aac ttc ttc |
|                                 |                                  | gtc gac gag tgc aga aag acc gtc ttc gcc ggc |
| DTX-K                           | AAAKC KLPLR IGPCG              | ggc gcc aag tac tgc aag ctg gcc ctg atc gag tgg |
|                                 | RKPIS FYWYM KAKQC              | cca tgc aag cgg aac aag atc gcc ttc tac aag ttc |
|                                 | LFWFDY SGGCG NANRF             | cgg tgc gga ggc aac gcc aac cgc ttc aag acc ttc |
|                                 | KTIBE CRRTC VG                 | gag taa tgc aga acc gcc tgc gag ttc aag |
| CONKI                           | KDRPS LCDLP ADSGS              | aag cag ccg ccc agc tgc tgc gac cct gcc gat |
|                                 | GTRAE KRIYY NARK                | ccc ggc aag cgc agg tgc cct gcc ttc ttc ctc ttc |
|                                 | QCDFR DTFTQ GGNEN              | ttc gcc tctgcc aag ggc gag agg ccg atc tac |
|                                 | NFRTF YDCQR TCLYR               | tca aac gcc gag ggc gag aag gag aac ttc ggg |
|                                 |                                  | cgg acc tac tgc cag aac gcc tgc ttc ttc tac ttc |
| Linker                          | GTAAA DYKDD DDKD                | ggt acc gcc ggc gcc gag tac aag gag gac gac |
|                                 | AAAG GCNC NEF                 | aag atc gag gcc gcc gcc ggc gcc gtc ttc gag |
| LOV2-Jx                         | VATTL ERIEK NFVIT              | tgg gct act aca ctt gaa cgt att gag aag aac ttt |
|                                 | DPFLP DNPPI FASDS              | gtc att act gac cca aga tgg cca gat aac ctt |
|                                 | FLQFR EYSRE EILGR              | at a ttc gcc tgc gat att ttc tgc gag aca gag |
|                                 | NCRLQ GQPET DRTAV              | tat agc gct gaa aat tgg gga aag acc tgc agg |
|                                 | RKKRD GIDNO TEVIT              | ttt cta caa ggt cct gaa act gtc gcc gcg aca gtc |
|                                 | QLNYX TKSGF KPVNL              | aga aac atc gag gcc ata gat aac cca aca gag |
|                                 | FHLQQP MRDQK GDVQY             | gtc act gtt cag ctg att att tat aca aag aat ggt |
|                                 | FIGVQ LDDGT HVRDA              | aag aac ttc ggc aac ctt ctc tgg cag cct att |
|                                 | AEREG VMLIK KTAEN              | cga gat cag aag gag gat ctc gac tac att ttt ggg |
|                                 | IDEA AKEL                      | ggt ctc gag aag gaa gtc cag atg att aag aat ggt |
|                                 |                                  | gca gaa aat att gat gag gcg gca aag ctt |
| PDGF-R-mCherry                  | RAVVG QDQF VIVVP               | cgc gtc gct gtt ggc cag cag cag gac atc |
|                                 | HLSLVF KVVVI SAILA             | gtc gtc gta cca cac ctc ttc ccc ttt aag gtt gtt gtg |
|                                 | LVVLVF IISLL ILILML           | gtt gct ctc ggc cct gct gct ctc gcc aac gcc |
|                                 | WQKPP PRIMI VISGEE            | atc tcc aac atc atc atc aag cct ctt ctt cgg cag aag |
|                                 | EDNMA IIEK MRFKV               | aaa cca ctt agg att ctt atg att gag aag ggc gag |
|                                 | HMEGS VNGH FEIEG              | gag gat aac atg gcc atc aac aag gag gag gcc tgc |
|                                 | EGEGR PYBTG QTAKL             | ttc aac ggc ggc gac gcc ggc ggc ggc gcc ggc gcc |
|                                 | KVTKG GLPFLF AMDIL             | cag gag ttc gag agg gag gag gag gcc ggc ccc |
|                                 | SPQFM YGSKA YVHKP             | cag gag ggc acc acc agg aag ctg ggg ctg aag |
|                                 | ADIPD YLKL FPEEGF              | cgg aag agg agg gcc gcc ctg gcc gcc ggt gcc |
|                                 | kWVFR MNFSD GGVVT              | gtc cct tcc gag atg tgc cag gcc gcc gcc gcc gcc |
|                                 | VQDQG SLQGD EFYK              | ttc cct gcc ggc ctg gcc gcc gcc gcc gcc gcc |
|                                 | VKLNG TNPS DOPJUN             | aac gcc ggc gcc gcc atc ttc gcc ttc gcc ttc |
|                                 | QKKTQ DWLASS SERMY            | gac gcc gcc ggc ggc ggc ggc gcc gcc gcc gcc |
|                                 | PEDGA LKGEIK QRLKL            | gac gcc ctc ctc ctc ctc ctc gcc gcc gcc gcc |
|                                 | KDGHH YDAEV KTTYK             | gcc gta atc cag aag aac acc atg ggc gag ggc |
|                                 | AKKPV QLPYA YNVNI             | ctc ctc gcc gag agg gcc gcc gcc gcc gcc tgg |
|                                 | KLDIT SHNED YTITVE            | aag ggc gag atc aag cag agg ctg aag ctg gag |
|                                 | QYBRA EGRHS TGMD              | ggc ggc cac tac gac gct gag tgc aag acc acc ttc |
|                                 | ELYK*                         | aag gcc aag aag cgc ctc gag ctc gcc gcc tgc |
|                                 |                                  | aac gcc aac gcc ctc gag ctc gcc gcc tgc |
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CONKI, Conkunitzin-S1; DTX, a-Dendrotoxin; LOV2, light-oxygen-voltage domain; PDGF-R, platelet-derived growth factor receptor.
We developed an analytical model for lumitoxin function, based on biophysical principles, and focusing on the hypothesis that the illumination of the lumitoxin would, as the LOV2-J2 unfold, modulate the local concentration of the peptide toxin near the cell surface when expressed on cellular membranes. In our theory, the signal magnitude is not determined by the detailed interactions between the LOV2 domain and the coupled effector domain (here, a peptide toxin), as in past LOV-based optogenetic tools, but rather by the difference in length and rotational freedom of the J2 helix between lit and dark states, which directly influences the difference in local concentration near the cell surface. Both the apparent forward and backward rates of the zDTX-containing lumitoxins are similar to the unfolding and folding kinetics of the isolated AsLOV2 domain, hinting at the rate-limiting step in light switching for lumitoxins utilizing AsLOV2 as the photoswitch. Our models, protein ligand swaps, and mutagenesis data suggest that the lumitoxin architecture is sufficiently modular for AsLOV2 properties such as kinetics, helix bending, and response to light, making it a potentially important modular and tunable architecture in the field of engineered light-activated proteins. Note that, in this context, the high light sensitivity of lumitoxins (activated by as little as 10 µW/mm²) relative to ‘traditional’ optogenetic tools such as ChR2 (routinely driven by >1,000 µW/mm²) may enable lumitoxins to be incorporated into existing experimental contexts without requiring disturbance of other engineered signaling pathways.

Systematic studies of a variety of different protein ligands, together with other structural features of lumitoxins such as linker length, composition and choice of membrane anchor, are needed to expand the family of lumitoxin reagents and improve its functional characteristic. Lumitoxins capable of blocking channels in response to light, in addition to mediating their actuation, would also be particularly useful. In the future, the lumitoxin architecture, or future variants thereof, might be utilized to tether arbitrary genetically encoded payloads beyond Kv channel modulating protein ligands—neuropeptides, growth factors, signaling domains—close to membrane-associated ion channels and receptors without requiring extensive re-optimization of the protein architecture.

Methods

Modelling tethered-particle motion and lumitoxin stimulation. A system of two spheres, representing a LOV2 domain (2 nm diameter) and a peptide toxin (0.75 nm diameter), connected to each other and a solid wall via linkers with a variable number of elements (0.2 nm diameter) representing linker amino acids, was simulated in MATLAB. Each linker element (n) was connected to its neighboring linker elements (n+1 and n−1) with springs (spring constant 0.5 N m⁻¹ nm⁻²) at the distance of a peptide bond (0.4 nm). Pairs of second nearest neighbour elements were also connected to each other with a spring (spring constant 0.25 N m⁻¹ nm⁻²) in order to maintain the equilibrium bond angle between them at 109 degrees. Fluctuations of this system because of Brownian motion took the form of r = F_random + F_electrostatic + F_tether + F_molecular, and were simulated for 150 ns for several combinations of lengths of linkers.

Modelling of lumitoxin stimulation was performed using MATLAB scripts numerically integrating a set of ordinary differential equations describing the thermodynamic model depicted in Fig. 1c. Simulation parameters were nTOxin = nChannel = 0.009; reaction volume = 0.009; reaction time between 1 × 10⁻¹ to 1 × 10⁻³; (corresponding to a 13-μm (inner radius) hollow sphere with 15 nm wall thickness); z = 2 × 10⁸ M⁻¹ s⁻¹; z* = 6.6 × 10⁷ M⁻¹ s⁻¹; β = 2 × 10⁷ M⁻¹ s⁻¹; κ_m(lit) = 2.5 × 10⁻¹ M⁻¹ s⁻¹; κ_m(dark) = 1.6 × 10⁻² M⁻¹ s⁻¹; κ_m = 2.55 × 10⁻⁵ M⁻¹ s⁻¹. For theoretical activation ratios (Fig. 3b) z ranged from 3 × 10⁵ to 4 × 10⁶ M⁻¹ s⁻¹, z* ranged from 1.7 × 10⁶ to 1.4 × 10⁶ M⁻¹ s⁻¹, and β ranged from 2 × 10⁵ to 4 × 10⁶ M⁻¹ s⁻¹.

Molecular biology and construct generation. Genes encoding for fusion proteins were assembled as a multicompartment cloning cassette from annealed oligonucleotides (IDT DNA) containing these elements (in order): BglII or Nhel—Secretion Signal/FLAG tag—HindIII—(zDTX or DTX-K or CONK1)—KpnI—Linker—LOV2-J2 (404-546)—NcoI—PDGF-R-mCherry—XbaI. PDGF-R-mCherry was derived from pFU-MVIIA-PC (Addgene)10. See Table 1 for sequence details. This cassette was inserted into the mammalian expression vector pCDNA3.1+ (Invitrogen) using Nhel/XbaI restriction sites. The respective genes coding for rat Kv1.2 (Kv1.2), rat Kv1.1 (Kv1.1) or Shaker were amplified from Kv1.2-2.0 plasmid, Shaker-pBlueScript (gifts from Roderick Mackinnon), or BacMam Kv1.1 (Invitrogen) and inserted into pcDNA3.1+ (pEGFP-N3 using BamHII/EcoRI or Nhel/EcoRI, respectively. Both zDTX–lumitoxin and Kv1.2 cassettes were also inserted into the bidirectional expression vector pBli-CMV1 (Clontech) using BglII/ XbaI and BamHII/NcoI sites, respectively, to drive expression from the same plasmid.

Mammalian cell culture. PC12 cells (ATCC) were maintained in DMEM (Cellgro), 10% fetal bovine serum (Invitrogen), 5% horse serum (Invitrogen), 1% penicillin/streptomycin (Cellgro) and 1% sodium pyruvate (Biowhittaker). For electrophysiological recordings and imaging, cells were plated on glass coverslips treated with Matrigel (BD Bioscience). Adherent cells were transfected using Lipofectamine LTX (Invitrogen) following the manufacturer’s instructions and recorded 36–48 h later.

For confocal microscopy, cells were fixed with 4% formaldehyde, permeabilized with 0.4% saponin and either stained with anti-FLAG M2 peptide antibody (Sigma A2220, 1:1,000 dilution) followed by mouse-Alexa488 secondary antibody staining (Invitrogen) or directly observed using mCherry fluorescence.

Electrophysiology and illumination. K⁺ currents were recorded from PC12 cells 36–48 post-transfection using whole-cell voltage clamp. Analog signals were filtered (1–5 kHz) using the built-in 4-pole Bessel filter of an Axopatch 200B patch clamp amplifier (Molecular Devices), digitized at 10 kHz (Digitdata 1440 A, Molecular Devices) and stored on a computer hard disk. The bath solution contained the following (mM): 125 NaCl, 2 KCl, 3 CaCl₂, 1 MgCl₂, 10 HEPES, 30 glucose, adjusted to pH 7.3 with NaOH. Electrodes were drawn from borosilicate patch glass (Warner Instruments) to a resistance of 5–10 MΩ.

Patches with access resistance of >50 MΩ were discarded from the data analysis. The reported n refers to the number of patched cells.

Cells were screened for mCherry expression using a 565-nm high-power LED (Thorlabs) filtered by a 560 ± 40 nm bandpass filter (Semrock) through a 40 × lens. Lumitoxins were stained with a 455-nm high-power LED (Thorlabs).

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Author contributions
D.S. and E.S.B. designed the experiments and wrote the manuscript. D.S. performed the experiments. F.C. and P.W.T. implemented the tethered particle simulation.

Additional information
Accession codes: Construct sequences have been deposited in the DDBJ/EMBL/GenBank nucleotide database under accession codes KF878105 (aDTX-Lumitoxin), KF878106 (CONK1-Lumitoxin) and KF878110 (DTX-K-Lumitoxin) and are available on request at http://syntheticneurobiology.org/protocols.

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