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From the Murky Depths to the Brain: A Tale of a Glowing Protein That Became the Core of a Seizure-Suppressing Molecular Machinery

[1]

A pH-Sensitive Closed-Loop Nanomachine to Control Hyperexcitability at the Single Neuron Level

Merolla A, Michetti C, Moschetta M, Vacca F, Ciano L, Emionite L, Astigiano S, Romei A, Horenkamp S, Berglund K, Gross RE, Cesca F, Colombo E, Benfenati F. A pH-sensitive closed-loop nanomachine to control hyperexcitability at the single neuron level. *Nat Commun*. 2024;15(1):5609.

Epilepsy affects 1% of the general population and 30% of patients are resistant to antiepileptic drugs. Although optogenetics is an efficient antiepileptic strategy, the difficulty of illuminating deep brain areas poses translational challenges. Thus, the search for alternative light sources is strongly needed. Here, we develop pH-sensitive inhibitory luminopsin (pHIL), a closed-loop chemo-optogenetic nanomachine composed of a luciferase-based light generator, a fluorescent sensor of intracellular pH (E2GFP), and an optogenetic actuator (halorhodopsin) for silencing neuronal activity. Stimulated by coelenterazine, pHIL experiences bioluminescence resonance energy transfer between luciferase and E2GFP which, under conditions of acidic pH, activates halorhodopsin. In primary neurons, pHIL senses the intracellular pH drop associated with hyperactivity and optogenetically aborts paroxysmal activity elicited by the administration of convulsants. The expression of pHIL in hippocampal pyramidal neurons is effective in decreasing the duration and increasing latency of pilocarpine-induced tonic-clonic seizures upon in vivo coelenterazine administration, without affecting higher brain functions. The same treatment is effective in markedly decreasing seizure manifestations in a murine model of genetic epilepsy. The results indicate that pHIL represents a potentially promising closed-loop chemo-optogenetic strategy to treat drug-refractory epilepsy.

Commentary

In the summer of 1961, Osamu Shimomura and his colleague Frank Johnson gathered jellyfish (*Aequorea victoria*) in Friday Harbor, WA, to find what made them glow. Incidentally, Shimomura realized that calcium ions in seawater in the sink caused a chemical reaction of the jellyfish's protein he and Johnson named aequorin, leading to the emission of blue luminescence. Subsequently, Shimomura and his colleagues found that the blue light emitted from aequorin matches the excitation wavelength of another protein of the jellyfish, the green fluorescent protein (GFP). Upon excitation, GFP emits the energy as a light in the green wavelength.[1] This naturally occurring phenomenon is now known as bioluminescence resonance energy transfer (BRET).

Fast-forward to the nineties, Martin Chalfie inserted the GFP gene next to the gene of the touch receptors in the transparent roundworm *Caenorhabditis elegans* and made the six touch receptor neurons of the worm glow green in UV light. Later on, Roger Tsien developed new GFP variants to extend the palette with more colors and enhance the emission intensity. In 2008, Shimomura, Chalfie, and Tsien were awarded the Nobel Prize in Chemistry for their discoveries. GFP proteins have become key tools for multiple applications, from tracking neuronal networks to sensing arsenic.[1] The light emitted from GFP was later harnessed for exciting a second fluorophore in fusion proteins by fluorescence resonance energy transfer (FRET).

Acid-Sensing Molecular Lamps for Seizure Suppression

The BRET and FRET of GFP are the basis for the closed-loop seizure-suppressing machinery described by Merolla et al.[2] The core of this machinery is an engineered triple-chimeric protein that translates intracellular acidosis in hyperactive neurons to silencing of neuronal activity. The protein consists of (1) a light generator—a super Renilla luciferase (RLuc8) which uses the systemically administered coelenterazine 400a to generate bioluminescence (in analogy with aequorin); (2) a GFP variant that is excited by coelenterazine via BRET, and the emission intensity increases as the pH drops; (3) the actuator —an enhanced form of halorhodopsin that translates the light energy (transmitted from the GFP variant via FRET) to activation of chloride-mediated hyperpolarizing current.

Following extensive calibration and testing in vitro, the investigators selectively expressed the triple-construct protein in excitatory neurons of the hippocampus in mice using a viral vector injected stereotactically. When coelenterazine was intravenously injected into the mice, luminescence could be detected in the hippocampal formation in both brain hemispheres without affecting hippocampal-dependent behaviors. The system performance in

seizure suppression was tested on seizures acutely evoked with pilocarpine and in a genetic epilepsy model (*PRRT2* knockout). In the pilocarpine-treated mice, the appearance of severe seizure behaviors was delayed and their severity was reduced by one-third as compared to control mice, expressing a fusion protein without the actuator. Mice treated with the closed-loop machinery were also less likely than controls to develop tonic-clonic seizures. In the genetic model, coelenterazine administration increased the latency to paroxysmal manifestations and considerably inhibited sound-evoked convulsive seizures.

The main novelty of the featured system is in eliminating the need for external devices, because the light generator is internal. More severe seizures trigger more pronounced shifts in pH which in turn lead to more efficient silencing of excitatory neuronal activity. The effect is selective to hyperactive pathological neurons. The background activity of the system is minimal and the actuator effect is almost independent of the chloride gradient. An advantage of the fusion protein over genetic therapies is the reversibility of its effects. In contrast to other modalities such as chemogenetics with inhibitory DREADDS, it does not require continuous monitoring to administer treatment on-demand.

Smaller Things

The envisioned translational product will likely consist of two components: the fusion luciferase-GFP-halorhodopsin protein, whose gene will be delivered to excitatory neurons, and the small-molecule luciferase substrate. The authors termed the fusion protein a "nanomachine," based on the broader definition of nanomaterials encompassing any nanoscale compound, including engineered proteins (although most nanomaterial definitions relate to particulate matters).[3] Another nano-dimension of this project could be the use of lipid nanoparticles (instead of a viral vector) for the delivery of the genetic payload into neurons.[4] The feasibility of encapsulating mRNA in lipid nanoparticles has already been demonstrated with the COVID-19 vaccines.

The concept of gene therapy is shared with two other recently described closed-loop sensors that detect excessive glutamate[5] or neuronal activity.[6] A drawback common to all three systems is potential immune reactions against the protein product. Another aspect, unique to the featured system, is the need for an external compound (the luciferase substrate) to be systemically and chronically administered, similar to most antiseizure medications. As the authors stated, this would require optimization of the administration route and pharmacology of coelenterazine. Coelenterazine is likely to be delivered by frequent dosing or a controlled-release formulation as whole-body imaging demonstrated a narrow time window for light generation. One additional challenge could be the ability of this compound to cross the blood-brain barrier (BBB). The molecular weight of coelenterazine 400a is 392 g/mol, which is larger than any currently approved antiseizure medication. Coelenterazine is an excellent example of a molecule that might be tested in the zebrafish model before clinical development, given the transparency of zebrafish and the value of this model in predicting the outcomes of clinical testing.[7]

Concluding Remarks

Despite the complexity of the featured molecular machinery, it offers a novel approach for "on-demand" seizure suppression. Similar to existing therapies that seemed distant visions only a decade ago, this jellyfish-derived system might lead to a novel means for treating epilepsy, probably with some modifications, and an exciting preclinical tool.

An "unsung hero" of this story is Prof Yashimasa Hirata at Nagoya University, who realized that isolating the glowing molluscan materials is a nearly impossible task and did not want to give it to a student who needed to demonstrate success to obtain a PhD thesis. Shimamura was then Hirata's assistant and was not in a rush. Later on, when Shimomura was recruited to Princeton, Hirata made sure that Shimomura was awarded a PhD from Nagoya University for his work.[1] This provides another lesson from the GFP story—be kind to your students, fellows, and employees, who might be awarded a Nobel Prize one day.

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