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Developmentally-Relevant Optogenetic Control of Synaptic Pruning via Light-Cleavable Hevin

Introduction
During the early stages of development, the brain over-creates synaptic connections between neurons to promote learning. As learning progresses, excess synapses are pruned through molecularly precise coordination of supporting cells (microglia and astrocytes) in the brain. Currently, this process can only be observed – no tool exists to engage this machinery on demand to granularly examine the effects of specific synapse degradation on brain development.

A recent discovery by Ramirez et al. elucidates how the SPARCL1 protein (Hevin) is both highly involved in synapse-pruning and uniquely bifunctional. Its intact form assembles synapses in the thalamocortical system, while its proteolytically-liberated C-terminal fragment signals thalamocortical synapse disassembly via the TLR4 receptor. Hevin cleavage is triggered by the ADAMTS4 protease, with no toolkit for precise control. We propose replacing Hevin's proteolytic switch with a photonic one – engineering a violet light-cleavable Hevin triggering C-terminal fragment cleavage on demand, placing this pruning pathway under optical control.

Goal
We hypothesize that replacing the ADAMTS4 cleavage site in Hevin with a photocleavable domain will enable light-dependent cleavage of the TLR4-binding C-terminal fragment, providing on-demand control over synapse elimination. This will enable a variety of studies on synaptic development and dynamics in *in vivo* systems.

Specific Aim 1: *Engineer and test a light-cleavable Hevin variant via rational design.*
We will insert the photocleavable protein PhoCl2f (Lu et al., 2021) at the ADAMTS4 cleavage site between Hevin's synaptogenic domain and its C-terminal fragment. For examination of production and construct cleavage, we will express constructs in HEK293. Western blot will be used to evaluate both protein yield and light-induced cleavage. Binding in a synaptogenic context will be evaluated via SPR to ensure the engineered protein retains native function.

Specific Aim 2: *Enhance signaling via combinatorial engineering of TLR4 binding affinity.*
Since PhoCl2f cleavage is only 88% efficient (Lu et al., 2021), enhancing cleaved protein affinity to TLR4 would ensure more can reach its target. A lack of binding information suggests a combinatorial approach. Site-saturation mutagenesis will mutate 15-20 TLR4-binding residues that are not required for synaptogenic function. Yeast display will be used for screening. Production, construct cleavage, and synaptogenic function retention will be evaluated as above.

Specific Aim 3: *Test the optimized, light-cleavable Hevin construct* in vivo.
The optimized construct will be packaged into an adeno-associated virus (AAV) for gene delivery in mice via injection. Unilluminated mice will be examined for synapse density to confirm non-spontaneous construct cleavage. Injected mice will receive illumination for different durations for synapse density analysis versus controls. Additionally, microglial and astrocyte upregulation will be analyzed for anomalies. Spatial penetration of cleaved Hevin will be analyzed by illuminating defined cortical regions.

Innovation, Outcomes, & Impact
This project produces the first tool to optically control synapse-pruning, and the first engineered Hevin with enhanced signaling. Long-term, this enables mechanistic study of neurodevelopment.

Background & Significance

Currently, neuroscientists understand some of the broader processes that define how the human brain develops – neurogenesis, migration, and differentiation. However, more granular and mechanistic studies are required to precisely understand the molecular events that define these processes. The work proposed here addresses one such gap: the molecular pathway through which astrocytes coordinate thalamocortical synapse elimination during the postnatal critical period. This process is directly relevant to not only understanding the process of development, but to many neurodevelopmental disorders including autism spectrum disorder (ASD) and schizophrenia.

Neurons are the primary signal carriers of the nervous system. They communicate via synapses, specialized junctions where neurotransmitters are transferred from the axon (a signaling branch extending from a neuron) of one pre-synaptic neuron to another post-synaptic neuron. The formation of a functional synapse requires precise molecular alignment of presynaptic machinery with postsynaptic receptor scaffolds, a process orchestrated by cell adhesion molecules including neurexins (presynaptic) and neuroligins (postsynaptic).

The thalamocortical system is a primary sensory relay circuit through which sensory information is transmitted to the cerebral cortex. The thalamus, a bilateral structure at the center of the brain, receives input from all major human sensory inputs and connects to corresponding areas of the cortex via thalamocortical axons. These axons form synapses on cortical neurons, establishing the first cortical processing stage for sensory signals. Proper development of this circuit is essential for sensory function. Sensory activity during a defined postnatal "critical period" is required to refine the initial connectivity established during embryonic development into the precise maps that characterize the adult cortex (Crair and Malenka, 1995).

The postnatal critical period is defined by a phase of rapid synaptogenesis followed by a phase of synapse elimination (pruning). The developing brain initially overproduces synaptic connections to maximizes the probability that appropriate connections will form, and subsequently eliminates weaker, less active synapses in an activity-dependent manner (Nelson et al., 2025). This pruning process refines neural circuits from broad and diffuse to sparse and optimized, and is distinct from homeostatic synaptic scaling – it is a developmental refinement mechanism that permanently eliminates defined synaptic populations rather than adjusting synaptic strength. In the mouse visual cortex, this period spans approximately postnatal days 1–21 and involves the coordinated activity of both neurons and non-neuronal glial cells. Disruption of normal synaptic pruning has been strongly implicated in neurodevelopmental and psychiatric disorders: insufficient pruning results in the excess synaptic density associated with ASD, while excessive or mistimed pruning has been linked to the cortical thinning and cognitive deficits observed in schizophrenia (Sekar et al., 2016; Nishi et al., 2025).

Two major glial cell types, astrocytes and microglia, collaborate to coordinate synapse formation and elimination during this critical period. Microglia are the brain's immune cells and have been recognized as the primary destroyers of synaptic material via phagocytosis (Stephan et al., 2012). Astrocytes are star-shaped cells that contact both neurons and synapses, positioning them to regulate synaptic chemistry and connectivity. Both cell types have been implicated in the same developmental processes - including synaptogenesis, synaptic pruning, and circuit maturation -

but how they communicate to coordinate these opposing activities had been unknown until recently (Verkhratsky and Nedergaard, 2018).

Hevin (gene name Sparcl1, also known as SPARC-like 1 and SC1) is an astrocyte-secreted matricellular glycoprotein of the SPARC family. Broadly, SPARC-family proteins contribute to extracellular matrix development and activity, growth factor activity, and cell adhesion (Bradshaw, 2012). Hevin was first identified as a positive regulator of excitatory synaptogenesis: astrocyte-derived Hevin drives the formation of structurally intact but functionally immature synapses, while its close relative SPARC antagonizes this effect (Kucukdereli et al., 2011). Mechanistically, Hevin bridges two otherwise incompatible trans-synaptic partners: presynaptic neurexin-1a (Nrx1a), and postsynaptic neuroligin-1B (NL1B). This bridging physically assembles thalamocortical glutamatergic synapses during the critical period of the mouse visual cortex (Singh et al., 2016). Hevin-null mice have fewer thalamocortical synapses and exhibit deficits in ocular plasticity, while SPARC-null mice show the opposite (Kucukdereli et al., 2011).

A recent study by Ramirez et al. (2026) reveals that Hevin is bifunctional: its intact form assembles synapses as described, but when proteolytically cleaved at amino acid 350 by the extracellular protease ADAMTS4, the liberated C-terminal fragment no longer possesses synaptogenic activity. Instead, this fragment signals to microglia through Toll-like receptor 4 (TLR4), inducing a pro-phagocytic microglial state characterized by enhanced thalamocortical synapse engulfment. Microglial TLR4 signaling is required for proper synapse elimination: mice in which TLR4 signaling is disrupted showed impaired thalamocortical synapse refinement during the critical period. This means that through controlled cleavage of a single secreted protein, astrocytes can sequentially drive both the assembly and the elimination of the same synaptic population - an interesting molecular strategy for coordinating circuit refinement. Hevin therefore occupies an unusual position as a single secreted protein capable of coordinating both sides of synaptic refinement – assembly in its intact form, and elimination once cleaved.

Innovation

Our project is innovative in two ways:

1. First optogenetic tool for on-demand control of the Hevin synapse-pruning mechanism. No tool currently exists to engage this mechanism with spatial and temporal precision. Existing studies of this pathway rely on constitutive genetic perturbations (knockout mice, full-length vs. truncated Hevin variants) that cannot resolve how timing or spatial extent affect growth. Replacing the ADAMTS4 cleavage site with PhoCl2f generates the first tool capable of triggering C-terminal fragment release at user-defined times and locations within an otherwise wild-type specimen.
2. First engineered variant of the Hevin C-terminal fragment with enhanced TLR4 binding affinity. The native Hevin C-terminal fragment signals microglia through TLR4, but its diffusion through the extracellular space and the incomplete photocleavage efficiency of PhoCl2f impose limits on signaling yield. No prior work has attempted to engineer the TLR4-binding activity of this fragment. This project generates the first affinity-matured Hevin C-terminal fragment variants, which may prove resourceful beyond this study for future studies on TLR4 signaling.

Prior Work

Prior approaches to characterizing synaptic pruning mechanisms have relied predominantly on gene knockouts. Studies using complement-deficient mice (C1q, C3, or C4 knockouts) established that microglia use complement-tagged synapses as phagocytic targets, but these manipulations are chronic and affect all complement-dependent processes throughout development. The Ramirez et al. (2026) study itself required generation of Hevin-null mice, TLR4 knockouts, and multiple AAV-delivered variants to figure out the synaptogenic and pro-pruning arms of Hevin function. While these approaches established the existence and necessity of the pathway, they cannot resolve questions requiring acute or spatial control. For example, these toolkits cannot answer whether initiating synapse pruning earlier or later in the critical period alters outcomes, or whether spatially restricted pruning in one cortical area affects neighboring areas. The lack of control on this pathway is the unmet need this project addresses.

The photocleavable protein PhoCl was originally engineered from the fluorescent protein mMaple (Zhang et al., 2017). Upon illumination with ~400 nm violet light, the PhoCl chromophore undergoes a reaction that breaks the protein backbone, producing a large N-terminal empty barrel fragment and a small C-terminal peptide fragment. Because the cleavage breaks a covalent bond, it is irreversible. This distinguished PhoCl from reversible optogenetic systems which return to baseline when illumination stops. However, PhoCl1 had a dissociation half-time of ~500 seconds, making it barely useful in actual contexts. Lu et al. (2021) addressed this engineering two improved variants: PhoCl2c, with increased cleavage efficiency, and PhoCl2f, with significantly faster dissociation kinetics.

PhoCl and its derivatives have been inserted into numerous proteins as photocleavable linkers. The original PhoCl1 paper demonstrated photocleavable Cre recombinase, Gal4 transcription factor, and viral protease activity (Zhang et al., 2017). Following work used PhoCl to enable single-molecule optogenetic release of membrane-anchored proteins (Kashyap et al., 2024). PhoCl has additionally been deployed in zebrafish embryos for *in vivo* optogenetic protein cleavage (Brown et al., 2022). Across these applications, PhoCl acts as a modular linker given that the insertion site is between domains.

Yeast surface display (YSD) has been used in the past to generate extracellular binding domains of eukaryotic proteins, as the secretory pathway of yeast supports disulfide bond formation and glycosylation. The Hevin C-terminal fragment is an extracellular domain; as such, Yeast Display is the most advantageous method to use for this project. Iterative rounds of yeast display have achieved picomolar affinity variants starting from micromolar leads in the past (Hackel et al., 2010). These precedents provide confidence that four rounds of YSD with decreasing TLR4 concentrations will be sufficient to enrich variants with meaningfully improved TLR4 binding relative to the wild-type C-terminal fragment.

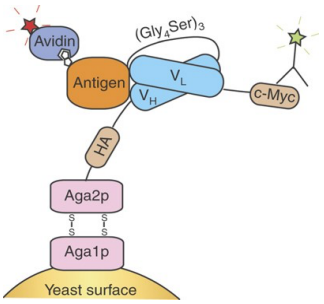

Figure 1. The yeast display process. Protein variants are expressed as fusions to the yeast surface protein Aga2p, enabling direct coupling of genotype to phenotype. Iterative rounds of fluorophore-labeled ligand incubation and FACS enrichment select for variants with improved binding affinity. Figure taken from Chao et al., 2006.

Approach

Aim 1: *Engineer and test a light-cleavable Hevin variant via rational design.*

As discussed above, the Hevin protein is currently cleaved via the ADAMTS4 protease. Currently, there is no mechanism that allows for on-demand engagement of this proteolytic activity. The Ramirez et al. study that elucidated the function of Hevin required the generation of multiple engineered mouse variants to investigate the overall function of the protein, which is time-consuming and can often require multiple tries to get right. Additionally, the study was only able to analyze how many synapses were present at the beginning and end of development compared to wild-type, and not additional effects such as spatially-specific synapse degradation or initiating synapse degradation at various periods of the developmental cycle.

Engineering the ADAMTS4 protease itself to spatially cluster and cleave the Hevin protein would be quite a challenge, as the mechanisms for co-location of ADAMTS4 and Hevin are still not fully understood. A simpler approach would be to engineer the Hevin protein itself for cleavage. As optogenetic tools are already widely-deployed in *in vivo* neurological studies, we settled upon engineering photocleavability into Hevin.

As there already exists an optimized photocleavable domain, PhoCl2f (Lu et al., 2021), work will instead focus on rationally engineering this domain into the Hevin protein. Prior work by Ramirez et al. and Singh et al. have identified that ADAMTS4 cleaves Hevin at a.a. 350, but a.a.s 330-550 (highlighted in Figure 2) form the domain required for synaptogenic function. Therefore, we will insert PhoCl2 into Hevin just before a.a. 330. We hypothesize that linkers will not be required as this region of the Hevin protein is largely disordered and flexible but will generate variants with 1-5 repeats of a GGS linker for redundancy.

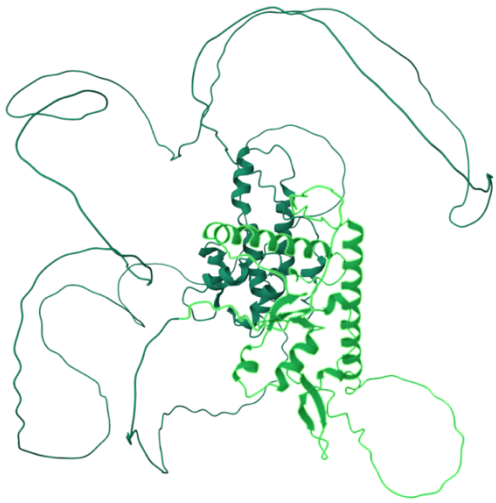

Figure 2. Alphafold structure of the Hevin protein with synaptogenic domain highlighted in bright green. Note the insertion site falls within a disordered region, minimizing structure disruption from PhoCl2f integration. Sequence originated from UniProt.

DNA inserts of each variant with an N-terminal FLAG-tag will be cloned into HEK293 expression vectors to confirm that they do not adversely impact protein production, can cleave when exposed to 405nm light, and retain their synaptogenic function. Standard protein purification protocols will be used to isolate Hevin; Western blot will be used to confirm the molecular weight lines up with expected results. A separate batch of purified protein will be expressed to 405nm light before running on a gel to observe whether two bands are visible, indicating cleavage. Finally, surface plasmon resonance will be utilized to ensure synaptogenic function remains – specifically by measuring binding between constructs and neurexin-1a / neuroligin-1, as occurs naturally. Wild-type Hevin will be used as a control in all experiments.

Aim 2: *Enhance signaling via combinatorial engineering of TLR4 binding affinity.*

While engineering PhoCl2 into the Hevin protein enables light-mediated cleavage of the C-terminal fragment, PhoCl2 itself only has 88% cleavage efficiency (Lu et al., 2021). Therefore, increasing the signaling effectiveness of the C-terminal fragment itself would ensure that whatever fragment is cleaved has the desired effect of initiating synaptic degradation. Since diffusion of the C-terminal fragment into the extracellular space limits the signaling effectiveness even in the wild-type, we reason that increasing the binding affinity of the C-terminal fragment to the TLR4 signaling receptor it activates would increase the signaling effectiveness.

No definitive structure for the Hevin C-terminal fragment / TLR4 complex has been determined in literature. Additionally, the binding interface of the Hevin C-terminal fragment has not been properly characterized. As such, a combinatorial approach is required to identify mutations that have a beneficial impact on C-terminal binding affinity.

Broadly, a site-saturation mutagenesis strategy will be employed to find favorable mutations. To identify which codons within the C-terminal fragment are most favorable for mutation, a filtering approach will be taken. Any codon within the synaptogenic region (highlighted in Figure 2) will be excluded to maintain synaptogenic function, which is crucial for *in vivo* studies. Next, the remaining residues will be analyzed for solvent exposure, with the top 15-20 being selected for saturation mutagenesis. NNK codons will be used to induce mutagenic saturation at the desired codons.

Figure 3. Sequence of the Hevin protein, with the synaptogenic domain highlighted in red. This domain spans residues 330–550 and is excluded from site-saturation mutagenesis to preserve function. Solvent-exposed residues outside this region are sites for combinatorial engineering. Sequence acquired from UniProt.

To select for mutants with higher binding affinity, yeast display will be utilized. Additional benefits of yeast display include preservation of the genotype-phenotype link for easier variant recovery, and compatibility with fluorescence-activated cell sorting (FACS).

The PhoCl2-Hevin protein will be expressed into a yeast display vector and mutated via SSM as discussed above. Fluorophore-conjugated TLR4 will either be generated via conjugation or purchased from a supplier. Four rounds of yeast display will be conducted. Decreasing concentrations of TLR4 will be applied to the yeast display pool, with binding variants sorted via FACS. At the end, individual variants will be screened against each other via serial dilution ELISA to determine best variant by EC50; kinetic screening will be used as a secondary filter for variants within 2-fold of the top EC50. Individual variants will also be screened to confirm protein production viability, light-activated cleavage, and synaptogenic function as in Aim 1.

Expected results: We expect to isolate C-terminal fragment variants with improved TLR4 binding EC50 relative to wild-type, carrying mutations at solvent-exposed residues outside the synaptogenic domain. *Potential challenge*: If commercially available fluorophore-conjugated TLR4 is unavailable for FACS, we will produce it recombinantly in HEK293 cells.

Aim 3: *Test the optimized, light-cleavable Hevin construct* in vivo.

While engineering the PhoCl2-Hevin variant *in vitro* allows for potential benefits for mouse developmental studies, final examination of the best-performing variants *in vivo* is required to examine that all necessary components function as desired. Expression of the variant, retention of synaptogenic function, non-spontaneous cleavage, and changes in synaptic density are all important properties to confirm.

Using the method outlined and validated in Ramirez et al., an adeno-associated virus vector (AAV) will be generated to deliver the enhanced-binding PhoCl2-Hevin variant *in vivo*. Mice will then be injected with the construct to generate an experimental pool. From this pool, mice will be sacrificed for brain tissue harvesting, with expression confirmed via anti-Hevin staining.

After variant expression is confirmed, synaptogenic function and non-spontaneous cleavage will also be confirmed. Mice expressing the variant and wild-type mice will be sacrificed for brain tissue harvesting at 15 days post-natal (P15). Thalamocortical synapse abundance will be examined via staining and fluorescence (or light) microscopy. We hypothesize that if the variant preserves synaptogenic function and does not cleave non-specifically, there should be no statistically significant difference in the amount of thalamocortical synapses observed. If a statistically significant difference is observed, additional studies with variants that remove the PhoCl2 domain and with the wild-type C-terminal binding structure will be conducted to determine which mutation caused the loss of function.

Once function preservation and non-specific cleavage are confirmed, additional experiments to confirm the effect of light-specific cleavage on synaptic populations. Wild-type mice and variant mice will be exposed to varying pulses of 405nm light from P1-P5, with mice being sacrificed on P15 for brain tissue harvesting. A statistically significant difference in the amount of thalamocortical synapses in the variant population would indicate successful engineering of photo-activated synapse cleavage.

Finally, additional studies on the spatial efficacy of the photoactivation would be conducted. Variant mice would be exposed to varying intensities of 405nm light in various locations from P1-P5. The mice would be sacrificed on P15 for brain tissue harvesting. Fluorescence microscopy would be used to examine thalamocortical synapse degradation within and outside the target location, enabling study of how target-specific the synapse degradation mechanism is.

Expected results: Illuminated variant mice are expected to show statistically significant reduction in thalamocortical synapse density relative to unilluminated controls, with spatially restricted illumination producing localized pruning. *Potential challenge*: Since 405 nm light does not penetrate deep tissue effectively, illumination will be delivered via fiber optic cannula, consistent with standard in vivo optogenetic delivery.

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