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Perspective

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Understanding neural circuit function through synaptic engineering

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Abstract

Synapses are a key component of neural circuits, facilitating rapid and specific signalling between neurons. Synaptic engineering — the synthetic insertion of new synaptic connections into in vivo neural circuits — is an emerging approach for neural circuit interrogation. This approach is especially powerful for establishing causality in neural circuit structure–function relationships, for emulating synaptic plasticity and for exploring novel patterns of circuit connectivity. Contrary to other approaches for neural circuit manipulation, synaptic engineering targets specific connections between neurons and functions autonomously with no user-controlled external activation. Synaptic engineering has been successfully implemented in several systems and in different forms, including electrical synapses constructed from ectopically expressed connexin gap junction proteins, synthetic optical synapses composed of presynaptic photon-emitting luciferase coupled with postsynaptic light-gated channels, and artificial neuropeptide signalling pathways. This Perspective describes these different methods and how they have been applied, and examines how the field may advance.

Sections

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Introduction

Synapses have an essential role in neural circuit function, enabling selective and dynamic signalling between presynaptic and postsynaptic neurons. Synaptic transmission is initiated by a prominent shift in the membrane potential of a presynaptic neuron, which translates into a transient membrane potential change or modulation in the postsynaptic partner (Fig. 1a). In chemical synapses, a large depolarization of the presynaptic neuron induces Ca^{2+}-dependent release of neurotransmitter from clear small vesicles; the neurostransmitter binds to adjacent receptors of the postsynaptic neuron, leading to ion channel opening and a postsynaptic potential[1]. In electrical synapses, gap junctions linking partner neurons enable ion flow between these neurons, propagating membrane potential fluctuations between them[2]. In neuropeptide-based transmission, the presynaptic neuron releases specific peptides from dense core vesicles in an activity-dependent manner, which spread by diffusion until they bind to particular postsynaptic receptors in multiple distant target neurons, modulating their electrical properties or synaptic strength[3] (Fig. 1a).

Several approaches have been developed for probing the logic and operation of neural circuits. The majority of these techniques consist of altering neuronal activity or synaptic transmission of entire cells. For example, pharmacological or genetic methods can be used to dampen or excite the membrane potential, block or enhance certain synaptic receptors, or alter neurotransmitter release. Once the neurotransmitter receptors of a certain neuron are deactivated, for example, all synaptic inputs that share that receptor type are eliminated. Such treatments, therefore, are aimed at neuronal manipulation rather than targeting specific synapses between two particular neurons[4] (Fig. 1b). Chemogenetic[5] and optogenetic[6] methods have dramatically expanded the possibilities for neuronal manipulation by adding precise spatiotemporal control. This allows highly specific targeting of neurons and their extremely rapid and brief activation or silencing. These methods also make it possible to elicit artificial neuronal activation and transmission patterns within neural circuits and to trigger individual synapses, by illuminating, for example, single spines[7]. When combined with neuronal monitoring, such as calcium or voltage imaging, the external control of neuronal activity could be used to emulate neuronal signalling and could even provide closed-loop feedback control[8] (Fig. 1c). Obviously, to control every single neuronal event requires complicated hardware, varyingly invasive external intervention and high illumination intensities, with possible side effects[9].

Synaptic engineering takes a different approach. Rather than controlling neuronal activity, synaptic engineering targets connectivity, building new synaptic connections between specific neurons and thereby synthetically altering the dynamics and flow of internally generated neuronal activity. This is performed by ectopic genetic expression of distinct synaptic components in the presynaptic and postsynaptic neurons. The requirement for both presynaptic and postsynaptic transgene insertions restricts the manipulation to single synaptic connections (Fig. 1b). Moreover, synaptic engineering enables fully autonomous function, with no need for real-time external monitoring or triggering, and no reliance on external software or hardware for implementing the new synaptic connection (Fig. 1c).

Thus, synaptic engineering makes it possible to modify existing paths of information flow or to construct new ones altogether, providing valuable insights into the sufficiency of certain patterns of connectivity for establishing particular circuit dynamics and behaviours. Although synaptic engineering is a relatively recent approach, several methods have already been successfully developed, validated and applied, with many of these being implemented in the nematode *Caenorhabditis elegans*. Increasingly, these methods are being deployed in mammalian systems. In this Perspective, we review existing methods for synaptic engineering and highlight the challenges and promises of their implementations. We compare current approaches and lay out potential near future directions for advancing the field.

Current strategies

In principle, synaptic engineering could be accomplished by intervening in the natural processes underlying the formation of synaptic connections. Synaptic specification and circuit architecture emerge during development and are later refined throughout the lifetime of the organism via complex interactions between genetic[10], neuronal[11] and biomechanical factors[12], in addition to stochastic processes of pattern formation[13]. In order to reprogram synaptogenesis, several major hurdles must be overcome: (1) for many, synapses in the instructive signals guiding synaptogenesis remain unknown; (2) even known signals tend to be redundant, making in vivo loss-of-function strategies insufficient to rewire circuits; and (3) some molecules and processes that drive synaptogenesis — such as morphogens, adhesion molecules, neurotransmitter synthesis and signalling receptors — affect multiple developmental events, so that their manipulation could lead to additional, unpredictable outcomes beyond altered synapse formation[14].

To bypass these challenges, current strategies for synaptic engineering are based on directly introducing new synaptic components into existing target neural circuits (in parallel to native synaptic connections), modifying in this way existing connectivity, and establishing new connections. To minimize undesired interactions with endogenous components of the system and to maximize experimental control, synthetic synapses make use of heterologous parts, consisting of natural or engineered exogenous proteins that are not normally produced by the host organism. These include natural[15] and engineered[16] variants of vertebrate gap junction connexin proteins, for inserting new electrical synapses between target neurons[4]; luciferase in conjunction with channelrhodopsin (ChR), for constructing novel synthetic optical synapses[17,18]; and orthogonal neuropeptide and cognate receptor pairs, for establishing new long-range signalling pathways between neurons[19,20].

Engineered electrical synapses

Gap junctions are intercellular channels that enable the passage of ions and small molecules between cells[21]. In the nervous system, gap junctions serve as electrical synapses that couple the activity of neurons, providing an effective means of signalling between them. Although gap junctions exist in many species, their molecular constituents differ between vertebrates and invertebrates[22]. Vertebrate gap junctions are primarily composed of proteins from the connexin family, and invertebrate gap junctions are formed by innexins. Biochemical and in vivo studies suggest that connexins and innexins do not cross-interact[23]. This provides an opportunity for establishing independent synthetic electrical connections without unintended crosstalk with endogenous gap junction components (Fig. 2a).

Mouse connexin 36 (Cx36), to our knowledge, was the first connexin to be successfully expressed in invertebrate *C. elegans* neurons (through the use of cell-specific promoters) and to form new electrical synapses between particular target neurons[15]. Through the regulation of their specific expression in the context of the known connectome,

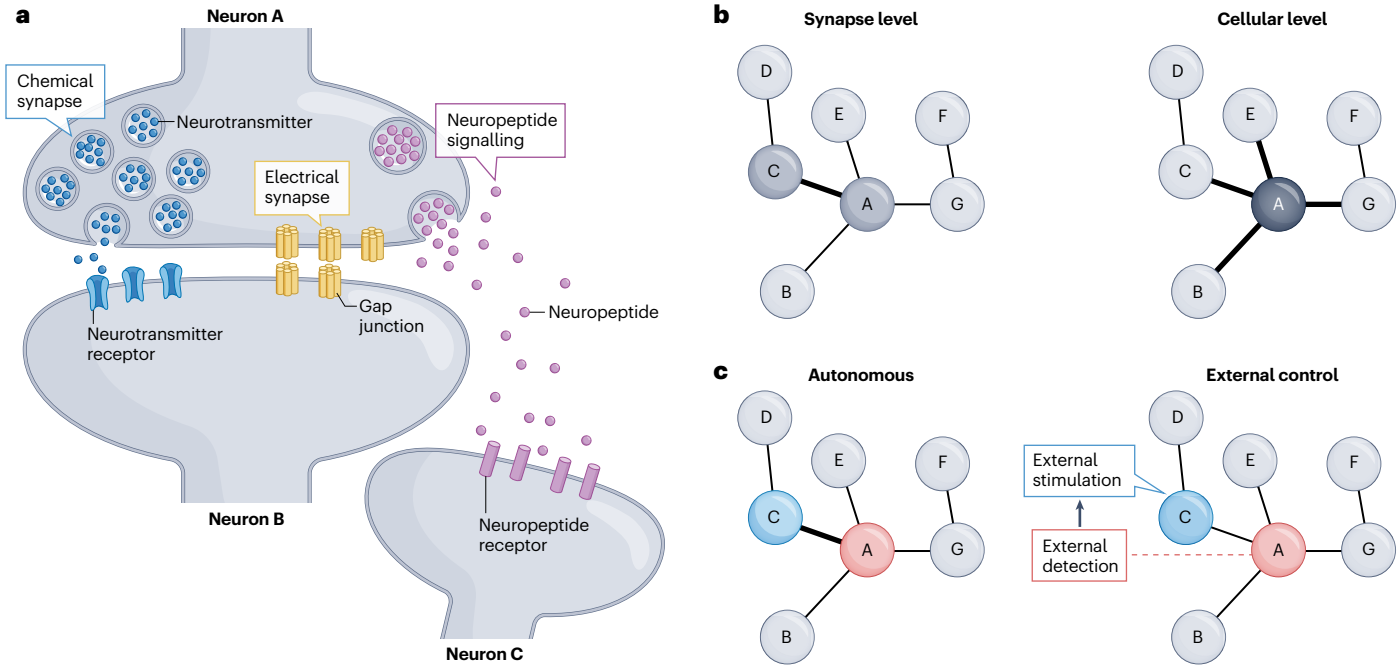

Fig. 1 | Distinct features of synaptic engineering. a, Three forms of synaptic connectivity: chemical synapses, electrical synapses and neuropeptide signalling. **b**, Synaptic engineering (left) targets the link between two specific neurons (for example, the connection between neurons A and C, denoted by the thick line) through expression of compatible synaptic genetic components in the two neurons (darkened neurons A and C). By contrast, cellular-level manipulations of activity or synaptic machinery (right), implemented by neuron-specific transgene expression (darkened neuron A), affect all inputs or outputs of the target neuron (thickened connections between neuron A and its synaptic partners, neurons B, C, E and G). **c**, Synaptic engineering confers on the neural circuit autonomous functional modification. For example, by inserting a new connection between neurons A and C, natural activation of neuron A (red) intrinsically leads to activation of neuron C (cyan). By contrast, neuronal manipulation techniques require external intervention, for instance, external activation of neuron C upon detection of natural activation of neuron A.

such engineered electrical synapses can be rationally designed to 'rewire' neuronal connections, including the creation of de novo electrical coupling for empirically testing how information flows through circuits and adjusts behaviours in specific target neuronal connections. For example, in *C. elegans*, the chemosensory neuron AWC normally inhibits interneuron AIY via chemical synapses (Fig. 2b). Consequently, the excitation of AWC results in avoidance behaviour owing to AIY inhibition[24]. Inserting an engineered electrical synapse between AWC and AIY (Fig. 2b) resulted in the inversion of inhibitory AWC–AIY transmission to excitation, sufficient to switch the behavioural response to AWC-sensed odours from attraction to aversion[15].

Since its first introduction[15], connexin-based synaptic engineering has been implemented in multiple studies of synaptic and neural circuit function in *C. elegans*, demonstrating the general usefulness of synaptic engineering for circuit neuroscience. This includes the investigation of the roles of endogenous electrical synapses in regulating specific behaviours, such as coincidence detection[25] and the defecation cycle[26], and the causative impact of synaptic modification in plasticity and learning, such as in cross-modal plasticity[27], temperature learning[28] and olfactory learning following exposure to pathogenic bacteria[29]. As testimony to the versatility of electrical synaptic engineering, connexins have also been useful for studying the development and regulation of innate electrical synapses[30], for probing neural circuit sexual dimorphism[31], for studying electrical coupling between symmetrical neuron pairs[32,33] and for circumventing neuronal damage[32].

Recent efforts have built on lessons from *C. elegans* to develop proteins that could constitute electrical synapses in vertebrates. A new system called long-term integration of circuits using connexins (LinCx) has been established using connexins from *Morone americana* (white perch fish)[16]. Through computational modelling of connexin hemichannel interactions, protein mutagenesis and functional assays, synthetic connexin variants that do not interact with endogenous proteins but are capable of forming electrical synapses among themselves and in specific combinations have been introduced in vertebrates. Use of these synthetic electrical synapses in mice demonstrated their capacity to alter information flow between different brain regions, with a clear behavioural impact[16], establishing the validity and potential use of engineered electrical synapses in vertebrates.

Connexin-based synaptic engineering can, thus, serve as a powerful approach for probing synaptic and neural circuit function, and for reshaping neural circuit connectivity in both invertebrate and vertebrate systems.

Engineered optical synapses

A different approach to synaptic engineering that was recently introduced is based on synthetic optical signalling between neurons, by coupling postsynaptic light-gated ChR ion channels with presynaptic light-producing luciferase enzymes[17,18] (Fig. 3a).

Light-activated ChR can be expressed in vivo to depolarize neuronal membrane upon specific illumination[34] and, thus, can function as a

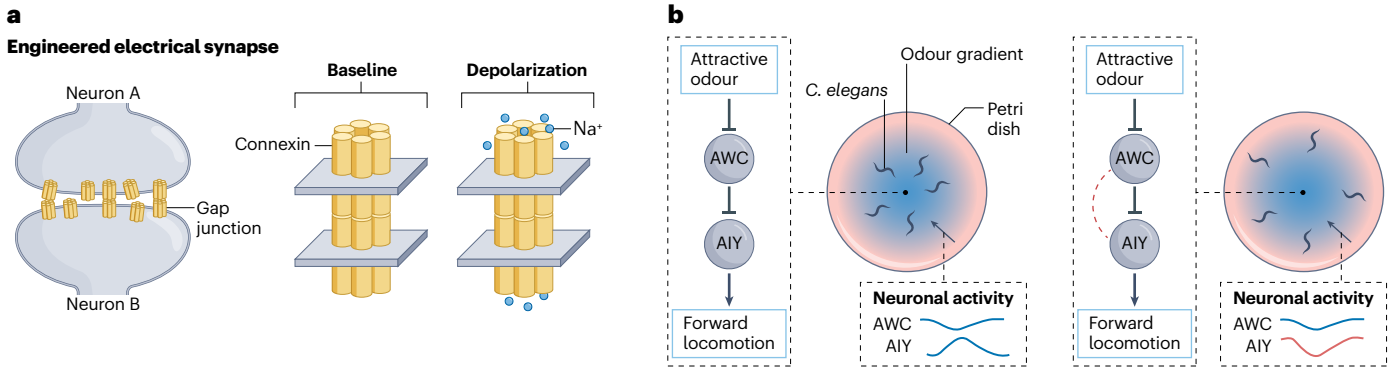

Fig. 2 | Connexin-based engineered electrical synapses. a, Engineered electrical synapses are created by cell-specific expression of connexin in two adjacent target neurons, A and B, which forms a gap junction between them. Changes in membrane potential in neuron A lead to current flow through the gap junction to neuron B, affecting the membrane potential of the latter. **b**, *C. elegans* navigation up an attractive odour gradient is controlled in part by inhibitory chemical synaptic signalling between AWC olfactory neurons and AIY interneurons[24] (left; *C. elegans* neuron names usually consist of three letters). Inserting an engineered electrical synapse between AWC and AIY (dashed connector on the right) flips the signalling between these neurons, biasing locomotion away from rather towards the source of odour and thereby switching attraction to avoidance[15].

postsynaptic optical receptor. The light sensitivity of ChR stems from a covalent attachment to a retinal chromophore[35], which absorbs light at a wavelength of approximately 470 nm, leading to the opening of the ion channel pore. The known repertoire of ion conductance for light-gated ion channels is very broad — including K^+, Na^+, Cl^- and Ca^{2+} ion conductances[34] — enabling considerable flexibility in optical synapse design. Moreover, several ChR variants have been discovered or engineered that display blue-shifted or red-shifted absorption[36–39], adding further diversity. All known ChRs rely on the same retinal group[40], which has a rather low extinction coefficient. However, certain ChR variants that exhibit a long open-state lifetime and elevated sensitivity have been derived, making these particularly useful for optical synapses[17,41].

To obtain trans-synaptic neuronal activity, the postsynaptic ChR must be combined with a presynaptic light emitter. Luciferase enzymes, which emit light upon oxidation of their substrate[42], generally termed luciferin, have proven to be an effective driver of postsynaptic rhodopsins[17,18] and are, thus, highly suitable for presynaptic optical transmission. Many different luciferases are known to emit light at various wavelengths along the entire visible spectrum[43]. The most powerful marine luciferases emit photons at approximately 470 nm with a near-perfect match to the ChR action spectrum[44,45]. Importantly, these enzymes are relatively small and, unlike firefly luciferase, do not depend on ATP as a substrate, which means that their activity does not pose an additional metabolic burden on the energy budget of the cells.

Two independent proof-of-principle experiments have demonstrated that an optical synapse can be generated using presynaptically secreted *Gaussia* luciferase or an engineered, calcium-sensitive nanoluciferase: the first approach, termed interluminescence, was applied in mice and consisted of a slow-burn luciferase, *Gaussia* luciferase, fused to the signal peptide sequence of the human pro-opiomelanocortin gene, enabling packaging into synaptic vesicles and activity-dependent secretion[18]. Once released, the secreted luciferase diffuses across the synaptic cleft and activates postsynaptic ChR. Such optical synapses were successfully constructed in cultured neurons, enabling both excitatory signalling and inhibitory signalling. They were then applied in vivo, linking presynaptic glutamatergic thalamic neurons and postsynaptic parvalbumin-positive neurons of the somato-sensory cortex. This was sufficient to produce a clear increase in gamma oscillations, indicative of successful activation of sensory processing neurons in the neocortex[18].

In the second approach, termed PhAST (for photons as synaptic transmitters), photon emission was directly coupled to Ca^{2+} concentration within the active presynaptic compartment[17]. This was achieved using an engineered enhanced nanoluciferase[45,46] split by a calcium-sensing domain[47] that is reconstituted to produce photons when Ca^{2+} concentrations rise, enabling optical signalling following presynaptic depolarization and Ca^{2+} influx. Such luciferases are ideal for establishing synthetic optical connections and, together with the long open-state lifetime of ChR2-HRDC (a ChR harbouring an H134R and D156C mutation), enabled the engineering of an optical synapse in *C. elegans*[17]. To assess the effectiveness of this approach, a de novo PhAST optical synapse was designed to enhance the relatively weak response of male *C. elegans* to noxious stimuli[31]. The new synapse linked ASH nociceptive neurons to AVA premotor neurons involved in initiating backward motion, producing the desired enhanced escape response[17] (Fig. 3b). This effect was similar to that observed for ASH–AVA engineered electrical synapses[31], demonstrating how engineered synapses can form new signalling pathways between neurons lacking innate synaptic connections.

These pioneering demonstrations of two different all-optical synapses in *C. elegans* and mice establish the efficacy of light as a synthetic neurotransmitter. The proof-of-principle experiments also promise new possibilities for novel engineering of synthetic neural circuits, facilitated by the range of available luciferase and ChR variants, with diverse emission and activation wavelengths and ion selectivity. In the future, luciferases with higher enzymatic activity and self-sustained autoluminescence[48] that do not depend on external luciferin delivery may help increase the versatility of optical synapses for synaptic engineering.

Engineered neuropeptide signalling

Neuropeptides serve multiple modulatory roles in the nervous system, enabling dynamic reconfiguration of circuit functionality that can alter diverse behaviours[49]. Compared with electrical or chemical junctional

synapses, neuropeptides are not as constrained by neural circuit architecture and can have relatively long-range effects on circuits and tissues that are not immediately adjacent to the secreting neuron. These attributes of neuropeptide signalling could be utilized for engineering modulatory synaptic pathways (Fig. 4a). This was demonstrated by the construction of an artificial neuropeptide-based transmission system named HySyn (hydra-derived synthetic synapse), combining a presynaptic carrier molecule for heterologous production of a cnidarian neuropeptide and a postsynaptic cognate receptor that is also a Ca^{2+} channel[19]. The divergent evolution of this neuropeptide–receptor pair was exploited to produce a synthetic synapse that is inert to endogenous neuropeptides. As the neuropeptide processing, transport and release mechanisms[50] and Ca^{2+} signalling are conserved[51], expression of neuropeptide ligand–receptor pairs in heterologous systems enables neuromodulatory control of intracellular calcium signals at nanomolar neuropeptide concentrations. The utility of HySyn was validated in cultured cells and in *C. elegans*. In the latter, HySyn was used to repair a broken serotonergic neuromodulatory pathway between the enteric NSM neuron and muscle cells (Fig. 4b). A similar approach was used in rats, in which the insect peptide allatostatin was ectopically expressed in viscerosensory neurons and paired with allatostatin receptors linked to G protein-coupled inward-rectifier potassium channels in the brainstem nucleus of the solitary tract, leading to in vivo modulation of blood pressure[20].

These experiments in rats and *C. elegans* demonstrated the ability of engineered neuropeptide signalling to create new functional, remote and distributed neuronal pathways, enabling the reconfiguration of neural circuits for in vivo dissection of the role of neuromodulation in neural circuit logic and connectivity[49]. The challenge of engineering neuropeptide signalling pathways boils down to selecting neuropeptides and receptors that are inert in the host organism when separately expressed. Once they are co-expressed, they become fully functional by harnessing the synthesis, release and postsynaptic response mechanisms shared by endogenous neuropeptide pathways.

Functional differences

The various approaches for synaptic engineering described so far offer a basic range of features that satisfy diverse design requirements. It is, therefore, useful to compare these alternative synaptic engineering strategies and consider how they may suit distinct applications (Table 1).

Kinetics

Different modes of synaptic transmission follow distinct time courses and kinetics. Engineered electrical synapses are, in principle, the fastest, as they directly couple connected neurons, enabling almost instantaneous signalling[52]. Optical synaptic transmission depends on the kinetics of a series of events (Ca^{2+} binding[17] or vesicle release[18], substrate oxidation and channel opening), but it still operates at relatively fast timescales, which can be approximated to several milliseconds[17,18]. In neurons with a high input resistance, a single open ion channel can elicit marked depolarization[53], depending on its conductance and open-state lifetime. In addition, photon absorption and quantum efficiency of the retinal chromophore set a lower limit on signalling speed[17]. Neuropeptide signalling is typically the slowest, as it entails presynaptic vesicle release (in the order of milliseconds), diffusion (which may take seconds or even more) of the neuropeptide, and a postsynaptic response (submilliseconds for an ion channel, several milliseconds for a metabotropic receptor, and seconds or minutes, or more, for triggered transcription)[3]. Thus, in terms of kinetics, engineered electrical synapses could be especially effective for rapid (submilliseconds) neuronal synchronization and intercellular coupling, whereas optical synapses may offer quick (in the order of 10–100 ms) focused signalling between neurons, and engineered neuropeptide-based transmission may be a good choice for applications requiring graded network-wide broadcasting and neuromodulation over a broad range of timescales.

Directionality

Engineered neuropeptide and optical synthetic connections are both inherently unidirectional, signalling from presynaptic to postsynaptic neuron. By contrast, an electrical synthetic synapse may be bidirectional if constituted from the same (homotypic) connexins in both neuronal partners[54]. Alternatively, certain combinations of dissimilar (heterotypic) connexins may give rise to a rectifying electrical synapse[55]. Such rectifying connexin-based engineered synapses have

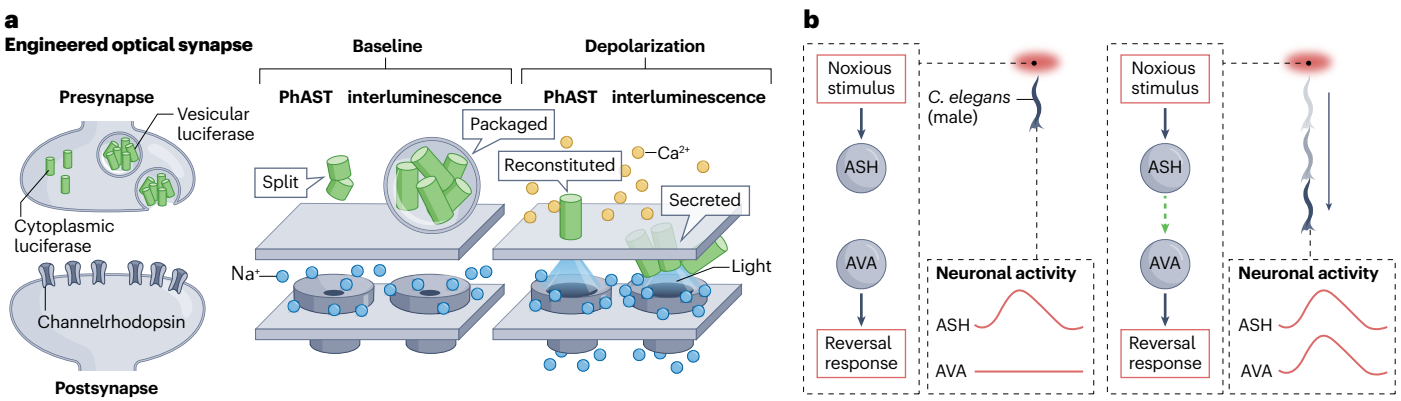

Fig. 3 | Luciferase–channelrhodopsin-based engineered optical synapses. **a**, Engineered optical synapses can be constructed by expressing split Ca^{2+}-dependent cytoplasmic (PhAST system) or vesicle-secreted (interluminescence system) luciferase in presynaptic neuron A and a light-gated ion channel, channelrhodopsin (ChR), in postsynaptic neuron B. Upon depolarization of neuron A, the luciferase emits photons that activate ChR, eliciting a postsynaptic potential in neuron B. **b**, Male *C. elegans* normally show weak avoidance responses to noxious stimuli (left). Insertion of a de novo optical synapse (indicated by green arrow) between the ASH nociceptive neuron and the AVA premotor neuron is sufficient to alter behaviour and produce a robust escape response[17] (right). PhAST, photons as synaptic transmitters.

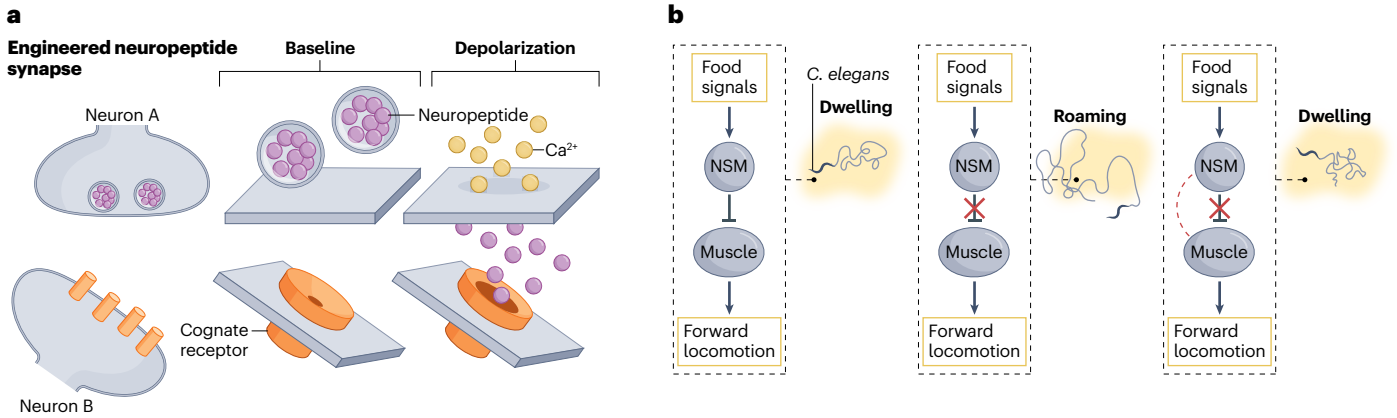

Fig. 4 | Engineered neuropeptide signalling. a, Engineered neuropeptide signalling is established by expressing a foreign neuropeptide and its cognate receptor in neurons A and B. Depolarization of neuron A leads to release of the ectopic neuropeptide from dense core vesicles, which reaches its ectopic receptors in neuron B by diffusion and activates neuron B (ref. 3). **b**, Serotonin secretion from food-sensing NSM neurons is important for regulating dwelling behaviour in the presence of food in *C. elegans* (left). Eliminating this signalling (indicated by the 'X' mark) leads to maladaptive roaming (middle). The break in information flow (indicated by the 'X' mark) can be circumvented by engineering a synthetic neuropeptide signalling pathway between NSM and muscle cells (right)[19].

recently been implemented[16]. The bidirectionality and rectification of engineered electrical synapses can produce different outcomes from optical or neuropeptide-based unidirectional synapses. For instance, an engineered optical synapse between a sensory neuron, A, and an interneuron, B, will reinforce A → B information flow. In comparison, an engineered electrical synapse will enable at the same time both A → B bottom–up information flow and B → A top–down feedback[56] from interneuron to sensory neuron.

Presynaptic signal

Optical and neuropeptide signalling are prompted by presynaptic depolarization. High presynaptic Ca^{2+} influx triggers photon production in the PhAST optical synapse[17] or initiates vesicle release of neuropeptide[19] or luciferase[18]. By contrast, electrical synapses can transmit both depolarization and hyperpolarization signals[54]. Therefore, engineered electrical synapses can be especially effective in enhancing analogue communication and promoting correlations between synthetically coupled neurons, whereas engineered optical or neuropeptide-based synapses are more suitable for transmitting discrete positive signals. Notably, optical synapses using Ca^{2+}-sensitive luciferases[17] can be used as spatiotemporal probes for presynaptic signalling, as their photon emission can be captured by camera[57].

Postsynaptic signal

An interesting feature of engineered electrical synapses is that the same synapse may elicit both excitatory and inhibitory changes in postsynaptic membrane potential. By contrast, a particular optical synapse or neuropeptide pathway will generate postsynaptic signals or modulation according to the properties of the specific postsynaptic receptor included in that synapse. Although the allatostatin system, for example, demonstrated neuropeptide-driven postsynaptic membrane potential inhibition[20], neuropeptide signalling can also modulate postsynaptic intrinsic electrical properties and alter presynaptic and postsynaptic signalling[3]. Theoretically, the optical synapse too may be designed to couple the presynaptic light signal to artificial light-activated neurotransmitter receptors[58] or even metabotropic opsins for neuromodulation[59], expanding the postsynaptic signalling repertoire of optical neurotransmission.

Considerations

Both engineered electrical and optical synapses require close physical proximity between target neurites to enable transmission. Recently, the list of all physically adjacent *C. elegans* neurons, the 'contactome', has been extracted from electron microscope images[60,61], providing a map of possibilities for electrical and optical coupling. By contrast, neuropeptide-based synaptic engineering is less restricted, but aspects such as the kinetics of peptide diffusion, receptor activation in specific tissues and integration of Ca^{2+} signalling over time[62] must be taken into account, especially in systems that are not as compact as *C. elegans*, or in systems wherein less is known about the physical adjacency of neurons.

A general limitation of synaptic engineering shared with other genetic approaches is the specificity of available promoters driving expression of synaptic components. Various intersectional solutions exist for combining promoters[63], but even if specific neuron populations are segregated, targeting individual neurons or neuron classes remains challenging. New methods for cell-specific gene expression will be required to achieve this level of resolution[64,65]. The specificity of synapse insertion may vary between systems according to available transgenic techniques (germ line versus somatic; promoter-driven versus site-specific transduction). Such considerations may influence the design according to the host system.

Whereas engineered electrical and neuropeptide-based synapses are fully self-contained, optical synapses require for their function the presence of auxiliary compounds (that is, bioluminescence substrates and sometimes also a retinal chromophore). This may pose certain limitations but may also serve as an effective control switch, enabling transient reconfiguration of the target neural circuit. Different systems may differ in the efficacy and manner of delivering such compounds. For example, many species endogenously produce the retinal chromophore required for optical synapse function, whereas *C. elegans* relies on the retinal chromophore being externally supplied as part of the experimental design.

Although the basic unit of manipulation for synaptic engineering is the single synapse connecting two neurons, it is often practical to consider the coupling of two neuron populations rather than two individual neurons, as the impact of a single synapse on circuit function and animal behaviour may be negligible. This has important consequences, especially for the design of electrical synapses between two neuronal populations, which may inadvertently lead to the formation of unintended connections within each population, altering off-target circuit function and undermining specificity. An elegant solution was provided by the LinCx synthetic connexins described in the 'Engineered electrical synapses' section, which were derived as two variants, $Cx34.7_{M1}$ and $Cx35_{M1}$ (ref. 16). Each variant can form heterotypic but not homotypic gap junctions. Thus, $Cx34.7_{M1}$ expressed in one neuronal population will not form gap junctions with endogenous connexins, or with other $Cx34.7_{M1}$-expressing neurons within that population, but it will readily participate in gap junctions with the synthetically designed partner connexin $Cx35_{M1}$. In this way, $Cx34.7_{M1}$-expressing neurons will exclusively connect with $Cx35_{M1}$-expressing neurons. This enables the establishment of engineered electrical synapses between two neuron populations while avoiding undesired connections within each population. However, if the goal is to achieve synchrony within a population, whereby each neuron may connect with every other neuron, then standard connexins (in invertebrate systems) or innexins or connexins that are incompatible with innate connexins (in vertebrate systems) should be used to form homotypic gap junctions.

A final point to consider is that as in any transgene, the ectopic expression of synaptic components could have additional effects on the cell beyond the intended ones. For example, connexin expression even in a single neuron may form hemichannels[66] that could alter its electrical properties. Therefore, it is important to compare the effects of the isolated synaptic components with those of the full system for every type of engineered synapse.

Future directions

The ability to design and insert specific new synaptic connections into neural circuits in vivo offers vast opportunities for investigating structure–function relationships in the nervous system. These include hypothesis testing about information flow in neural circuits, probing the causative role of synaptic modification during learning, comparing alternative forms of communication between the same neurons and more. The approaches developed so far provide considerable flexibility in the implementation of new engineered synapses, enabling diverse functional interventions and the prospect of a causative account of neural circuit operation. To conclude this Perspective, we highlight potential next steps in the conceptual development of synaptic engineering that could substantially advance and broaden its impact.

Expanding synaptic engineering

Existing methods for synaptic engineering could be readily expanded by combining further variants of connexin, luciferase, ChR, neuropeptides and neuropeptide receptors, providing a broader range of kinetics and transmission properties. In this manner, the diversity of engineered synapses may begin to approach that of natural synapses[67], providing hints about the roles of such diversity in circuit function and enabling sensitivity analyses of the outcomes of subtle differences in neural communication. To this end, systematic testing and tuning of the underlying variants will need to be performed. In addition, several recent techniques for synaptic manipulation have emerged that could potentially facilitate the development of new forms of synaptic engineering. For example, synthetic adhesion molecules based on the bacterial receptor–ligand pair barnase–barstar fused with neuroligin–neurexin synaptic adhesion molecules have been shown to induce synaptic organization in cultured neurons[68]. Delivering these molecules in an intact brain using cell type-specific promoters[68] could form the basis for a new synaptic engineering approach, providing novel sites for engineered connectivity. This approach must be carefully tested to determine the extent to which such perturbation of connectivity may impact the entire network. Other prospective components for synaptic engineering include the induction of synapse formation by de novo neurotransmitter synthesis[69] or the switching of ion selectivity of ligand-gated ion channels, for example, from anion to cation[70]. These latter approaches target presynaptic or postsynaptic neurons rather than a specific synaptic connection, but could be combined with other techniques of synaptic engineering to provide specificity.

On–off switching

A potentially useful feature of engineered synapses would be the ability to switch them on or off. Unlike optical synapses, whose reliance on external substrates provides switching ability, current implementations of engineered electrical and neuropeptide connections persist throughout the entire lifetime of the host organism and cannot be enabled or disabled. There are many advantages to switching. First, to discern putative developmental effects, it is important to introduce (switch on) the engineered synapse only in adulthood or at a specific developmental stage. Second, the insertion of an engineered synapse could alter neural dynamics, eliciting plasticity changes that may lead to further indirect circuit modifications, making it difficult to discern the direct impact of the engineered synapse. Brief temporally controlled activation of the engineered synapse could make it possible to pinpoint the specific contribution of the new connection to circuit function before any plasticity processes take place. At the same time, it could enable the study of how new changes in connectivity are integrated into a dynamic neural circuit. Last, more advanced synaptic engineering designs may require sequential activation or deactivation of alternative synaptic pathways or the implementation of artificial forms of activity-dependent rewiring. These features may be required for the engineering of novel dynamic decision-making behaviours and even new forms of learning. By combining engineered synapses with several existing tools used in other systems, on–off switching may be

Table 1 | Comparison of current synaptic engineering approaches

Feature	Synaptic engineering approach		
	Electrical	Optical	Neuropeptide
Kinetics	Very fast	Fast	Slow
Directionality	Bidirectional or rectifying	Unidirectional	Unidirectional
Presynaptic signal	Depolarization or hyperpolarization	Depolarization	Depolarization
Postsynaptic signal	Excitatory or inhibitory	Excitatory or inhibitory	Excitatory, inhibitory or modulatory
Considerations	Physical contact; within versus between populations	Light scattering; luciferin requirement	Diffusion limit

straightforward to implement, for example, using selective promoters active only during certain time windows of development or adulthood[71] or promoters responsive to external conditions, such as heat-shock[72] or the presence of specific chemicals[73]. For even quicker and more flexible temporal control, techniques based on protein degradation[74] or phosphorylation reactions[75] could provide a good solution. Adding inducible switching to synaptic engineering will provide tighter control of the system and will enable a broader range of applications.

Multiplexing

Most implementations of synaptic engineering so far have aimed to build a single synaptic connection or pathway between two specific neurons or subsets of neurons. However, more complex structures may be formed by simultaneously inserting multiple independent synthetic synapses in various locations in the circuit. Ultimately, an entire neural circuit could, in principle, be reconfigured by the addition and removal of a series of identified synaptic connections[76]. The capacity and possibilities for multiplexing engineered synapses vary between different engineering approaches and different target circuit configurations. For example, to establish multiple synthetic electrical synapses in parallel, it may be necessary to construct each individual synaptic connection using specific incompatible connexin variants[77] (for example, Cx36 for one synapse and Cx43, also shown to be functional in *C. elegans*[30], for another) and, similarly, orthogonal neuropeptide–receptor and luciferase–ChR pairs for engineered neuropeptide-based and optical synapses, respectively. This poses certain challenges, especially for potentially elaborate circuit configurations, owing to, for example, spectral overlap or a limited number of known variants of ChR. It would be helpful to establish for this purpose a repository of verified synaptic parts, as practised in the broader field of synthetic biology[78].

Conclusions

Synaptic engineering is emerging as an effective approach for probing neural circuit structure and function through building of modular components that enable the creation of new neuronal relationships. It facilitates the examination of existing neural circuits and makes it possible to address questions about new circuit configurations. To use this approach most effectively, it is important to appreciate the strengths and weaknesses of different types of available synaptic engineering tools and to consider how synaptic engineering can best complement other approaches for studying neural circuits.

In addition to its merits as a basic research tool, synaptic engineering could also lead to the development of interesting applications. For example, it could be used for the implementation of engineered behaviours in host organisms designed to perform certain tasks (for example, detect and attack a certain pathogen)[79] and in general could serve for the implementation of synthetic biology in multicellular organisms[80]. Synaptic engineering could also be explored as a potential future strategy for treating damaged neural circuits[32,81]. Ultimately, one can envision future large-scale synaptic engineering designs, including the construction of novel elaborate neural circuits, enabling the in vivo study of theoretical network topologies[82], serving as a powerful test of our understanding of neural circuit operation.

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