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Review

Tools and methods for cell ablation and cell inhibition in *Caenorhabditis elegans*

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To understand the function of cells such as neurons within an organism, it can be instrumental to inhibit cellular function, or to remove the cell (type) from the organism, and thus to observe the consequences on organismic and/or circuit function and animal behavior. A range of approaches and tools were developed and used over the past few decades that act either constitutively or acutely and reversibly, in systemic or local fashion. These approaches make use of either drugs or genetically encoded tools. Also, there are acutely acting inhibitory tools that require an exogenous trigger like light. Here, we give an overview of such methods developed and used in the nematode *Caenorhabditis elegans*.

Keywords: cell ablation; optogenetics; chemical ablation; channelrhodopsins; animal rhodopsins; photosensitizer; laser ablation; genetic ablation; hyperpolarization; synaptic vesicle release

Introduction

Eliminating neurons, and/or inducing the inhibition of their function, is a fundamental approach in the neurosciences, in order to understand the role of a neuron within the circuit(s) it is embedded in (Pei *et al.* 2008; Wiegert *et al.* 2017). This is a particularly fruitful and important approach in the nematode *Caenorhabditis elegans*, with its compact nervous system of a mere 302 neurons, where in many cases elimination of single neurons (or a class of neurons) has profound impact on circuit function and/or behavior (Qi *et al.* 2012; Lin *et al.* 2013b). Therefore, many approaches and (mostly genetically encoded) tools have been developed over the past 4 decades, to enable the inhibition of excitable cells. These range from pharmacological treatment to laser ablation, from expressing proteins inducing cell death, to photosensitizers allowing to eliminate individual cells or cell populations, to chemically or light-activated proteins that either hyperpolarize excitable cells, signal to inhibitory G protein pathways, or inhibit the release of neurotransmitter by destroying proteins required for synaptic transmission or by blocking synaptic vesicle (SV) mobility (Fig. 1a). Another approach is to (optogenetically) activate neurons that have inhibitory action (Liewald *et al.* 2008; Steuer Costa *et al.* 2019). The genetic encodability of such tools is particularly attractive, because it can be specific down to individual neurons, but also allows to address whole neuron classes as well as animal populations. The tools used for disruption or inhibition of (excitable) cell function can act at different spatial scales, affecting either the entire animal or parts of its body, down to individual cells or even synapses (the latter requires synapse-specific illumination and is possible, but not widely applied due to the technical difficulty of this approach; Fig. 1b). The tools described in this review are further characterized by the timescales of how fast (and tightly controllable) their use is in the onset, which can be submillisecond for microbial rhodopsin tools (Bergs *et al.* 2018), but also hours when it requires the expression of proteins (Fig. 1c). Last, the tools are characterized by the duration of their action as well as their reversibility: Some tools stop their action as soon as the stimulus is off, e.g. light, or can be very long lasting, e.g. if proteins need to be synthesized de novo to revert the manipulation. Other tools' actions are permanent, e.g. if the cell is physically or genetically ablated. In the following sections, we briefly describe the tools and some of their applications. We did not attempt to be fully comprehensive, as these approaches cover a vast field of applications and some have been used for several decades.

Pharmacology

The most straightforward possibility to silence neuronal activity or locomotion is through the use of drugs. Often, these act in a reversible way, if the animal is removed from the drug source, though prolonged exposure to some drugs will cause cellular damage or even death. Sodium azide (NaN_3) causes rapid

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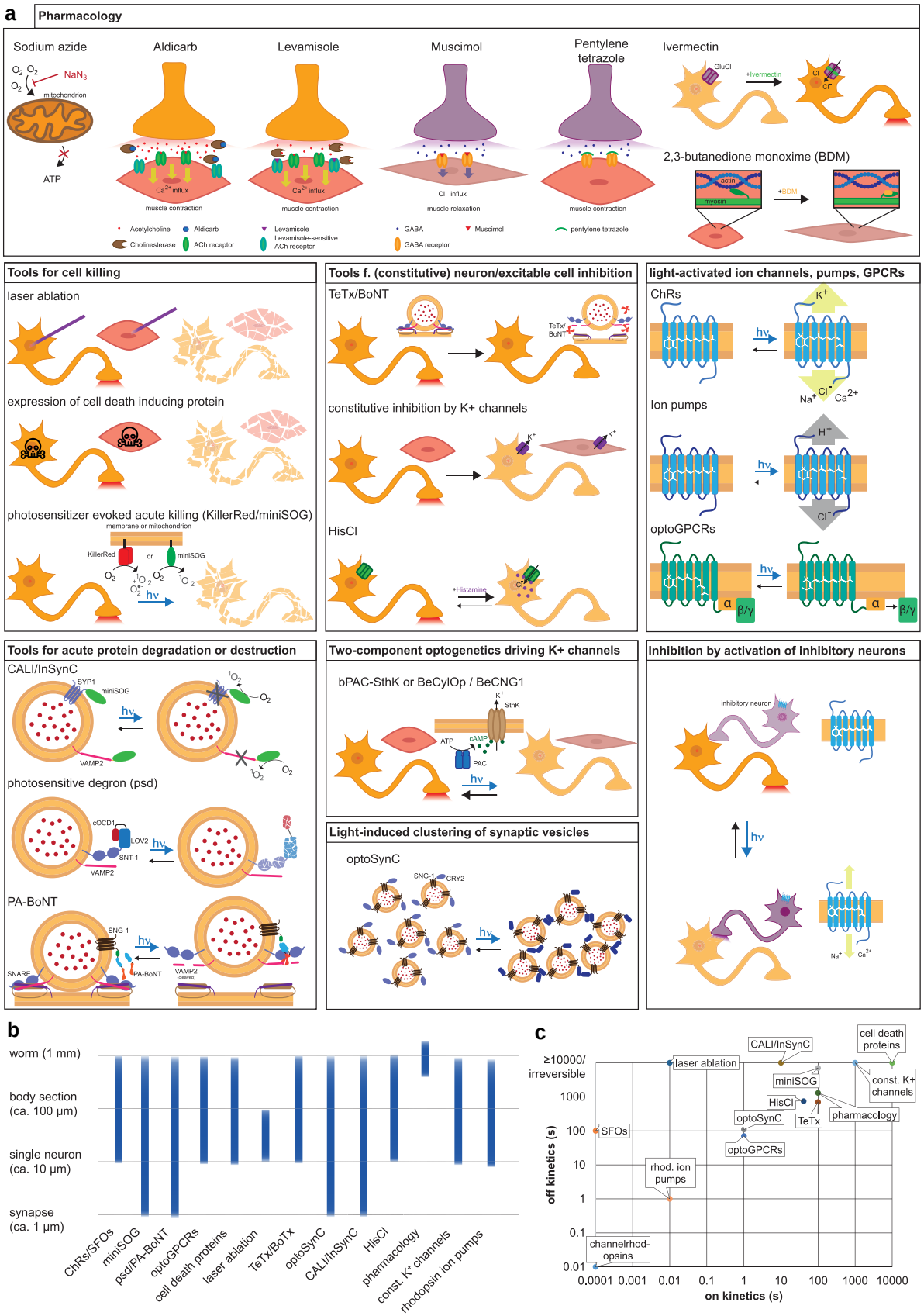

Fig. 1. a) Pharmacological and other silencing approaches, physical ablation, and (optogenetic) tools for inhibition of excitable cells or cell ablation, covered in this review. b) Spatial scales at which the inhibitory tools and approaches can be used. c) Temporal scales for on and off times/recovery rate of the tools and approaches. The given times are only roughly indicated for comparability. Irreversible tools are shown at the top of the chart, for simplicity.

paralysis due to its in inhibitory effect on cellular respiration. This causes a quick depletion of cellular energy equivalents (mostly adenosine triphosphate, ATP) and thus cessation of muscular activity, as well as synaptic transmission, that requires ATP for transmitter loading of SVs as well as for SV recycling. The neuromuscular junction can be used as a target for immobilizing drugs, mostly by activating nicotinic acetylcholine receptors (nAChRs) or inhibition of ACh degradation. These drugs, levamisole [a levamisole receptor agonist (Lewis *et al.* 1980a, 1980b)] and aldicarb [inhibiting ACh esterase (Miller *et al.* 1996)], cause or lead to excessive stimulation of nAChRs and thus evoke muscle hypercontraction. A light-activated version of ACh, AzoCholine, has also been used to enhance cholinergic function, though this does not lead to full paralysis on its own; yet, AzoCholine can enhance the effects of aldicarb in a light-dependent manner (Damijonaitis *et al.* 2015). Also, the inhibitory side of the neuromuscular junction (NMJ) can be used to induce paralysis, e.g. the $GABA_A$ receptor agonist muscimol (flaccid paralysis) or the gamma-aminobutyric acid (GABA) antagonist pentylenetetrazole (PTZ; causing overexcitation of muscles; Schaeffer and Bergstrom 1988; Avery and Horvitz 1990; Abraham *et al.* 2011). A very strong form of rapid paralysis is induced by ivermectin, which is agonist for glutamate-gated chloride channels (GluCls), residing on (cholinergic) motor neurons (Avery and Horvitz 1990; Cully *et al.* 1994; Arena *et al.* 1995; Dent *et al.* 1997; Hibbs and Gouaux 2011). Also, muscle can be inhibited by direct drug action, likely on the actin-myosin contractile apparatus, by using butanedione monoxime (BDM) (2,3-BDM; Miriam Goodman and Martin Chalfie, personal communication; see also Emtage *et al.* 2004 and Bounoutas *et al.* 2009).

Laser ablation

Cell ablation is used to understand the role of cells in a specific biological context. Cells can be ablated by intense light illumination, provided the light wavelength is absorbed by the tissue. Laser light is focused to a spot of the dimensions of a cell nucleus, where it causes heating and pressure increase. Light energy is typically provided in a pulsed fashion (3–70 ns duration, at <20 Hz), such that heating remains local and does only damage the cell of interest and not cells in the vicinity (Bargmann and Avery 1995). The first approaches for cell ablation utilized visible laser light (ca. 440 nm, directly absorbed, but possibly also by causing 2-photon excitation), or UV laser irradiation of the nucleus of the respective cell, which leads to cell death within comparably short time periods, and thus the loss of the function of the respective cell. This approach is quite powerful and has been widely used over the decades, since its first establishment in the late 1970s and early 1980s (White and Horvitz 1979; Sulston and White 1980). Considerations of how to set up a laser ablation system, and experimental parameters, are well summarized in 2 methods book chapters, for further reading (Bargmann and Avery 1995; Fang-Yen *et al.* 2012). Often, pulsed nitrogen-pumped tunable dye lasers, producing microjoule-level UV pulses with nanosecond durations, are used. These rather specialized lasers may require specific microscope setups and are rather expensive. Alternatively, femtosecond laser pulses of ca. 50 nJ, in the near-infrared, for multiphoton absorption, can be used at 1 kHz or at up to 80 MHz (Chung and Mazur 2009; Fang-Yen *et al.* 2012). Laser ablation has been used on various neuron types in *Caenorhabditis elegans* to identify cells involved in neuronal regulation of development (Bargmann and Horvitz 1991b) or behaviors such as chemotaxis (Bargmann and Horvitz 1991a; Bargmann *et al.* 1993), thermotaxis (Mori and Ohshima 1995), and locomotion (Gray *et al.* 2005). However, there are limitations to this approach: (1) It requires practice and experience to master, and it is of low throughput; (2) it does not allow to eliminate genetically defined cell populations, unless they are just a few; and (3) it does not allow to generate populations of animals lacking the cell of interest.

Genetically encoded cell death

Because of the limitations of laser ablation studies for high throughput, other approaches were chosen and/or developed. Proteins like caspases, or constitutively active ion channels, can lead to cell death or necrosis and thus cell loss. Consequently, genetic cell ablation methods have become widely used. Cells can be targeted for elimination by expressing cytotoxic proteins using cell-specific promoters. Several such proteins were established, including caspases, and split versions thereof, for combinatorial expression (CED-3 and human caspase 3; Chelur and Chalfie 2007; Bendesky *et al.* 2011; Glauser *et al.* 2011; Ikeda *et al.* 2020; Atanas *et al.* 2023; Chandra *et al.* 2023), CSP-1 (Denning *et al.* 2013), mammalian caspase 1 (also known as interleukin 1 beta convertase, ICE; Zheng *et al.* 1999; Kim *et al.* 2009; Bendesky *et al.* 2011; Oranth *et al.* 2018), or apoptosis regulators such as EGL-1 (Conradt and Horvitz 1998; Chang *et al.* 2006; Rauthan *et al.* 2007), a member of the BH3-only protein family.

Dominant alleles of *mec-4* and *deg-1*, which encode the hyperactive degenerin/ENaC family of epithelial sodium channels, lead to the necrotic death of specific sets of neurons (Chalfie and Wolinsky 1990; Driscoll and Chalfie 1991; Bianchi *et al.* 2004). Ectopic expression of the dominant allele *mec-4(d)* can disrupt the function of various cells (Harbinder *et al.* 1997; Shinkai *et al.* 2011; Teo *et al.* 2022). The ability of these dominant alleles to induce cellular degeneration depends on *mec-6* (Chalfie and Wolinsky 1990; Driscoll and Chalfie 1991; Harbinder *et al.* 1997), which encodes a putative chaperone for MEC-4 (Chen *et al.* 2016a).

PEEL-1 is a native *C. elegans* sperm-derived toxin that is normally counteracted in the embryo by its antidote, ZEEL-1 (Seidel *et al.* 2008). Ectopic expression of PEEL-1 can also be used to induce cell ablation (Seidel *et al.* 2011; Frøkjær-Jensen *et al.* 2012; Chen *et al.* 2016b)

Acutely induced cell death

Caspases and other cell ablation proteins may act as soon as they become expressed, which is only ill-controllable, and the lack of cells during development of an animal can induce compensatory/homeostatic plasticity mechanisms. Therefore, there is a need for tools that enable acute destruction of cells in a short period of time, yet still retain the benefit of genetic encodability to systemically address cell populations, as well as entire animal populations. To this end, photosensitizers based on light-absorbing proteins have been developed. These generate, in response to blue light, reactive oxygen species (ROS) that act in a destructive way on any oxidizable (amino acid) residues in the vicinity of the photosensitizer chromophore. This can occur through methionine oxidation, or by inducing cross-linking, and depending on subcellular localization, will damage nearby protein and can even induce cell death (Jacobson *et al.* 2008). Such tools include the green fluorescent miniature singlet oxygen generator (miniSOG); the red fluorescent KillerRed, which produces superoxide anion radicals; and SuperNova. Here, we discuss their development and their applications in *C. elegans*.

KillerRed

KillerRed, a derivative of the hydrozoan chromoprotein anm2CP, which is a homolog of green fluorescent protein (GFP), was initially developed for light-induced cell ablation of bacteria and eukaryotic cells, as well as for targeted protein inactivation (Bulina *et al.* 2006b). Subsequent adaptations enabled the use of KillerRed for effective neuronal inactivation in *C. elegans*, which can be induced by a 5 min illumination period, leading to morphological changes appearing after hours and persisting for several days (Kobayashi *et al.* 2013; Williams *et al.* 2013). Since its introduction, KillerRed-induced neural dysfunction has been employed in numerous studies (Lee *et al.* 2014; Luo *et al.* 2014; Berendzen *et al.* 2016; Shao *et al.* 2016; Young *et al.* 2019; Pohl *et al.* 2023), highlighting its utility in neurobiological research. However, it is noteworthy that certain types of neurons, including AWB, AFD, and PDE, exhibit resistance to KillerRed-induced cell inactivation (Williams *et al.* 2013). Additionally, when KillerRed is expressed in mechanosensory neurons, normal mechanosensation is disrupted even without light exposure (Takemoto *et al.* 2013). Several variants of KillerRed have been developed. KillerOrange, which is activated by both blue and green light, has been developed and tested in vitro (Sarkisyan *et al.* 2015), offering greater flexibility when concurrent manipulation of multiple proteins or cell types is required, due to the broader color spectrum. Since KillerRed tends to form homodimers, which might interfere with the normal function of the fusion proteins (Bulina *et al.* 2006a), monomeric variants called SuperNova, SuperNova Green, and SuperNova2 have been established in bacterial and cultured cell systems (Takemoto *et al.* 2013; Riani *et al.* 2018; Gorbachev *et al.* 2020). SuperNova exhibits proper localization with targeted proteins, which increases its potential to disrupt synaptic proteins or specific neurotransmitter receptors for functional analysis.

miniSOG

The miniSOG is a green fluorescent flavoprotein, which was engineered from the *Arabidopsis* phototropin 2 (Shu *et al.* 2011). While KillerRed is relatively large (237 amino acids) and requires dimerization, miniSOG only consists of 106 amino acids and is acting as a monomer. miniSOG was originally developed for use in correlative light and electron microscopy (Shu *et al.* 2011), by inducing the formation of an electron-dense polymeric precipitate from diaminobenzidine. As miniSOG produces a sufficient amount of ROS, specifically singlet oxygen, upon blue light illumination, it could also be used for light-induced cell ablation. By targeting miniSOG to the outer mitochondrial membrane, the production of ROS upon illumination results in cell degeneration and death (Qi *et al.* 2012). Since its development, mito-miniSOG was used in different ablation experiments in *C. elegans*, such as the study of a salt sensory circuit (Leinwand and Chalasani 2013) or identification of VAV-1 acting in signaling in the sensory and interneuron ALA (Fry *et al.* 2014). To cause cell ablation, mito-miniSOG requires 0.5–1.5 h blue light illumination, which can be limiting for its use in *C. elegans* as this organism can be killed by extended blue light exposure (Edwards *et al.* 2008). Additionally, if mito-miniSOG is highly overexpressed, it might have deleterious effects in absence of blue light, as ROS generation can be leaky under ambient light (Qi *et al.* 2012). Development of a (plasma-)membrane-targeted miniSOG allowed highly efficient ablation of multiple cell types, including neurons, muscles, and the epidermis (Xu and Chisholm 2016). Therefore, a mutant version of miniSOG (Q103L), showing enhanced ROS generation in vitro (Westberg *et al.* 2015) and enhanced cell killing efficiency in vivo, was targeted to cell membranes by using membrane targeting signals such as the pleckstrin homology (PH) domain from rat PLC-δ (Audhya *et al.* 2005) or the predicted myristoylation signal sequence from *C. elegans* NCS-2, myr (Xu and Chisholm 2016). miniSOG (Q103L), targeted to membranes, yielded a 10-fold increased efficiency compared to mito-miniSOG for cell ablation (Xu and Chisholm 2016). Besides identifying a role of the epidermis in locomotion (Xu and Chisholm 2016), this miniSOG variant was used to ablate AS motor neurons, thereby disrupting locomotion patterns (Tolstenkov et al. 2018).

Tools for silencing of excitable cell activity and synaptic transmission

Neurons and muscles are excitable cells and responsible for the action sequences that drive behavior or other organismic activities, like gut movements or pharyngeal pumping. Therefore, inhibition of such cells can be used to reduce or even abolish the behavior driven by the respective cell. Many tools have been developed over the past decades that enable excitable cell inhibition. This includes tools for constitutive inhibition which allows to silence cells on a permanent basis, as soon as they begin expressing the respective protein.

Constitutively open potassium channels

Since potassium ions have a steep downhill concentration gradient from the cytosol to the extracellular medium, expressing a constitutively open K^+ channel will allow positive charge to constantly leak from the cell and thus hyperpolarize its membrane potential. In excitable cells, this typically causes inactivation. This class of protein tools comprises several potassium channels of the 2-pore domain potassium (TWK) channel family. The first mutations discovered that led to a constitutively open TWK channel were *twk-18(gf)*, the constitutive allele *e1913*, encoding the mutation G165D, and the temperature-sensitive allele *cn110* (M280I), which leads to complete paralysis above 25°C (Hosono *et al.* 1985; Reiner *et al.* 1995; Kunkel *et al.* 2000). The mutant protein was used as a selection marker for single-copy transgene insertions using MosSCI (Frokjaer-Jensen *et al.* 2008) but has also been used to study neuronal/circuit function in the locomotion nervous system in order to silence premotor interneurons (Kawano *et al.* 2011) and to study the sleep neuron RIS (Koutsoumparis *et al.* 2022). The latter work also used a second TWK channel g.o.f. mutation, i.e. *egl-23(gf)*, and both proteins were further used in Busack and Bringmann (2023) for the same purpose. Recently, the TWK channel g.o.f. mutant, *twk40(gf)*, was used to study the premotor interneuron AVA (Meng *et al.* 2024).

Tetanus toxin/botulinum neurotoxin

A conventional method for inducing long-term disruption of synaptic transmission involves genetic expression or direct application of *Clostridium botulinum* or tetanus neurotoxins (Brooks *et al.* 1957; Molgo *et al.* 1990; Sweeney *et al.* 1995). These potent inhibitors of synaptic transmission are metalloproteases that target SNAREs, i.e. proteins required for SV fusion. Tetanus toxins (TeTx), which are zinc-dependent endoproteases, and several clostridial toxins cleave synaptobrevin at unique sites, via their catalytic light chains (Schiavo *et al.* 1992; Blasi *et al.* 1993; Arata *et al.* 1997). While TeTx may lack the temporal precision of chemogenetic or optogenetic approaches, it can effectively silence neurons over extended periods, especially those projecting primarily to a single downstream target (with collateral effects on other neurons). However, these toxins also target other cellular

counterparts of synaptobrevin, such as cellubrevin, which are ubiquitously expressed (McMahon *et al.* 1993). Notably, TeTx has been observed to impede cell membrane repair in fibroblasts and *Xenopus* oocytes, implying that molecules akin to synaptobrevin play roles in regulating additional fusion events (Steinhardt *et al.* 1994). Thus, the action of TeTx implicates that the protease cleavage of other targets contributes to the transmission abnormalities observed in toxin-treated cells.

In *C. elegans*, TeTx from the *tdc-1* promoter was used to inhibit RIM and RIC neurons, resulting in defects similar to those observed in *tdc-1* mutants (Sordillo and Bargmann 2021). In another study, TeTx was used to block DCV release in AVK neurons, partially mitigating increases in speed and body bending upon AVK photoactivation in *flp-1* mutants (Aoki *et al.* 2023). Additionally, TeTx was used to analyze the synaptic output of RMG, by utilizing the Cre/Lox system to express the light chain (LC) of TeTx, which revealed distributed synaptic outputs for aggregation with contributions from both RMG and ASK/ASJ neurons (Macosko *et al.* 2009).

Botulinum neurotoxin (BoNT) is a lethal toxin responsible for causing botulism, a condition characterized by paralysis resulting from food-borne contamination (Davletov *et al.* 2005). BoNTs consist of a ~50 kDa LC and a ~100 kDa heavy chain (HC), linked by a disulfide bridge (Montecucco and Schiavo 1994). The LC serves as the catalytic domain, while the HC facilitates the delivery of BoNT into neurons. BoNTs are primarily known for their ability to block synaptic transmission at the NMJ (Montecucco and Schiavo 1994). Specifically, BoNT/A targets the C-terminal 9 amino acids of synaptosome-associated protein of 25 kDa (SNAP-25). The resulting truncated SNAP-25 may persist to interact with plasma membrane syntaxin, thereby disrupting the normal process of vesicle fusion and leading to potent toxicity.

While regulated promoters or recombinase systems enable some level of temporal control over toxin expression (Yamamoto *et al.* 2003; Nakashiba *et al.* 2008; Airan *et al.* 2009), achieving rapid and localized control remains challenging at present. However, a photoactivated version of BoNT (PA-BoNT) has been generated and used to silence motor neurons (see PA-BoNT).

InSynC/chromophore-assisted light inactivation

The ability to generate ROS via miniSOG has further been taken advantage of for developing an optogenetic tool for neuronal silencing. The chromophore-assisted light inactivation (CALI) of synaptic proteins was developed by using miniSOG and targeting it to the presynaptic terminal using proteins such as synaptobrevin (VAMP2) or synaptophysin (SYP1). This allows inactivating the SNARE complex following blue light illumination (Ache and Young 2005). This approach was also used in *C. elegans* to target neurons via synaptobrevin or synaptotagmin (Lin *et al.* 2013b; Hermann *et al.* 2015). Using CALI as a neuronal silencer, however, comes with the same disadvantages as miniSOG used for cell ablation: The generation of damaging radicals may have off-target effects, and it is not known whether CALI has long-lasting effects on synaptic strength. As SNARE proteins are damaged by ROS generation, recovery from the inhibiting effect requires de novo synthesis of targeted proteins.

Photosensitive degron

To overcome the disadvantages of miniSOG-enabled synaptic silencing, a photosensitive degron (psd) was developed (Hermann *et al.* 2015). The psd comprises 2 domains: a photo-switchable LOV2 domain, which can undergo light-dependent conformational changes (Kennis *et al.* 2004), and a C-terminal degradation sequence derived from mouse ornithine decarboxylase (cODC). This sequence contains a Cys-Ala motif which is recognized by the proteasome (Takeuchi *et al.* 2008). Fused to a protein of interest (POI), such as synaptotagmin (SNT-1) for neuronal targeting, allows the degradation of the POI upon blue light illumination and thereby inhibition of synaptic transmission. The resulting silencing effect of psd is comparable to inactivation via miniSOG but enables more accurate targeting without off-target effects. However, expression of the degron-tagged POI in the presence of the endogenous protein is not sufficient to eliminate POI function, which is why one needs to work with a rescuing transgene in a mutant background lacking the endogenous protein. Alternatively, the psd must be introduced into the endogenous locus (Hermann *et al.* 2015; Jánosi *et al.* 2024).

PA-BoNT

To achieve the rapid and localized control of *C. botulinum* neurotoxin (BoNT), a photoactivatable form of BoNT serotype B (BoNT/B) LC protease was engineered (Liu *et al.* 2019). To this end, the *Avena sativa* AsLOV2-derived improved light-inducible dimerization system iLID (Guntas *et al.* 2015) was used for generation of a light-dependent reconstitution of BoNT/B, which was split into N- and C-terminal fragments. This resulting PA-BoNT could cleave significant fractions of VAMP2, yet some remaining uncleaved VAMP2 still enabled fusion of SVs. Thus, a variant of PA-BoNT was engineered that was directly targeted to SVs, vPA-BoNT. This was achieved by fusion of the C-terminal BoNT fragment to the C-terminus of synaptogyrin (SNG-1) and coexpressing it with the N-terminal BoNT fragment fused to iLID (Liu *et al.* 2019). Photoactivation of vPA-BoNT resulted in specific cleavage of VAMP2, at a spatial and temporal resolution comparable to the psd. Although PA-BoNT does not require constant illumination for long-term silencing, its recovery requires the de novo synthesis of SNAREs.

OptoSynC

To supplement the optogenetic toolbox with a light-activated neuronal silencing tool that allows for temporally and spatially precise silencing with a fast recovery time, optoSynC (optogenetic synaptic vesicle clustering) was developed. OptoSynC comprises 2 primary constituents: the *C. elegans* SV protein synaptogyrin SNG-1 and the *Arabidopsis thaliana* cryptochrome 2 (CRY2). While CRY2 serves as the photoactivatable element that enhances its capacity to form homo-oligomers after photoactivation, SNG-1 directs the tool to the SV membrane. Due to the abundant presence of SNG-1 in the SV membrane, fusing CRY2 to it enhances the likelihood of efficient SV clustering, due to increased avidity. Moreover, SNG-1 is dispensable for neuronal activity or synaptogenesis (Abraham *et al.* 2006). Since SNG-1 is not involved in the fusion process, optoSynC operates mechanistically differently from other optogenetic silencers, i.e. by achieving clustering of SVs in the reserve and recycling pools. Light-induced silencing of synaptic transmission using optoSynC was shown in different neuron classes and down to single neurons (Vettkotter *et al.* 2022). Formation of CRY2 homo-oligomers was facilitated by using CRY2olig(535) (Taslimi *et al.* 2016), a truncated variant with reduced dark activity [this term refers to background activity of some optogenetic tools that can occur already without illumination, e.g. for light-activated enzymes like bPAC; see Indirectly gating potassium channels with light (2-component optogenetics)]. Additionally, the E490G mutation enhances oligomerization (Taslimi *et al.* 2014). OptoSynC is highly light sensitive, has fast onset (only few seconds), and recovers within minutes.

Indirectly gating potassium channels with light (2-component optogenetics)

The use of constitutively active K^+ channels for silencing prompted the idea that this approach could also be used to generate regulatable tools, i.e. if K^+ channels could be acutely gated by the experimenter. One approach that was explored is the use of cyclic nucleotide–gated K^+ channels, e.g. the cAMP-gated K^+ channel SthK from *Spirochaeta thermophila*, or the cGMP-gated K^+ channel BeCNG1 from *Blastocladiella emersonii*. These channels could be combined with light-activated adenylyl and guanylyl cyclases, respectively. Such proteins, like *Euglena* or *Beggiatoa* photoactivated adenylyl cyclase (EuPAC or bPAC, respectively) or the engineered IlaC, as well as *Blastocladiella* or *Catenaria* nucleotidyl cyclase opsins (CyclOps) have been well established in *C. elegans* (Weissenberger *et al.* 2011; Ryu *et al.* 2014; Gao *et al.* 2015; Steuer Costa *et al.* 2017; Henss *et al.* 2022). The combination with SthK or BeCNG1 demonstrated light-activated K^+-mediated inhibition; however, these tools so far were either not very powerful: BeCNG1, its activation by BeCyclOp, both expressed in body wall muscle (BWM), caused a moderate body length increase of ca. 2% due to reduction of the muscle tone (note, the strongest effects evoked by Cl^- or K^+ conducting (channel)rhodopsins are ca. 8% body length increase; Zhang *et al.* 2007; Bergs *et al.* 2018). Or, the effects were too strong, since SthK, expressed in cholinergic motor neurons or in muscles, already responded to endogenous levels of cAMP, or background cAMP generated by bPAC in the dark; nonetheless, photoactivation of bPAC could evoke additional effects (Henss *et al.* 2022).

Histamine-gated chloride channel

Histamine is used as a neurotransmitter in many animal species; however, it is not found in *C. elegans* (Chase and Koelle 2007; Hobert 2013). Histamine receptors in insects, such as the *Drosophila* histamine-gated chloride channel HisCl1/2, function as ligand-gated chloride channels (Gisselmann *et al.* 2002; Liu and Wilson 2013). Because histamine evokes no endogenous effects in *C. elegans*, these characteristics render HisCl1/2 an exemplary tool for orthogonal neural inhibition, allowing for the selective silencing of neurons by hyperpolarization, without disrupting overall neural function. Pokala *et al.* (2014) demonstrated that these channels could be expressed in *C. elegans* neurons and that administration of exogenous histamine effectively inhibited activity of neurons expressing HisCl1/2. Various applications of the histamine-HisCl1 system facilitated studies on synaptic transmission through silencing neurons (Nelson *et al.* 2014; Hoerndli *et al.* 2015; Ghosh *et al.* 2016; Michelassi *et al.* 2017; Lopez-Cruz *et al.* 2019; Choi *et al.* 2021; Setty *et al.* 2022; Huang *et al.* 2023) or muscle cells (Ravi *et al.* 2018; Molina-García *et al.* 2020; Zhan *et al.* 2023). Compared to optogenetic silencers such as NpHR (Zhang *et al.* 2007; Bergs *et al.* 2018), this system provides significant convenience for prolonged neural inhibition, because it requires the presence of a chemical, not light. Yet, compared to optogenetic approaches, the HisCl1 system has limitations in assays requiring precise temporal control of neural (in)activity. Recently, HisCl1 has also been applied as a negative selection marker to facilitate generation of single-copy transgene insertions (Abiusi *et al.* 2017; El Mouridi *et al.* 2021; Mueller *et al.* 2023).

Targeted protein degradation via the auxin-inducible degron

Genetically encoded tools for targeted protein degradation (TPD) exploit endogenous protein degradation machineries, such as the ubiquitin–proteasome system, or the lysosome pathway, allowing researchers to exogenously induce protein degradation in a spatiotemporal manner. If this is applied to proteins of the synaptic transmission machinery, or ion channels required for neuronal depolarization, it may allow to silence neuronal function. This has been shown in some cases, targeting the UNC-31 protein, required for dense core vesicle fusion, or the UNC-7 gap junction subunit (Liu *et al.* 2017; Cornell *et al.* 2022). In this section, we discuss the development of auxin-inducible degron (AID), and its applications in exploring the function of synaptic proteins in *C. elegans*. For more comprehensive discussions on other TPD tools, readers are referred to previous reviews (Nance and Frøkjær-Jensen 2019; Zhang *et al.* 2022).

Auxins, a class of plant growth factors, are used in plants to regulate gene expression posttranslationally, by inducing the interaction of a degradation substrate, exposing the "auxin inducible degron" sequence, and the F-Box protein TIR1, eventually leading to protein degradation (Teale *et al.* 2006). In 2 pioneer studies (Nishimura *et al.* 2009; Kanke *et al.* 2011), the AID system was successfully transplanted from plants into cultured vertebrate cell lines and yeast. To this end, TIR1 needs to be expressed in the system of choice, and the AID target sequence needs to be introduced into the target as well. Zhang *et al.* (2015) employed the AID system in *C. elegans*, demonstrating rapid and reversible depletion of AID-targeted proteins. Limitations of the AID system, sometimes observed across various organisms, are leaky degradation, independent of the presence of auxin, and inefficient depletion (Natsume *et al.* 2016; Kerk *et al.* 2017; Daniel *et al.* 2018; Schiksnis *et al.* 2020). Additionally, auxins have been reported to induce undesired effects in worms, such as enhanced resistance to endoplasmic reticulum (ER) stress and increased lifespan (Bhoi *et al.* 2021; Loose and Ghazi 2021). Some approaches for refinement have been reported in mammalian cells, aiming to reduce basal degradation and to accelerate depletion (Li *et al.* 2019; Sathyan *et al.* 2019). Further improvements in *C. elegans*, as in the AID2 system, employed AtTIR1(F79G), the mini IAA7 degron, and 5-phenyl-indole-3-acetic acid (5-Ph-IAA) to reduce auxin-independent degradation and to enhance the loss-of-function phenotype of AID-targeted proteins (Uchida *et al.* 2018; Yesbolatova *et al.* 2020; Hills-Muckey *et al.* 2021; Negishi *et al.* 2022; Sepers *et al.* 2022). Xiao *et al.* (2023) established an expandable FLP-ON::TIR1 system, addressing the challenge that cell type–specific expression of TIR1 often results in less efficient protein degradation. The AID system was used to investigate loss-of-function phenotypes of various *C. elegans* proteins, including neuronal ones (Kerk *et al.* 2017; Liu *et al.* 2017; Yu *et al.* 2017; Shen *et al.* 2018; Zhang *et al.* 2018; Cornell *et al.* 2022; Stojanovski *et al.* 2023; Turner *et al.* 2023; Stefanakis *et al.* 2024).

Rhodopsin-based, light-controlled silencing

Microbial opsins are integral membrane proteins with 7 transmembrane helices found in a subset of archaea, bacteria, and even lower eukaryotes like algae. Acting as light-driven ion channels and pumps, these proteins either provide perception of electromagnetic radiation, which allows the organisms to adapt to their environment through phototaxis, or build up ion gradients to convert light energy into a chemical equivalent (Oesterhelt and Tittor 1989; Béjà *et al.* 2000). Absorption of photons triggers isomerization of the cofactor retinal and a conformational change of the protein, leading to either channel opening and passive ion transport or induction of pumping activity—a mechanism common to all microbial rhodopsins (Nagel *et al.* 2002).

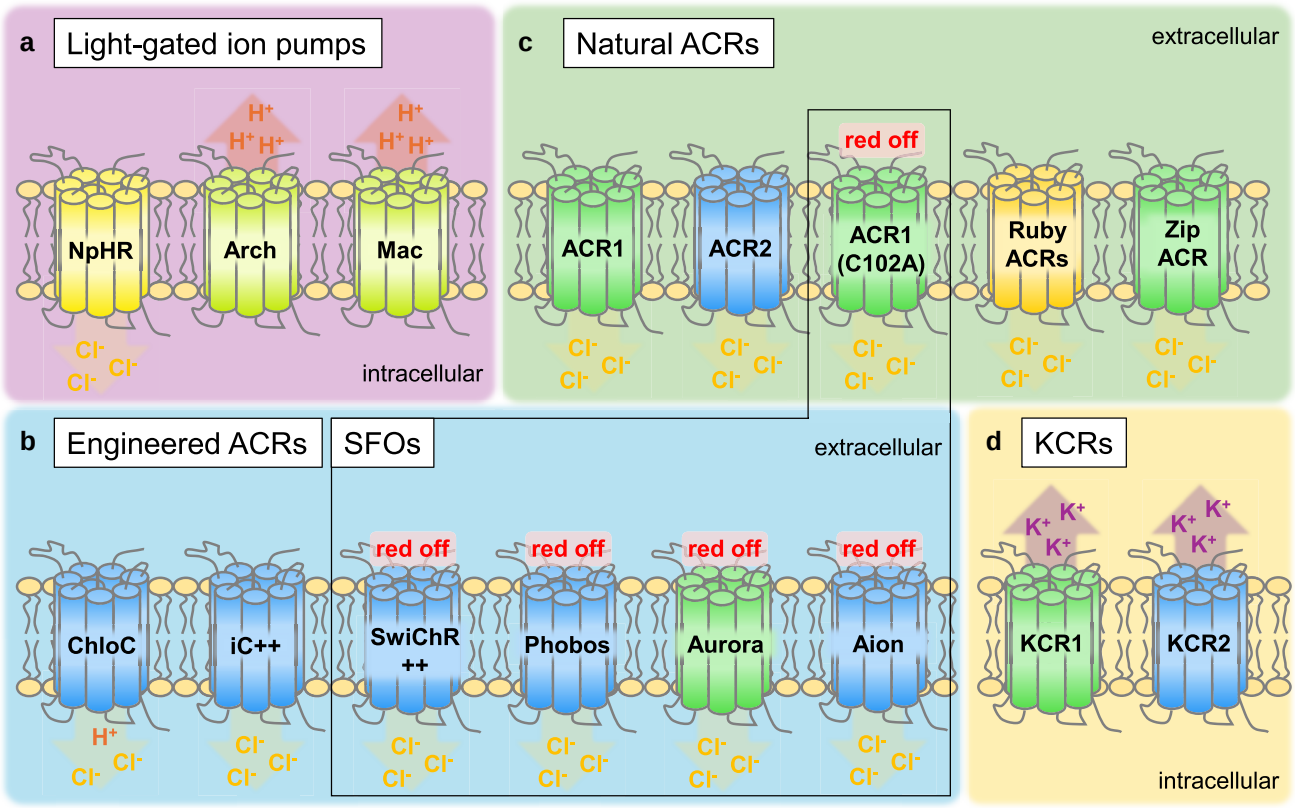

Fig. 2. Classes of rhodopsin-based optogenetic silencers. a) Light-driven ion pumps. b) eACRs and step function opsins for long-lasting photoinhibition. c) Natural anion channelrhodopsins. d) KCRs. Tools are color-coded according to their absorption maximum. Ions conducted or pumped, and direction based on electrochemical gradient, or pump vectoriality, are indicated.

Halorhodopsin

In optogenetics, the most used rhodopsin-based tool, channelrhodopsin-2 (ChR2), a depolarizing nonselective cation channel, is complemented by a growing repertoire of optogenetic silencers. Here, halorhodopsin (NpHR) from the archaeon *Natronomonas pharaonis* is one of the most frequently used light-driven ion pumps for hyperpolarization, which—when stimulated by yellow light—pumps chloride ions into the cell cytoplasm (Fig. 2a) (Han and Boyden 2007; Zhang *et al.* 2007). Just as ChR2 enables activation, halorhodopsins provide silencing of excitable cells, like neurons, following brief light stimuli with high spatio-temporal precision. Being spectrally distinct to ChR2 (absorption maximum at 470 nm as opposed to 590 nm for NpHR), multiplexing with NpHR is possible, providing independent access to both de- and hyperpolarization in the same cell. In contrast to channelrhodopsins, where absorption of 1 single photon allows the passage of many ions, for NpHR, only 1 ion is moved per photon (and photocycle), as is generally the case for light-driven ion pumps. Hence, plasma membrane localization is crucial to achieve robust light-induced effects. The initial accumulation tendency of NpHR in the ER was overcome for mammalian cells by adding ER export motifs resulting in the improved version eNpHR2.0, while limitations in membrane localization were countered by addition of Golgi export and membrane trafficking signals, then termed eNpHR3.0 (Gradinaru *et al.* 2008, 2010). *C. elegans* was the first model organism in which NpHR was established in vivo and in which triggered behavioral outcomes were characterized (Zhang *et al.* 2007). Here, muscle activity was probed in swimming and body length analysis assays, when NpHR was expressed from the *myo-3* promoter (BWMs), or indirectly, when expressed in cholinergic motor neurons, from the *unc-17* promoter. When stimulated by light, activation of NpHR immediately arrested swimming behavior and resulted in relaxation of muscles leading to increased body length of the animals by up to 7.5%. Patch-clamp recordings in BWMs of dissected animals revealed light-evoked outward currents of 265 pA. As mentioned above, combined expression of NpHR and ChR2 enabled to counteract ChR2-driven body contraction by activation of NpHR with yellow light. Further studies using NpHR in *C. elegans* demonstrated, among other effects, a significant decrease in the release of SVs upon light-induced hyperpolarization of cholinergic motor neurons (Liu *et al.* 2009). Furthermore, using NpHR allowed to remote-control temperature-seeking behavior in a bidirectional manner together with ChR2 (Kuhara *et al.* 2011) and helped to investigate the gentle touch circuit (Husson *et al.* 2012).

However, as the trafficking motifs mentioned above are not conserved, usage of improved NpHR versions did not overcome aggregation issues in the worm (Husson *et al.* 2012), which narrows the applicability of NpHR to cells with strong promoters. Thus, subsequent *C. elegans* studies established the light-activatable proton pumps Mac from *Leptosphaeria maculans* and archaerhodopsin (Arch) from *Halorubrum sodomense* as alternatives for optical silencing, where hyperpolarization is achieved via an extrusion of protons (Chow *et al.* 2010). The 2 inhibitory tools, maximally absorbing at 550 nm (Mac) and 566 nm (Arch), respectively, show improved plasma membrane expression and slightly higher photocurrents compared to NpHR (Husson *et al.* 2012). This way, it was possible to investigate the nociceptive ASH circuit, where Mac and Arch were used to interfere with downstream signaling while upstream neurons were stimulated by ChR2. An improved variant

of Arch, ArchT, was also implemented in *C. elegans* (Okazaki and Takagi 2013; Busack *et al.* 2020; Maluck *et al.* 2020).

Engineered anion channelrhodopsins

Since active transport restricts the utility of rhodopsin-based hyperpolarizing ion pumps, for which continuous and intense illumination is required, a lot of effort was devoted to invert the ion selectivity of ChR2 and turn it into a hyperpolarizing channel (engineered anion channelrhodopsins, eACRs; Fig. 2b). Substitution of Glu-90 in the central selectivity filter and near the retinal Schiff base led to a hyperpolarizing Cl^--conducting variant (ChloC) (Wietek *et al.* 2014). Additional point mutations within the proton pathway resulted in the variants iChloC (improved ChloC) and iC++ and eliminated the residual proton conductance of ChloC (Wietek *et al.* 2015; Berndt *et al.* 2016). Further introduction of point mutations within the so-called DC-gate improved conductivity and operational light sensitivity, as the closing kinetics of the channel were significantly decelerated (step function opsins). These attempts resulted in the slow-cycling variants SwiChR++ (Berndt *et al.* 2016), the color-tuned eACRs Aurora and Phobos based on red light–activatable ReaChR (Lin *et al.* 2013a) and the aforementioned iC++, respectively (Wietek *et al.* 2017). Most recently Aion with a closing time constant of around 15 min was engineered, based on Phobos (Rodriguez-Rozada *et al.* 2022). All these tools enable silencing without the need for continuous illumination and, moreover, allow to trigger channel closure by way of a second illumination pulse of longer wavelength.

Natural anion channelrhodopsins

Inspired by the determinants of Cl^- conductance as found during molecular engineering of existing ChRs, researchers performed BLAST searches of known genomes. This revealed naturally occurring light-activatable Cl^--conducting ChRs in the cryptophyte *Guillardia theta* that were then termed anion channelrhodopsins (ACRs, Fig. 2c). Optimized by evolution, the first 2 described tools—*Gt*ACR1 and *Gt*ACR2—proved to be superior to both eACRs and depolarizing ChR2 in terms of steady state current amplitudes (Mahn *et al.* 2018). While ACR1 exhibits larger plateau currents at an absorption maximum of 515 nm, ACR2 demonstrated faster kinetics, maximally absorbing at 470 nm. Due to their exceptional high light sensitivity and superior conductance, ACRs finally constitute equal rank to the most powerful depolarizing tools in the inhibitory range for optogenetic applications. Further screening studies identified 2 additional ACRs with fast kinetics, namely, ZipACR (from *Proteomonas sulcata*) and RapACR (from *Rhodomonas salina*), that showed even higher photocurrents, enabling up to 100 Hz spike suppression (Govorunova *et al.* 2017, 2018). Furthermore, RubyACRs (from *Labyrinthulea*) were identified: These are spectrally red-shifted with absorption maxima at 590–610 nm (Govorunova *et al.* 2020). ACRs have been proven to function as potent neural inhibitors in various model animals (Mauss *et al.* 2017; Mohamed *et al.* 2017; Mohammad *et al.* 2017; Mahn *et al.* 2018).

In *C. elegans*, ACR1, ACR2, and ZipACR, as well as the slow-closing (step function) variant ACR1(C102A), were first introduced into BWMs and characterized in a comparative study using behavioral readout (Bergs *et al.* 2018). In body length analyses, upon light stimulation, ACR-expressing animals demonstrated exceptional effects at low light intensities that could be repetitively induced with high stability. Patch-clamp recordings revealed large peak photocurrents of up to 1530 pA (ACR2)—5–10 times larger compared to NpHR. Further *C. elegans* studies applied ACRs to neurons of locomotory and behavioral regulatory circuits (Tolstenkov *et al.* 2018; Xu *et al.* 2018; Aoki *et al.* 2023) or tested their applicability for long-term inhibition (Yamanashi *et al.* 2019). In addition to that, ACR2 was used in a tandem configuration together with the red light–activatable depolarizer Chrimson (termed BiPOLES) and combined with the genetically encoded voltage indicator QuasAr2 to generate an optogenetic voltage clamp in live worms (Bergs *et al.* 2023).

Potassium channelrhodopsins

Although ACRs are highly effective and reversible silencers of excitable cells (Govorunova *et al.* 2015; Mohammad *et al.* 2017), they have certain limitations, especially in cells (e.g. cardiomyocytes) or subcellular compartments (e.g. axons) with high intracellular chloride concentrations, where they may cause depolarization (Mahn *et al.* 2016; Malyshev *et al.* 2017; Ott *et al.* 2024). Moreover, upon prolonged gating of their artificial chloride conductance, they trigger unwanted and long-lasting downstream effects, putatively due to the exhaustion of the endogenous Cl^- gradients and the time it takes the cell to recover them (Bergs *et al.* 2018).

Since repolarization of membrane potentials generally happens through efflux of K^+ via voltage-gated potassium channels (Hodgkin and Huxley 1952; Johnstone *et al.* 1997; Jan and Jan 2012), inhibition of depolarization may occur by artificial manipulation of K^+ currents. To achieve this, major efforts have attempted to generate K^+-selective light-gated channels for example by fusion of light-reactive elements such as the LOV2 domain or coexpression of K^+ channels with light-absorbing activators (Alberio *et al.* 2018; Bernal Sierra *et al.* 2018) [see Indirectly gating potassium channels with light (2-component optogenetics) for a discussion of the use of the SthK channel in *C. elegans*]. Engineered K^+-selective light-gated channels have major drawbacks, namely, poor surface expression (Cosentino *et al.* 2015), slow kinetics, or unwanted activation of cellular signaling pathways (Beck *et al.* 2018; Henss *et al.* 2022). Therefore, the recent discovery of natural light-gated channels with a high selectivity of K^+ over Na^+ proved a significant improvement for precise inhibition of excitable cells (Govorunova *et al.* 2022) (Fig. 2d). These K^+-selective channelrhodopsins [potassium channelrhodopsins (KCRs)] have been shown to efficiently inhibit action potentials upon illumination in cultured mammalian neurons and cardiomyocytes.

Structural analysis of the novel K^+ channel signature motif in pore-lining amino acid residues of the *Hyphochytrium catenoides* KCRs (HcKCR1/2) led to the discovery of improved variants with even higher K^+-selectivity by either phylogenetic analysis (WiChR; Vierock *et al.* 2022) or point mutation (Tajima *et al.* 2023). A recent study has also shown the capabilities of KCRs in small model organisms in which they enabled silencing of cells with high intracellular chloride in which ACRs were ineffective (Ott *et al.* 2024). Yet the study only briefly assessed their use in *C. elegans*. Here, KCRs were expressed pan-neuronally and not selectively in stimulatory cholinergic or inhibitory GABAergic neurons in which measurement of body length could indicate de- or hyperpolarization (Liewald *et al.* 2008). The observed impact on crawling speed may also arise from depolarization and thus hypercontraction of muscle cells (Tolstenkov *et al.* 2018). Nevertheless, the results obtained from *Drosophila melanogaster* promise rapid and reversible inhibition of excitable cells in invertebrates (Ott *et al.* 2024). While KCRs seem to be auspicious tools for many applications, the residual Na^+ conductivity is substantial. The KCR with the currently highest relative permeability ratio of K^+ over Na^+ of 80 (WiChR; for stationary photocurrents) still conducts significantly more Na^+ than mammalian voltage–gated

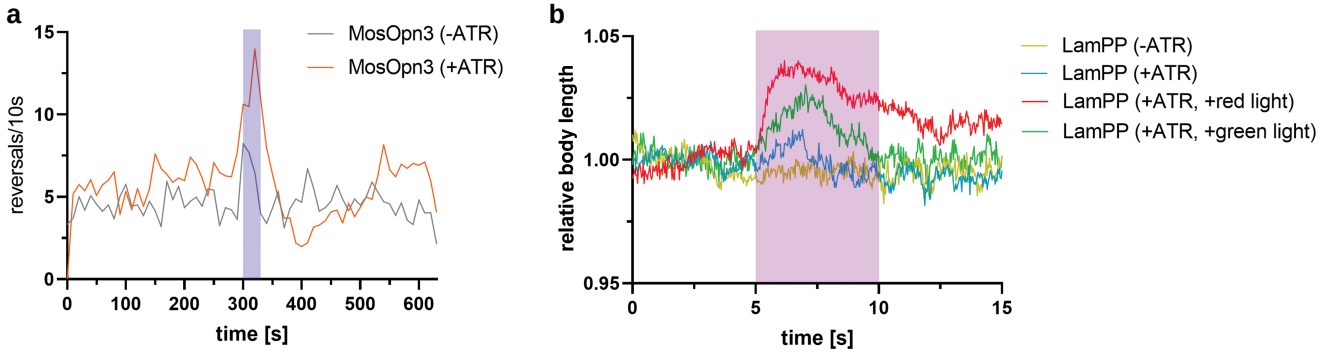

Fig. 3. Examples of light-sensitive GPCRs (rhodopsins), used for inhibition: a) light-evoked reversals of animals expressing MosOpn3 in ASH neurons. The multiworm tracker (Swierczek *et al.* 2011) was used to observe the crawling behavior of worms expressing MosOpn3 in ASH neurons. Upon blue light illumination (30 s, 100 µW/mm^2, 470 nm, shaded area, 300–330 s), worms treated with ATR (100 µM) showed an increase in reversals, indicating an avoidance response upon MosOpn3 activation. b) LamPP activation in cholinergic neurons induces a wavelength-dependent increase of body length, due to muscle relaxation. Body length of animals either without ATR (–), or treated with ATR (+), were compared. Additionally, animals treated with ATR were either kept in the dark until the measurement, or preilluminated with red light (620–660 nm; overnight), or illuminated with green light (520–535 nm) for 30 s prior to the start of the experiment. Violet light illumination (5 s, 100 µW/mm^2, 373–387 nm, shaded area, 5–10 s) of animals without ATR evoked no change in body length, while ATR-treated worms exhibited a slight increase. To convert the active ATR-bound state back to its inactive state, worms were illuminated with green light before the start of the measurements, resulting in a body length increase upon subsequent violet light illumination. To test whether red light illumination of the LamPP active state causes photoregeneration, worms were fed ATR under red light illumination overnight. These animals showed an increase in body length upon violet light illumination.

potassium channels (P_K/P_{Na} ~100–1000; LeMasurier *et al.* 2001; Mironenko *et al.* 2021). This may pose a problem in some cases and must be remembered when using these tools, especially when applying continuous stimulation, working with a highly negative membrane potential or in conditions with high external Na^+ (Govorunova *et al.* 2022; Vierock *et al.* 2022).

Inhibition via light-activated G protein-coupled receptors (animal rhodopsins) with $G_{i/o}$ coupling specificity

Reversible regulation of cellular activity can also be achieved through G protein–coupled receptors (GPCRs). Different light-reactive, inhibitory GPCRs have been previously expressed and characterized in neurons of *C. elegans*. One of the most commonly used inhibitory GPCRs is bovine rhodopsin (bRho), which endogenously couples to transducin, a $G\alpha_{i/o}$-type protein, although previous studies have shown that bRho is also able to activate other G proteins of the same family (Kanaho *et al.* 1984). Cao *et al.* (2012) demonstrated that pan-neuronal expression and illumination of bRho in *C. elegans* efficiently inhibits locomotion, the effect of which is relatively long-lasting compared to the duration of the stimulus, as 1 s high-intensity light stimulation was sufficient to fully inhibit movement of the animals for ca. 20 min. Normal crawling behavior resumed after ca. 80 min. Analysis of various mutant strains provided evidence that the signaling occurs selectively through GOA-1, the *C. elegans* $G\alpha_{i/o}$ ortholog, and is dependent on cAMP-specific phosphodiesterases (Cao *et al.* 2012).

Expression and activation of a different GPCR, mosquito Opn3 (MosOpn3), in ASH neurons elicited light-dependent avoidance behavior (see Fig. 3a for quantification of light-evoked reversals of animals expressing MosOpn3 in ASH), which is in agreement with activation of ODR-3-dependent signaling, a *C. elegans* G protein which shows similarity to members of the $G_{i/o}$ family. MosOpn3 is bleach-resistant, which could enable repeated stimulation with less reduction in the intensity of the response (Koyanagi *et al.* 2022). Similar effects were shown by these authors when bRho was expressed in ASH, arguing that it is a $G_{i/o}$-coupled GPCR. ASH neurons can also be photoactivated by channelrhodopsin (Schmitt *et al.* 2012), a depolarizing light-gated channel, indicating that there must be a "sign inversion" of GPCR-induced signals downstream of ODR-3, leading to depolarization of the neuron. Hilliard *et al.* (2005) also showed that repellent-induced Ca^{2+} signals in ASH were diminished in *odr-3(n2150)* mutants. Earlier work has shown that the TRP channels OSM-9 and OCR-2 are required for Ca^{2+} transients and depolarization of ASH (Colbert *et al.* 1997; Tobin *et al.* 2002). How exactly ODR-3 signaling, supposedly via lipid mobilization of polyunsaturated fatty acids (PUFAs), activates OSM-9/OCR-2 (Kahn-Kirby *et al.* 2004) is not well understood, and whether ODR-3 is a genuine $G_{i/o}$ protein, i.e. inactivating adenylate cyclase, has not been shown in vitro. bRho requires supplementation of 11-cis-retinal, or as a substitute, 9-cis-retinal, for its activity in *C. elegans* neurons. In contrast, MosOpn3 is functional both in its 11-cis-retinal- and its 13-cis-retinal-bound form. Thus, it is also active when all-*trans*-retinal (ATR) is fed to the worms as it is in equilibrium with the 13-cis isomer (Cao *et al.* 2012; Koyanagi *et al.* 2022).

The same study also investigated the UV-sensitive bistable lamprey parapinopsin (LamPP) in *C. elegans*. The peculiarity of LamPP is that it exhibits large spectral differences between the inactive and active state, allowing sustained $G_{i/o}$-pathway activation and controlled deactivation (Koyanagi *et al.* 2004; Eickelbeck *et al.* 2020). Thus, expression of LamPP in *C. elegans* cholinergic motor neurons allowed color-dependent control of behavior (Koyanagi *et al.* 2022). We could show that activation of LamPP also induces color-dependent changes of body length (Fig. 3b). Red light illumination of the LamPP active state led to photoregeneration and a state with an absorption maximum almost identical to the dark state (Koyanagi *et al.* 2004). Hence, worms were fed under red light, which resulted in relaxation, and body length increase upon subsequent violet light exposure (Fig. 3b). Green light, right before the measurement, which should convert LamPP back to its active state, resulted in reduction of crawling upon illumination with violet light and a significant increase in body length, which returned to baseline levels. However, most animals showed reduced locomotion during the entire duration of observation, indicating continued activity and $G_{i/o}$ signaling in cholinergic motor neurons.

Another rhodopsin mediating inhibition in *C. elegans* was recently described, the *Platynereis dumerilii* opsin (PdCO), which is a

bistable opsin that can be turned on and off with 405 and 525 nm light, respectively (Wietek *et al.* 2024). PdCO was expressed pan-neuronally, and as it couples to $G\alpha_o$, and through $G\beta/\gamma$ may activate GIRK (G-protein activated, inward rectifier potassium) channels, caused rapid and reversible reduction of locomotion speed by ca. 75%, in an ATR-dependent manner.

An approach related to activation of inhibitory GPCRs has been recently introduced by Lockyer *et al.* (2023). The authors could inhibit $G\alpha_q$ signaling in *C. elegans* neurons as well as in other organisms by using an engineered version of a mammalian $G\alpha_q$-specific regulator of G protein signaling (RGS2), modified with a cryptochrome CRY2 domain, that could be recruited to plasma membrane-bound CIBN protein in a light-induced manner. Using this so-called CRY2-CIB1 system (Kennedy *et al.* 2010), the authors were able to reduce motility of the worms in a light-dependent way (Lockyer *et al.* 2023).

Activation of $G_{i/o}$ protein signaling for cellular inhibition is useful especially when intending to target specific intracellular pathways, inhibiting activity without directly affecting membrane potential, or to mimic endogenous GPCR-induced inhibitory processes. GOA-1 was found to be expressed in all or nearly all neurons in *C. elegans* (Mendel *et al.* 1995); thus, the potential application of GOA-1 addressing tools is not limited to only some cells. However, due to the potentially different intracellular pathways in different cell types, being mediated and amplified by various intracellular effectors, the effects as well as the mechanism of inhibition might differ slightly when a light-reactive GPCR is utilized in different cells. Hence, results obtained with such tools, as well as their G protein coupling preference, need to be evaluated carefully for the cell type under study.

Silencing by optogenetic activation of inhibitory neurons

Another intriguing possibility to inhibit neuronal or muscle function is through activation of upstream inhibitory neurons, for example, by expression of cation ChRs (CCRs) in GABAergic motor neurons (Liewald *et al.* 2008; Bergs *et al.* 2018). GABA release upon CCR gating will lead to an inhibition of BWMs and thus relaxation in animals treated with ATR (Schultheis *et al.* 2011; Lu *et al.* 2022). Next to directly targeting synaptic transmission by optogenetics, release of inhibitory neuromodulators from interneurons may be triggered to achieve a widespread and long-term inhibition of excitable cells. Examples include the photo-evoked release of FLP-11 neuropeptides from the RIS neuron to inhibit motor neurons or the release of serotonin from sensory neurons to inhibit interneurons (Flavell *et al.* 2013; Steuer Costa *et al.* 2019). The major advantage of this approach is the use of endogenous transmission to study the effect of inhibition in a more physiological paradigm. However, in order to draw causal conclusions, one must keep in mind the complexity, interconnectivity, and feedback mechanisms of most neuronal signaling pathways, which requires combining optogenetic experiments with a range of other assays and (genetic) manipulations, as exemplified in this work by Sordillo and Bargmann (2021) and further summarized in a review by Fang-Yen *et al.* (2015).

Conclusions

The range of methods and approaches to inhibit or eliminate (excitable) cells in *C. elegans*, or individual proteins within these cells, is very broad, and it may thus be challenging to choose the right method. However, this should be decided based upon the respective process or cell type that is targeted and whether the manipulation needs to be fast, i.e. to observe acute effects, or to avoid compensatory changes to occur. Also, it may be important to consider whether the manipulation needs to take place during or following the end of development, e.g. depending on whether the cell or protein targeted is essential for the same. While some of these methods can be applied to single or few cells at a time, other methods, which include a genetically encoded tool, allow to eliminate entire cell types and can be applied in populations of animals. For some methods, temporal control of the moment of inhibition or ablation is poor, i.e. defined by the time of expression, while others can be precisely timed by administration of a chemical compound, or with light. Some methods are permanent, others are immediately reversible, and even others require long recovery times, e.g. when de novo protein synthesis is needed. It may be advisable to test more than one approach to achieve the desired outcome. We hope this review provides readers with a good basis for their decision.

Data availability

Strains and plasmids are available upon request, as far as they have been generated by the authors or former Gottschalk lab members. The authors affirm that all data necessary for confirming the conclusions of the article are present within the article, figures, and tables.

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Conflicts of interest

The authors declare no conflicts of interest.

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