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Genetically encoded neural activity indicators

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ABSTRACT

Recent years have witnessed the fascinating development of imaging approaches to studying neural activities; this progress has been based on an influx of ideas and methods from molecular biology and optical engineering. Here we review the design and application of genetically encoded indicators for calcium ions, membrane potential and neurotransmitters. We also summarize common strategies for the design and optimization of genetically encoded neural activity indicators.

KEYWORDS

calcium imaging, neural activity, membrane potential probe, neurotransmitter reporter

1 Introduction

Imaging the activity of a specific group of neurons in real time has long been a goal of neuroscientists. Many chemical indicators have been developed to monitor neuronal activities by converting intrinsic parameters of neuronal activity into optical signals such as fluorescence changes.

A major goal of neuroscience is to determine the precise functions of different neuronal cell types [1, 2]. However, except for a few semi-genetically encoded indicators, chemical indicators are unable to target specific cell types in the living mammalian brain [3]. Furthermore, the loading procedure has limited the usage of chemical indicators. Genetically encoded neural activity indicators (GENAIs) are a class of biological molecules that can indicate neural activity, usually through changes in fluorescence. Compared with synthetic chemical indicators, GENAIs can be expressed in cells, thus facilitating long-term observation of individual cells [4] while enabling the targeting of specific cell types through genetic techniques.

GENAIs can be classified into several groups according to their usage (Fig. 1):
Figure 1 Classification and common strategies of neural activity indicators. (a) GENAIs can be classified into calcium indicators (GECIs), voltage indicators (GEVIs) and synaptic transmission indicators (GESTIs) by their target physical parameter. These signals could be transformed into fluorescence signal by (b) amplifying the signal induced structural variation by conformation-sensitive fluorescence protein such as circularly permuted FP (cpFP, upper) or fluorescence resonance energy transfer (FRET, lower). (c) Hybrid neurotransmitter indicator CNiFER senses its ligand by GPCR and indirectly reports such signal by genetically encoded calcium indicator TNXXL. (d) Archaerhodopsin based GEVI could directly transform the voltage variance signal to optical signal without other additional structure.

(1) Genetically encoded calcium indicators (GECIs), which sense the concentration of Ca²⁺ ions, a second messenger that faithfully indicates neural activity;
(2) Genetically encoded voltage indicators (GEVIs), which can be used to visualize changes in neuron membrane potential.
(3) Genetically encoded synaptic transmission indicators (GESTIs), which sense the presence of common neurotransmitters and provide a fluorescence readout.

GENAIs usually indicate neural activity through fluorescence changes. Because most signals generated by GENAIs are recorded by optical systems, either traditional cameras or sophisticated microscopes, optical performance is the most critical feature of GENAIs. Some important factors are summarized below (Fig. 1).

Signal intensity and signal-to-noise ratio. The contrast of an image is proportional to the intensity difference ΔI between two image areas, divided by the average image brightness I, as shown in the following equation:

\[ C = \frac{I^1 - I^0}{I} = \frac{\Delta I}{I}. \]

In fact, contrast is often the only output that can be measured from a GENAI, because normalizing the captured intensity to an absolute level of neural activity is difficult. Neural activities are usually recorded as ΔF/F, the ratio of the fluorescence intensity to the average recorded intensity. Without a high-intensity fluorescence signal source, the measured neural activity is easily overwhelmed by the noisy background: the signal I may be too weak to be distinguished from background noise. In addition, GENAIs themselves must maintain a high signal-to-noise ratio (SNR) to distinguish their on and off states. For a neural indicator designed for in vivo recording, labeling specificity also substantially contributes to the final contrast: poor labeling specificity or densely labeled neurons lead to high background and neuropil contamination. GENAIs combined with selective expression methods and sparse labeling techniques can circumvent this problem and therefore are especially useful in recording axonal or dendritic activities.

Temporal resolution. Real-time imaging of certain biological processes requires rapidly responding indicators. In many attempts to improve GENAIs, the major focus has been enhancing temporal resolution. If the temporal resolution of the indicators is insufficient, information loss occurs and may preclude certain experimental designs.

Dynamic range. Dynamic range (DR) refers to the range of target physical values that can be measured by a GENAI. In most cases, DR is limited at one end of the range by saturation and at the other end by random noise or uncertainty in signal levels, which are often referred to as
the sensitivity of an indicator. If the DR of the indicator cannot cover the entire range of a changing biological parameter, the indicator is unable to perfectly record the entire biological process.

**Photostability.** For most GENAIs using fluorophores as an output, the readout relies on fluorescence excitation by a light source. Although techniques such as non-linear multiphoton excitation can partially decrease phototoxicity and the chance of photobleaching, photostability remains a key factor for fluorophores used in long-term observation. Beside the techniques involving decreasing the photon energy of the input light or the intensity or time-span of light exposure, indicator design using more robust fluorophores that are less prone to bleaching is another practical method to obtain high photostability.

Although all GENAIs have the same goal in transforming neural activity into measurable optical changes, different strategies have been used to achieve this goal (Fig. 1). For most commonly used GENAIs, the design principle is based on either amplifying the variance of the protein structure under changing physical parameters through conformation-sensitive fluorescence protein (cpFPs, e.g., cpGFP) or fluorescence resonance energy transfer (FRET), or through a hybrid sensor system that transcodes the target parameter representing neural activity into another physical form that is easier to visualize. Conformation-sensitive fluorescent proteins can transcode changes in the conformations of other protein domains in response to neural activities into changes in the absorption and emission properties of fluorophores. The most common GFP-based biosensor in this class relies on a circularly permuted GFP (cpGFP) developed by Baird et al. [5]. As shown in Fig. 1(b) (upper), the calmodulin (CaM) and M13 domain in a GCaMP molecule, a widely used calcium indicator, switches between non-binding (left) and binding conformations (right), and this change triggers fluorescence emission by cpGFP.

FRET, another way to amplify the conformational changes of protein domains (Fig. 1(b), lower), involves energy transfer between two light-sensitive molecules (chromophores) [6]. A donor chromophore, initially in its excited electronic state, may transfer energy to an acceptor chromophore through nonradiative dipole-dipole coupling. Unlike intensity-shift indicators, most FRET-based indicators change in spectrum when they are activated. FRET is highly sensitive to the relative orientation of chromophores as well as the distance between them [7]. However, FRET-based neural activity indicators require at least two fluorophores, and the relatively large sizes of fluorescent protein domains sometimes hinder expression vector construction and protein trafficking.

Some hybrid genetically encoded neural activity indicators function indirectly through transcoding the target physical parameter to be measured into an intermediate form, which in turn is transformed into an optical signal. These indicators often consist of a pair of separated sensing and reporting units working cooperatively. **Figure 1(c)** shows examples of the hybrid sensor strategy: a calcium indicator indicates acetylcholine (ACh) signal indirectly through its increasing fluorescence intensity in response to a calcium signal produced by the ACh receptor pathway [8]. Some other hybrid sensors use synthetic dye to label the genetically encoded tag. However, use of this class of hybrid sensors is limited, owing to the relatively low SNR and low tissue penetrance of synthetic dye [9]. Beyond these categories, some other genetically encoded indicators can directly convert activity signals to fluorescence output. These indicators are mainly archaeorhodopsin or green-absorbing proteorhodopsin (GPR) based; examples include Archer and QuasAr voltage indicators.
2 Genetically encoded calcium indicators

Quantification of free [Ca\textsuperscript{2+}] changes can be used as a reliable proxy for neural activity, because dramatic changes result from action potential firing and synaptic transmission. Chemical calcium indicators can sense the concentration changes of Ca\textsuperscript{2+} ions, thus indirectly indicating neural activity. In early practice, such quantification was widely used in functional in vivo imaging; however, the complicated loading process and lack of cell-type specificity have strongly limited its use. As an alternative, GECIs can be easily expressed in a specific group of neurons through genetic techniques and subsequently enable long-term observation, as do other GENAIs (Fig. 2).

Many GECIs use CaM as a sensing module (GCaMP [9–13], Pericams [14], Cameleons [15] and FIP-CB\textsubscript{SM} [16]), whereas most of the remaining GECIs use TnC, the skeletal muscle troponin C (TN-L family [17–19], Twitch’s [20]) to indicate changes in Ca\textsuperscript{2+} concentration (Table 1).

2.1 Calmodulin based calcium indicators

CaM has a relaxed linear structure in the absence of Ca\textsuperscript{2+} binding (Fig. 2). As the concentration of cellular calcium increases, calcium binding CaM moves closer to M13, and the conformation of chimeric proteins switches from a dumbbell-like extended structure to a compact globular structure [21]. This conformational change in protein structure can be transcoded into changes in signal intensity by cpEGFP or FRET. The GCaMP family is the best-known CaM-cpEGFP based indicator. Its improved versions, the GCaMP6 series, are able to monitor synaptic calcium transients in individual dendritic spines over the course of weeks [11]. The most recently described member of the GCaMP family, the jGCaMP7 has several derivatives for specific purposes: jGCaMP7f (“fast”) has an improved SNR and fast kinetics, whereas jGCaMP7s (“sensitive”) has slower kinetics but higher sensitivity; jGCaMP7b (“baseline”) has a high fluorescence baseline for neuropil imaging, and jGCaMP7c (“contrast”).

![Figure 2](https://mc03.manuscriptcentral.com/brainsa) Genetically encoded calcium indicators. (a) A cpFP-calmodulin based GECI, the calcium binding cause structural variance of CaM and M13 domain and amplified through a circularly permuted GFP. (b) FRET-calmodulin based GECI, the calcium-induced structural variance is reported by a FRET pair. (c) Skeletal muscle troponin C (TnC) based GECI, the calcium-induced structural variance is reported by FRET between two fluorescent protein domains.
has a very low baseline fluorescence level (Dana et al. https://web.archive.org/web/20180912222414/https://www.janelia.org/jgcamp7-calcium-indicators). To decrease scattering and absorption in in vivo imaging, red-shifted variants of calcium indicators based on the same concept have also been developed [22–24]. Among them, cp-mApple based indicators have the drawback of photo-switching problems when they are illuminated with blue light, whereas cp-mRuby based indicators are more compatible for simultaneous use with ChR2 [23].

Beyond the cpFP based CaM-M13 indicators, another important class of widely used calcium signal indicator tools is a series of fluorescent indicators called Cameleons (Fig. 2). Cameleons consist of two fluorophores surrounding a CaM domain and an M13 domain. When these domains are brought together through CaM-M13 interaction in presence of Ca\(^{2+}\), FRET between the blue or cyan mutant GFP and the green or yellow GFP dramatically increases and indicates calcium concentration changes and neural activity [15, 23, 25].

### 2.2 Troponin C based calcium indicators

Skeletal muscle TnC based calcium indicators are another large family of GECIs (Fig. 2). Most TnC based GECIs use FRET as their reporting domain. In some cases, CaM based GECIs have insufficient calcium sensitivity or have a decreased dynamic range in certain targeting experiments [17]; in such cases, TnC based calcium indicators are an acceptable alternative.

### 2.3 Specialized GECIs

Some application-specific GECIs have also been developed in recent years. By combining the photo-regulated features of photoactivatable (pa) or photoconvertible (pc) FPs with allosterically modulated cpFPs, Hoi et al. developed a “highlightable” GECI that can convert output spectra by illumination [26]. Through library screening and structure-guided mutagenesis, Fosque et al. further developed a calcium modulated photoactivatable ratiometric integrator (CaMPARI), which can provide a snapshot of activated neurons by permanently marking activated indicator molecules under violet light illumination. With short timescale violet light illumination, CaMPARI can be used to record neuron activity, thus providing a powerful tool for optical functional mapping [27].

Because CaM based GECIs such as GCaMPs may interfere with gating and signaling of L-type calcium channels and therefore disrupt Ca\(^{2+}\) dynamics and gene expression, Yang et al. developed an improved indicator, GCaMP-X, by incorporating an additional apoCaM-binding motif. GCaMP-X exhibits far fewer side-effects than regular GCaMP indicators while retaining good calcium-sensing properties [28]. Because many GECIs use CaM as their sensing domain and may have the same drawbacks, this work may inspire the creation of a series of GECIs with few side-effects.
3 Genetically encoded voltage indicators

GEVIs indicate neural activities by allowing for visualization of neuronal membrane potential (Fig. 3). The first generation GEVIs, including FlaSh [29], SPARC [30] and VSFP1 [31], are based on voltage-gated ion channels. In 1997, Siegel and Isacoff described the design of the first GEVI. By fusing a C-terminally disordered GFP into a voltage-sensitive Shaker K⁺ channel, they produced an indicator that shows fluorescence changes in response to potential changes [29]. In 2001, Sakai et al. designed VSFP1, which is based on FRET of a pair of GFP mutants (CFP and YFP) fused to a potassium channel. The FRET efficiency of the chromophore dipole changes when a potential change causes the S4 of the channel to rotate and changes the orientation between chromophores [30]. In 2002, Ataka and Pieribone designed SPARC by inserting GFP into a sodium channel [30]. These early GEVIs showed potential but had poor membrane targeting performance [32, 33].

3.1 Voltage-sensitive fluorescent proteins

A major class of GEVIs, the voltage-sensitive fluorescent proteins (VSFPs), including VSFP2 and its variants, are based on the paddle domain of voltage-gated phosphatase (VSP), mainly Ci-VSP (Fig. 3). In 2007, Dimitrov et al. designed VSFP2.1 by using Ci-VSP and a fused FRET pair, which is similar to VSFP1 but exhibits efficient membrane targeting [34]. In 2008, Lundby et al. reported VSFP3.1, which is monochromatic and replaces the slow FRET-based fluorescence reporting in VSFP2s with voltage-dependent fluorescence intensity [1, 2]. In 2012, Jin et al. described ArcLight indicators, which have substantially improved ΔF/F and consequently better voltage sensitivity [35]. However, the kinetics of ArcLight variants, even that of their fast components, is still relatively slow. In 2014,
Pierre et al. introduced ASAP1, in which a different VSP from chicken is used. They chose a VSD from *Gallus gallus* because of its shorter S3-S4 loop, and they fused a cpGFP into *Gallus gallus* VSD. Their results showed that ASAP1 has ultrafast temporal resolution and can track spike trains of up to 200 Hz (Table 2) [36].

### 3.2 Opsin-based voltage indicators

Another class of GEVIs using an opsin-based mechanism was introduced after VSFPs (Fig. 3). The first prototype, PROPS, was described by Kralj et al. in 2011 in *E. coli*. PROPS is based on a GPR in bacteria that contains a Schiff base moiety with pH-dependent fluorescence. PROPS shows different fluorescence intensity at different induced transmembrane voltages [39]. However, PROPS does not localize to the eukaryotic membrane and cannot function as a GEVI [40]. Subsequently, Kralj et al. introduced Arch D95N, based on Arch, in which bacterial GPR is replaced with Archaerhodopsin from *Halorubrum sodomense*. The photocurrent in wild-type Arch is eliminated in Arch D95N, and this improved version can be used in mammalian neurons [40]. The fluorescence of original Arch must be excited

**Table 2** Summary of genetically encoded voltage indicators.

| GEVI name       | Year | Sensor                  | Reporter** | ΔF / F (%) | t<sub>on</sub> (ms) | t<sub>off</sub> (ms) | References   |
|-----------------|------|-------------------------|------------|------------|----------------------|----------------------|--------------|
| FlaSh           | 1997 | Shaker (K⁺ channel)     | GFP        | −5.1       | 85                   | 160                  | [29]         |
| VSFP            | 2001 | Rat Kv2.1 (K⁺ channel)  | CFP/YFP    | 1.8        | 0.74                 |                      | [31]         |
| SPARC           | 2002 | Rat Na⁺ channel         | GFP        | ~0.5       | 0.8                  |                      | [30]         |
| VSFP2.1         | 2007 | Ci-VSP                  | CFP/YFP    | 8.6        | 15                   | 75                   | [34]         |
| VSFP3.1         | 2008 | Ci-VSP                  | CFP        | ~0.6       | 1.3                  |                      | [37]         |
| Mermaid         | 2008 | Ci-VSP                  | mUKG/mKOx  | ~28        | 11.8                 | 70                   | [38]         |
| PROPS           | 2011 | GPR                     | Same as left| 150       | 4.7                  |                      | [39]         |
| Arch            | 2011 | Archaerhodopsin 3       | Same as left| 40        | 0.5                  |                      | [40]         |
| Arch D95N       | 2011 | Archaerhodopsin 3       | Same as left| 60        | 41                   |                      | [40]         |
| ArcLight Q239   | 2012 | Ci-VSP                  | pHluorin   | ~39        | ~21’                 | ~22’                | [35]         |
| ElectricPk      | 2012 | Ci-VSP                  | cp-EGFP    | −1.2       | 2.24                 | 2.09                 | [41]         |
| VSFP-Butterfly 1.2| 2012 | Ci-VSP                  | mCitrine/mKate2 | 4.2 |                      |                      | [42]         |
| Mermaid2        | 2013 | Ci-VSP                  | mUKG/mKOx  | 48.5       | ~1.6’                | 10.3                 | [43]         |
| MacQ-mCitrine   | 2014 | *L. maculans* rhodopsin | MacQ/mCitrine| ~20        | ~6.5’                 | ~9.6’                | [44]         |
| QuasAr1         | 2014 | Archaerhodopsin 3       | Same as left| 32        | ~0.064’               | ~0.10’              | [45]         |
| QuasAr2         | 2014 | Archaerhodopsin 3       | Same as left| 90        | ~2.5’                 | ~1.7’                | [45]         |
| Archer1         | 2014 | Archaerhodopsin 3       | Same as left| 85        |                      |                      | [46]         |
| Archer2         | 2014 | Archaerhodopsin 3       | Same as left| 60        |                      |                      | [46]         |
| ASAP            | 2014 | Gg-VSP                  | cp-EGFP    | −17.5      | ~8.6’                 | ~13’                | [36]         |
| Ace2N-mNeon     | 2015 | Acetabularia acetabulum rhodopsin | Ace rhodopsin/mNeonGreen | −18        | ~0.68’                 | ~1.0’              | [47]         |
| FlicR1          | 2016 | Ci-VSP                  | cp-RFP     | 6.6        | 1.1’                  | ~1.6’               | [48]         |
| ASAP2f          | 2016 | Gg-VSP                  | cp-EGFP    | ~25        | 5.8’                  | 8.0’                | [49]         |
| Archon1         | 2018 | Archaerhodopsin 3       | Same as left| 43        | ~0.83’                 | ~1.5’               | [50]         |
| Archon2         | 2018 | Archaerhodopsin 3       | Same as left| 19        | ~0.25’                 | ~0.23’              | [50]         |

* These numbers are estimated with \( \tau = \frac{\alpha \tau_{fast}}{\alpha \tau_{fast} + (1-\alpha) \tau_{slow}} \), where \( \alpha = \frac{\text{fast component amplitude}}{\text{total amplitude}} \).

** FRET donors and acceptors are separated with a slash.
under high-intensity illumination, thus posing a substantial autofluorescence problem. The mutants Archer1 and Archer2 were later introduced by Flytzanis et al. to improve the properties, especially the fluorescence baseline and dynamic range of Arch [46]. Compared with fluorescent protein-based GEVIs, Arch-based GEVIs typically require 300–800 W·cm⁻², whereas brighter fluorescent protein-based indicators require 10 W·cm⁻² [51]. To circumvent the problem of the low brightness of Arch-based GEVIs, FRET designs have been developed by fusing Arch or its mutant with a chromophore, such as QuasAr2-mOrange [51], to enhance the signal intensity and achieve high signal intensity, SNR and temporal resolution. Moreover, some opsin-based GEVIs retain their endogenous proton-pumping capability and produce a 1020 pA outward photocurrent.

3.3 Recent progress in action potential imaging

In recent work, in 2018, Piatkevich et al. developed a robotic cell picker to screen hundreds of thousands of proteins in a few hours and produced the high-performance opsin-based GEVI Archon1 [50]. Archon1 is improved in multiple aspects and exhibits excellent localization, SNR, sensitivity, response speed and photostability, as well as full compatibility with optogenetic control [50]. Because action potential has a millisecond timescale, currently, only state-of-the-art sCMOS cameras can provide a sample rate (~1000 FPS) permitting in vivo action potential imaging. However, these devices are generally large and are unable to be integrated into head-mountable devices for imaging in freely moving animals, and they usually have a limited field of view. To remedy these defects, some ongoing projects aim to build miniature microscopes capable of high-speed action potential imaging (https://web.archive.org/web/201809122222322/http://grantome.com/grant/NIH/R21-EY028381-01).

4 Genetically encoded synaptic transmission indicators

GESTIs are a class of neural activity indicators that provide a signal response to specific neural transmitters (Fig. 4). These sensors can be further categorized into bacterial periplasmic binding...
protein (PBP) based sensors, hybrid sensors and GPCR-based sensors on the basis of their design principles (Table 3).

### 4.1 PBP-based synaptic transmission sensors

PBPs compose a large superfamily of members that bind various chemicals including neurotransmitters (Fig. 4). One of these proteins, glutamate-binding PBP (Glt-I) has been used to build a synaptic transmission sensor. FLIPE [52], GluSnFR and superGluSnFR [53, 54] resulted from early attempts to construct a FRET-Glt-I glutamate sensor. However, the SNR of FRET-based Glt-I sensors was insufficient to resolve individual responses [53] and hampered in vivo observation. iGluSnFR, based on combining the glutamate-binding protein Glt-I with cpEGFP, became the first practical sensor used to visualize glutamate dynamics in vivo [55]. Although PBP based GESTIs cannot be easily modified to sense different neurotransmitters, the sensitivity, temporal resolution and SNR of some of its improved variants have made it a workhorse in glutamate functional imaging in the past few years.

### 4.2 Hybrid synaptic transmission sensors

Attempts to address the limitations of single GESTIs have yielded several hybrid sensors (Fig. 4). As a natural receptor for neurotransmitters, GPCRs have outstanding ligand specificity and can be easily modified to apply a sensor design to other homologous receptors. However, the ligand induced conformation changes of GPCRs are relatively weak, thus causing difficulties and insufficient SNR in signal amplification. To avoid this problem, cell-based neurotransmitter fluorescent-engineered reporters (CNiFERs) were introduced by Nguyen et al. to report on extracellular ACh [8] and were extended to dopamine and norepinephrine reporting by Muller et al. [56]. CNiFERs are cultured cells engineered to express the GPCR of the target molecule and the calcium indicator TN-XXL. GPCRs in CNiFERs transform neurotransmitter signals into increased cytosolic [Ca^{2+}] via the IP pathway, and report on [Ca^{2+}] through readout of the calcium indicator TN-XXL [8, 56]. CNiFERs can indicate subtype activity; however, they still require a complex injection process to be loaded to target sites in the brain; moreover, they are unable to label specific genetically defined cell types and cannot provide sufficient temporal resolution to directly monitor the transmitter release events on sub-second time scales [57].

Another problem in GPCR-based GESTI design is that FRET pairs used to amplify conformational variance signal are large and thus cause protein trafficking problems. Because chemical dyes are much smaller than fluorescent proteins, some hybrid GPCR-based GESTIs use the biarsenical chemical dye FlAsH (fluorescein arsenical hairpin binder) to act as one of the fluorophores in the

| GESTI      | Year | Ligand   | Sensing | Reporting          |
|------------|------|----------|---------|--------------------|
| GPCR-cam   | 2003 | NE & PTH | GPCR    | FRET pair          |
| FLIPE      | 2005 | Glutamate| Glt-I   | FRET pair          |
| GluSnFR    | 2005 | Glutamate| Glt-I   | FRET pair          |
| Adrenergic | 2006 | NE       | GPCR    | FRET pair          |
| TANGO      | 2008 | GPCR     | GPCR    | Reporter genes     |
| M1-CNIFER  | 2010 | ACh      | GPCR    | TN-XXL             |
| M1, M3, M5 | 2010 | ACh      | GPCR    | FRET pair          |
| M1-mAChR   | 2012 | ACh      | GPCR    | FRET pair          |
| iGluSnFR   | 2013 | Glutamate| Glt-I   | cpEGFP             |
| D2-CNIFER  | 2014 | DA       | GPCR    | TN-XXL             |
| α1A-CNIFER | 2014 | NE       | GPCR    | TN-XXL             |
| iTANGO2    | 2017 | GPCR     | GPCR    | Reporter genes     |
| GACH       | 2018 | ACh      | GPCR    | cpEGFP             |
| dLight1    | 2018 | DA & other| GPCR    | cpEGFP             |
| GRAB-DA    | 2018 | DA       | GPCR    | cpEGFP             |
FRET pair [13, 58], thereby decreasing the indicator size.

4.3 GPCR-activation-based sensors

Among the GESTIs, GPCR-activation based sensors (GRABs) are a promising series of GESTIs that consist of a ligand specific GPCR and a reporter to amplify the structural variation of the 7-TM rhodopsin domain of these GPCRs (Fig. 4). Currently, most GRABs are based on subfamily A17 rhodopsins, such as the 5-hydroxytryptamine receptor, dopamine (DA) receptor, adrenergic receptor and subfamily A18 rhodopsins such as ACh receptors. Unlike traditional synaptic transmission indicators, GPCRs are natural homologous receptors for many neurotransmitters, and their design can be easily applied to other cognate GPCRs for sensing their ligands.

Beyond the GESTIs used to amplify the ligand-binding signal via the intrinsic GPCR signaling pathway described in Section 4.2, the TANGO assay developed by Barnea et al. provides another practical method to monitor subtype specific GPCR activity by cleaving and releasing the transcriptional activator tTA fused to ligand-activated GPCRs via a TEV protease-human β-arrestin chimeric protein. The tTA dependent reporter gene can thus be expressed in response to subtype-specific GPCR activation [59]. The improved iTANGO2 assay achieves a higher temporal resolution (on a scale of minutes) than the original TANGO assay (on the scale of several hours) by adding a light inducible protease system to the ligand-gated gene expression system [60]. However, limited by the gene expression, the temporal resolution of reporter gene-based methods is still incompatible with the fastest single molecule indicators. FRET is a common method to construct high temporal resolution indicators. Efforts to design dual-fluorescent protein GPCR indicators have resulted in several FRET-based GESTIs. FRET-based GRABs have superior temporal resolution [18, 61], but because of the relatively small conformational changes of GPCRs, most of these indicators do not have ideal temporal-resolution and sensitivity. As mentioned in Section 4.2, the size of these dual-FP FRET indicators is still a problem for in vivo study. In recent studies creating a high-temporal solution GPCR-based GESTI, a conformationally sensitive fluorescent protein has been inserted in the third intracellular loop of GPCRs, because the intracellular loop between the TM and TM undergoes a relatively large conformational change upon ligand binding. In addition, these GPCR-based sensors must be carefully designed to avoid perturbing the intrinsic physiology in the cell of interest [9]. Jing et al. have constructed the ACh indicator GACH, which has high sensitivity (EC50 = 1 μM), high SNR (=14) and fast kinetics ($\tau \approx 200–800$ms ) while maintaining an endogenous receptor level on/off specificity and photostability [62]. A similar approach has been applied to the construction of the DA indicator [57, 63] and several other indicators including norepinephrine, serotonin, melatonin and opioid [57]. These single-FP based GRABs enable spatio-temporally precise measurements to indicate their corresponding neurotransmitters.

5 Discussion

The development of GENAIs in recent decades has provided powerful tools for cell-type-specific neuroscience studies. These indicators have been conceived, constructed and improved to meet the growing requirements of functional imaging. Efforts to improve GENAIs have mainly focused on their optical properties including photosensitivity, fluorescence intensity, signal sensitivity, dynamic properties and compatibility with optical neuronal activity manipulating methods. However, for many real-life applications, all these requirements cannot be satisfied simultaneously,
and certain applications may require distinct properties that could be achieved by a single indicator. The next generation GENAIs could be optimized for specific uses, such as the JGCaMP7 series variants, as recently reviewed by Luo et al. [2]. These variants with different sensitivity, temporal resolution and baseline intensity can satisfy the diverse requirements of applications such as neuropil observation and wide-field imaging.

Finally, given how the field has developed in recent years, further developments of GENAIs that are ultrafast, ultrasensitive and compatible with manipulation methods are likely to boost further study of brain function.

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