Title: Current Status and Future Strategies for Advancing Functional Circuit Mapping In Vivo

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Abstract: *The human brain represents one of the most complex biological systems, containing billions of neurons interconnected through trillions of synapses. Inherent to the brain is a biochemical complexity involving ions, signaling molecules, and peptides that regulate neuronal activity and allow for short- and long-term adaptations. Large-scale and non-invasive imaging techniques such as functional Magnetic Resonance Imaging (MRI) and electroencephalography (EEG) have highlighted brain regions involved in specific functions and visualized connections between different brain areas. A major shortcoming, however, is the need for more information on specific cell types and neurotransmitters involved, as well as poor spatial and temporal resolution. Recent technologies have been advanced for neuronal circuit mapping and implemented in behaving model organisms to address this. Here, we highlight strategies for targeting specific neuronal subtypes, identifying, and releasing signaling molecules, controlling gene expression, and monitoring neuronal circuits in real-time in vivo. Combined, these approaches allow us to establish direct causal links from genes and molecules to the systems level and ultimately to cognitive processes.*

Recent advances in deconstructing neuronal circuits in vivo involve the synergy of four areas: cell-specific expression of proteins using molecular genetics, monitoring neuronal activity and neuromodulators over time, triggering the release of specific transmitters, and tracking behaviors. Using model organisms like rodents allows one to target distinct cell types with protein-based tools such as fluorescent proteins and light-activated ion channels. Viruses encoding these tools are injected into the brain, and their expression is restricted to specific cell types under the control of Cre or Flp recombinase in transgenic rodent driver lines (Gong et al., 2007; Weinholtz and Castle, 2021). For example, tracing experiments label distinct neuronal subtypes with fluorescent proteins, allowing axonal projections to be traced by various volumetric imaging techniques (Oh et al., 2014; Glaser et al., 2023). This type of circuit mapping can scale to subcellular levels and reveal intricate anatomical features, such as dendritic and axonal arborization, identifying convergent or divergent connections from one brain region to another.

While these imaging techniques provide details on the connectome, they lack dynamic information on activity or the biological compounds that regulate these signals. Recent advances in sensor engineering and caging of neuromodulators enable us to monitor intra- and extracellular signaling molecules with high spatial and temporal resolution in head-fixed or even freely moving animals. For example, genetically encoded fluorescent sensor proteins can monitor calcium, voltage, dynorphin, dopamine, norepinephrine, acetylcholine, orexin, and more (Patriarchi et al., 2018; Adam et al., 2019; Feng et al., 2019; Jing et al., 2020; Abraham et al., 2021; Duffet et al., 2022; Zhang et al., 2023).

Concurrently, several imaging techniques have been developed to monitor these sensors. Miniaturized fluorescence microscopes are head-mounted onto freely moving rodents for *in vivo* detection (Ghosh et al., 2011; Cai et al., 2016). Two-photon microscopy through transcranial windows of head-fixed animals provides sub-cellular resolution. It can be complemented by Gradient Refractive Index (GRIN) lenses or prisms to image deep brain areas (Levene et al., 2004; Andermann et al., 2013). Fiber photometry detects bulk fluorescence through implanted optical fibers, targeting specific neuronal subpopulations and analytes (e.g., voltage, Ca^{2+}, neurotransmitters, neuromodulators) (Gunaydin et al., 2014) These tools allow for a distinct assessment of the roles of cell types, neuronal circuits, neurotransmitters, and neuromodulators in cognitive functions. For example, they revealed the dynamics of dopamine

release from the ventral tegmental area during reward paradigms, the firing-dependent volumetric release of acetylcholine in the entorhinal cortex, and orexin dynamics in somatosensory cortex and basal forebrain during wake-sleep cycles (Patriarchi et al., 2018; Jing et al., 2020; Duffet et al., 2022).

Optogenetic (Yizhar et al., 2011) and chemogenetic (Vardy et al., 2015) actuators, such as channelrhodopsins and DREADDS, link circuits to behaviors by directly manipulating neuronal activity. Membrane potentials can be depolarized or hyperpolarized, and light-activated ion channels can trigger neurotransmitter release. Intracellular signaling cascades can be modulated by designer G-protein coupled receptors (GPCRs) such as DREADDs, by light-activated adenylyl cyclases such as bPAC (Stierl et al., 2011) or light-activated GPCRs such as parapinopsin (Copits et al., 2021) and OPN3 (Mahn et al., 2021). Combining these techniques allows simultaneous monitoring and manipulation of targeted neuronal circuits. For instance, one can trigger neuromodulator release from a specific cell type using channelrhodopsin and simultaneously monitor the activity of connected downstream neurons using calcium imaging or sensors for neuromodulators. This combined approach enables the reconstruction of detailed cellular and molecular maps of brain circuitry and informs our understanding of the interplay between brain regions during cognitive processes.

The past 15 years saw tremendous progress in functional circuit mapping in model organisms. In the context of fear memory, specific groups of neurons (known as engram cells) in the entorhinal cortex and within various regions of the hippocampus are thought to encode contextual information. In contrast, engram cells in the amygdala primarily encode fear-associated information. A series of mapping experiments revealed that these distinct cell ensembles form a complex network encapsulating the fear memory (Josselyn and Tonegawa, 2020). Similarly, *in vivo* calcium imaging of the CA1 region in the hippocampus of freely moving rats visualized and tracked hundreds of place cells, crucial for spatial navigation and memory, over weeks. This innovative approach revealed that place cells could maintain their location specificity over extended periods, providing insights into hippocampal circuit organization, which is vital for memory, navigation, and learning (Wirtshafter and Disterhoft, 2022).

Other mapping studies have shown the importance of mPFC's dopaminergic afferent connection with the ventral tegmental area (VTA) in managing social avoidance and stress resilience. Furthermore, efferent connections from mPFC to other regions like the basolateral amygdala (BLA) were critical in reversing stress-induced behavioral deficits and producing rapid antidepressant effects. Manipulation of the mPFC's various circuits influenced anxiety and depression-like behaviors, indicating the central role mPFC plays in regulating affective states (Liu et al., 2021).

Another study monitored thousands of striatal spiny projection neurons of the direct (dSPNs) and indirect (iSPNs) pathways in the basal ganglia. The results showed that L-DOPA can cause imbalances in the basal ganglia when treating Parkinson's due to the differential responses of dSPN and iSPN pathways. Thus, future treatments need to consider the activity rates and the spatial-temporal coordination of neurons within these circuits (Parker et al., 2018).

These examples show that the future of circuit mapping relies on increasingly precise and multifaceted methods matching the complexity of brain circuits. This review highlights recent technological advances in 1. genetically encoded sensors, 2. recording neuronal activity with mini-microscopes, 3. protein integrators of neuronal activity, 4. CRISPR/Cas9 gene editing, and 5. time-locked release of neuropeptides from nanovesicles (**Fig. 1**). These topics were discussed at a corresponding symposium at the 2023 annual meeting of the Society for Neuroscience.

Next generation sensor design: Overcoming challenges in protein engineering.

One key to circuit mapping is monitoring the release of neuromodulators and neuropeptides from genetically defined neuron populations. Fluorescent sensor proteins are ideally suited for

this task because they can be engineered to bind specific ligands, and their expression can be spatially restricted by Cre-Lox recombination or cell-specific promoters. Existing sensors for calcium and neurotransmitters have provided new information on neuronal circuits. However, much work remains to improve the brightness, sensitivity, kinetics, dynamic range, spectral tuning, and specificity of sensor proteins. For example, *in vivo* imaging of individual neurons through transcranial windows or miniaturized microscopes requires highly optimized sensors with large signal amplitudes. For imaging neuronal activity through calcium transients, this threshold was initially reached with the development of GCaMP6 from GCaMP3 (Chen et al., 2013).

In addition, there is a vast array of targets for future sensor development. Here, we consider the family of opioid receptors and peptides. Despite the large body of research on opioid receptor function, there is a limited understanding of the endogenous opioid peptides released in neuronal circuits. Fluorescent opioid sensors could identify the specific types of neurons that release endogenous opioids during pain responses, reward, or stress. One example is the recently developed sensor kLight1.2, based on the kappa-opioid receptor. The sensor could detect dynorphin release in the medial prefrontal cortex of mice under morphine withdrawal (Abraham et al., 2021). A similar prototype sensor based on the mu-opioid receptor, however, failed to generate a sufficient signal response, highlighting the need for further optimization (Patriarchi et al., 2018).

Optimizing fluorescent sensors for detecting neuropeptides via mutagenesis has been a significant challenge due to the immense mutational landscape of proteins. Targeting only five residues by randomized mutations can result in 3.2 million variants. This number far exceeds the current throughput of screening approaches for most sensor proteins (usually in the low hundreds). Newly developed high-throughput systems such as BeadScan (Koveal et al., 2022) and Opto-MASS (Rappleye et al., 2022) and video-based pooled screens (Tian et al., 2023) can functionally test signal amplitudes, kinetics, and ligand selectivity of semi-randomly mutated variants in minutes. For example, Opto-Mass tested 21,000 variants of mLight and identified a significantly improved variant called uMASS, which features improved membrane trafficking as well as five times larger response amplitudes towards the synthetic opioid [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO).

uMASS inherits the ligand binding promiscuity of the mu-receptor. Accordingly, it is highly sensitive to Met-enkephalin but also detects endorphin, Leu-enkephalin, and dynorphin with lower affinities. Future opioid sensors for each type would allow us to distinguish neurons producing different opioids. Given that different opioids can have complementary or opposing effects, multiple opioid-specific fluorescent sensors could uncover competitive receptor binding or the co-release of opioids. High-throughput screening platforms such as Opto-MASS can be adapted to screen sensor libraries while several competing agonists are applied sequentially. The screen could identify variants with distinct selectivity profiles, such as high met-enkephalin and low endorphin affinity or vice versa.

These high-throughput approaches create large datasets that link mutations to sensor functions. Implementing machine learning (ML) to these datasets could significantly advance sensor engineering. In one recent example, ~1,000 mutations of GCaMP were used to train ML ensembles to predict response amplitudes and kinetics of known GCaMP mutations (Wait et al., 2023). The final ML ensembles incorporated the five amino acid properties out of 554, with the highest predictive power for the function of GCaMP variants. The ML model was applied to a library of 1,423 previously untested GCaMP mutants. Variants with high fluorescence and kinetic responses were subsequently tested. Two variants, called ensemble-GCaMPs (eGCaMP+ and eGCaMP2+), have generated the largest dynamic range of any calcium indicator, while eGCaMP+ is also the fastest known variant in these tests (Wait et al., 2023).

These ML models can be applied to map sequence-function relationships in new sensors instead of more complex structure-function relationships. These approaches will

become invaluable to significantly accelerate sensor engineering when combined with high-throughput platforms. Such next-generation methods are urgently needed: ~6,000 neuropeptides have been discovered in vertebrate and invertebrate organisms, with most functions only weakly defined at the circuit level (Wang et al., 2015). Beyond neurotransmitters and neuromodulators, sensors that detect changes in neurotrophic factors, such as BDNF (Brain-Derived Neurotrophic Factor), could provide insights into neural development, neural plasticity, and neurodegenerative processes. A sensor for cortisol could help us better understand the brain's response to stress. Furthermore, developing sensors to detect signaling molecules involved in immune responses, like cytokines, could be revolutionary. Traditional (slow) trial-and-error mutagenesis for engineering and optimizing these sensors would require tremendous resources and time commitments. Further advances in protein engineering are critical to overcome these challenges (**Fig. 2**).

Functional Imaging of neuronal circuits in freely moving rodents with Miniscopes

While monitoring the release of neuromodulators in real time using sensors provides information on when and where these molecules are released, it is also essential to study the effect on the activity of individual neurons. One of the most frequently used fluorescent sensors is GCaMP for measuring changes in intracellular calcium, which occurs downstream of action potentials. Due to the slower time scales of calcium sensor kinetics compared to voltage sensor kinetics, imaging acquisition rates are lower than for voltage indicators and allow lower excitation light power, reducing hardware demands and photobleaching. Combining fluorescent biosensors with miniaturized microscopes using gradient refractive index (GRIN) lenses instead of traditional objective lenses enables probing deeper brain regions like the hippocampus, thalamus, striatum, orbitofrontal cortex, and brainstem. Microscope miniaturization can be combined with robust behavioral paradigms to allow the investigation of circuits underlying functions such as learning and memory (Ghosh et al., 2011; Cai et al., 2016).

The use and development of the open-source UCLA Miniscope is one example of how in vivo imaging can be applied to answer complex questions about memory dynamics *in vivo*. For instance, Miniscopes recorded active neurons as mice explored varying contexts (**Fig. 3**). These tests proved that memories that occur within a day are more likely to share an overlapping neural ensemble, leading to behavioral linking: the recall of one memory likely triggers recall of the other memory that occurred close in time. Additionally, specific memory-linking deficits in aged mice were rescued using artificial ensemble activation via chemogenetic stimulation by DREADDS (Vardy et al., 2015). These experiments provided novel and critical insight into how the brain organizes and integrates different experiences across time. A future question is how memories are encoded across even longer time scales. A recent preprint details how strong aversive experiences drive ensemble reactivation of a neutral memory formed two days prior, effectively transferring fear from the aversive to the neutral context (Zaki et al., 2023). Such findings indicate that memory linking can occur between events rather than only at initial memory encoding, suggesting a mechanism that allows the brain to associate memories across time.

There is a need to increase access to these powerful imaging technologies, as evidenced by the growing number of users involved in the UCLA Miniscope project. To remove barriers to technology adoption and iteration, all aspects of the Miniscope–from the hardware components to the software used for analysis–were selected based on their widespread availability. The success of the UCLA Miniscope project has prompted the initiation of many others, including NINscope (de Groot et al., 2020), cScope (Scott et al., 2018), MiniFAST (Juneau et al., 2020), Miniscope3D (Yanny et al., 2020), and Mesoscope (Rynes et al., 2021). These collective efforts have engendered innovative approaches that leverage 3D imaging, variable sampling rates, and different fields of view.

Though powerful in providing anatomically precise, circuit-level investigations of brain function, existing tools have limitations. Miniscope designs generally use a one-photon wide field image similar to a tabletop fluorescent microscope and produce images that are projections from the distal tip of the GRIN lens. These one-photon imaging approaches are prone to out-of-focus light from other depth planes scattering and producing noise in the field of view. Image analysis requires spatiotemporal unmixing of the 2D images to differentiate cells and separate signal from noise. While the currently used algorithms can extract calcium activities from individual neurons fairly reliably amidst high noise levels, ensuring that the same neuronal populations are recorded across imaging sessions remains challenging. Due to the chromatic aberration of GRIN lenses that are typically used in Miniscope, Miniscopes are typically only able to record one wavelength of light at a time, precluding the possibility of recording multiple fluorophores simultaneously in the same field of view. In contrast, two-photon microscope systems can measure multiple signals simultaneously and do not suffer from contamination by out-of-focus light scattering; however, their requirement for head-fixed preparations hinders use in many behavioral paradigms.

To overcome these limitations, there are continual developments in open-source single-photon and two-photon Miniscopes with multiwavelength recording capabilities (Zong et al., 2021)(Zong et al., 2022).This allows for simultaneous imaging of two dynamic signals, such as dopamine and calcium, using dLight (Patriarchi et al., 2018) and GCaMP (Chen et al., 2013) or a static signal with a dynamic signal (e.g., tagging cells with constitutively active indicators like tdTomato and monitoring changes with GCaMP). Innovative software, such as CaImAn (Giovannucci et al., 2019) and Minian (Dong et al., 2022), for calcium imaging analysis, have been developed to be combined with programs like DeepLabCut (Mathis et al., 2018) relating behavioral responses to neuronal activity. As these approaches allow researchers to modulate and monitor neuronal signaling in behaving animals with high spatial and temporal resolution, it is critical to continue promoting affordable and user-friendly implementation as new technologies develop.

Integrators and Tickertapes for imaging brain-wide neuronal activity

In parallel with the development of new sensors and imaging systems is the search for techniques to monitor the activity of an unlimited number of individual neurons that can be recorded simultaneously. With current imaging techniques, light scatter constrains live-tissue imaging to depths of typically < 500 nm for two-photon microscopy and < 1 mm for three-photon. Efforts to image deeper require the destruction of overlying tissue and sometimes sacrificing single-cell resolution. On the other hand, brain-wide maps of macromolecular signals (e.g. gene expression or protein levels) can be generated in fixed tissues. Still, these maps probe only a single point in time and do not capture physiological signals such as calcium or neuromodulators (**Fig. 4A**). Thus, neuroscientists face a tradeoff between dynamical and spatial information.

Several recently developed techniques show a path toward transcending this tradeoff. The key feature is to create long-lived chemical traces that record transient events within individual cells. The encoding occurs *in vivo*, but the readout is measured *ex vivo*. While whole-brain recordings are not yet possible, there are exciting advances in several areas.

First, light-gated integrators store a snapshot of neural activity in a long-lived chemical signal (Fig. 4B). As their name suggests, these tools build up a signal proportional to the cumulative activity during an interval defined by a light pulse. In one technique, a light-gated voltage integrator and a light-gated voltage sample-and-hold reporter store long-term records of electrical activity via the conformation of a microbial rhodopsin (Venkatachalam et al., 2014). Alternatively, a light-gated calcium integrator, CaMPARI, undergoes an irreversible green-to-red transition in the simultaneous presence of calcium and violet light (Fosque et al., 2015). These

tools enable *ex vivo* mapping of circuits that had been active during the illumination epoch. However, they do not provide any downstream genetic access to these cells.

The ST-Cal-Light (Lee et al., 2017b; Hyun et al., 2022), FLiCRE (Kim et al., 2020), and scFLARE2 (Sanchez et al., 2020) light-gated calcium integrators also sense cumulative calcium during an optically gated interval. These tools also provide a transcriptional readout (**Fig. 4C**). With these tools, a transcription factor is tethered to the cell membrane by a linker cleaved in the simultaneous presence of blue light and calcium. FLiCRE requires three genetic components (two for the integrator and one for the readout). The scFLARE2 integrator has a single sensing component (but still requires another gene as the readout). This approach allows the coupling of the Ca^{2+} activity signal to a wide range of subsequent measurements or perturbations. For instance, an optogenetic actuator or silencer could be expressed to test the causal roles of the tagged neurons. Alternatively, structural markers could be expressed to assess their connectomes or ribosome pulldown tags to measure their ensembles of translated proteins.

SPARK (Kim et al., 2017) and iTANGO (Lee et al., 2017a) reporters operate on a similar principle to FLiCRE and scFLARE but are sensitive to the activation of G-protein coupled receptors (GPCRs). These reporters have a modular design so that they can be coupled to various upstream receptors, as well as arbitrary transcriptional readouts. Integrators have been developed for dopamine receptors D1 and D2, the beta-adrenergic receptor, and others.

Existing light-gated integrators have two key limitations. First, these tools only provide a single readout. Ideally, one wishes to map changes in signal associated with a particular experimental condition, but the existing tools can be confounded by basal activity unrelated to the experiment. Readouts at multiple times would also permit comparison of patterns of neural activation under different conditions within the same animal. Second, existing integrators require blue or violet light for the optical gate. Due to strong blue light scattering in tissue, this limits the recording depth to ~1 mm. In principle, red or infrared-gated integrators could record throughout a mouse brain, with light sources mounted outside the skull.

Several recent efforts have sought to provide multi-time-point measures of neural activity and to overcome the depth limitations of light-gated integrators. Protein tickertapes comprise linearly growing protein fibers that incorporate colored bands during epochs of neural activity, which are then read *ex vivo*. Based on linear crystals of the Pak4 kinase, one approach has an absolute time resolution of ~30 minutes, but the crystals deform the cells and thus are incompatible with *in vivo* use (**Fig. 3D**) (Lin et al., 2023). Another approach is based on filaments of *E. coli* isoaspartyl dipeptidase but has a time resolution of days and does not have an absolute time-base (Mohar et al., 2022). In both cases, expression of the tags is driven by neural activity-dependent genes (e.g. cfos), which are downstream of action potentials.

Pulse-chase labeling with HaloTag-ligand dyes also provides a means to capture aspects of neural activity at multiple time points. In one recently demonstrated technique, dubbed DELTA, protein turnover was mapped brain-wide via pulse-chase labeling of HaloTag-protein fusions with multiple colors of brain-permeant HaloTag-ligand dyes. In another technique, dubbed EPSILON, newly surface-exposed AMPA receptors were identified via pulse-chase labeling with multiple colors of membrane-impermeable dyes (Kim et al., 2023). EPSILON could identify synapses potentiated during a particular chemically gated window *in vivo*, providing an approach to mapping the physical basis of memory formation.

The field of light- and chemical-gated integrators and tickertapes is still in its infancy. Nonetheless, these tools promise to provide a view that complements existing in vivo and ex vivo tools for functional circuit mapping, revealing brain-wide maps of multiple chemical signals as a function of time.

CRISPR/Cas9 gene editing approaches to study gene function

While genetically encoded fluorescent sensors can be targeted to specific types of neurons using specific promoters and Cre-lines of mice, it is also essential to determine the causal roles of specific genes in neural circuit dynamics. The basis for the functional, anatomical, and molecular diversity in the brain is fundamentally linked to the gene expression diversity of cells within neuronal circuits. Consequently, gene editing technologies such as CRISPR-Cas9, which modify gene expression, are critical for complementing the connectome with genetic information (Charpentier and Marraffini, 2014; Heidenreich and Zhang, 2016). Combined with neurophysiological tools and behavioral analyses, gene-editing technologies will provide new insights into the genetic basis of brain function (Savell and Day, 2017).

Gene-modifying approaches such as knock-in and knock-out transgenic mice, virally mediated gene delivery of RNA interference (RNAi) constructs, targeting of DNA nucleases, including zinc-finger nucleases (ZFNs) and transcription activator effector nucleases (TALENs), have helped to discover new genetic contributions to brain function (Urnov et al., 2005; Limaye et al., 2009; Wang et al., 2013). Yet, the high costs, time for generation, temporal limitations, and complexity of some of these methods restrain their application. Recently, using CRISPR technology combined with Cas bacterial enzymes like Cas9 has addressed many shortcomings of earlier methods.

CRISPR-Cas9-based gene-editing approaches engineered for eukaryotic systems are based on the guidance of Cas9 nucleases to a DNA target site of interest using single-guide RNAs (sgRNAs) (Jiang and Doudna, 2017; Kalamakis and Platt, 2023). Once the sgRNAs deliver Cas9 to the site of interest in DNA, the DNA is unwound and cleaved near a protospacer adjacent motif (PAM) (**Fig. 4A**). Cellular mechanisms of either homology-directed repair (HDR) or non-homologous end joining (NHEJ) are recruited to repair the double-stranded break (DSB) in the DNA (Day, 2019). HDR pathways are usually recruited in dividing cells and use DNA template strands to precisely edit and repair the DSBs. In non-dividing cells (like neurons), NHEJ mechanisms are instead usually recruited, resulting in random insertions, deletions, or base-pair substitutions to correct the DSB, thus disrupting gene expression.

In addition, catalytically inactive (“dead”) Cas9 (dCas9) is altered to prevent DNA cleavage while retaining sgRNA-directed guidance. Thus, dCas9 can guide fused effector proteins to specific DNA sequences (**Fig. 4B**) (Gilbert et al., 2013). These approaches have been further expanded by CRISPR interference (CRISPRi) to repress gene transcription and CRISPR activation (CRISPRa) that can activate transcriptional states (Pickar-Oliver and Gersbach, 2019; Savell et al., 2019; Duke et al., 2020). Recently, CRISPR/Cas9 systems have been used to shuttle other epigenetic effector proteins to alter gene expression (Choudhury et al., 2016; Choi et al., 2023).

Applying these CRISPR-Cas9 approaches *in vivo* has expanded our knowledge of genetic contributions to brain function. For example, two adeno-associated virus (AAV)-mediated gene delivery can express the CRISPR/Cas9 system in the adult mouse brain knocking out genes of interest (Swiech et al., 2015). The development of *Cre*-inducible Cas9 knock-in mice further revolutionized genome editing and its application across systems (Platt et al., 2014). It allowed crossing these mice with *Cre*-driver lines to specifically express Cas9 in cell types of interest in the nervous system. Thus, by virally delivering sgRNAs into targeted brain regions, it can induce rapid genome alterations in a cell-type specific manner (Yamaguchi et al., 2018; McQuillan et al., 2022).

In 2020, a conditional, single AAV-based approach was used to express a smaller Cas9 and sgRNAs in adult mammals (Hunker et al., 2020). The resulting gene mutagenesis was as efficient as gene disruption in knockout mice. Juarez *et al.* recently combined these approaches with in vivo opto-electrophysiology, fiber photometry, and two-photon slice imaging to elucidate how ion channels contribute to patterns of VTA physiology and reinforcement learning (Juarez et al., 2023). Notably, the conditional, single-AAV-based method could be combined with *Cre*-recombinase or *Flp*-recombinase systems. A compelling finding of this study was that neurons

in the lateral hypothalamus produce both stimulating neurotensin and inhibitory GABA (γ-aminobutyric acid). Notably, these signals are used in a coordinated manner to control neurons in the VTA that regulate dopamine release (Soden et al., 2023).

Advances in CRISPR-Cas9 approaches are now opening the door for ways to manipulate the genome of adult animals to better model human conditions (Cox et al., 2015). Methods that shift the DSB repair mechanism from NHEJ to HDR are being refined to introduce single nucleotide polymorphisms (SNPs) in the adult nervous system (Scholefield and Harrison, 2021). This could broaden the application of CRISPR to activate or repress genes of interest and model human disease-associated SNPs in animals. A critical feature for future applications is the temporal control of CRISPR-Cas9 function, which is under development and includes optogenetic or pharmacological induction of gene editing (Savell and Day, 2017; Choi et al., 2023).

Photosensitive Nanovesicles: A Promising Tool for Precise Neuromodulation

A missing tool in the arsenal of measuring the release of neuromodulators, studying the activity on neurons, and mapping brain-wide circuits is the ability to control the release of a specific neurotransmitter or neuromodulators within a targeted brain region. Recent advances have improved the ability for spatially and temporally precise delivery of neuro-active compounds.

It is now possible to remotely control the release of neuroactive compounds using external stimuli such as magnetic fields (Rao et al., 2019), ultrasound (Airan et al., 2017; Wang et al., 2018),' or light (Rapp and DeForest, 2021). Among these methods, light-based modulation offers high temporal and spatial resolution. One approach is to cage a desired neuroactive molecule by blocking a group key for its biological activity, where the molecule remains inactive until photo-uncaging (Ellis-Davies, 2007; Taura et al., 2018). While various caged compounds exist, such as glutamate or GABA (Ellis-Davies, 2020), their use is limited by residual activity, low solubility, and uncaging efficiency(Silva et al., 2019), and the risk of off-target effects (Maier et al., 2005; Noguchi et al., 2011). Along the same line, studying the role of neuropeptides (e.g., oxytocin, vasopressin, or somatostatin) in brain circuits and behavior has been particularly challenging due to the lack of specific methods for localized uncaging or release (DeLaney et al., 2018). While several caged neuropeptides have been reported, their application *in vivo* is limited by instability due to peptidase degradation (Ma et al., 2023; Xiong et al., 2023b). An alternative approach involves targeting neurons that endogenously produce these neuropeptides using cre-recombinase and expressing opsins to either opto-stimulate or inhibit the neuron and the subsequent neuropeptide release (Arrigoni and Saper, 2014; Dao et al., 2019). However, a major limitation of this approach is that neuropeptides are often co-released with classical neurotransmitters (Merighi, 2002; Hökfelt et al., 2003), making it challenging to isolate and study their specific functions.

A new technology based on photosensitive nanovesicles has been developed to address these limitations. Photosensitive nanovesicles encapsulate neuroactive molecules in self-assembled nanoscale vesicles, such as those made by a bilayer of phospholipids (liposomes) and a light-sensitive component for the photosensitive release. This innovative approach addresses the limitations of traditional pharmacology and provides a versatile platform to decipher the role of neuropeptides and neuromodulators. Specifically, the physical separation of neuroactive molecules from the surrounding brain environment improves *in vivo* stability compared with caged compounds.

Plasmonic gold-coated nanovesicles, in combination with Cell-based Neurotransmitter Fluorescent Engineered Reporter (PACE), have been used to investigate neuropeptide signaling in the mouse neocortex *in vivo* (**Fig. 6A,B**) (Xiong et al., 2022). Near-infrared light stimulation triggered the release of femtoliters to picoliters of Somatostatin-14 (SST) from nanovesicles in the brain, which was subsequently detected by activation of SST2 CNiFERs

tuned to nanomolar concentrations of SST. PACE revealed a reduced yet coordinated SST transmission within 130 µm, with significantly diminished and delayed transmission at greater distances. PACE provides a novel method for examining the scale and timing of neuropeptide volume transmission and signaling within the brain.

Another class of photosensitive nanovesicles involves photoswitchable azobenzenes. Azobenzene groups have gained significant attention in the field of photoswitches (Bahamonde et al., 2014; Cabré et al., 2019; Morstein et al., 2019, 2020, 2022; DiFrancesco et al., 2020; Kellner and Berlin, 2020; Mukhopadhyay et al., 2022). These compounds exhibit reversible trans-cis photoisomerization. Recently, researchers incorporated azobenzene into lipids by replacing one of the hydrophobic tails on the phospholipid, leading to the development of photoswitchable lipids, including the photoswitchable phosphatidylcholine derivative azo-PC (Pernpeintner et al., 2017; Urban et al., 2018, 2020; Pritzl et al., 2020, 2022). Azo-PC enables optical control of membrane organization and permeability, providing a versatile framework for delivering neuroactive compounds. Building upon the concept of photoswitchable lipids, a new class of photoswitchable nanovesicles termed “azosomes” have been developed. Azosomes consist of liposomes formulated with azo-PC. When the azobenzene undergoes reversible isomerization, it increases the permeability of the membrane and allows the contents to diffuse out (**Fig. 6C-E**). Computational studies suggest that trans-configuration may decrease the thickness of the lipid bilayer, thereby increasing permeability for encapsulated molecules.[109] The ability to switch the release of molecules on and off using short light pulses (< 3 seconds) demonstrates the potential of azosomes for neuromodulation. Recently, azosomes loaded with the D1 agonist SKF-81297 were shown to modulate striatal neurons *in vitro* upon light stimulation (Xiong et al., 2023a).

To explore the utility of azosomes *in vivo*, they are being tested in awake, behaving mice using fiber photometry to control the release of agonists and simultaneously monitor neural activity. For control, calcein-azosomes were infused into one hemisphere and calcein-liposomes (non-photoswitchable) into the opposite hemisphere. A dual optic fiber delivered light stimulation and monitored fluorescence on both hemispheres. The hemisphere implanted with calcein-azosomes (but not calcein-liposomes) exhibited a fluorescence increase upon light photo-switching, confirming the photo-release of calcein *in vivo.* To study the photo-release of SKF-81297 from azosomes *in vivo*, D1-medium spiny neurons (MSNs) in the dorsomedial striatum, a region involved in locomotor activity control, were targeted. While monitoring D1-MSN Ca^{2+} dynamics (with DIO GCaMP expressed in a D1-Cre mouse) and the distance traveled, it is possible to simultaneously photo-release an azosome’s cargo, record neuronal activity, and monitor motor behavior. Additionally, we are exploring the use of azosomes to release neuropeptides, expanding the repertoire of neuroactive compounds that can be remotely released.

In summary, photosensitive nanovesicles possess several features that make them promising tools for the controlled release of neuroactive compounds and precise neuromodulation. Photosensitive nanovesicles can be loaded with fluorescent dyes, drugs, or neuropeptides, allowing for versatile applications. Harnessing the ability to remotely and precisely control the release of neuroactive compounds *in vivo* will further elucidate the *in vivo* function of several neuropeptides in the future.

Conclusions:

Revealing the brain's intricacy, with its expansive neural network, synaptic connections, and fined-tuned genetic and biochemical regulations, remains challenging. Details at the cellular and molecular levels and their control of cognitive function remain especially elusive. However, this gap is continuously narrowing thanks to transgenic model organisms and techniques like fluorescence imaging, CRISPR/Cas9 gene editing, protein tickertapes, and time-locked release of neuromodulators, allowing real-time *in vivo* functional circuit mapping. Notably, using protein-

based tools in genetically-defined neuronal subpopulations has illuminated the granular control of circuit function by a diverse array of cell types and neuromodulators. Concurrent advancements in imaging, epitomized by head-mounted Miniscopes, promise deeper insights into previously inaccessible brain regions. New fluorescent sensors, especially for neuropeptides, are of high priority to maximize the synergistic effects of these developments. Emerging high-throughput techniques for sensor optimization, combined with machine learning, are ideally suited to expedite advancements. Additionally, the revolutionary CRISPR/Cas9 gene editing techniques are increasingly used *in vivo* to delineate the genetic underpinnings of brain function, while photosensitive nanovesicles promise increased precision in neuromodulation. One major challenge for the future is overcoming the tradeoff between spatial and temporal information. *In vivo* imaging, electrophysiology, and fiber photometry enable dynamic yet spatially restricted monitoring, while *ex vivo* imaging can provide spatially expansive yet primarily static information. Collectively, the emerging tools and methodologies highlighted in this review are elucidating the brain's functional architecture and outlining future innovations in circuit mapping.

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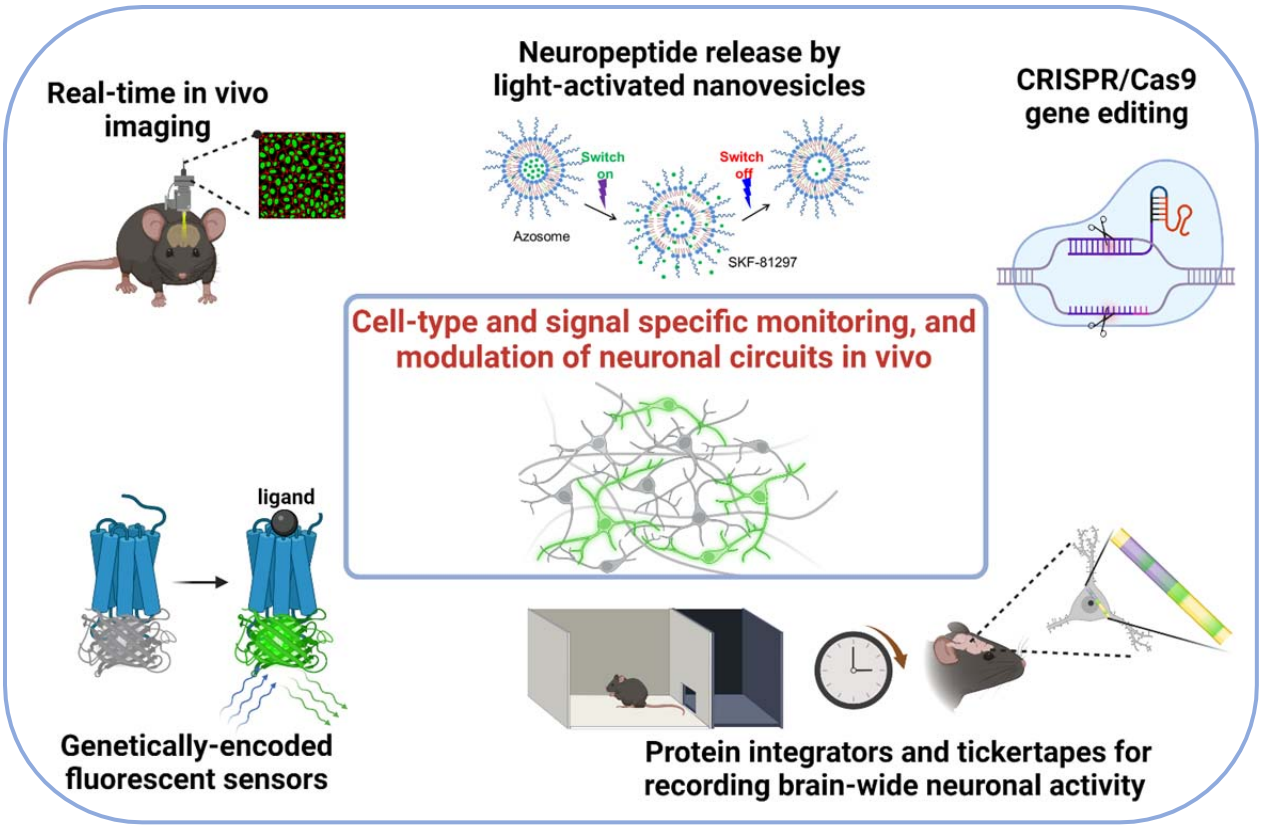

Figure 1. Comprehensive *in vivo* circuit mapping requires the modulations and monitoring of genetically defined neuron populations. This review highlights recent advances in techniques to study neuronal circuits on the genetic, molecular, and cellular levels. Applying these methods *in vivo* could link circuit functions to cognitive processes and disease mechanisms. (Figure created with BioRender.com)

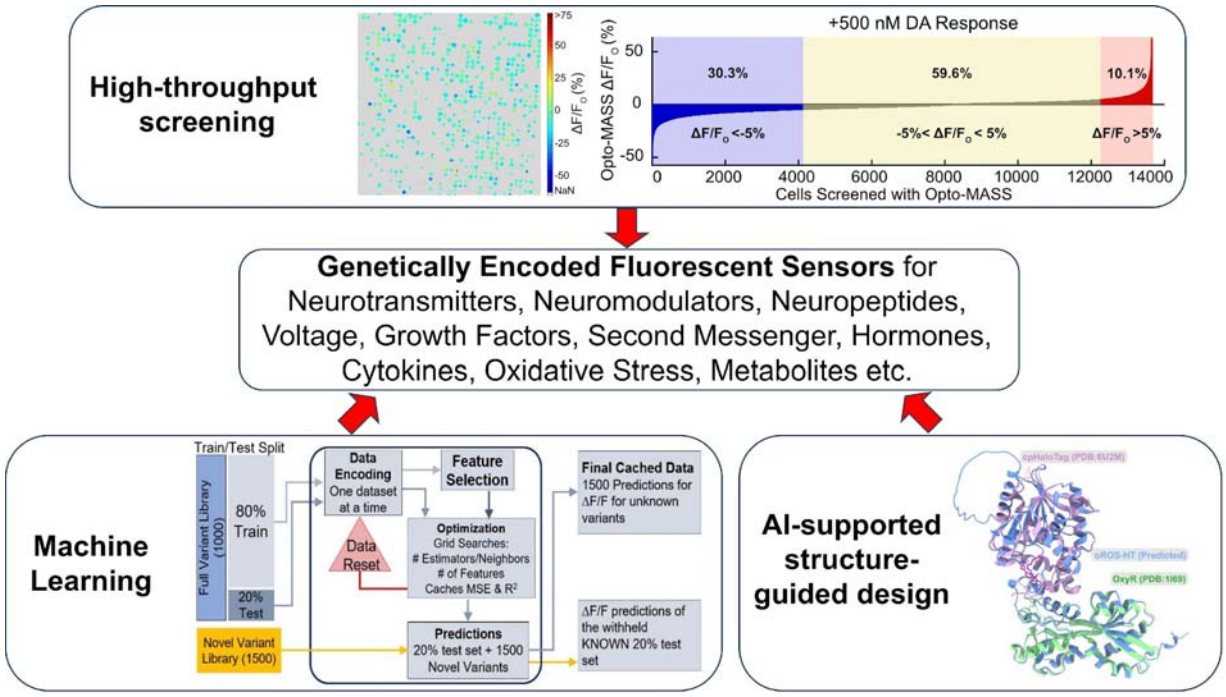

Figure 2. Next-generation approaches for engineering genetically encoded fluorescent sensors. Thousands of biological compounds regulate brain activities and cognitive functions. Fluorescent protein sensors for these compounds can be designed following similar design principles. However, optimizing dynamic range, signal sensitivity, and kinetics is hampered by the immense mutational space of each sensor protein. Therefore, further advancements in protein engineering are required by integrating high-throughput functional testing of large variant libraries, data-driven approaches, and AI-supported structure-guided design. The figure has been adapted from (Rappleye et al., 2022; Wait et al., 2023) with permission.

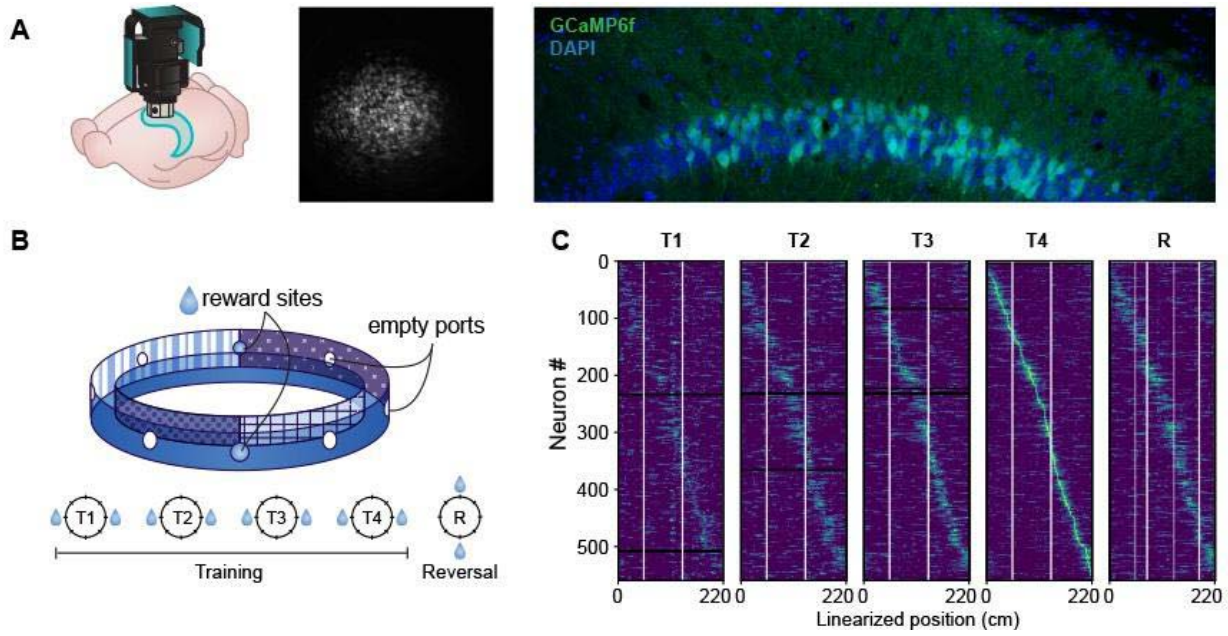

Figure 3. A. Technologies such as Miniscope calcium imaging allow real-time observations of neuronal ensembles *in vivo*. while mice perform behavioral tasks (**B**). **C.** Computational analysis of the imaging sessions allows to identify the dynamic activity of hundreds of neurons.

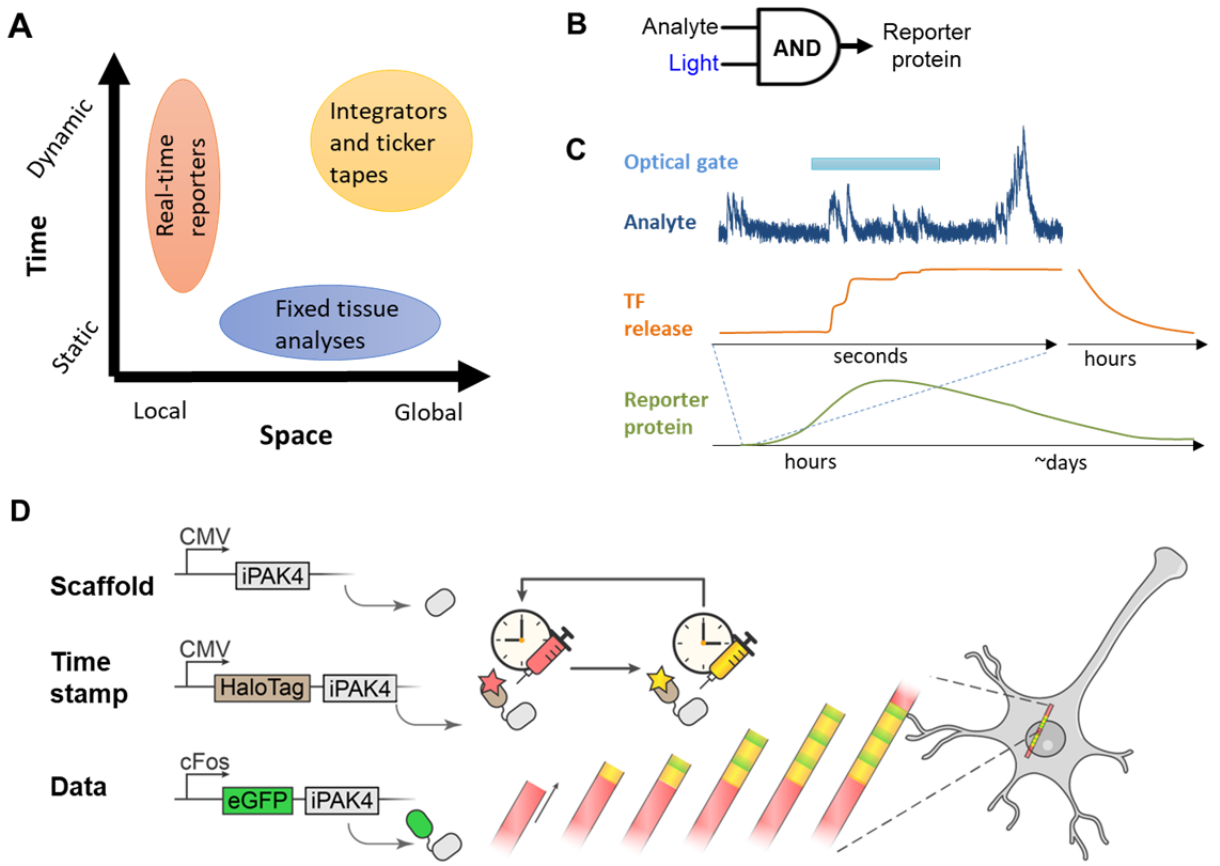

Figure 4. Integrators and tickertapes transcend the tradeoffs between *in vivo* and *ex vivo* imaging. A. Real-time reporters probe neural dynamics but with a limited field of view; *ex vivo* analyses can access the whole brain but provide static snapshots. Integrators and tickertapes can provide dynamic, brain-wide information. **B.** Light-gated integrators convert the cumulative level of an analyte during an illumination epoch into a transcriptional readout. **C.** Schematic operation of a light-gated integrator. Dynamic fluctuations in an analyte (e.g., Ca^{2+}) are converted into transcription factor (TF) release during an illumination epoch. The TF drives expression of a reporter protein. **D.** Example tickertape recorder. A linearly growing scaffold is marked at periodic intervals via alternating injection of two colors of HaloTag-ligand dye. An activity-dependent promoter (e.g., cFos) drives expression of a GFP-labeled monomer which introduces green stripes into the fiber. Localization of the green stripes relative to the red/yellow color transitions identifies the timing of neural activity. Figure (D) was adapted with permission from (Lin et al., 2023)

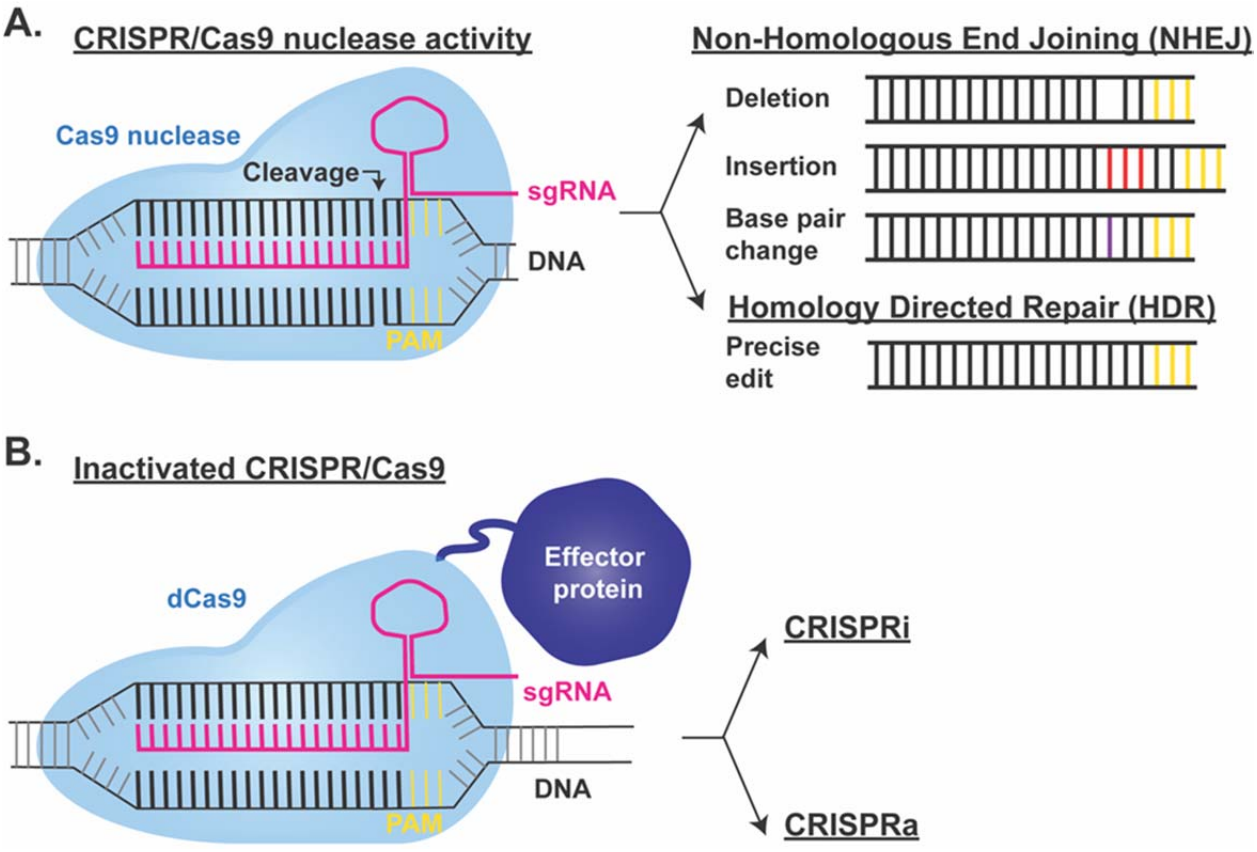

Figure 5. Capabilities of the CRISPR/Cas9 toolbox. A. Systems using CRISPR/Cas9 components with Cas9's nuclease activity intact will cause DNA cleavage and error-prone NHEJ, most likely employed in neurons or precise editing with HDR. **B.** CRISPR/Cas9 systems with inactive nuclease activity, or dCas9, can be fused with effector proteins to further modify gene expression.

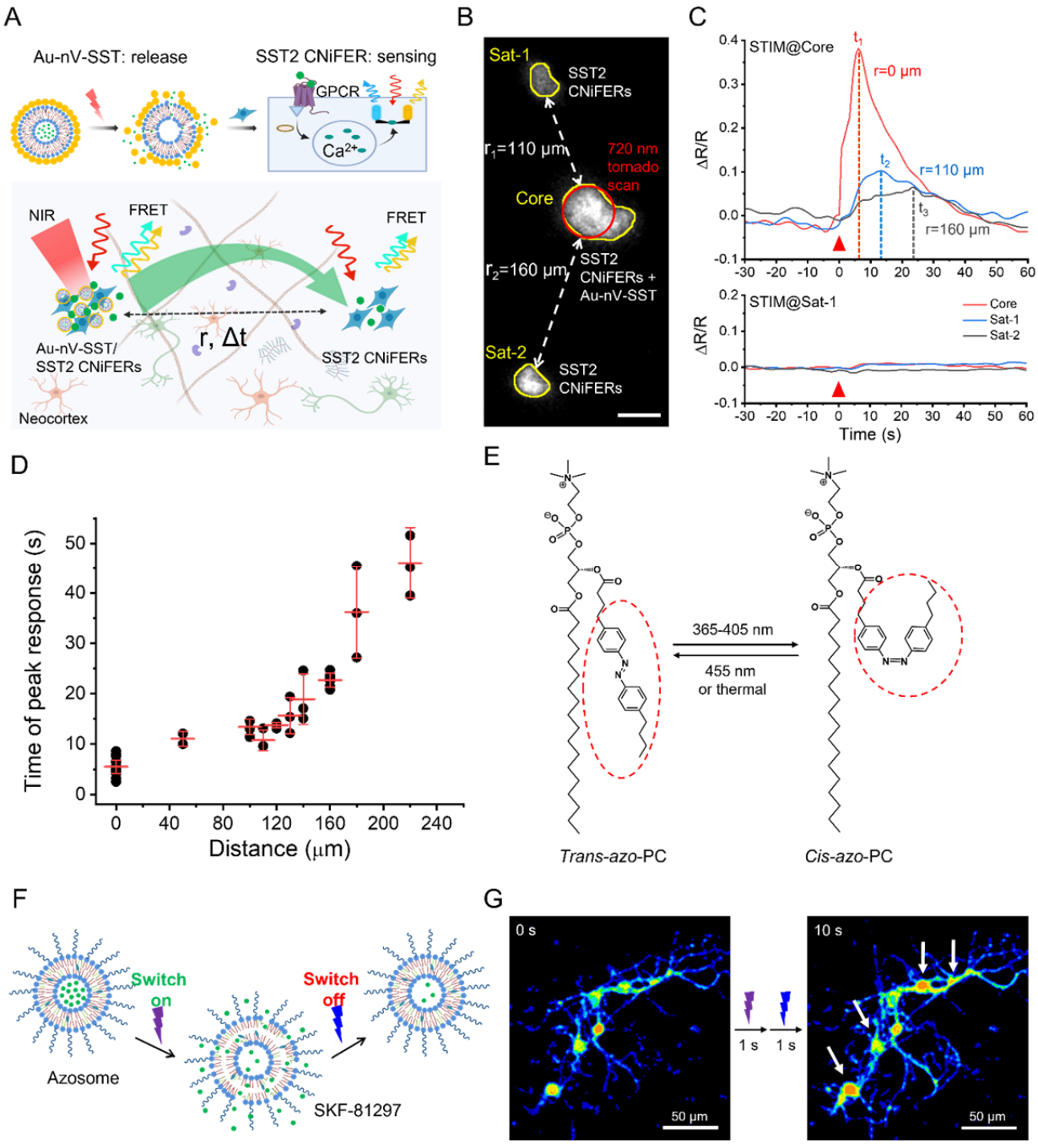

Figure 6. Photosensitive nanovesicles for precise neuromodulation.
A. Schematic of neuropeptide transmission measurement by plasmonic gold-coated nanovesicles (Au-nV-SST) and two clusters of SST2 CNiFERs. **B.** Two-photon fluorescent image of SST2 CNiFERs at 200μm in the mouse cortex. (Ex: 900 nm; Em: 520-560 nm). **C.** Response curves for SST2 CNiFERs when stimulated (STIM) at different regions (core vs satellite 1/sat-1). **D.** Peak response time of SST2 CNiFER implants at defined distances from the core implant. **E.** Schematic of the photoisomerization of azo-PC. **F.** Schematic of the photoswitchable release of SKF-81297 from azosome. **G** Real-time fluorescent images of cultured primary mouse striatal neurons before and after the irradiation of sequential 365 nm light and 455 nm light. Fluo-4 was used as the Ca2+ indicator. Scale bar: 50 μm. Figures

(A,B,C) were adapted with permission from [95]. Figures (D,E,F) were adapted with permission from (Xiong et al., 2023).