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Investigating learning-related neural circuitry with chronic *in vivo* optical imaging

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Summary

Fundamental aspects of brain function, including development, plasticity, learning, and memory, can take place over time scales of days to years. Chronic *in vivo* imaging of neural activity with cellular resolution is a powerful method for tracking the long-term activity of neural circuits. We review recent advances in our understanding of neural circuit function from diverse brain regions that have been enabled by chronic *in vivo* cellular imaging. Insight into the neural basis of learning and decision-making, in particular, benefit from the ability to acquire longitudinal data from genetically identified neuronal populations, deep brain areas, and subcellular structures. We propose that combining chronic imaging with further experimental and computational innovations will advance our understanding of the neural circuit mechanisms of brain function.

Introduction

A central goal of neuroscience is to achieve a mechanistic understanding of the relationship between neural circuits and behavior, including how the brain changes adaptively during learning. Long-term, repeated optical measurement of cellular activity in the living brain (in vivo), referred to here as chronic imaging, has become an important method for investigating the neural basis of behavior in animal models because of its ability to track the activity of identified types of neurons and glia in various brain areas with cellular resolution (Lütcke et al. 2013; Margolis et al. 2014; Crowe and Ellis-Davies 2014; Hamel et al. 2015; Clopath et al. 2017). Although other imaging methods exist to measure brain activation longitudinally, including fMRI and emerging technologies such as optoacoustic imaging, these methods currently lack the ability to resolve individual neurons, which is crucial for identifying the cellular and circuit mechanisms of learning-related neural changes (Jonckers et al. 2015; Ovsepian et al. 2017). In recent years, technical advances in neural activity sensors and

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optical microscopy have converged with novel behavioral assays and data analysis methods to make chronic in vivo imaging of neural activity an essential technique for the study of the neural circuitry underlying learning and memory formation. Since the initial applications that tracked changes in the fine-scale structure of neuronal dendritic spines in the mouse cerebral cortex (Grutzendler et al. 2002; Trachtenberg et al. 2002; Chen and Nedivi 2010), chronic in vivo imaging has been used to investigate a number of important problems in neuroscience, including synaptic formation (Holtmaat et al. 2006), cell fate in neurogenesis (Pilz et al. 2018), genetic activity markers (Wang et al. 2006), changes in disease, aging, or injury models (Hill et al. 2017, 2018; Akassoglou et al. 2017; Eyo et al. 2018; Real et al. 2018). Exciting advances in chronic imaging have also been made in a number of species from flies to non-human primates (Sadakane et al. 2015; Huang et al. 2018). In this review, we focus on novel applications of chronic *in vivo* imaging of neuronal activity in mice, where it is possible to leverage genetic accessibility, optical accessibility, and behavioral assays, in order to investigate the neural basis of mammalian behavior. One key feature of chronic optical imaging is the ability to resolve both active and sparsely active or "silent" neurons (Shoham et al. 2006; Margolis et al. 2012; Ovsepian 2019), which is critical for identifying how neuronal population activity changes with learning. We first cover the experimental methods that allow optical access to brain areas and cellular populations of interest, followed by advances in our understanding of the neural basis of learning-related activity changes enabled by chronic optical imaging, as well as emerging computational methods for analyzing the large resulting datasets.

Methods: imaging wide and deep

The last decade has seen a dramatic increase in the area of tool development for neuroscience, or neurotechnology (Jorgenson et al. 2015; Alivisatos et al. 2015), including advances in both electrophysiological and imaging methods for chronic recording of neural activity (Lütcke et al. 2013; Fu et al. 2016; Steinmetz et al. 2018; Piatkevich et al., 2019). Improved fluorescent sensors for the optical detection of neural activity, along with new microscopy techniques, are the primary factors that have driven the field forward by increasing the capacity for sensitive *in vivo* measurements of large populations of neurons.

Genetically encoded calcium indicators (GECIs) are the most commonly used class of fluorescent protein-based neural activity sensors because of the large relative change in intracellular calcium concentration that occurs with action potential firing and the resulting high signal-to-noise of the detected signals (Grienberger and Konnerth 2012). GECIs with improved sensitivity, brightness, kinetics, and expression properties have revolutionized the field, allowing many new applications for measuring neural activity *in vivo* (Kerr and Denk 2008; Hires et al. 2008; Margolis et al. 2011; Tian et al. 2012; Lin and Schnitzer 2016; Yang and Yuste 2017). The GCaMP family of GECIs, based on green fluorescent protein (GFP), has been widely adopted because of the large calcium-dependent fluorescence changes, photostability and capacity for imaging calcium signals in subcellular structures such as axons and dendrites (Chen et al. 2013b; Dana et al. 2019). Red-shifted GECIs have also undergone major improvements and now rival green GECIs in their ability to detect neuronal activity (Dana et al. 2016). This is important because the longer wavelength excitation and emission light used for red GECIs scatters less in tissue, allowing cellular imaging deeper

in the intact brain. Calcium indicators have two main caveats. First, calcium signals are a relatively slow readout of neuronal activity because calcium entry during a single action potential lasts approximately 100 ms, with GECI fluorescence emission typically taking hundreds of ms to return to baseline, limiting the measurement of detailed temporal activity patterns during trains of action potentials. Second, calcium indicators buffer intracellular calcium, potentially influencing natural intracellular calcium dynamics (Steinmetz et al. 2017; Bootman et al. 2018; McMahon and Jackson 2018). In spite of these limitations, GECIs have become an essential tool for in vivo cellular imaging, and currently remain the most popular neural activity sensors among a growing number of promising voltage and neurotransmitter indicators (discussed further in the Outlook section, below).

While the first chronic imaging studies using GECIs in visual, motor, and somatosensory cortex tracked population activity over an impressively long time period (Figure 1A) (Mank et al. 2008; Margolis et al. 2012), it has remained an important goal to increase the number of imaged neurons in order to understand the nature of neuronal population activity across widespread brain areas. To achieve this, advanced versions of two-photon microscopes have been developed with larger or multiple fields of view laterally (across the brain surface) (Lecoq et al. 2014; Chen et al. 2016; Sofroniew et al. 2016; Stirman et al. 2016), or rapid scanning of volumes axially (through the depth of the brain) (Song et al. 2017a; Lu et al. 2017; Nöbauer et al. 2017). In addition, newly developed optical implants have enabled hugely expanded views of neural tissue, rendering up to ~36 mm^2 of the cortical surface optically accessible compared to ~1 mm^2 in original glass coverslip-based windows (Figure 1B). Removable or penetrable windows have also been developed that allow access for drug application or introduction of recording electrodes (Goldey et al. 2014; Roome and Kuhn 2014). The combined advances in microscopy and optical implants have allowed an astonishing increase in the number of neurons possible to image in a single mouse, from a few in pioneering studies (Mank et al. 2008; Andermann et al. 2013) to an estimated one million with the recently developed Crystal Skull and See-Shell methods (Kim et al. 2016; Ghanbari et al. 2019).

Neurons within deep brain structures have been essentially hidden from view since the fluorescence excitation light needed to reach them is heavily scattered within brain tissue, especially at shorter wavelengths. Typical two-photon imaging (820–980 nm wavelength) is limited to less than 1 mm below the brain surface (Denk and Svoboda 1997; Helmchen and Denk 2005). Red fluorescent probes improve imaging depth by using longer wavelength excitation (1000–1100 nm) (Dana et al. 2016). Recently established three-photon excitation (1300 nm wavelength) (Ouzounov et al. 2017) can visualize neurons through all layers of mouse cortex to the hippocampus, more than 1 mm below the brain surface. However, even with these developments, optical access to many regions of the intact mammalian brain cannot be achieved. The introduction of chronically implanted optical relay lenses (e.g., GRIN lenses) and optical chambers (created from a glass coverslip fused to a guide tube or cannula) has been a major advance for deep brain imaging (Barretto et al. 2009; Dombeck et al. 2010). Although invasive, requiring removal or displacement of the overlying neural tissue, such implants are currently the only available method to optically access deep brain areas beyond the light penetration depth of multiphoton excitation. Optical chambers have better optical quality than GRIN lenses and have provided high-resolution data from

hippocampus and striatum (Sato et al. 2016; Bloem et al. 2017), but are also more invasive because of their typically three times larger diameter. Transcortical GRIN lenses have been successfully used to perform chronic optical imaging of neurons within structures such as hypothalamus, hippocampus, and amygdala (Ziv et al. 2013; Bocarsly et al. 2015; Grewe et al. 2017), several mm below the brain surface (Figure 1C), and can be used with either two-photon or one-photon fluorescence excitation (Jung et al. 2004). Microprisms can also be used for specialized applications (Figure 1D), allowing an *en bloc* view of neuronal populations across different cortical layers, vertically oriented apical dendrites, or hard to reach locations such as insular or entorhinal cortex (Andermann et al. 2013; Low et al. 2014; Livneh et al. 2017). Together, the parallel improvements in fluorescent activity indicators, microscopes, and optical implants represent major advances for the study of *in vivo* neural function using chronic optical imaging techniques.

Stability and plasticity

How has chronic *in vivo* imaging of cellular activity been used to investigate unanswered questions in neuroscience? One fundamental question is the degree of stability of single neuron versus population activity over extended time periods. The balance between stability and plasticity could relate to the capacity of neural circuits to faithfully encode and store information, yet also adapt to a changing environment and learn new information (Lütcke et al. 2013; Margolis et al. 2014; Clopath et al. 2017). A number of longitudinal studies have provided insight into the nature of stability of neural function on many levels, from synapses (Ziv and Brenner 2018) to whole-brain representations (Kolasinski et al. 2016).

Chronic imaging of GECIs examines this question on the neuronal population level. One emerging view from studies of visual, somatosensory, motor, and association areas of the cerebral cortex is that neuronal population activity is largely stable over days even though the activity of individual neurons that comprise these populations is variable (Mank et al. 2008; Margolis et al. 2012; Mayrhofer et al. 2015; Clopath et al. 2017) (but see counterexamples, below). For example, the overall proportion of neurons active in response to touch in primary sensory cortex (S1) of behaving mice is stable, but the identities of the individual neurons that are active can change from day to day (Peron et al. 2015; Chen et al. 2015a). Similar results have been found in visual and motor cortex (Huber et al. 2012; Peters et al. 2014; Poort et al. 2015). The same is true of neurons in posterior parietal cortex during performance of a visually-guided virtual reality navigation task, even after learning, when behavioral performance had stabilized and the neural representation had become more refined (Driscoll et al. 2017). These data suggest that the activity of individual neurons is more variable or flexible over time than the population as a whole, implying that unknown population-level homeostatic mechanisms normalize overall population activity (LeMessurier and Feldman 2018). There are also open questions about the distribution of activity within neuronal populations. In superficial layers of sensory cortex, for example, a small fraction of neurons responds to stimuli reliably and robustly, while most other neurons are more variable and less excitable (Shoham et al. 2006; Barth and Poulet 2012; Margolis et al. 2012; Ovsepian 2019). In general, the role of individual neurons versus populations in neural signaling is an important question for understanding both local circuit computations and how information is transmitted between brain areas.

There are also striking examples of long-term stability of neuronal activity, indicating that not all single neurons are variable. Chronic two-photon imaging data from neurons in the singing-related area HVC of the songbird show maintenance of the precise sequence of population activity across several weeks (Katlowitz et al. 2018) (Figure 2A–C). A high degree of stability was also found in chronic electrophysiological recordings of cortical and subcortical neurons in rats performing natural behaviors and motor tasks (Dhawale et al. 2017). It will be important to determine whether certain subsets of neurons are more stable than others by using high density electrical recordings (Steinmetz et al. 2018) or large field-of-view neuronal population imaging (Lecoq et al. 2014; Chen et al. 2016; Sofroniew et al. 2016; Stirman et al. 2016), and to gain information from various brain regions during both learning and stable behavioral performance.

The determinants of neuronal stability and flexibility are still unknown. One possibility is that the inherent dynamics of a given neural circuit, and its modulation during behavior, determines its degree of stability. A chronic calcium imaging study of hippocampus in behaving mice addresses whether different hippocampal subregions in the same animal show different degrees of stability (Hainmueller and Bartos 2018). The authors used an optical cannula to access three regions of hippocampus, CA1, DG and CA3 and imaged neuronal activity during performance of a visually guided task in virtual reality. Remarkably, DG was the only one of the 3 hippocampal subregions to show stability of spatial coding across 3 days (Figure 2D–F), while CA1 and CA3 were more variable (similar to (Ziv et al. 2013)). These results indicate that even closely connected sub-regions of a larger interconnected neural structure can show varying degrees of stability. Whether the differences in flexibility relate to the role of these sub-circuits in cognitive flexibility or the capacity for learning remains to be determined. It is reasonable to posit that stability and flexibility both play important roles for brain function.

Neural circuit changes during learning

How the brain changes with learning is another fundamental question that chronic optical imaging is uniquely suited to investigate. As behavioral tasks are learned and refined, multiple brain areas participate in adaptive synaptic and population-level changes in neuronal function (Romo and de Lafuente 2013; Le Merre et al. 2018; Crochet et al. 2018). Many learning paradigms in mouse models, especially those combined with imaging, involve presentation of a sensory stimulus followed by a response generated by the subject that produces a reward (usually food or water). This broad categorization of sensorimotor learning can be conceptualized as three phases of learning: perceptual, sensorimotor association, and skill learning (Makino et al. 2016). By tracking changes in functional properties of population activity over time, chronic imaging experiments can investigate the progression of neural changes within targeted brain regions through these distinct phases of learned behavior. Recent chronic imaging studies in mice have begun to make insights into longstanding issues in neuroscience, including the extent of plasticity that takes place in primary sensory areas of the cerebral cortex, how information is routed from one brain area to downstream target areas during learning, and how subsets of neurons undergo learning-related changes in selectivity for sensory, behavioral, and cognitive or

reward-related features of task performance. For further consideration of data from in both non-human primates and mice, we refer the reader to (Makino et al. 2016).

Major efforts have been made to optimize experimental approaches in head-restrained mice that combine cellular resolution optical imaging with behavioral paradigms implemented around a stationary microscope, allowing high-resolution imaging during locomotion and sensory-guided decision-making tasks (Komiyama et al. 2010; Guo et al. 2014; Dombeck and Tank 2014). In a complementary approach, miniaturized head-mounted microscopes allow chronic imaging of population activity during more naturalistic behaviors (Flusberg et al. 2008; Cai et al. 2016; Jacob et al. 2018), albeit with lower optical resolution, and therefore more challenging cell identification across days (Ziv et al. 2013). The following examples illustrate key features of changes in cortical and subcortical neuronal population activity during learning, measured using either head-restrained or miniature head-mounted chronic cellular imaging techniques.

Early sensory areas of the cerebral cortex send axonal projections to many downstream target areas, and also receive feedback inputs from “higher order” areas, but it has remained unclear if certain pathways are particularly important for learning-related neural processing. In the primary somatosensory cortex (S1) of mice, intermingled populations of layer 2/3 neurons project to either S2 or M1, and can be distinguished using retrograde tracers (Chen et al. 2013a; Yamashita et al. 2013; Chen et al. 2015a; Tervo et al. 2016; Chatterjee et al. 2018). When mice learn to discriminate between textures with their whiskers, S2- and M1-projecting neurons undergo different types of functional changes: the fraction of M1-projecting neurons sensitive to touch increases, while the S2-projecting neurons remain the same in number but become more discriminative between stimuli (Chen et al. 2015a) (Fig. 3A). Strikingly, learning-related activity changes persist outside of the behavioral task for M1-projecting neurons, while the activity of S2-projecting neurons is discriminative only during task performance, highlighting the specificity of behavior-related neural dynamics for distinct populations of projection neurons within S1. These findings indicate that learning-related plasticity can occur within specific types of projection neurons, even at primary cortical stages of the sensory pathway. However, the relative contribution of the encoding of sensory stimuli versus changes in the learned behavioral strategy (i.e., stronger or more efficient whisker movements toward target objects) to S1 plasticity remains to be determined (Peron et al. 2015; Chen et al. 2015a).

A striking example of learning-related changes in early sensory encoding comes from work in mouse primary visual cortex (V1). When mice are trained to discriminate visual stimuli of different orientation for water rewards, individual V1 neurons show prominent changes in orientation selectivity with learning (Fig. 3B) (Poort et al. 2015). Subsets of neurons become selective for either the rewarded or non-rewarded visual stimuli (with many still visually responsive but non-selective). Neuronal populations as a whole show progressive improvement in neural discriminability that parallel the increased behavioral performance with learning (Fig. 3B, right). These results, and those of studies in other sensory modalities (Shuler and Bear 2006; Hui et al. 2009; Gdalyahu et al. 2012; Kato et al. 2012; Lacefield et al. 2019), support the concept that neuronal selectivity for sensory stimuli can change dramatically depending on the stimuli’s behavioral salience (rewarded or unrewarded).

Such results indicate that cognitive aspects such as reward or salience can strongly influence neural plasticity that takes place during learning. Indeed, reward may even be represented by dedicated neuronal subpopulations, as recently found with in vivo imaging in hippocampus (Gauthier et al. 2018). In a noteworthy example from mouse visual association cortex, (Ramesh et al. 2018) found that neurons recruited to become active during learning were part of reward-related (value-coding) rather than stimulus-related neuronal subpopulations (ensembles). Imaging studies have also revealed extensive learning-related changes in neuronal population activity in subcortical brain areas with key roles in valence encoding, such as the amygdala. Here, pairing an auditory tone with an aversive foot shock led to a near-total switch in auditory-responsive neurons, with neurons initially activated by the conditioned tone losing responsiveness, and previously inactive neurons becoming active (Grewe et al. 2017) (Fig. 3C). These results emphasize the importance of behavioral salience for learning-related neural signaling and population-level functional plasticity.

While we have largely focused on functional studies of neural activity, structural plasticity of neurons, especially on the level of dendritic spines, is another type of experience-dependent and learning-related plasticity that has been extensively studied using chronic two-photon imaging (Trachtenberg et al. 2002; Holtmaat and Svoboda 2009; Berry and Nedivi 2017). Motor learning affects spine formation and maintenance in motor cortex but not in other cortical areas, while spines in sensory cortex undergo plasticity after sensory discrimination training (Yang et al. 2009; Fu et al. 2012; Kuhlman et al. 2014). Auditory fear conditioning leads to increased spine formation in primary auditory cortex (A1) and extinction leads to elimination of the newly formed spines (Lai et al. 2018). Notably, the opposite effects are seen in frontal association cortex, where extinction of learned associations causes spine formation (Lai et al. 2012). Learning-related changes in spines have been shown to be influenced by inhibitory interneuron activity in motor cortex, suggesting an interplay between excitatory and inhibitory neurotransmission in dendritic spine plasticity (Chen et al. 2015b). However, while it is likely that structural spine dynamics relate to neural activity during learning, the exact relationship has yet to be determined. Fewer in vivo studies have investigated the functional properties of dendritic spines because of the high level of mechanical stability needed to image these micron-scale structures. Thus, most data on spine function has been acquired in anesthetized animals or in ex vivo preparations (Yasuda et al. 2004; Chen et al. 2013b; Berry and Nedivi 2017). However, newer head fixation methods such as using an air supported platform has achieved stability necessary to resolve spines in awake behaving animals (Pryazhnikov et al. 2018), suggesting that functional imaging of dendritic spines will be an active area of future research.

Chronic optical imaging of axonal calcium signals in behaving mice has been performed in several studies (Glickfeld et al. 2013; Broussard et al. 2018; Dana et al. 2019), which has revealed progressive, circuit-specific changes during learning (Burgess et al. 2016; Kupferschmidt et al. 2017). In the future, it may be possible to combine cellular resolution presynaptic axonal and postsynaptic dendritic imaging (Takahashi et al. 2016; Lacefield et al. 2019) to define changes in the learning-related input-output properties of neural circuits. This could enable novel investigations of many outstanding questions related to how brain areas interact during learning and decision-making, including the influence of feedback

signals from higher-order cortical areas, or subcortical structures such as thalamus and amygdala, and the effects of neuromodulator signals on neuronal population activity.

It is important to mention that in vivo imaging, in particular studies of dendritic spine structural dynamics, have provided valuable information on potential mechanisms of neural dysfunction. Alterations in dendritic spines have been associated with neurodevelopmental, neurodegenerative, and neuropsychiatric disorders, as reviewed elsewhere (Knobloch and Mansuy 2008; Glausier and Lewis 2013; Martínez-Cerdeño 2017). In Alzheimer's disease, for example, there is a decrease in spine density that becomes more pronounced near plaques that are characteristic of the disease (Spires et al. 2005). Furthermore, whisker stimulation-related plasticity is impaired in aged compared to young mice, suggesting impairments in spine dynamics during normal aging as well (Voglewede et al. 2019). Further experiments designed to simultaneously acquire in vivo functional and structural data, in addition to further chronic neuronal population imaging studies in mouse disease models, would provide important information on the relationship between structural and functional neural circuit plasticity in both the healthy and diseased brain.

Analysis of chronic imaging data for understanding neural coding and behavior

Chronic cellular imaging experiments can produce massive datasets, including hours of movies from thousands of neurons measured repeatedly in multiple imaging sessions. Thus, optimizing data analysis is critical to avoid major bottlenecks for such experiments (Paninski and Cunningham 2018). Beyond measuring first-order features, such as the fraction of active neurons or their tuning properties, sophisticated analysis methods are essential for inferring the dynamic properties of neuronal population activity from calcium imaging data. A key first step is to estimate the underlying neural activity from the fluorescence signal, which is not trivial since calcium fluorescence signals are relatively slow and noisy compared to action potential firing, and optical signals from individual cells can be obscured by their neighbors. A number of computational methods based on template matching, deconvolution, approximate Bayesian inference, and matrix factorization have been developed to infer spiking activity of neurons from calcium imaging data. For further information, the reader is referred to recent reviews on the topic of calcium imaging analysis methods and pipelines (Stringer and Pachitariu 2019; Pnevmatikakis 2019). One popular analysis framework (Pnevmatikakis et al. 2016) uses a constrained nonnegative matrix factorization approach to identify the locations of neurons and demix the ones that are spatially overlapping, while simultaneously deconvolving their spiking activity from the spatiotemporal structure of the population calcium recordings. A limitation of these methods is the requirement of user intervention (e.g., setting parameters), and certain assumptions imposed on the model of calcium signal generation, or on the dynamics of fluorescence measurements. Such limitations can present particular challenges for analysis of chronic cellular imaging data, because the same cells must be correctly identified and tracked, and calcium signals extracted, across multiple days in order to provide meaningful measurements of long-term stability or plasticity. Newly developed analysis frameworks have started to take these

specific issues related to chronic imaging into account (Giovannucci et al. 2019), and will undoubtedly be a topic of further development.

Data-driven methods, supervised learning techniques, and machine learning have great potential for improving analysis of chronic cellular imaging data by minimizing user intervention and increasing the scalability and flexibility of the frameworks (Sasaki et al. 2008; Patel et al. 2015; Speiser et al. 2017; Theis et al. 2016). One study that compared the performance of various generative and supervised spike inference algorithms (including deep neural networks) applied to specific datasets, concluded that many algorithms yield similar performance for inferring spike rates, but that each offers unique advantages and disadvantages in terms of speed and generalizability (Berens et al. 2018). Other recent work has argued that spike inference using simple non-negative deconvolution meets the performance of supervised methods, and recommended this as the preferred choice due to its simplicity and efficiency (Pachitariu et al. 2018). Regardless of the specific algorithm used, a key issue for chronic cellular imaging datasets is the accuracy and robustness of the analysis of the same cells across multiple imaging sessions. One study found that an algorithm first trained on simultaneous recordings of spikes and calcium data performed well on inferring spike rate when applied to new datasets (Theis et al. 2016). The same approach could be used to analyze data from the same neuronal populations from one day to the next, enhancing the efficiency and power of chronic imaging data throughput, thereby speeding insights into mechanisms of neural plasticity.

Calcium imaging can also be performed across large-scale, spatially separated brain areas. Spatiotemporal features of such widefield calcium signals are generally analyzed directly (without spike inference). Network analysis methods originally developed for human neuroimaging experiments (Bassett and Sporns 2017; Khambhati et al. 2018) have been applied to imaging data from various optical sensors in mice, including calcium indicators, to make insights into dynamic interactions between brain regions on the mesoscale level (Xie et al. 2016; McVea et al. 2016). For example, spectral analysis of resting-state cortical networks, constructed based on wide-field calcium imaging data, found frequency-dependent activity clusters in specific cortical regions (Vanni et al. 2017). Other studies applied visibility graph (Lacasa et al. 2008) in combination with machine learning techniques to investigate the temporal characteristics of wide-field cortical calcium dynamics related to behavioral state (Zhu et al. 2018). An emerging view from these and other studies (McGinley et al. 2015; Musall et al., 2019; Stringer et al. 2019) is the importance of global modulatory influences driven by arousal and movement on behavior-related neural dynamics. However, little is still known about how large-scale networks change during learning (Makino et al. 2017), or how such large-scale changes are related to changes in individual neurons or tractable neuronal populations. Artificial intelligence (AI) and deep learning techniques capable of automatically learning patterns and representations in data may be particularly well-suited for investigating neural mechanisms of behavior across the multiple spatial and temporal scales available in chronic imaging experiments, perhaps by generating models that are predictive of learning rate. To reach this stage, however, machine-learning approaches will have to establish their robustness for the analysis of neural data (Vogt 2018). It remains an active goal to bridge gaps in our understanding of how neural circuits operate on multiple levels of brain function, on the temporal scale

between short-term (milliseconds or seconds scale) and long-term (days or weeks scale) changes in neuronal activity, and on the spatial scale between brain regions and local neuronal populations. Chronic imaging, combined with experimental and computational advances, has great potential for further mechanistic discovery of neural circuit function.

Outlook: multimodal interrogation of neural circuits

Rapid progress in the development of additional neural sensors has been an exciting area of advance. High-performance genetically encoded voltage indicators (GEVIs) have been a long-sought goal as a more direct and faster readout of neuronal activity (Knöpfel et al. 2003). Recent improvements in GEVIs have been dramatic, including the capacity for *in vivo* imaging, and their sensitivity is beginning to rival that of GECIs for detecting single action potentials (Hochbaum et al. 2014; Gong et al. 2015; Song et al. 2017b; Piatkevich et al. 2019). Imaging the millisecond kinetics of GEVI signals from neuronal populations requires fast frame rates generally beyond the capacity of two-photon laser-scanning microscopy, and the high light levels needed for fluorescence excitation can lead to photobleaching and photodamage (Yang and St-Pierre 2016; Xu et al. 2017; Bando et al. 2019). Despite these challenges, chronic cellular resolution imaging of GEVIs has the potential to provide unprecedented information on plasticity- or learning-related changes in the temporal patterns of neuronal firing. GEVIs, like the most sensitive GECIs, are also capable of detecting slower subthreshold voltage changes, although most population imaging studies have focused on the faster signal associated with action potential firing.

Additionally, new classes of genetically encoded indicators for detecting neurotransmitters (e.g., glutamate, GABA (Marvin et al. 2013, 2019)) and neuromodulators (e.g., dopamine, acetylcholine, or norepinephrine (Patriarchi et al. 2018; Jing et al. 2018; Feng et al. 2019)) represent major advances with potential for further circuit discovery. In future chronic imaging experiments, it may be feasible to investigate multiple aspects of neural circuit activity simultaneously by imaging different color indicators expressed in distinct cell types or cellular compartments. For example, simultaneous imaging of axons and dendrites or cell bodies could be performed using green and red GECIs, respectively, to distinguish pre- and postsynaptic sites of plasticity. Neuromodulation of neuronal population activity could be investigated with co-expression of GECIs or GEVIs and neuromodulator sensors in neurons (for example, GCaMP6 and dLight1 (Patriarchi et al. 2018)). Novel applications of viral vectors (Bedbrook et al. 2018) and a large repertoire of transgenic reporter mice (Dana et al. 2014, 2018; Madisen et al. 2015; Wekselblatt et al. 2016; Daigle et al. 2018) allow flexibility in restricting expression of genetically encoded indicators to specific cell types, subcellular structures, and brain regions (Broussard et al. 2018; Dana et al. 2019). Employed together with sophisticated behavioral paradigms in both head-fixed and freely moving mice, the growing toolkit of genetically encoded indicators holds great potential for extending our knowledge of neural circuit function and plasticity both on a fast time scale of single behavioral trials and over longer time scales during learning and memory formation. Many previously intractable questions in neuroscience are now addressable, from the functional architecture of memory, neural circuit mechanisms of injury or disease, and even undiscovered functional cell populations and projections, which will in turn lead to new insights into the functioning of the brain and open up new areas of inquiry.

All-optical circuit interrogation using optogenetics to manipulate neuronal activity combined with functional imaging to simultaneously monitor activity has become an achievable goal (Prakash et al. 2012; Carrillo-Reid et al. 2019; Marshel et al. 2019). Optogenetics can be used to selectively modulate the activity of neurons embedded within neural circuits (Deisseroth 2015). Combining optogenetics with two-photon imaging has recently been used to recapitulate activity in neuronal ensembles that can mimic visual input (Carrillo-Reid et al. 2019; Marshel et al. 2019). Future experiments combining optogenetics with GEVIs will certainly lead to additional understanding of how neural networks encode stimuli and integrate synaptic input.

Ongoing and future experiments aim to achieve an expanded view of large-scale neuronal population activity with cellular resolution, and a fine-scale view of activity within subcellular compartments, such as axons and dendrites. Combined imaging of voltage, calcium, neurotransmitters, and neuromodulator indicators could be performed on both the cellular and subcellular levels during innate and learned behaviors. Together, neurotechnology developments for chronic optical imaging, including new indicators and next-generation optical systems, have great promise for providing novel insight into neural circuit function and plasticity.

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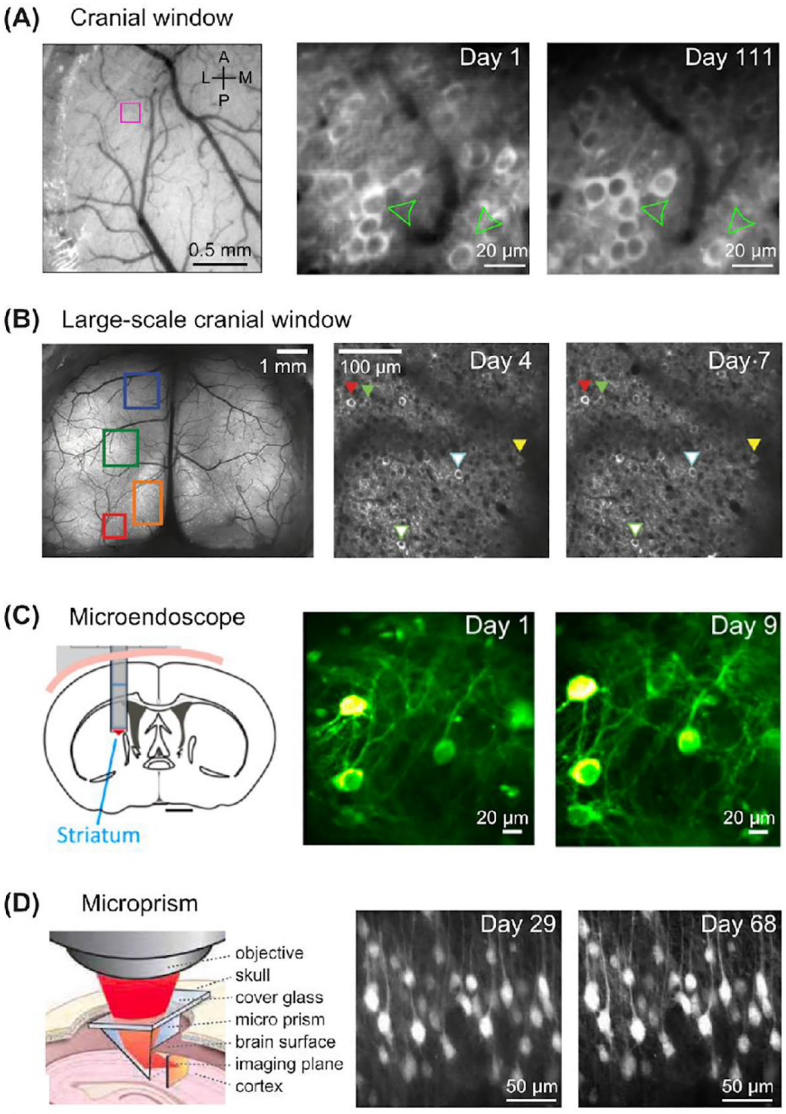

Figure 1.

Chronic imaging approaches for tracking neuronal activity using two-photon imaging of genetically encoded calcium indicators (GECIs). **A.** Left: Top view of the cerebral cortex through a cranial window implanted above the dura mater. Right: layer 2/3 cortical neurons expressing YC3.60 followed over 111 days. Adapted from Margolis, Lütcke et al. (2012) (Margolis et al 2012). **B.** Left: Top view of cortex through a large-scale “Crystal Skull” cranial window. Right: Chronic imaging of one of many optically accessible cortical neuronal populations expressing GCaMP6s. Adapted from Kim et al. (2016) (Kim et al. 2016). **C.** Left: Deep brain structures can be imaged through an implanted GRIN lens. Right: Neurons in striatum expressing GCaMP6s. Note that neurons were followed for a longer time period than shown here. Adapted from Bocarsly, Jiang et al. (2015) (Bocarsly et al. 2015). **D.** Left: Axially oriented brain structures can be imaged through a prism implanted facing the side of the area of interest. A microprism can also be used for deeper brain structures. Right: Neurons in neocortex expressing GCaMP3 imaged over multiple weeks.

Apical dendrites are visible in the x-z plane. Note that the original image was cropped for display. Adapted from Andermann et al. (2013) (Andermann et al. 2013).

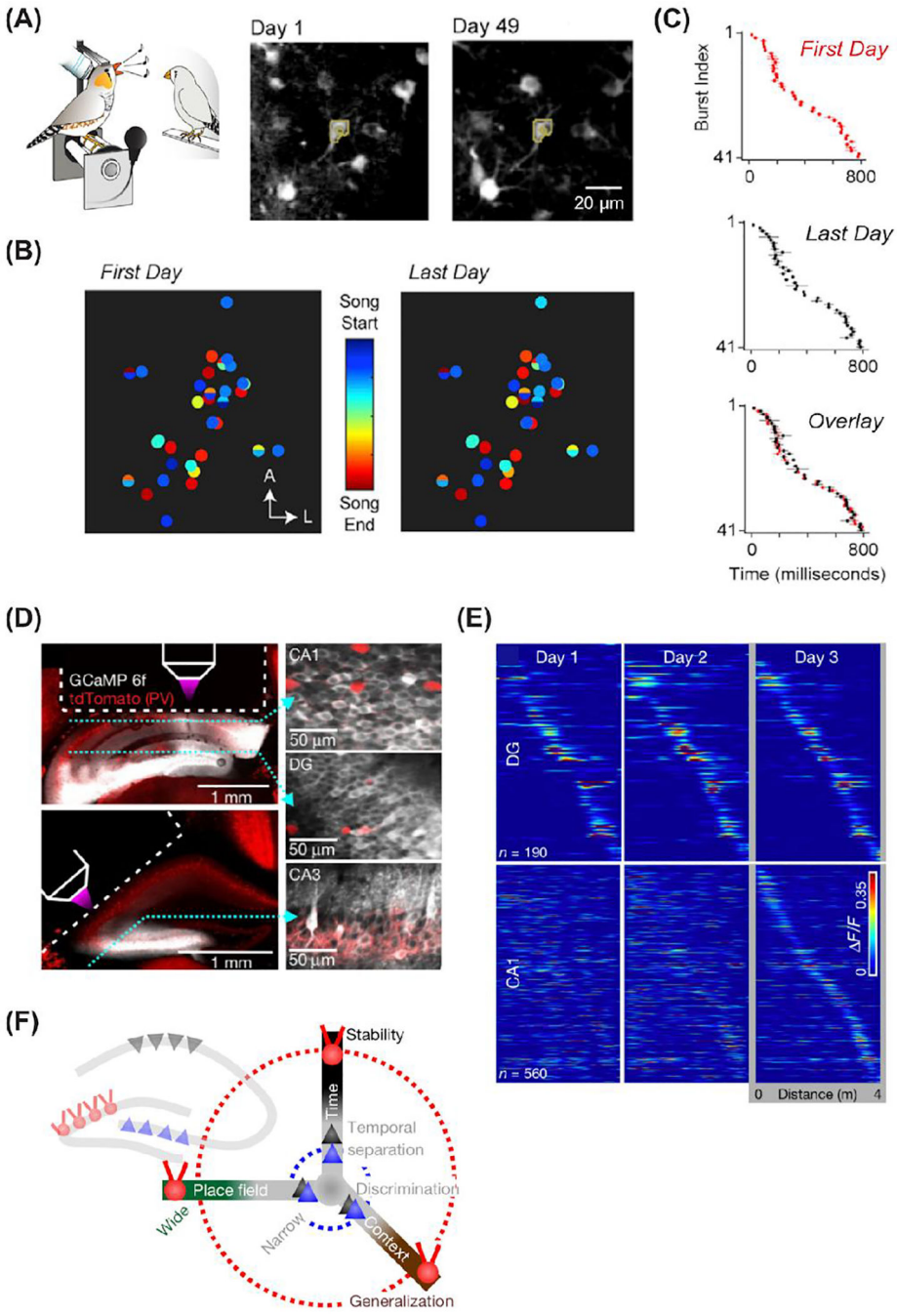

Figure 2.

Stability and flexibility of behavior-related neuronal population activity measured with chronic imaging. **A.** Example of stability in songbird experiment. Two-photon images of neurons expressing GCaMP6f tracked over 49 days. **B.** Comparison of song timing maps for a population of neurons. **C.** Stability of burst index measures. A-C adapted from Katlowitz et al. (2018) (Katlowitz et al. 2018). **D.** Subregion-specific stability in hippocampus. Chronic imaging of CA1, DG, and CA3 sub-regions. **E.** Heat maps of neuronal calcium signals across three days showing higher stability for DG than CA1. **F.** Schematic of differences in

stability, width, and generalization for CA1, DG, and CA3. D-E adapted from Hainmueller and Bartos (2018) (Hainmueller and Bartos 2018).

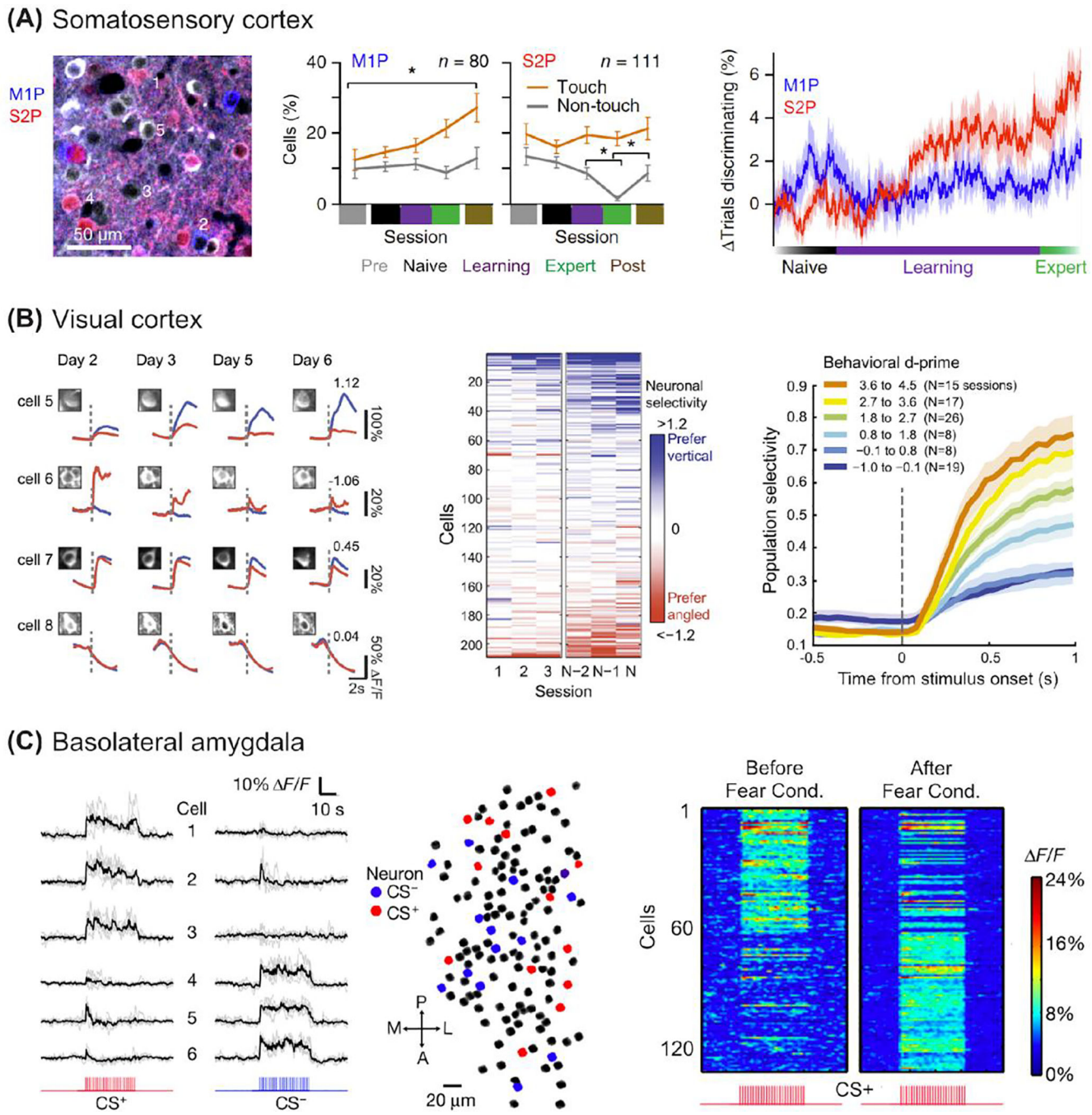

Figure 3.

Learning-related changes in neuronal population activity measured with chronic cellular imaging. **A.** Projection neurons in primary somatosensory cortex (S1) show distinct learning-related activity during performance of a tactile discrimination task. Left: Identification of M1-projecting (M1P) and S2-projecting (S2P) neurons via retrograde tracers. Middle: The fraction of neurons classified as touch or non-touch as a function of Naive, Learning, and Expert behavioral phases. Right: The change in discrimination accuracy of M1P and S2P neurons for Go and NoGo tactile stimuli (P100 vs. P1200

textures) through learning. Adapted from (Chen et al. 2015a). **B.** Neurons in primary visual cortex (V1) show diverse learning-related activity during performance of a visual discrimination task. Left: Example calcium signals from four neurons across four imaging sessions in response to a vertical, rewarded stimulus (blue) or an angled, non-rewarded stimulus (red). Middle: Selectivity of neurons across the first three and last three training sessions. Right: Neuronal population selectivity as a function of learning. Each curve depicts the time course of selectivity at a range of behavioral d'. Adapted from (Poort et al. 2015). **C.** Neurons in basolateral amygdala change selectivity with auditory fear conditioning. Calcium signals of cells responsive to two different auditory tones (CS+ or CS−) before pairing the conditioned stimulus (CS+) with a foot shock using a fear conditioning paradigm. Right, Cell population responses in an example mouse to the CS+ tone before and after fear conditioning. A, anterior; L, lateral; M, medial; P, posterior. Adapted from (Grewe et al. 2017).