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REVIEW

Functional multineuron calcium imaging for systems pharmacology

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Abstract Functional multineuron calcium imaging (fMCI) is a large-scale technique used to access brain function on a single-neuron scale. It detects the activity of individual neurons by imaging action potential-evoked transient calcium influxes into their cell bodies. fMCI has recently been used as a high-throughput research tool to examine how neuronal activity is altered in animal models of brain diseases, for example stroke, Alzheimer's disease, and epilepsy, and to estimate how pharmacological agents act on normal and abnormal states of neuronal networks. It offers unique opportunities to discover the mechanisms underlying neurological disorders and new therapeutic targets.

Keywords Receptors/ion channels · Pharmaceuticals · Optical sensors · Fluorescence/luminescence

Introduction

Functional imaging is a promising strategy for experimental access of the developmental, physiological, and pathological processes of the brain. One of these strategies is calcium imaging, which optically records intracellular calcium dynamics. The calcium ion is an intracellular messenger involved in many neuronal functions, for example secretion, neurite motility, gene expression, and synaptic plasticity. Neuronal electrical activity evokes calcium influx, and, in particular, an action potential reliably leads to a transient calcium increase in the cell body. Functional multineuron calcium imaging (fMCI) takes advantage of this fact. By optically referring to the timings of somatic calcium transients of individual neurons, it reconstructs the spiking activity of a large neuron population. The remarkable advantages include:

1. simultaneous recordings from up to 10,000 neurons in a local circuit;
2. single-cell and single-spike resolution
3. identifiable locations and types of neurons (even neurons that are inactive during the recording period).

fMCI is, therefore, a powerful tool for recording neuronal network activity in intact and abnormal brains. In this review, we describe the principle and recent technical advances in fMCI and introduce examples of practical applications of fMCI in diseased or damaged brain models, providing a framework for future neuropathological pharmacology.

fMCI for neuronal circuits

The nervous system functions through a huge network that consists of a large amount of various types of neurons. Physiological studies have been made to investigate the electric properties of individual neurons and synapses, yet the intrinsic structure and dynamics of neuronal circuits remain to be fully understood. This might be attributable, at

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least partly, to a lack of practical approaches in monitoring and analyzing large-scale neuronal network activity. Thus, our previous understanding of neuronal circuitry and its dynamics is mostly based on predictive insights, which are collected a posteriori from independent pieces of information about the morphology and excitability of neurons. Otherwise, it is based on recordings of field activity, i.e., averaged net responses of a local neuron population, which does not carry information about individual neuron "personality". In network systems, however, neurons act in a cooperative manner and thereby exhibit nonlinear assembly dynamics which cannot be predicted by simple summation of individual neuron dynamics. Therefore, we need a novel experiment that monitors activity simultaneously from a large population of neurons at single-cell resolution.

Over recent decades, large-scale brain imaging techniques have been dramatically developed and improved, including functional magnetic resonance imaging, positron-emission tomography, imaging of intrinsic optical signals, and voltage-sensitive dye-based imaging [1–5]. These techniques have been used for studying different aspects of brain function and have readily contributed to the discovery of important macroscopic features of neuronal behavior at the system level; a significant drawback of these techniques is, however, their poor spatial resolution.

With regard to spatial resolution, fMCI provides one of the most useful techniques, which can monitor neuronal activity with single-cell resolution. An action potential elicits a transient large influx of calcium ion into the soma through voltage-gated calcium channels. fMCI captures this calcium transient as an increase in somatic fluorescence of chemical or genetically-encoded calcium sensors. If an appropriate sensor is selected, it gives a fine signal-to-noise (*S*/*N*) ratio; a single action potential evokes a 2−30% increase in the fluorescent intensity (Fig. 1a). Thus fMCI can even reconstruct the spontaneous firing pattern of individual neurons, because the excellent *S*/*N* ratio makes the across-trial averaging unnecessary; note that voltage-sensitive dye imaging requires multiple trials of imaging to reliably detect neuronal signals because of its poor *S*/*N* ratio (<1% change of signals). fMCI data also include spatial information, i.e., the locations of individual neurons, and in some cases it can even identify the cell types based on the temporal patterns of calcium activity. Cell types can also be identified by post hoc histological labeling with cell-specific markers. After fMCI, neurons are identified by staining with fluorescent Nissl or immunostaining with anti-NeuN or anti-GABA antibodies [6, 7]. In addition, sulforhodamine 101 is a useful fluorescent compound that selectively labels astrocytes even in living tissues [8].

The original idea of fMCI was introduced by Yuste and Katz [9]. They labeled cortical slices prepared from juvenile rats with an acetoxymethyl (AM)-ester derivative of a calcium indicator and imaged the activity of dozens of neurons. AM-ester is a residue which enhances biological membrane permeability. On entering the cell AM-ester is rapidly hydrolyzed by endogenous cytoplasmic esterases and converted into an active form of the indicator. The active form has ionic charges, which make the indicator membrane-impermeable, and thus the indictor accumulates inside the cell up to a concentration higher than the extracellular concentration of the AM-ester form. Thus, a simple method for labeling cells is to incubate brain slices or cell cultures in a solution containing the AM-ester form of the indicator dye. For example, Oregon green 488 BAPTA-1 AM is one of the most widely used synthetic calcium indicators, because it has a high *S*/*N* ratio and rapid kinetics of signal rise and decay (~500 ms) following single action potentials [7].

Several genetically engineered calcium indicators have been developed through a combination of fluorescent proteins and calcium-sensitive proteins [10]. These protein sensors can be selectively expressed in defined cell populations through specific promoters and enable long-term imaging in vivo at the cellular level. Although genetically encoded sensors have started to be used for recording of neuronal activity, their low sensitivity and low expression levels have not allowed detection of single action potentials. Recent genetic improvements overcame this problem, providing potential tools for future fMCI studies [11, 12]. The expression level of these calcium indicators, however, may vary between cells, in particular when the genes are introduced by electroporation. Thus, calcium sensors for the ratio imaging, for example FRET-based indicators, will be more practical for spike decoding [13].

An established approach for fMCI uses one-photon or multi-photon excitation, for which confocal or two-photon microscopy, respectively, concentrates excitation light on to a focal spot in the specimen and collects the emission light from the spatially confined spot. Although normal epifluorescence microscopy suffers from light scattering, confocal microscopy uses a pinhole to pick up photons emitted only from the laser focus. Recent technical advances have improved the spatiotemporal resolution to enable monitoring of the population activity of neurons. Nipkow-disk confocal microscopy uses multisite focal illumination, and thus detects fluorescence virtually en masse from the entire field of view, while conventional confocal microscopy can only scan a small spot in the field at a time. A rotating spinning disk used in Nipkow-type microscopy uses tens of thousands of multiple pinholes, through which the laser is split into small rays to fully cover the field. The fluorescence light is detected by a back-illuminated electron-multiplying charge-coupled device camera. Nowadays, this optical system enables scanning as fast as 2000 Hz (unpublished data). Nipkow-disk microscopy also

helps reduce photobleaching and phototoxicity. This seems to be because trials are rapidly averaged to generate an image. For each trial, the intensity of the small laser is very weak; note that the photobleaching is reduced sub-linearly with light intensity. Thus, Nipkow-disk fMCI allows continuous imaging for a long time. Figure 1c shows an example of fMCI-monitored spontaneous neuronal activity in a hippocampal cultured slice, in which more than 100 CA1 neurons were continuously recorded for an hour. By using a low-magnification objective, fMCI can monitor about 1,000 cells and even more than 10,000 cells in a hippocampal and cortical slice, respectively [14, 15].

fMCI is used to record spatiotemporal activity patterns of neural circuits with single-cell resolution. Compared with multi-electrode recordings [16], fMCI has an advantage that all cells in a field of view can be monitored, regardless of whether or not they fire spikes. Monitoring the multineuronal activity with fMCI has revealed that cortical microcircuits generate non-randomly organized spatiotemporal patterns of spontaneous activity [6, 17, 18] and that patterns of externally evoked activity statistically resembles those of spontaneous activity [19].

fMCI for in vivo preparations

In the last decade, fMCI has started to be used in in vivo experiments. In vivo studies are of importance in investigating more natural and physiological neuronal responses. One of the technical problems in in vivo fMCI was the depth to be focused in tissues. Even in mouse neocortex, most neurons are located hundreds of micrometers below the pial surface, and excitation and emission light is severely scattered through the tissue. Two-photon microscopy solves this problem [20]. Two infrared photons produced by an ultra-short pulsed laser (~80−250 fs) are simultaneously absorbed to excite a fluorophore that normally absorbs at visible wavelengths. Compared with visible light, infrared light has greater tissue penetration ability, and moreover, quadratic dependence of two-photon excitation leads to a finer spatial confinement of the excitation volume. As a result, two-photon excitation occurs even hundreds of microns from the tissue surface and enables inherent optical sectioning, which mitigates the effects of light scattering and does not require a detector pinhole used in confocal microscopy. In comparison, confocal microscopy enables study of depths less than 100 μm in optically dense tissues, for example the brain.

An effective method has been developed to load large numbers of cells with AM-form calcium dyes in vivo [21]. With this procedure, thousands of cortical neurons in a living brain are labeled by injection of concentrated calcium indicator solution through a micropipette [22, 23]. Another versatile method is local electroporation of dextran-conjugated or the salt form of calcium indicators [24]. This is applicable not only to image at the cell population level, but also to visualize subcellular processes such as dendritic spines or axon terminals.

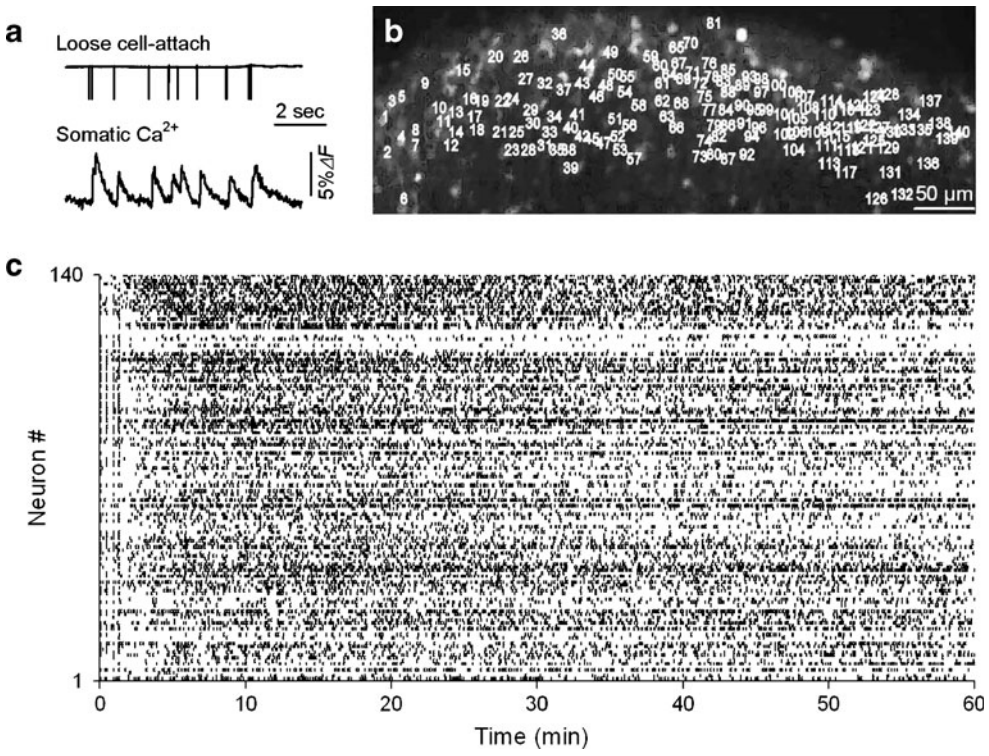

Fig. 1 (**a**) Simultaneous cell-attached electrical recording of spikes (*upper*) and imaging calcium transients (*lower*) obtained from hippocampal CA1 neuron loaded with Oregon green 488 BAPTA-1 AM. Note a series of action potentials was faithfully reflected as timings of calcium transients. (**b**) Locations of fMCI-imaged cells within a CA1 region of a cultured slice. (**c**) Reconstructed pattern of spontaneous spikes of 140 neurons, indicated in (**b**), during a period of 60 min

Excellent applications of fMCI in vivo were carried out in the sensory cortex of mammalian brain. The cerebral cortex is functionally organized, particularly in higher animals, and adjacent cortical neurons show similar responses to a specific sensory input, forming a cortical column. Ohki et al. [22] depicted the functional map of cat primary visual cortex with single-cell resolution and demonstrated that functional neurons were spatially arranged with sharp borders at single cellular levels. fMCI was also applied to rat barrel cortex and monitored the neuronal population responses to whisker stimulation [25]. The authors revealed that, despite their sparseness and variability, population responses showed a clear spatial organization on the columnar scale.

In recent years, the in vivo fMCI technique has been applied to behaving animals. Conventional fMCI setups were too large for animals to carry on their heads, and thus, in vivo fMCI was limited in animals under anesthesia. To overcome the problem, Helmchen et al. [26] developed a compact two-photon microscope 7.5 cm long, which weights 25 g, and which rats can carry on their heads. Excitation light is delivered through a single-mode optical fiber, and images are scanned with resonant vibrations of the fiber tip using a piezo-electronic actuator. More recently, a further improved version of this miniaturized microscope was introduced by Engelbrecht et al. [27]; this includes a gradient-index microlens, and weighs only 0.6 g. These two-photon mini-microscopes recorded calcium signals of cerebellar Purkinje cells from anaesthetized animals, but they still suffered from motion artifacts and failed in functional imaging during active behavior.

An alternative smart approach of fMCI with behaving animals is to use normal two-photon microscopy with a head-restrained animal on the treadmill system. Dombeck et al. [28] applied this system to mobile mice on a Styrofoam ball and simultaneously monitored cellular-level activity of cortical neurons during running or walking behavior. More recently, they designed a "virtual reality" system in which data of treadmill movement are linked online to visual cues projected on a screen in front of the mouse [29]. The visual display was updated in real time on the basis of the movements of the animal. This setup will be combined with in vivo fMCI.

High-throughput and advanced use of fMCI

Automated reconstruction of neuronal activity

fMCI data sizes are usually big, so that manual procedures of spike reconstruction are labor-intensive and inevitably include artificial errors. In order to solve this problem, several methods have been developed to automatically detect neuronal events from raw fluorescence traces, including template-matching algorithms [30], reverse correlation [31], threshold crossing of $\Delta F/F$ or its derivative [17, 18] and a novel detection algorithm for rodent cortex in vivo [32]. Sasaki et al. [7] introduced a new computational framework that can reconstruct spike timings more precisely without any subjective factor. In this algorithm, spike patterns of individual neurons are reconstructed by principal-component analysis with support vector machine. The latter is a machine learning process to automatically find an optimum threshold to extract neuronal spikes. Because calcium transients have a much slower kinetics than action potentials, fMCI cannot resolve complex temporal firing patterns such as burst firing. Temporal deconvolution can reconstruct firing rate changes from calcium signals, which increase the effective temporal resolution by a factor of ~100 compared with the decay of calcium transients [33].

Probing neuronal circuitry by fMCI

Because fMCI data convey both spatial location information and temporal activity patterns of the imaged neurons, fMCI is suitable for probing neuronal connectivity. To find cells participating in network synchronization, Bonifazi et al. [14] designed a method to map the functional connectivity that denotes the statistical similarity of activity patterns between neuron pairs. With this online analysis of fMCI data, they identified "hub" cells with high connectivity and experimentally tested their effects on network dynamics. As for structural connectivity, some studies have used fMCI to reveal the connectivity of functional synapses in local circuits. With fMCI, Peterlin et al. [34] identified the postsynaptic cells that were activated after patch-clamp stimulation of presynaptic neurons. Conversely, by monitoring postsynaptic inputs with patch-clamp recordings, Aaron and Yuste [35] imaged presynaptic source cells, which fired spikes immediately before synaptic input events that were recorded in patch-clamped postsynaptic neurons. This method is named reverse optical probing (ROPing). Because it refers to an inverse correlation between synaptic events in a postsynaptic cell and spontaneous calcium transients in imaged cells, the detection power depends on the number of spikes in presynaptic candidates during the observation period. It also suffered severely from background synaptic activity that occurs in a postsynaptic cell. To improve the signal-to-noise ratio of ROPing, we activated presynaptic candidates by glutamate locally injected through a micropipette and monitored postsynaptic inputs in a patched neuron [36]. As the stimulating pipette was moved over the field, we identified as many as 96% of the presynaptic cells in the imaged field with a 1% false positive error. Nikolenko et al. [37] introduced a systematic

probing strategy of functional synapses. They combined fMCI with high spatial resolution stimulation of two photon-activated caged glutamate compounds. They used multiplexed laser beams through diffractive optical elements to increase the focal spots of two-photon excitation, by which they reliably excited neurons of interest. Serial activation of individual neurons provided a comprehensive input map, i.e., synaptic strength from all location-identified neurons in the field of view.

fMCI application in disease evaluation and drug screening

fMCI is valuable in investigating the functional activity of neurons in diseased brain states, for example stroke, neurodegenerative pathologies, and seizures. We describe example pioneer studies with functional imaging of pathological conditions.

Intracranial hypertension, ischemia, and stroke

Intracranial hypertension occurs in pathologic conditions, for example brain edema, tumor, and intracranial hemorrhage, which result in brain dysfunction. It has yet to be clarified whether the pressure change per se directly perturbs neuronal activity. Although in vivo studies examined the effect of artificially increased intracranial pressure on neuronal activity, these experiments could not exclude the secondary effect of brain herniation or reduced cerebral blood flow. Thus, pressure-elevation circumstances must be replicated as an in vitro experimental model. A useful model is a hermetically sealed perfusion system that can regulate water pressure. This sealed chamber, however, does not allow physical access of a recording electrode to preparations inside the chamber, and, thus, it is almost impossible to electrophysiologically record neuronal activity under high-pressure conditions. Because fMCI uses optical sensors, it can access neurons even in a glass-sealed chamber. Using fMCI, we succeeded in recording neuronal activity under high pressure conditions [38] and found that high-pressure conditions even at 100 mmHg do not alter the network excitability. Thus, the acute hyperbaric circumstance itself is unlikely to exert a detrimental effect on neuronal function.

Increased intracranial pressure is known to lead to brain herniation and reduced cerebral blood flow, resulting in severe ischemia. Ischemia, which also occurs following thrombosis or embolism, causes a disturbance in blood supply and subsequent disruption of brain function. Ischemia is often modeled in vitro by oxygen–glucose deprivation (OGD). Brain tissues are perfused with external saline that lacks a glucose and oxygen supply. We investigated the acute effect of OGD on hippocampal network activity with fMCI (Fig. 2a). OGD increased the frequency of spontaneous spikes of CA3 neurons within a couple of minutes. Accordingly, fMCI enables quantification of the sensitivity of individual neurons to OGD. In addition to the acute effect of OGD, transient ischemic preconditioning induces tolerance to subsequent lethal ischemia and OGD. Using fMCI in cultured cortical neurons, Tauskela et al. [39] monitored neuronal activity during the preconditioning by drugs that increase neuronal excitability and examined the resulting tolerance to OGD exposure.

Stroke is a rapid loss of brain function in response to a cerebrovascular dysfunction. Recovery after stroke damage depends on surviving brain regions that take over the role of the lost network function. Winship and Murphy [40] performed in vivo fMCI in the forelimb and hindlimb somatosensory cortex of mice and addressed stroke-induced changes in sensory-evoked function and spatial organization of individual neurons. They found that receptive fields in peri-infarct neurons were greatly enhanced and that a month after stroke, neurons that were originally selective for a single limb began to process information of multiple limbs. After a longer recovery period, these cells developed a more specific response and exhibited defined limb activity.

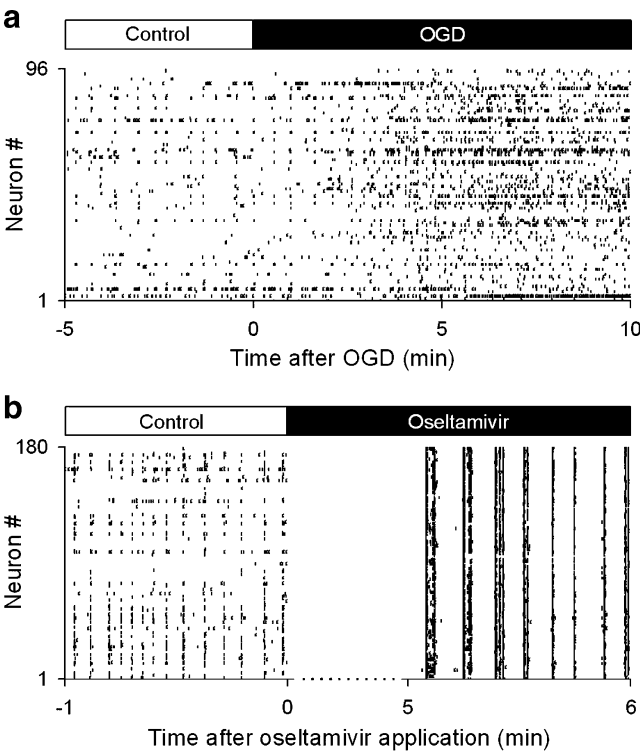

Fig. 2 (**a**) Effects of OGD on CA3 network activity. OGD caused a gradual increase of neuronal activity. (**b**) Effect of oseltamivir on CA3 network activity. After application of oseltamivir, CA3 network showed synchronized bursty activity in virtually all neurons

Alzheimer's disease

Alzheimer's disease (AD), a common form of dementia, is a degenerative and terminal disease for which there is currently no cure. A pathologic hallmark seen in AD is extracellular plaques of amyloid-β protein (Aβ) and intracellular neurofibrillary tangles of tau protein, both of which accumulate in subregions of the brain. Different animal models of AD help to understand the role of these proteins and to locate possible drug targets and novel therapeutic strategies [41]. Recently, fMCI was used to record from neurons around amyloid plaques in transgenic AD mice. In vivo two-photon multicolor imaging was combined with labeling of plaques with thioflavin S, a fluorescent marker [42]. Using this approach, Busche et al. [43] found clusters of hyperactive neurons close to plaques in Aβ-depositing cortex. They concluded that a redistribution of synaptic drive between silent and hyperactive neurons, rather than an overall decrease in synaptic activity, provides mechanisms underlying cortical dysfunction in AD. In addition to these neuronal aberrations, synchronous hyperactivity and inter-cellular calcium waves were observed in cortical astrocytes of AD mice [44].

Epilepsy

Epilepsy is a chronic neurological disorder, characterized by recurrent unprovoked seizures, which result from abnormal, excessive, or synchronous neuronal activity in the brain. Electrophysiological recording, functional magnetic resonance imaging, and positron-emission tomography are widely used to monitor epileptic activity, but they lack the resolution and so cannot reveal the activity pattern of neuron populations at the cellular level during seizures. fMCI is a novel method enabling characterization of epileptiform events with single-cell resolution in an experimental model of seizure [45]. In our experience, epileptiform discharges are often found to evoke strong calcium transients, so individual spikes in each discharge event are almost inseparable, because of the slow kinetics of calcium signals. Moreover, such globally synchronized activities increase the calcium fluorescence not only in the cell body but also in the surrounding area, which includes dendrites and axons. The latter component often contaminates the somatic signal, leading to false positive spikes. Thus, the data must be carefully interpreted; note that subtraction of the background fluctuation sometimes works to clean up the signal [46]. It reveals that, in experimentally conditioned seizures in cortical slices, layer 5 neurons had an important role in generating neocortical seizure. Epileptic discharges are initiated, in part, by a local depolarization shift that drives a group of neurons into synchronous bursting [47]. In vivo fMCI demonstrates that astrocytic calcium activity preceded paroxysmal depolarization shifts of neurons during 4-AP-induced seizure-like activity [48]. Furthermore, anti-epileptic drugs, such as valproate, gabapentin, and phenytoin, reduce both the amplitude of neuronal discharge and astrocytic calcium activity. This suggests that pathological activation of astrocytes may have a central role in the genesis of epileptiform discharges, being a possible target for treatment of epileptic disorders.

Others

Using in vitro fMCI, Usami et al. [49] addressed the pharmacological effects of oseltamivir on neural circuit activity. Oseltamivir, an anti-influenza drug, inhibits virus neuraminidase and suppresses the growth of influenza virus. A mammalian homologue of neuraminidase regulates sialylation of hippocampal CA3 neurons. The sialylation is believed to modulate the properties of cell adhesion molecules and voltage-gated sodium channels. With fMCI, we examined the pharmacological effects of this drug on the activity of neuron populations (Fig. 2b). After bath application of oseltamivir, CA3 neurons showed a gradual increase in activity rates and finally started to show globally synchronized "population burst" activity, which abruptly occurred a few times per minute.

Another pharmacological study with in vivo fMCI has revealed that the transition of awake states to anesthetic states induces changes in neuronal activity [32]. After administration of ketamine, an anesthetic drug, the activity of cortical neurons were suppressed at lower spike rates, and the activity patterns were switched from bursty activity to correlated activity.

Conclusion and perspective

fMCI provides a unique opportunity to image the electrical activity of a population of neurons and even the calcium activity of non-neuronal cells such as astrocytes. It attains both high temporal and spatial resolution and detects a series of spikes from individual neurons. Thus we know when, where, and how a given neuron acts in a brain circuit. By taking these advantages of fMCI, a number of in vitro and in vivo studies shed light on the fundamental properties of neuronal network activity. Recent applications of fMCI have revealed neuronal activity under pathological conditions in experimental models. Some diseases arise from deep brain structures, such as limbic cortex and basal nuclei. Although two-photon microscopy can access tissues as deep as several hundreds of micrometers, much deeper than confocal microscopy, in vivo fMCI is still restricted to imaging superficial layers of the brain, i.e., the neocortex [50]. One of the promising approaches used to explore

deeper brain regions is a stick-like objective, which can be inserted into the brain to a depth of up to 4000 μm below the pial surface. With this method, Kuga et al. [51] monitored cerebrovascular blood flow in the hippocampus with single-capillary resolution. The diameter of the lens used here was less than 1 mm, and thus physical damage to the tissue was small. Moreover, microendoscopes with gradient-index lenses have now been improved to reach a numerical aperture of 0.82 and thus achieved spatial resolution at cellular and subcellular levels [52, 53]. These new techniques will soon enable in vivo fMCI from deeper brain areas, which may offer an opportunity to find the new aspects of neurological disorders that occur in the deep brain, for example Parkinson's disease with progressive neurodegeneration of the basal nuclei.

Appendix

Publication list of methods and tools for details

Loading procedure for calcium indicators

In vitro fMCI

(Bath application) Ikegaya et al, 2005 [54]; Takahashi et al., 2007 [55]
(Pressure injection) Stosiek et al., 2003 [21]

In vivo fMCI

(Pressure injection) Stosiek et al., 2003 [21]; Nimmejahn et al., 2004 [8]; Ohki et al, 2005 [22]
(Electroporation) Nevian and Helmchem, 2007 [56]; Nagayama et al., 2007 [24]

fMCI data analysis

Cell identification

(Fluorescence contour) Crepel et al., 2007 [57]
(Correlation based) Ozden et al. 2008 [58]
(Spatio-temporal independent component analysis) Mukamel et al., 2009 [59]

Extraction of calcium signals (spike detection)

(Temporal deconvolution) Yaksi and Friedrich, 2006 [33]
(Template matching) Kerr et al., 2005 [30]; Greenberg et al., 2008 [32]
(Machine learning) Sasaki et al., 2008 [7]
(Spatio-temporal independent component analysis) Mukamel et al., 2009 [59]

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