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Annual Meeting Mini-Symposium

Novel Approaches to Monitor and Manipulate Single Neurons *In Vivo*

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The complexity of the vertebrate brain poses an enormous challenge to experimental neuroscience. One way of dealing with this complexity has been to investigate different aspects of brain function in widely different preparations, each best suited to address a particular question. Accordingly, cellular questions are typically addressed with intracellular recordings in *in vitro* preparations such as brain slices or neuronal cultures, whereas network behavior and sensory or motor response properties are analyzed *in vivo*, often with extracellular recordings. This division of labor has proved to be an experimentally effective strategy. However, although there seems to be no limit to the wealth of data that can be generated in this way, integrating results derived in different preparations comes with its own set of challenges. The enormous difficulties encountered when one attempts to link cellular phenomena such as synaptic plasticity to systems properties such as spatial memory (Martin et al., 2000) have shown us that close collaboration between molecular–cellular and systems neuroscience is required (Tonegawa et al., 2003) and that we need more convergence of experimental techniques to analyze the cellular basis of neural function under more natural conditions. Studying neurons under naturalistic conditions is, however, easier said than done. A return to *in vivo* preparations will only be successful if we are able to solve the technical problems that led previous researchers to abandon the study of intact brains in the first place. Thus, studying neurons at the cellular level in vertebrate brains is today first and foremost a technological challenge. Here we highlight recent efforts to improve our ability to analyze functions of single neurons *in vivo*. Given the "mini-review format," we cannot aim for completeness but must focus on the techniques featured in the accompanying mini-symposium without meaning to imply that other novel developments are less significant.

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Monitoring neurons with improved cellular resolution

Structural plasticity and synaptic function

Synapses are the smallest units of organization in neural networks, and they are thought to encode memories. What happens at synapses when we learn? To understand synaptic dynamics in intact animals, it will be necessary to monitor the structure and function of individual synapses *in vivo* over times of milliseconds to months. This goal has been made attainable by the invention of two-photon laser scanning microscopy (2PLSM) (Denk et al., 1990), which allows imaging in the scattering environment of the intact brain (Denk and Svoboda, 1997). In addition, fluorescent proteins [with their large extinction ratios, quantum efficiencies, and resistance to photobleaching (Tsien, 1998)] are ideal for *in vivo* imaging and can be genetically targeted to neurons of interest (see below) (Feng et al., 2000). Long-term 2PLSM imaging of green fluorescent protein (GFP)-expressing neurons is used to track structural plasticity *in vivo* (Grutzendler et al., 2002; Trachtenberg et al., 2002). Whereas dendritic arbors in the adult brain are essentially stable, a fraction of dendritic spines appear and disappear over time periods of days (Trachtenberg et al., 2002). The turnover of dendritic spines is increased after novel sensory experience, suggesting that spine addition and subtraction, together with synapse formation and elimination, is a substrate of experience-dependent plasticity (Trachtenberg et al., 2002) (Fig. 1*A*). Fluorescent proteins have been engineered to indicate aspects of cellular activity, including pH changes (Miesenbock et al., 1998), Ca^{2+} concentration (Miyawaki et al., 1997), membrane potential (Siegel and Isacoff, 1997), and others (Miyawaki, 2003). Thus, aspects of synaptic function such as glutamate release (Oertner et al., 2002) will become accessible *in vivo*. Two-photon imaging can be easily combined with fluorescence lifetime measurements for quantitative fluorescence resonance energy transfer imaging (So et al., 2000). These techniques may help to decipher the cascades of signal transduction underlying synaptic plasticity *in vivo*.

Network analysis

Understanding neuronal computations requires a multilevel approach, and, hence, tools for analysis of network activity need to complement the synaptic and cellular analysis methods. Network

activity has "microscopic" (individual neurons) and "macroscopic" (neuronal ensembles) aspects, which should preferably be monitored simultaneously. Current techniques [extracellular multielectrode recordings, functional magnetic resonance imaging, imaging of intrinsic optical signals, and voltage-sensitive dye-based imaging (Orbach et al., 1985; Grinvald et al., 1988; Raichle, 1998; Shoham et al., 1999; Nicolelis and Ribeiro, 2002)] are well suited for monitoring activity at the macroscopic level but are restricted in their ability to read out the activity of individual neurons. The divide between the macroscopic and the microscopic brain activity can be bridged by the recently developed multi-cell bolus loading (MCBL) technique, which provides a targeted staining of neuronal populations with calcium-sensitive dyes (Stosiek et al., 2003). The cells are stained by a brief bolus injection of a membrane-permeant calcium indicator dye (e.g., Calcium Green-1 AM) into extracellular space and imaged by means of two-photon laser scanning microscopy. This technique allows simultaneous functional analyses of many individual neurons, i.e., a monitoring of brain activity at both the microscopic and macroscopic level (Fig. 1*B*). To clarify the identity of the imaged cells, mixtures of a Ca^{2+}-sensitive dye and different cell-specific markers (e.g., an astrocyte marker sulforhodamine 101) (Nimmerjahn et al., 2004) may be used for bolus injections. Identified cells can be further subjected to two-photon targeted patching (TPTP) (Margrie et al., 2003) to measure electrophysiological signals underlying their activity. MCBL is also very useful for monitoring activity in awake, behaving animals (Helmchen et al., 2001; Adelsberger et al., 2004), thus providing a versatile tool for analysis of intact neuronal circuits.

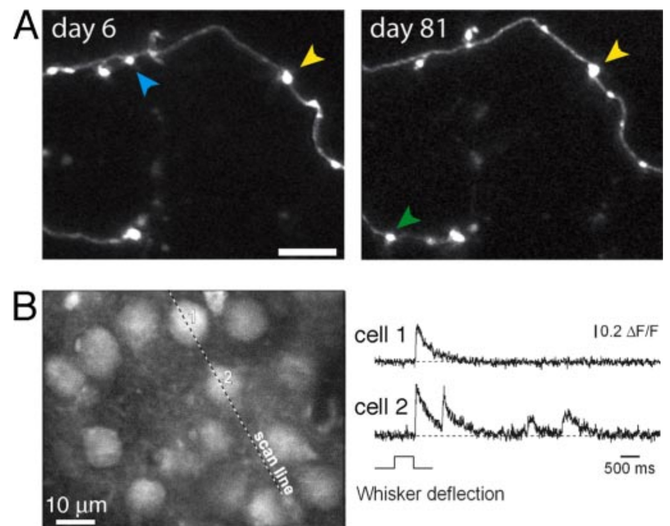

Figure 1. Monitoring neurons with improved cellular resolution. *A*, Long-term *in vivo* imaging of GFP-expressing axons in the adult mouse barrel cortex. Note gained (e.g., green arrowhead), lost (blue arrowhead), and stable (yellow arrowheads) putative synaptic terminals (A. Holtmaat, personal communication). *B*, Ca^{2+} transients evoked by whisker deflection. Left, A high-magnification image of layer 2/3 neurons *in vivo* (depth, 130 μm) in the barrel cortex of a 13-d-old mouse. Right, Line-scan recordings of Ca^{2+} transients evoked in two neurons by a deflection of the majority of whiskers on the contralateral side of the mouse's snout. The position of the scanned line and the cells analyzed are indicated (left) (modified from Stosiek et al., 2003).

Monitoring neural activity under natural conditions

Extracellular recordings

Although recent advances in electrode fabrication and microdrive techniques have led to the ability to record from many neurons in freely behaving animals (Bragin et al., 2000; Serruya et al., 2002; Nicolelis et al., 2003), many challenges remain in the effort to develop chronic recording systems with the flexibility and selectivity of acute recording systems. For mechanical stability, implanted multielectrode arrays are usually constructed from electrodes (e.g., microwires) that are optimal for multiunit (multi-cell) rather than single-unit (single-cell) signals (Gray et al., 1995). These signals can be decomposed into putative single-unit signals using spike-sorting techniques, but this method is relatively insensitive to smaller neurons and neurons with low firing rates. To avoid these difficulties, chronic recordings could be made with large numbers of electrodes optimized for single-unit recording that are individually moved in the brain to achieve high-quality single-unit signals on every electrode. We and others have recently made advances toward this goal by developing a motorized microdrive in which electrodes can be individually and remotely manipulated to isolate single neurons (Fee and Leonardo, 2001; Cham et al., 2004).

Another limitation of extracellular recording techniques is that neurons are usually found during recording process by searching for spontaneous spikes as the recording electrode is slowly advanced through the brain. Unfortunately, in some cases,

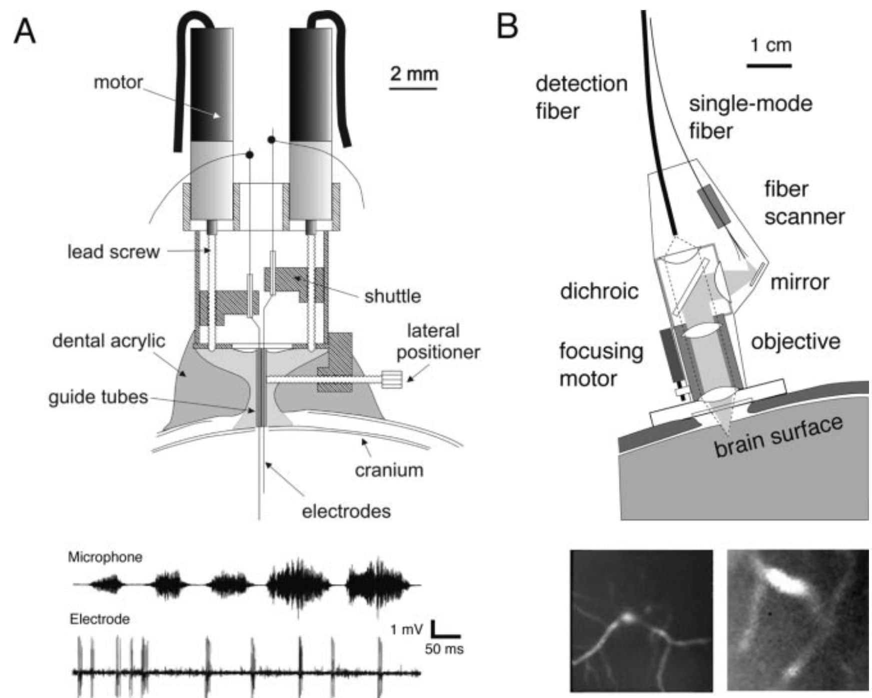

Figure 2. Monitoring neural activity under natural conditions. *A*, Top, Overview of motorized microdrive. Electrodes are driven independently by small brushless DC motors. Bottom, Sample recording from a single neuron in the nucleus robustus archistriatalis of a zebra finch during singing (adapted from Fee and Leonardo, 2001). *B*, Top, Principal design of the miniaturized two-photon microscope. Excitation light and fluorescence emission light are guided through optical fibers. The two-photon fiberscope is mounted above a small cranial window and can be carried by freely moving rats. Bottom, Two-photon fiberscope-acquired image of a layer 2 neuron filled with Calcium Green-1 (left) and small blood vessels labeled via tail-vein injection of FITC– dextran (right) (adapted from Helmchen et al., 2001).

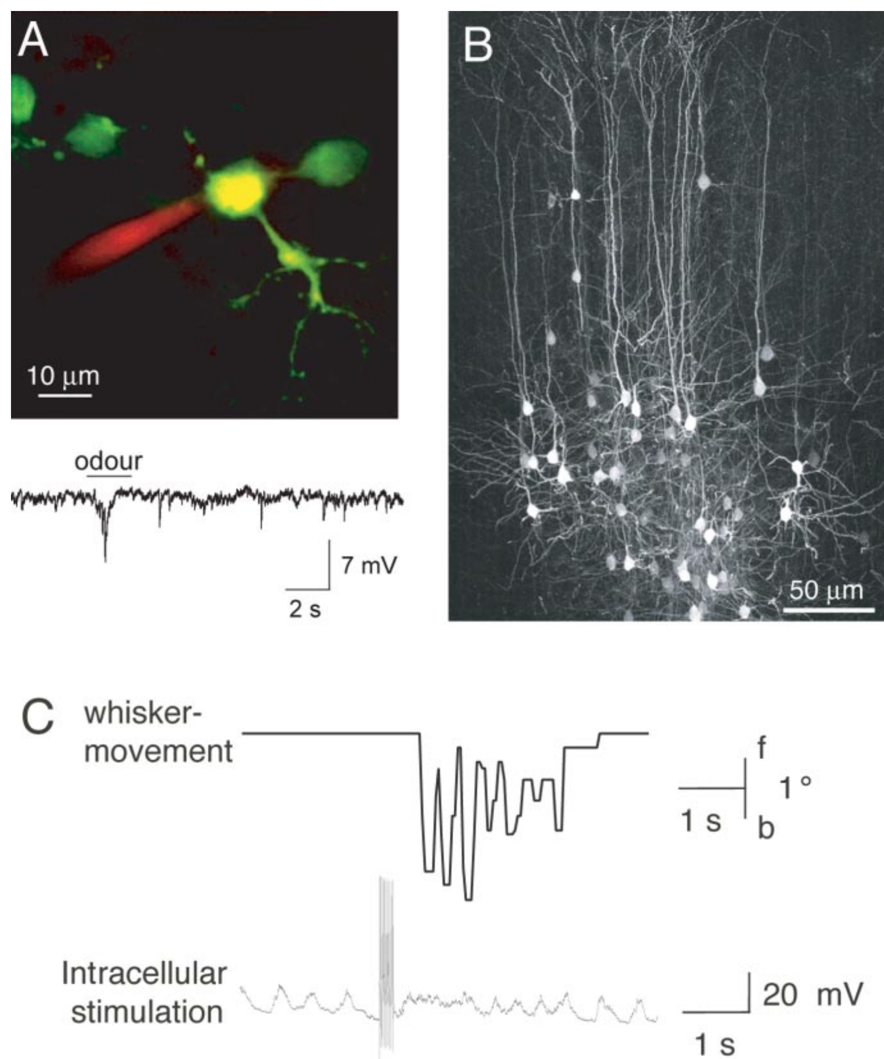

Figure 3. Targeting and manipulating single neurons. *A*, Top, Targeted whole-cell recording from a calretinin-positive interneuron in the glomerulus layer of the mouse olfactory bulb (postnatal day 21). Image is an overlay of two channels (red and green) showing the illuminated patch pipette filled with Alexa 594 attached to the GFP-labeled interneuron. Bottom, Trace shows membrane potential in response to odor presentation (2 sec). *B*, Enhanced GFP-expressing lentivirus was injected into the parenchyma of rat layer 2/3 somatosensory cortex (postnatal day 28). After a 2 week expression period, the rat was killed, and enhanced GFP fluorescence was visualized from100 μm paraformaldehyde-fixed brain sections. The image is a maximal projection of 40 confocal sections, separated by 2 nm. *C*, Intracellular stimulation of the rat primary whisker motor cortex. Top trace, Position of whisker E1 during an intracellular stimulation trial. Bottom trace, Membrane potential recordings during current injection (10 action potentials at 50 Hz); the stimulation current is not shown.

neurons exhibit little or no spontaneous activity and cannot be found using this approach. We recently used the classic technique of antidromic activation (Swadlow, 1998) to find premotor neurons in the songbird brain that are completely silent in the awake bird, except when the bird sings. Furthermore, during the song, these neurons generate a single brief burst of spikes (Hahnloser et al., 2002). The discovery of extremely sparse activity in a critical population of premotor neurons in the songbird, as well as other studies (Beloozerova et al., 2003), suggest that a combination of remote manipulation of selective electrodes, and antidromic identification techniques, should be useful to study brain circuitry in freely behaving animals.

Microscope miniaturization

The development of miniaturized fiber-optic devices for imaging neurons at microscopic resolution in brains of awake, freely moving animals is both an important goal and a major technological challenge. Two types of fiber-optic fluorescence microscopes have been developed. The first type delivers excitation light through a single-core fiber, in which case two-dimensional images are obtained by a scanning mechanism in the microscope headpiece (Delaney and Harris, 1995; Helmchen et al., 2002). Using such a fiberscope, we could resolve neurons and dendrites in cortical layer 2 of anesthetized rats and small blood vessels in freely moving animals (Helmchen et al., 2001) (Fig. 2*B*). A particular challenge in this case has been the use of two-photon excitation through optical fiber to gain imaging depth (Helmchen et al., 2002). In standard glass fibers, nonlinear effects cause broadening of the ultrafast light pulses and thus reduce two-photon excitation efficiency (Helmchen et al., 2002). Fortunately, radically new types of optical fibers (with hollow air-filled cores) now permit distortion-free propagation of femtosecond pulses (Göbel et al., 2004a). A second type of fiber-optic microscope is based on so-called coherent optical fiber bundles, which contain many thousands of fiber cores and transmit entire images, albeit with low spatial resolution (Hirano et al., 1996; Knittel et al., 2001; Göbel et al., 2004b). Fiber bundles are now available in small diameters and can be attached to small cylindrical lenses suitable for endoscopic imaging. These ultrathin (<1 mm diameter) microscope probes will extend imaging to deeper brain structures (Jung and Schnitzer, 2003; Levene et al., 2004). Microscope miniaturization together with the improvements in methods for fluorescence labeling *in vivo* (Stosiek et al., 2003) promises to shed light on cellular activity in behaving animals.

Targeting and manipulating single neurons

Targeted recordings

An increasing refinement of recording specificity has been a leading theme in the development of the whole-cell patch-clamp recording technique (Hamill et al., 1981). When combined with differential interference contrast or fluorescence microscopy, the readily visible tip of the patch pipette could be targeted not only to a specific cell genetically manipulated to express a particular fluorophore but also to multiple locations on the same neuron (Stuart et al., 1993). The stability of whole-cell recordings combined with the possibility of rapidly loading fluorescent indicators (Helmchen and Waters, 2002) has therefore made blind whole-cell recordings attractive to *in vivo* experimentalists (Pei et al., 1991; Jagadeesh et al., 1993). Whole-cell recordings can be obtained from the anesthetized and awake preparation, superficial and deep structures (Margrie et al., 2002), extremely small cells (Chadderton et al., 2004), and even from the dendrites of large cortical neurons (Larkum and Zhu, 2002). One major drawback of blind whole-cell recordings is the random but not necessarily represen-

tative sampling of neurons, which severely limits the interpretation and the rapidity of data acquisition in neuronally heterogeneous brain regions. Recently, it has become possible to target electrophysiological recordings to genetically labeled neurons *in vivo*. To this end, two-photon microscopy is used to guide a whole-cell patch pipette filled with a fluorescent dye onto a GFP-expressing neuron in the intact brain (Margrie et al., 2003) (Fig. 3*A*). This TPTP technique was initially used to record from the sparsely distributed parvalbumin-positive interneurons in the somatosensory cortex. TPTP experiments on GFP-expressing parvalbumin-positive interneurons provided the first piece of direct evidence in support of a role of electrical coupling in these cells during sensory processing *in vivo* (Margrie et al., 2003). More recently, TPTP has been used to target cortical cells that have been altered in their intrinsic and synaptic conductances (Komai et al., 2004) by lentivirus-based genetic means (see Single-cell genetics).

Single-cell genetics

The establishment of novel recording and stimulation techniques makes it highly desirable to develop methods that would combine the optical and electrophysiological single-cell analyses with the genetic means to manipulate individual neurons. Our approach for providing such a methodology, termed "single-cell genetics," is based on stereotactic delivery of lentiviruses (Naldini and Verma, 2000). Lentiviral infection can be titrated to affect small numbers of neurons (on the order of tens to few hundreds) within intact brain networks (Fig. 3*B*), providing an excellent spatiotemporal control of the onset of the genetic manipulations. Importantly, the lentiviral vector-encompassed genetic information is stably integrated into the host cell genome, allowing for subsequent functional analysis as early as several days and as late as weeks or months after the viral delivery. We optimized the use of the self-inactivating lentiviral vectors (Lois et al., 2002) specifically for combination with cortical physiology, primarily by developing pyramidal neuron-specific expression from synapsin I or α-CaMKII (Ca^{2+}/calmodulin-dependent protein kinase II) promoters (Dittgen et al., 2004). Coexpression of GFP and Cre recombinase from these vectors can now be used in mice with floxed genes for creating gene knock-out, whereas coexpression of GFP and double-stranded short-interfering RNAs is an efficient method for gene knock-down in the infected population of pyramidal neurons in the otherwise intact brain (Elbashir et al., 2001; Pfeifer et al., 2001; Licznerski et al., 2003; Qin et al., 2003). The advantages of this approach lie in the ease of its application, as well as in the fact that modification of a small neuronal population avoids lethality of phenotype or activation of compensatory mechanisms that are often associated with standard genetics approaches affecting the whole brain or particular brain regions.

Reverse physiology

It is unlikely that, if one had access to all the spike trains in all brain cells of an animal of choice, it would be possible to derive mechanistic explanations for the animal's mental events and behavioral performance. Thus, in addition to the correlative analysis of single-neuron activity, we need to study the brain by altering single-neuron activity. On long timescales, such manipulations might be done very elegantly by genetic approaches. On short timescales, microstimulation techniques have been applied (Tehovnik, 1996; Cohen and Newsome, 2004) and have, for example, provided very direct evidence for the involvement of certain sets of cortical neurons in visual and tactile neural representations (Salzman et al., 1990; Romo and Salinas, 2001). The major drawback of microstimulation techniques is their lack of cellular specificity, i.e., the stimulated cellular elements are not identified and estimates of the number of stimulated neurons vary by more than an order of magnitude (Tehovnik, 1996). To overcome such limitations, intracellular stimulation has been applied recently in the vibrissa motor cortex (Woody and Black-Cleworth, 1973; Brecht et al., 2004). Quite unexpectedly, we found that single cells could drive noticeable movements. These evoked movements were spatiotemporally complex and varied as function of the lamina of the stimulated neuron (Fig. 3*C*). We hope to extend this work to a "reverse physiology approach," in which one studies the perceptual and motor correlates of experimentally evoked spikes rather than (as is done conventionally) analyzing the occurrence of spikes as a correlate of external or internal events.

Summary

As yet, most *in vivo* records of cellular activity in the vertebrate brain come from unidentified cells, and most manipulations of neural activity affect the whole brain or large populations of neurons in particular brain regions. The combined application of the optical, electrophysiological, and genetic techniques outlined here will allow us to refine our focus to the structure and function of identified neurons in the intact brain. This is the only means by which we can determine with any precision the cellular basis of brain function.

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