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Intracellular recording in behaving animals

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Electrophysiological recordings from behaving animals provide an unparalleled view into the functional role of individual neurons. Intracellular approaches can be especially revealing as they provide information about a neuron's inputs and intrinsic cellular properties, which together determine its spiking output. Recent technical developments have made intracellular recording possible during an ever-increasing range of behaviors in both head-fixed and freely moving animals. These recordings have yielded fundamental insights into the cellular and circuit mechanisms underlying neural activity during natural behaviors in such areas as sensory perception, motor sequence generation, and spatial navigation, forging a direct link between cellular and systems neuroscience.

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Introduction

Extracellular recordings in awake animals have yielded a rich set of discoveries involving patterns of spiking activity that underlie specific behaviors [1–7]. Intracellular recordings have been employed to investigate the cellular mechanisms underlying such firing patterns, but technical issues have limited the extent to which intracellular studies could be carried out in awake animals. Anesthetized or sleeping preparations, which in general have smaller and fewer mechanical disturbances caused by animal movements, are sufficient for investigating some aspects of neural processing, such as feature selectivity in primary sensory areas. However, for studying other phenomena, including active sensation, birdsong or place cell activity, the animal must be awake or even moving around an environment. Here we review technical advances that have allowed intracellular recordings involving increasing degrees of mobility and highlight some recent cases where a deeper understanding of underlying mechanisms has directly resulted from these recordings.

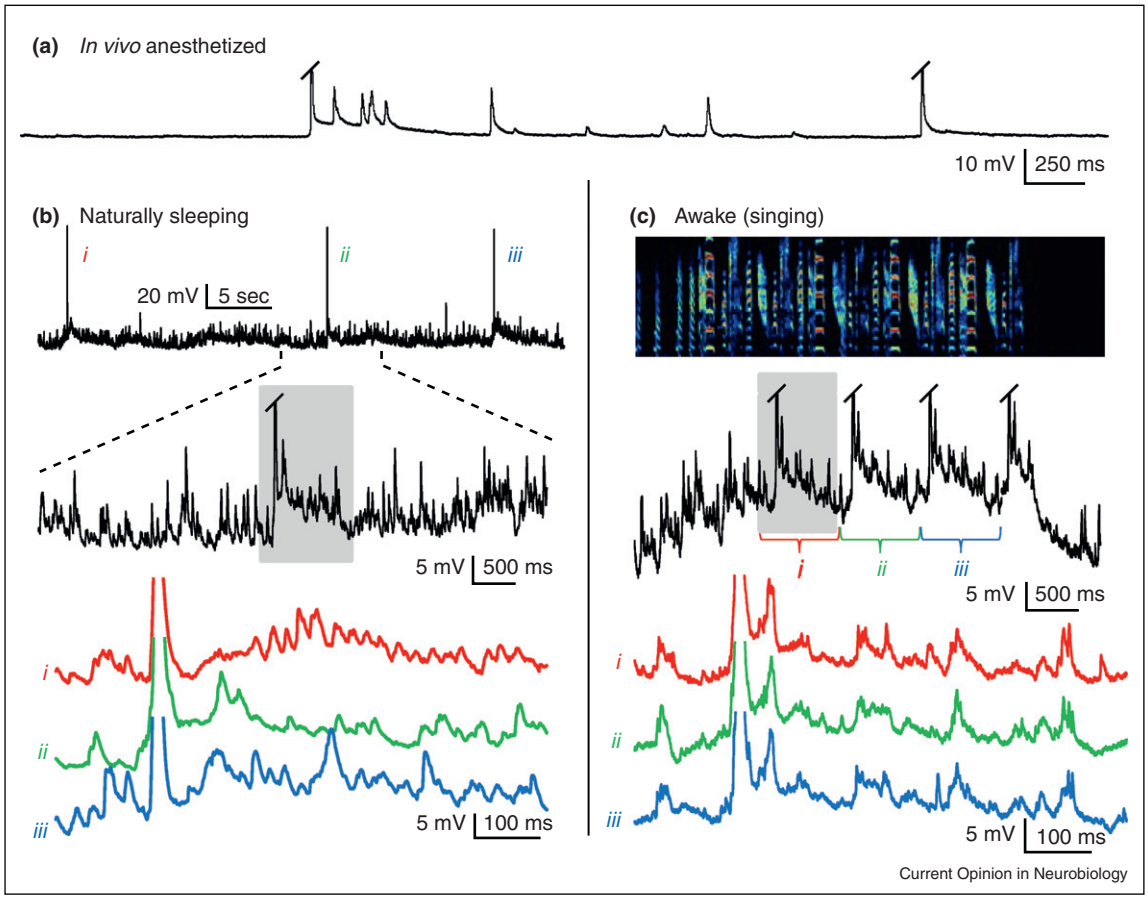
Why study the awake brain with intracellular recordings?

Electrophysiological recordings in anesthetized preparations have revealed a tremendous amount about the function and organization of neural processing centers. For instance, orientation tuning in primary visual cortex was discovered in an anesthetized preparation [8]. However, while some aspects of neural activity appear to be preserved across anesthesia and awake states, many features appear to uniquely correspond to the awake brain (Figure 1). On the one hand, recent work has shown that the shape of orientation [9,10•] and other [11] tuning is similar in the awake and anesthetized brain, thus detailed intracellular studies of the synaptic mechanisms underlying such tuning in anesthetized animals [12–15] should apply directly to awake conditions. On the other hand, it was also shown that response magnitudes are strongly modulated by behavior in the awake animal [10•,16,17••], highlighting the dynamic nature of processing in the awake state.

A number of previous studies have demonstrated that anesthesia can significantly affect aspects of coding, such as the extent to which neurons respond to sensory stimuli [18–20]. Recently, the same neurons were recorded in barrel cortex during wakefulness and under the influence of the rapidly reversible anesthetic isoflurane [21•]. Under anesthesia, neurons exhibited prominent slow-wave membrane potential (V_m) fluctuations, similar to those which occur during certain stages of natural sleep [22,23]. However, when the animal was awake, V_m bistability was abolished and the neuron entered a desynchronized state [22,24] (but see [25]). Electrophysiological characteristics during wakefulness may be affected by neuromodulators such as acetylcholine [26] or norepinephrine [21•], which have also been shown to play an important role in the generation of fast oscillations [27] as well as the gating of plasticity processes [28].

Given the aforementioned differences between anesthetized and awake states, many researchers have sought to use awake preparations. But because awake animals often exhibit significant movements beyond those associated with heartbeats and breathing, awake intracellular recordings are in general challenging, as the recording pipette must maintain direct physical contact with the recorded neuron's membrane. The intracellular voltage, however, provides data that can distinguish between proposed

Figure 1



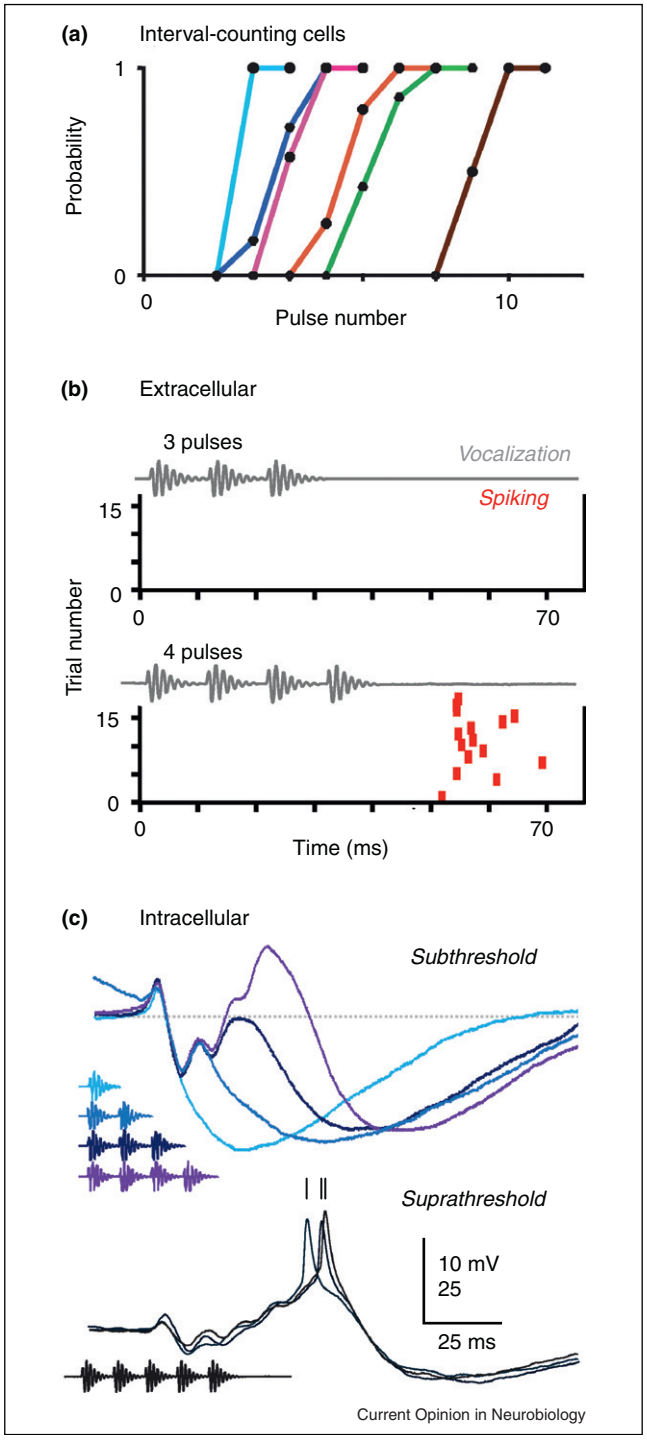
Intracellular dynamics of an identified neuron type recorded *in vivo* across different conditions – anesthetized, naturally sleeping, and singing. Each trace is taken from a distinct antidromically identified RA-projecting HVC [HVC(RA)] neuron in an adult zebra finch. **(a)** Under isoflurane anesthesia (1.5% in oxygen), large, infrequent postsynaptic potentials are observed, sometimes resulting in action potentials (truncated and demarcated with diagonal lines). **(b)** Membrane potential (V_m) of an HVC(RA) neuron in a naturally sleeping, head-fixed bird. **(c)** V_m of an HVC(RA) neuron in an awake, singing bird (spectrogram of song above). Both the **(b)** sleeping and **(c)** awake states were associated with considerably more frequent synaptic events than observed under anesthesia. Although the general shape of the subthreshold activity surrounding spiking events was similar in these two conditions (note the shaded epochs), the fine structure (i–iii) of the spike-aligned subthreshold activity was uncorrelated during sleep and nearly perfectly correlated during singing.
Panel **(a)** is courtesy of D. Vallentin; panels **(b)** and **(c)** are modified from [44••].

models of circuit function. Examples (from awake animals) of such data unattainable with other methodologies include subthreshold excitatory and/or inhibitory responses to internal [29–31,32•,33] or external (i.e. sensory) [34–38] stimulation as well as spontaneous V_m dynamics [39] and intrinsic properties [40,41]. Intracellular methods also allow experimental control of V_m to further probe mechanisms [42••,43••,44••,45••].

Case study: feature selectivity

An example that illustrates what intracellular recordings can tell us about an ethologically relevant sensory computation is complex auditory feature selectivity in frogs. Frog calls often consist of a series of regularly timed sound pulses that females can distinguish on the basis of differing pulse number. A possible neural substrate of this ability consists of single neurons in the auditory midbrain that begin to spike only after a threshold number of pulses has occurred with a specific interpulse interval (Figure 2a) [46]. An example 'interval-counting cell' fires after exactly four pulses, but not after three (Figure 2b). What gives a neuron such remarkable specificity? There are many potential models using specific combinations of intrinsic and extrinsic (synaptic) conductances. For instance, neurons that respond to a smaller number of pulses could have a more hyperpolarized spike threshold or be more intrinsically excitable than those responding to a larger number of pulses. Alternately, short-term dynamics of the synaptic inputs onto these neurons could determine their response properties. A direct approach to finding the actual solution involves obtaining the V_m from an interval-counting neuron during the presentation of a

Figure 2



Intracellular recordings reveal processes underlying feature selectivity for a complex sensory stimulus. A population of neurons within the torus semicircularis of the frog midbrain responds selectively to a specific number of sound pulses with a specific interpulse interval. **(a)** Probability of spiking versus the number of pulses for six such interval-counting neurons. **(b)** Raster plot showing the responses of an interval-counting neuron to three (no evoked spikes) and four (15 evoked spikes in 18 trials) sound pulses. **(c)** Whole-cell recording from another interval-counting neuron. At top are the mean intracellular responses to 1–4 sound pulses. Note that repeated pulses were associated with excitatory synaptic facilitation which eventually depolarized the neuron above its resting potential (represented by the dotted gray line). Below is an overlay of three individual trials showing suprathreshold responses to five sound pulses. Vertical lines above designate the timing of spiking responses. Panels **(a)** and **(b)** are modified from [46]; panel **(c)** is modified from [43••] and augmented with additional data courtesy of G. Rose.

call. Indeed, such recordings support a mechanism in which successive excitatory postsynaptic potentials (EPSPs) are strongly facilitating, allowing the neuron to overcome a substantial inhibitory conductance (Figure 2c) [43••]. By contrast, the difference between resting V_m and spike threshold was not significantly correlated with the number of pulses needed to fire the neuron [43••]. This ability to explain the origin of a complex sensory representation within a neuron holds promise for understanding higher-order processing centers in other systems.

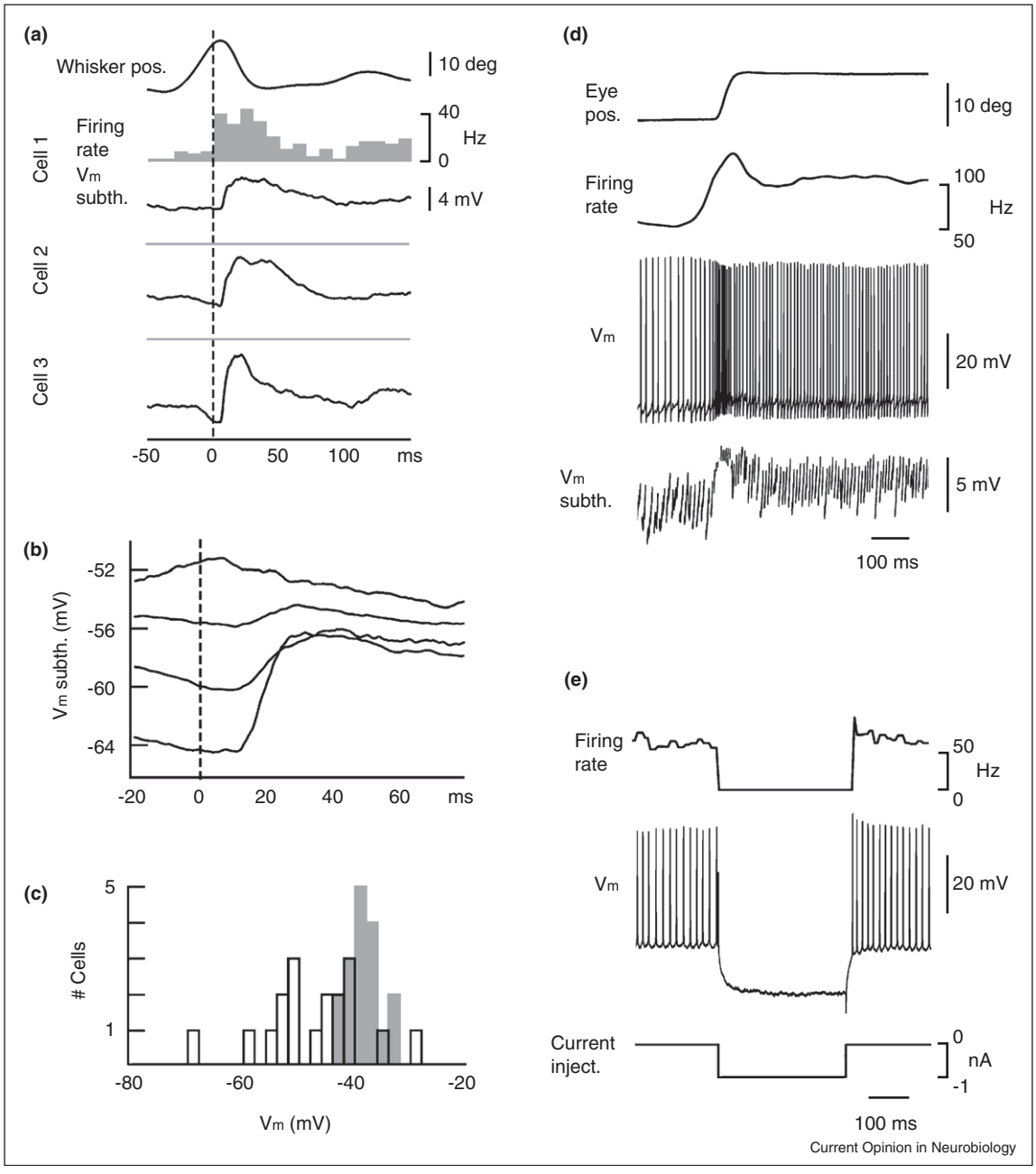
Experimental subjects as active participants

Although the previously mentioned recordings in frog midbrain were made in an awake (and paralyzed) preparation, the specific contributions of wakefulness to the underlying processes remain unclear in that example. However, in the following cases, the experimental subject's own behavior is involved in shaping and/or generating the neuronal inputs themselves.

Active sensation

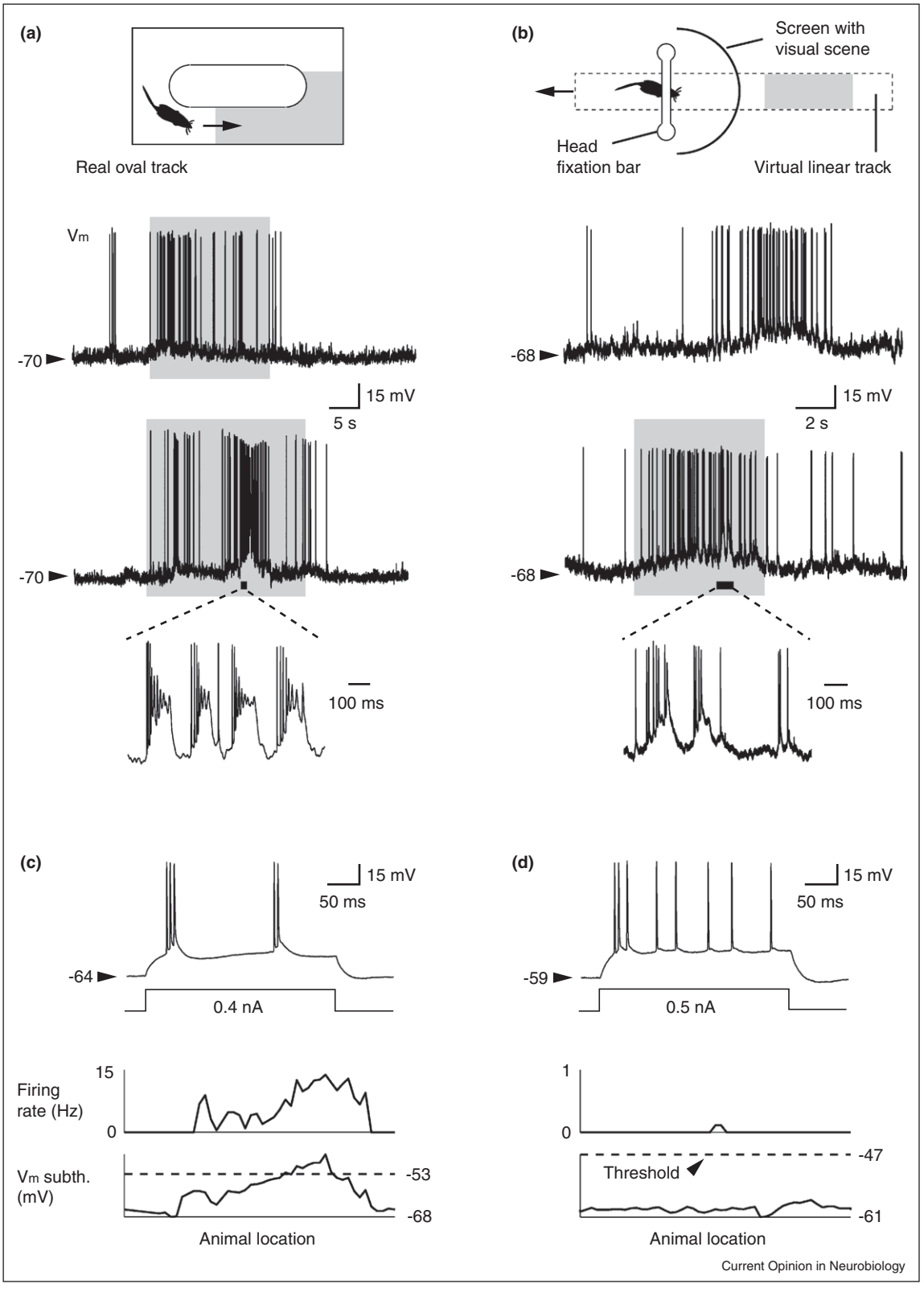
Animals do not exclusively experience their environment passively; they can also actively gather information by exploring the outside world with their senses. One example in which active sensation has been well-characterized is the electrosensory system of mormyrid fish. These fish possess an electric organ that generates a weak electrical field (called an electric organ discharge) and electroreceptors on the skin that respond to changes in the field caused by interaction with nearby objects. Intracellular recordings from neurons in the electrosensory lobe of paralyzed fish have allowed detailed studies of interactions between motor signals (i.e. efferent copies of the motor commands that drive the electric organ discharge) and sensory signals conveyed by electroreceptors [47]. While paralysis blocks the electric organ discharge itself, the fish continues to actively emit the motor commands that normally trigger discharge. This preparation allows one to observe responses due to motor signals either in isolation or in combination with artificial electrosensory stimuli that mimic the fish's own electric organ discharge. The stability of this preparation has allowed for intracellular studies of the integration of sensory responses and motor commands in tiny (<5 μm diameter) cerebellar-like granule cells [48]. The sparse response of these inhibitory neurons to combinations of sensory and motor inputs could enable them to form a specific 'negative

Figure 3



Mechanisms of sparse coding and persistent activity in awake behaving animals. **(a–c)** Intracellular recordings of neurons from the barrel cortex of head-fixed mice as they moved their whiskers into contact with an object. **(a)** The response to object contact of three neurons, all of which displayed a V_m deflection but only one of which (cell 1) fired spikes. **(b)** Experiments in which current injection was used to alter the V_m level before object contact, showing that contact drove this neuron to a particular reversal potential. **(c)** The distribution of contact-induced reversal potentials (open bars) and spike thresholds (gray bars) across neurons shows why spiking activity is sparse in response to object contact: the reversal potentials for most neurons are below their spike thresholds. **(d and e)** Intracellular recordings of neurons from the hindbrain of a head-fixed goldfish. **(d)** When the fish moved its eye from one position to another, there was a persistent change in firing rate and subthreshold V_m such that the activity level was correlated with eye position. **(e)** The effect of hyperpolarizing current injection on V_m and firing rate was transient, with a return to their original values when the current was removed. This shows that persistent activity is not maintained by an intrinsic mechanism involving plateau potentials; instead the result is consistent with a network mechanism in which synaptic inputs maintain the cell's activity level.
Panels **(a–c)** are modified from [45••]; panels **(d)** and **(e)** are modified from [42••].

Figure 4



Whole-cell recordings of hippocampal CA1 place cells in real and virtual environments. **(a)** Intracellularly recorded CA1 place cell from a freely moving rat as it ran around an oval-shaped track (top) and through the cell's place field (gray). **(b)** Intracellularly recorded CA1 place cell from a head-fixed mouse as it ran on a spherical treadmill linked to a virtual reality system that allowed it to move along a virtual linear track (top) and through the cell's

image' that subtracts the expected from the actual signals from each discharge, thus allowing the fish to detect novel, behaviorally relevant features of the environment. In a conceptually similar series of elegant experiments, intracellular recordings have revealed a novel, single-neuron mechanism of corollary discharge in an invertebrate preparation [49,50••].

Active sensation strategies can also be seen in rodents, which rely heavily on whisker-based touch information to explore objects in the environment. To physically sample the surroundings, rodents sweep their whiskers forwards and backwards at 5–15 Hz [51,52]. Consistent with previous extracellular recordings of spiking phase [53], intracellular recordings in head-fixed, whisking rodents showed that the V_m of each barrel cortex neuron oscillates with a different preferred phase relative to the whisk cycle [54,55], which could underlie object position coding [56]. Furthermore, it has been shown by intracellular, extracellular, and imaging methods that in both anesthetized and awake rodents layer 2/3 cortical neurons display minimal amounts of spiking overall in response to whisker deflection, whether in passive or active sensing mode [35,45••,54,57–61], as well as spontaneously [62]. Recently, in order to investigate the cellular basis of the sparse response to object contact during whisking, in which only ~10% of layer 2/3 neurons fire, intracellular recordings in head-fixed mice were employed [45••] (Figure 3a). Specifically, it was found that each object contact results in synaptic input that drives each neuron to a particular reversal potential that is a combination of excitatory and inhibitory conductances (Figure 3b). This potential differs between cells and for most neurons is below the spike threshold, explaining why only a fraction of cells respond to contact with spiking (Figure 3c).

Persistent activity

In addition to sensory processing, mechanistic explanations for other neuronal phenomena have depended on awake intracellular recordings. One notable case in point is persistent activity, which is correlated with, and potentially required for, short-term memory [63,64]. Persistent activity has been described in goldfish hindbrain Area I neurons, in which each brief saccadic command is converted into a sustained change in firing rate that allows the eye to maintain a fixed position in space [65] (Figure 3d). How can a neural circuit generate this sustained activity? One possibility is that the neurons are capable of maintaining various stable V_m states due to mechanisms intrinsic to the cell [66], such as dendritic plateau potentials [67]. Another is that persistent activity occurs because of recurrent synaptic activity which results in the continual activation of the network from other neurons within that circuit [68]. To distinguish between these two models, intracellular recordings were performed in a goldfish that was performing horizontal eye movements while it was head-fixed and its body restrained in a water tank [42••]. Current injection into the recording pipette was unable to induce changes in V_m that outlasted the current pulse (Figure 3e), suggesting that membrane multistability does not explain the persistent firing activity of these neurons. In addition, the variance in V_m increased linearly with eye position, consistent with an increase in synaptic feedback from other integrator neurons.

Recordings in moving animals

In the examples described so far, the animals could perform the behaviors while fixed in place and sometimes even paralyzed or partially dissected (as in some invertebrate cases [69••]). However, there are situations where the topic of investigation clearly necessitates significant bodily movement, such as studies of locomotion. Furthermore, sensory responsiveness can be affected by animal movement. The firing rate of the visual orientation-tuned neurons discussed earlier increased when mice transitioned from being stationary to running, suggesting a dynamic modulation of 'gain' dependent on different awake states [10•], and similar modulations have been described in walking versus still [16] and flying versus resting insects [17••,33]. For intracellular recordings to survive such movements requires special attention to mechanical stabilization of the electrode and nervous tissue.

Head-fixed animals

A general approach involves fixing a solid part of the body as near as possible to the recording site securely in place, but unlike in the fixation schemes described above, the animals are expected to move the rest of their body. Fixation of several vertebrae allowed intracellular recording from spinal cord neurons of cats walking on a standard, linear treadmill [70]. Along these lines, whole-cell recordings have recently been obtained during 'tethered' flight in head-fixed flies [17••,33]. As an alternative to this passive stabilization approach, an active system for moving the electrode in synchrony with brain movement was developed, yielding sharp microelectrode recordings from rats running on a linear treadmill [71•].

(**Figure Legend Continued**) place field (gray). **(a and b)** V_m during two passes (middle) through each place field shows a sustained subthreshold depolarization under the spiking inside the field (gray boxes correspond to periods when the animal was inside the place fields shown above). Inside the place fields, the cell also fired bursts with large, slow depolarizations (bottom). **(c and d)** Intrinsic firing pattern in response to a current step (top) for two CA1 pyramidal neurons in rats before they explored a novel maze. During freely moving exploration, the neuron with the intrinsically bursting firing pattern **(c)** became a place cell with a place field (middle) and a sustained subthreshold depolarization under the spiking (bottom). The other neuron **(d)**, which had a more regular-spiking firing pattern, became a silent cell that fired very few spikes in the maze (middle) and had a flat subthreshold V_m as a function of the animal's location in the maze as well as a higher spike threshold (dotted line) than the place cell (bottom).
Panels **(a)**, **(c)** and **(d)** are modified from [82••]; panel **(b)** is modified from [77••].

One variant of tethering that has received renewed attention involves a 'spherical treadmill' (usually a light ball floating on a thin layer of air inside a bowl), which allows more freedom of movement than other apparatuses such as linear treadmills while still keeping the animal in place. This allows insects [72–74] and rodents [75,76•,77••] to walk in two-dimensions. With the addition of a visual 'virtual reality' system [78] – in which a visual scene surrounding the animal moves in response to rotation of the spherical treadmill – rodents can navigate through simulated spatial environments such as mazes [75,76•,77••]. Using such a system, it was recently demonstrated that intracellular recordings could be obtained in head-fixed mice running in a virtual maze [77••].

Freely moving animals

What about unrestrained movement? A few years ago, it was discovered that rigidly fixing the electrode to the skull could yield long-duration (up to 1 h) whole-cell recordings in freely moving rats [79•,80,81•,82••]. Passive stabilization of electrodes in a chronically implanted microdrive has also allowed repeated sharp intracellular recordings in freely moving birds over several days [44••]. We now describe in more detail studies that have relied on these methods that allow more freedom of movement, beginning with birdsong.

Motor sequence generation

The concept of sequential neuronal activity mediated through groups of synaptically connected neurons has existed for decades [83,84], and a role for synaptic chains has been proposed in several brain regions, including the neocortex [85], but demonstrating the existence of these chains has proven to be difficult [86,87]. One circuit proposed to contain chains of synaptic activity [44••,88,89] is HVC, a forebrain motor structure which acts as a 'clock' for the production of song in the zebra finch [4,90,91]. To address the presence of synaptic chains within that nucleus, intracellular recordings were made in antidromically identified premotor neurons within HVC [44••,92] (Figure 1) using a chronically implanted motorized microdrive which enabled recordings in the freely moving zebra finch [44••]. These recordings revealed rapidly rising V_m directly preceding spiking activity, consistent with neurons being driven by a large synaptic input rather than a slow ramp or subthreshold rhythm. Furthermore, steady current injection (both hyperpolarizing and depolarizing) did not significantly change the timing of the firing, consistent with the idea that the neurons were being driven by a transient burst of input from the previous link the synaptic chain [44••]. Both of these observations were consistent with the presence of premotor activity which is driven through direct synaptic connections within HVC, but additional experiments are needed to further characterize these interconnections. Despite this fact, these data represent the most compelling evidence of a chain-like synaptic organization within a forebrain structure, which may represent a generic circuit strategy in cortex.

Spatial navigation

A rodent's ability to navigate to a specific location is dependent on the hippocampus [93,94]. Within a given environment (e.g. a maze), hippocampal CA1 pyramidal neurons divide into two groups: place cells, each of which fire when the animal is in a particular location (called the cell's place field) and which are probably used to solve navigational tasks [6,93], and silent cells, which fire few or no spikes [95]. What makes a place cell spike where it does? Observing place cells requires an animal capable of moving around an environment (but see [96]), either real or simulated [76•,77••], and intracellular recordings of place cells have been obtained in both freely moving rats [81•,82••,97] and head-fixed mice in a spherical treadmill virtual reality system [77••]. These recordings revealed that a sustained subthreshold V_m depolarization underlies spiking within the place field (Figure 4a and b), which rules out several models of place field origin, for instance those involving solely a localized increase in fluctuations (in both depolarizing and hyperpolarizing directions) that cross spike threshold [77••]. Further analysis showed that the shape of this depolarization is asymmetric [77••], as predicted [98]. Additional findings detectable only with intracellular methods included spikelets [81•], which add another dimension to mechanisms of neuronal integration, and large, putatively calcium-based events [77••,82••,99,100] (Figure 4a and b), which could trigger plasticity [101,102] underlying spatial memory formation in the hippocampus [103–105]. Lastly, what makes a neuron a place cell versus a silent cell? Comparison of place (Figure 4c) and silent (Figure 4d) cells revealed unexpected differences in excitability suggesting that intrinsic properties determine which cells become place cells in a novel environment [82••]. Together with studies in which excitability was molecularly manipulated [106], this suggests that intrinsic excitability biases which cells will participate in new memory traces.

The virtues of reality and virtual reality

Virtual reality systems [75,76•,77••,78] hold the promise of studying many natural behaviors in preparations that allow the use of larger electrophysiology, imaging [107], and other equipment that need not be carried by the animal itself, combined with the manipulability of virtual environments. For instance, the existence of place cells in such preparations [76•,77••] could allow experiments that separate the contributions of self-motion and external sensory information to spatial firing. The absence of vestibular cues required for normal place cell activity in freely moving animals [108,109], though, suggests a need for further investigation into which additional features of place cells and spatial behaviors are preserved with virtual approaches [75,76•,77••]. For these and other (e.g. social interactions) cases that may require more

degrees of behavioral freedom, freely moving methods for intracellular recording [44••,79•,80,82••] and imaging [110,111] can be employed.

Conclusions and future directions

There are a number of exciting avenues that are just beginning to be explored using awake intracellular recordings. Recent technical achievements have allowed for intracellular recordings to be understood in the context of the anatomical circuit within which these neurons are embedded. For instance, the application of *in vivo* imaging to target neurons for electrophysiological recordings [112] enables investigators to study specific cell types in head-fixed animals during natural behaviors [17••,113]. In addition, advanced methods have emerged for tract tracing [114,115] or circuit reconstruction [116,117] in order to establish the synaptic partners associated with the dynamics observed *in vivo*. Plasmids can be introduced within the recording pipette which label monosynaptically connected neurons [118•,119]. If presynaptic neurons express a calcium indicator [120], one may (in theory) observe the input information that is presented to a single neuron. The combination of awake intracellular recording with such approaches could help achieve the ambitious goal of understanding the mechanisms underlying the input/output transformation of each cell in the circuit responsible for a specific behavior.

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In an insect olfactory preparation, the authors perform *in vivo* intracellular recordings of a postsynaptic neuron in conjunction with multielectrode recordings of its presynaptic neuron population to address the connectivity underlying the sparse firing of the postsynaptic neuron. Through spike-triggered averaging of the postsynaptic V_m with respect to spontaneous spikes of the presynaptic projection neurons, they determine that each of these postsynaptic neurons is connected to approximately half of its input population. They show that the effective size of each input is small and estimate the number of simultaneous inputs needed to fire a target cell.

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