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REAL-TIME CLOSED-LOOP ELECTROPHYSIOLOGY: TOWARDS NEW FRONTIERS IN *IN VITRO* INVESTIGATIONS IN THE NEUROSCIENCES

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INTRODUCTION

In any behavior relevant to survival, the central nervous system (CNS) of man and animals faces an uninterrupted and rapidly changing stream of sensory information. In addition, sensory inputs are constantly perturbed by the impact of motor behavior on the environment, so that sensory-motor integration and processing is often identified as a "circular reaction" after the work of J. Piaget.

Interestingly, such a picture is functionally reflected at distinct levels of organization underlying the physiology of the nervous system: from ion channels to recurrent neuronal networks. Therefore, in analogy to behavior, sensation and motion, the input to the system depends on its output for the majority of biophysical processes. For instance, (i) the electric potential across a patch of excitable membrane is determined by the instantaneous fraction of open protein channels and by the distribution of ions these channels are selective to. On the other hand, the stochastic transitions between open and closed state for each channel are affected by the local electric field of the membrane in the form of diverse state-dependent kinetics (13, 29). (ii) The flow of charge between distinct compartments of a given neuron depends on the actual membrane voltage, which is in turn the result of the spatial distribution of charges. (iii) The signal transduction via ionotropic postsynaptic receptors can be limited by their internal state (e.g. the Mg^{2+}-block for $NMDA_R$). Indeed, these may act as coincidence detectors between synaptic currents and the local membrane depolarization, which is in turn determined by the activation of synaptic receptors. (iv) The temporal fluctuations of the membrane voltage, determined by the barrage of excitatory and inhibitory synaptic inputs (EPSPs and IPSPs), can "shunt" the contribution of further chloride-mediated IPSPs, because of a transient reduction of their ohmic driving force (i.e. $E - V_m$). This makes the actual value of the synaptic current to be proportionally dependent on the membrane potential. (v) The highly irregular spiking of cortical neurons *in vivo*, thought to arise from a stream of stochastic synaptic inputs, is substantially determining the background synaptic noise to other neurons and thus to itself, given the high degree of convergence in the cortical connectivity. (vi) Finally, in large recurrent networks, the firing rate of one neuron is influencing its own synaptic inputs, because of autaptic and synaptic recurrent pathways.

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Therefore, recording neurons *in vivo* and during behavior could in principle allow studying emergent dynamics in the real-world environment. However such inputs are difficult to constrain, fully control and parameterize. In addition, neurons are receiving and sending input from multiple unknown brain regions and it is challenging to record simultaneously from several neurons in one or multiple networks.

On the other hand, *in vitro* preparations have unique advantages in terms of accessibility, visibility and control of the cellular and subcellular physico-chemical conditions. Indeed, past studies have extensively explored neural information processing in single neurons and simple synaptic microcircuits, largely neglecting closed-loop influences. For instance, neurons and synapses are usually fed by periodic step-like episodes and their response is only correlated to different experimental conditions. These are clearly unrealistic and artificial conditions, just marginally relevant to understand behaviorally – related phenomena.

Thus, it is necessary to embed molecules, ion channels, single neurons and networks into a pseudo-environment, in order to address emergent dynamics in neuronal ensembles in the appropriate context of realistic input-output dependences. In such a way, inputs can be precisely controlled, multiple neurons simultaneously recorded at several levels of resolution, and the collective response of a neuronal population looped back in real-time, allowing the system to act on its "environment".

In the literature of the past few years, a clear trend towards unconventional technological approaches to *in vitro* (and *in vivo*) neuronal electrophysiology emerged. Thanks to recent advances in analog electronics, digital signal processing (DSP) and real-time (RT) computing, the input-output "loop" has been closed, stimulating the system according to its current state. Such a closed-loop approach is specifically targeted at system neuroscience by investigating cellular processes under more realistic conditions. This is expected to blur the borders between behavior and single-cell electrophysiology, allowing to peek into inner processes of the brain, hardly accessible by other means.

The present paper aims at identifying such a scientific trend in the Neurosciences, reviewing a few examples of the innovative technological directions mentioned above, such as the Dynamic-Clamp technique (25) and the use of hybrid silicon-neuronal circuits (17). At the same time, our discussion is focused at outlining the common background and motivation of the examples of choice, while emphasizing the relevant scientific results.

METHODS

In the following, we briefly review the technical details of each of the techniques whose results will be discussed in the Results section, while providing a short introduction to the biological preparations usually employed in these studies.

In vitro *models of the nervous system*

Acute brain tissue slices and cultures of dissociated cells represent extremely common choices for investigating the electrophysiology and activity-dependent plasticity in networks of synaptically interacting neurons in the CNS.

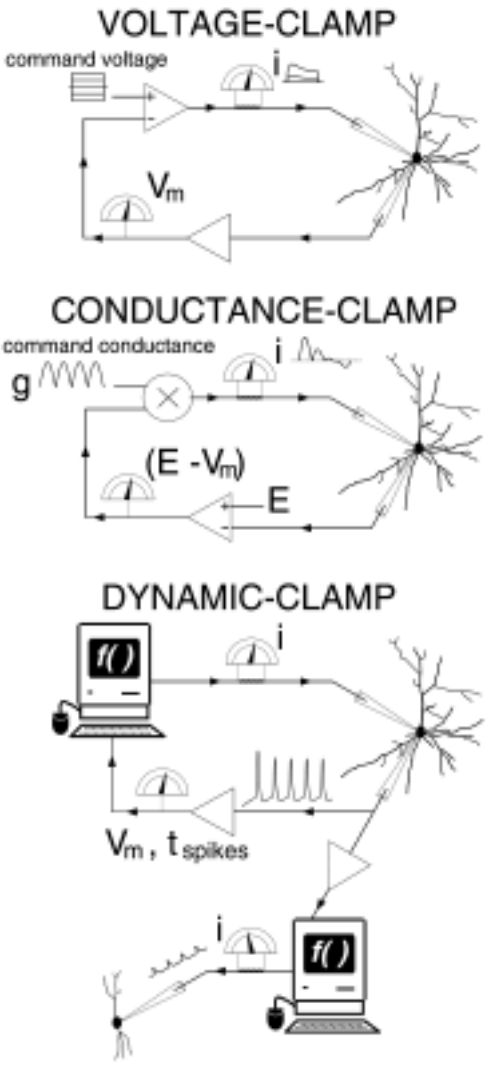

Fig. 1. - *Single-cell Closed-Loop Electrophysiology*.
The sketch compares the state-of-the-art experimental techniques employed to probe closed-loop single-cell electrical properties. (A) The voltage-clamp approach, pioneered by Hodgking and Huxley (1952) (12), can now be regarded as an important first example of closed-loop hardware development in cellular electrophysiology. Its contributions to the discovery of subcellular mechanisms, underling voltage-gated ion currents and the action potential generation, have been fundamental to Neurosciences (26). As a natural evolution of such a framework, in recent years it became possible to inject current waveforms *i(t)*, characterized by an instantaneous dependence on the actual membrane voltage V_m, simultaneously recorded. Nowadays, the availability of cheap A/D-D/A converters and fast digital computers makes it possible to emulate membrane conductance changes *g(t)*, characterized by an arbitrary time-course (B), as well as by computer-synthesized voltage-dependent kinetics (C). The relevance and applicability of these techniques has only recently become established. For the sake of simplicity, we reported the details of voltage-, conductance- and dynamic-clamp in whole-cell configuration based on two electrodes. The single-electrode case can be employed equivalently thanks to bridge electronic circuitry with both patch-clamp and sharp-electrode techniques (3).

With regards to the studies focused on single-cell properties, acute tissue slices from the brain and from the spinal cord allow the experimenter to investigate with high fidelity the cytoarchitecture of the corresponding CNS area. Thanks to infrared contrast (DIC) videomicroscopy, it is nowadays routinely possible to visually identify the morphology and location of a target neuron to record from. Together with the simultaneous micromanipulation of glass pipettes, such an approach makes possible to electrically access distinct compartments of the same cell (e.g. soma-to-dendritic), or synaptically connected neuronal pairs simultaneously.

On the other hand and with emphasis to the experimental attempts focused on network-level investigations, many authors regard dissociated cultures of neurons as one of the most appropriate experimental model system. These cultures organize and develop *ex vivo* into large networks with random connectivity. In addition, because of the relative absence of predefined constraints and the simplified 2D organization, cultures are ideal for studying the universals governing expression, formation, conservation and plasticity of neural activity. Similarly to acute slices, functional chemical synapses are extensively represented in these networks, and sensitive to changes in neural activity.

The voltage-clamp *technique*

The *voltage-clamp* became an extremely well known and accepted electrophysiological technique (14). It played an instrumental role in the discovery of the large diversity of ion channels and in the dissection of their voltage-dependence. As opposed to *current-clamp* that involves no stimulation/measurement-loop, *voltage-clamp* is an extremely effective electrophysiological strategy, consisting of feedback regulation of the voltage measured in an isopotential compartment by a glass electrode, through appropriate continuous current injection (Fig. 1A). It is very easy to forget that by no other means the subcellular basis of the action-potential generation mechanisms would have been quantitatively understood, given the strong dependence of the membrane potential on the membrane currents and vice versa.

In general terms, the *voltage-clamp* is a proportional negative-feedback regulation control with variable gain, whose active command current can indeed be interpreted as the true membrane current that is needed to compensate for, in order to maintain a fixed membrane voltage set to a command value $V_m=V_{cmd}$. In other words, any deviation of the recorded voltage from the command level is corrected by compensatory depolarizing or hyperpolarizing current injection.

$$i(t) = gain \cdot \left(V_{cmd}(t) - V_m(t)\right)$$

The conductance-clamp *technique*

Although the injection of a synthetic synaptic input, in terms of a *conductance* change, rather than of a current pulse, requires no more than the Ohm-law and basic analog electronic circuitry, extremely similar to the voltage-clamp, it was only at the beginning of the 1990s that *conductance-clamp* was proposed as a practical electrophysiological method. Pioneered independently by H.P.C. Robison and by E. Marder (28, 31, 32), the *conductance-clamp* is now even commercially available. It usually consists of an analog "four-quadrant" hardware multiplier, which emulates the ohmic dependence on the postsynaptic voltage V_m of the net current *i*, caused by a conductance change *g(t)* (Fig. 1B). By an additional analog adder, it is possible to study the impact of synaptic conductance inputs to neuronal integration, under a variety of configuration, even employing excitatory and inhibitory components.

In general terms, the *conductance-clamp* is the synthesis of an analog waveform, resulting from the product of an external waveform *g(t)* and the membrane voltage $V_m(t)$, measured instantaneously and referenced to a voltage that mimics the synaptic reversal potential:

$$i(t) = g_{ex}(t) \cdot \left(E_{ex} - V_m\right) + g_{inh}(t) \cdot \left(E_{inh} - V_m\right)$$

The dynamic-clamp *technique*

During the last decade, it has been further possible to replicate and extend the *conductance-*

clamp approach outlined in the previous section, employing cheap general-purposes digital instead of expensive dedicated analog hardware (25, 31, 32). As a consequence of the availability of increased digital computing power in personal computers and of fast A/D-D/A conversion boards, it is therefore possible to replicate digitally the analog multiplier. In addition, any mathematical function can be implemented thanks to the ease of programming simple arithmetic operations on the values digitized by the A/D stage, to be output by the D/A stage.

In general terms, the *dynamic-clamp* is the online synthesis of a digital waveform, resulting from the product of one or more (voltage-dependent) conductances $g_i(V_m, t)$ and the membrane voltage V_m at every sampling time. Indicating by $f(\ )$ any generic function of time and membrane voltage, whose computation does not require a time interval longer than the sampling period, we can write:

$$i(t) = f(t, V_m)$$

Accurate dynamic-clamp performance requires uninterrupted, rapid sampling of the membrane potential and fast computation of the current $i(t)$ to be injected. For a voltage-dependent conductance, the injected current is determined by a set of differential equations that describe the voltage and time dependence of the conductance. For instance, emulating a voltage-dependent sodium current and indicating by E_{Na} the Nerst-equilibrium for Na^+ ions across the membrane, requires

$$i(t) = g(t, V_m) \cdot (E_{Na} - V_m) \ ,$$

where $g(t, V_m)$ is fully simulated by the computer and effectively injected into the neuronal membrane at the site of the glass pipette localization.

For a synaptic conductance, the current injected by the *dynamic-clamp* is computed on the basis of presynaptic input that is recorded from another neuron, or generated by a model neuron or by a descriptive model of typical *in vivo* input.

The hybrid *approaches*

As a drawback of programmability and general-purposes, real-time digital computers are slow. This substantially limits the complexity of dynamic real-time loops (i.e. $f(\ )$) to be implemented. This is especially true when the *dynamic-clamp* approach, outlined above, is employed to connect distinct neurons by synthetic synapses (Fig. 1C) or to connect a real neuron to a computer-simulated clone. In the last case, hybrid synaptic microcircuits are obtained by artificial spiking neurons, which are functionally and bidirectionally interfaced to real neurons. In such a context, mathematical equations describing the model neurons must be numerically integrated in real-time, making the task of closing the loop extremely demanding. In recent years, dedicated DSP processors have been considered, running modified version of general purposes neuronal simulators, or dedicated very-large-scale-integrated (VLSI) analog electronics. Exploiting the results of alternative VLSI electronic designs, such as the use of field-effect-transistor as analog components, working under the so-called "subthreshold" or "weak-inversion" regime, special ad hoc *in silico* neuromorphic hardware were developed. Thus, ad hoc implementation of synaptic or intrinsic voltage-dependent conductances could be employed as an alternative to desktop computers. However, in some case these VLSI dedicated hardware simulators must be artificially slowed down to provide a real-time interfacing to real neurons, greatly affecting their convenience.

Substrate arrays of microelectrodes (MEAs)

The *ex-vivo* developing neuronal cell cultures enables further extensive sampling and manipulation of the physico-chemical variables, with unparalleled access and visibility, chronically and non-invasively. In fact, although most of the standard electrophysiological approaches can be employed in the study of large random cortical networks developing *ex vivo*, studies characterized by strong interests towards the network-level characterization of the neuronal population, applied

multi-electrode stimulation and recording techniques (34). In such conditions, the dissociated cortical cells are plated directly onto substrate-integrated arrays of microtransducers, which can be used to non-invasively record and stimulate the electrical activity of single cells and ensembles of cells. These devices, being the results of more than 20 years of research (11, 12, 21, 34), are obtained by means of modified silicon/glass substrate micromachining and microphotolitography that are extremely common and very similar to the technique for designing integrated microelectronic circuits, and are even commercially available from various sources, inspiring innovative and fruitful microelectronics-neurobiology interfacing scenarios.

MEAs, multielectrode closed-loop protocols and in vivo / in vitro *embodiment*

Very recently, a few authors proposed to provide a (distributed) capacitive electrical stimulation (35), by means of the same substrate electrodes detecting extracellular spiking activity. Similarly to the *conductance-clamp* technique, at the level of a large neuronal population, this implies to shape the spatio-temporal pattern of electrical pulses delivered by appropriate multichannel insulation electronics, as a function or as a consequence of the global network activity (20, 35) (Fig. 2A). Such a scenario necessarily requires the use of fast computers, implementing arbitrary complex functions between the detected distributed network activity and the stimulation pattern to be delivered. Nevertheless, this perspective opens the way to a novel interaction between theoretical and experimental approaches.

Some laboratories further proposed to close the loop without employing arbitrary mathematical functions to be computed and updated in real-time in analogy to the single-cell closed-loop approaches. Instead, they attempted to exploit the "circular reaction" naturally emerging in a simplified physical environment, as a consequence of an artificial embodiment (24). The use of a simple robotic actuator provided with sensors capable to provide a functional feedback on the overall performance of the recreated behavioural task, was attempted not only in cultured cells coupled to MEAs, but even *in vivo* (16, 36).

In conventional *in vivo* electrophysiology experiments, the group of M. Nicolelis exploited the multiunit/multielectrode signals detected from the motor cortex of chronically implanted primates (Fig. 2B) (36). Similarly to the artificial embodiment mentioned above for *in vitro* preparations, the occurrence times of extracellularly detected action potentials are fed into adaptive computer algorithms in charge of providing a control signal to set velocity and force to the robotic actuators. In the context of the *in vivo* experiments, the loop is closed by artificial visual or tactile feedbacks, informing the animal about the performance of the brain-derived motor-control. On the other hand, in *in vitro* experiments, no proper sensory feedback can be exploited and arbitrarily distributed patterns of electrical stimuli are employed as an alternative.

Iterative stimulation protocols, employing on-the-fly response analysis

Finally, the use of iterative stimulation protocols was proposed by just a few authors as an alternative technique. This employs non-real-time computation, which can still be regarded as a closed-loop strategy with very long reaction time constants, without the heavy constraints of computation-intensive real-time loops.

One example of such an approach is given by the selection of the relevant features (qualitatively and quantitatively) of a physical stimulation of a sensory system, on the basis of the neuronal responses, analyzed on-the-fly, right after each stimulation episode (10).

Another example is represented by the possibility of synthesizing artificial feedforward networks of interacting neurons, in single-cell experiments, while feeding again and again the neuronal response as its input (27). In abstract terms, this can be formalized as a sequence of stimulation/response analysis like

$$i_0(t) \rightarrow V_0(t) \quad i_1(t) = F\{V_0(t)\} \rightarrow V_1(t) \quad i_2(t) = F\{V_1(t)\} \rightarrow V_2(t) \quad \ldots \quad i_n(t) = F\{V_{n-1}(t)\} \rightarrow V_n(t)$$

where $F\{\ \}$ indicates, for instance the "conversion" of an evoked spike-train into a synthetic sequence of synaptic activations, leading to a set of excitatory postsynaptic current waveforms each occurring in correspondence to the evoked spikes. Similarly, a recurrent synaptic architecture

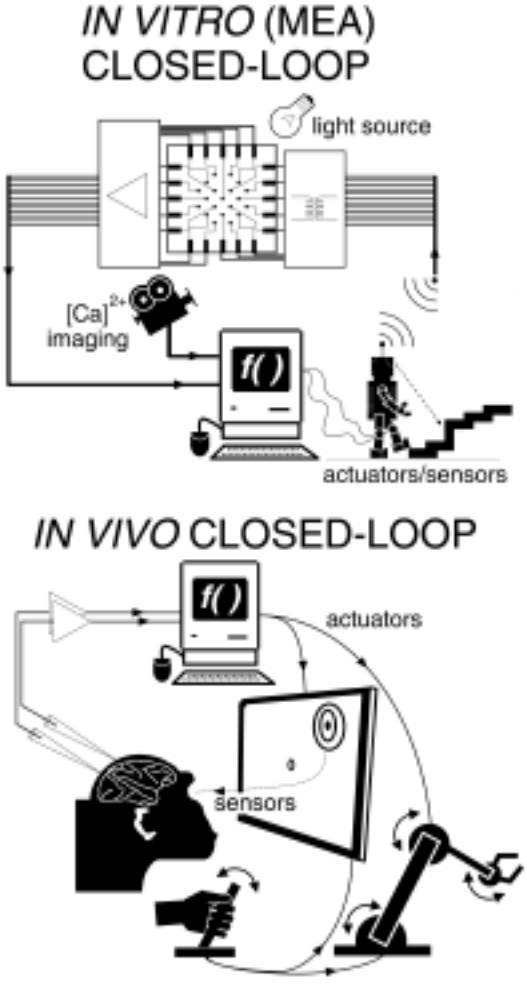

Fig. 2. - *Multi-electrode closed-loop electrophysiology and artificial embodiment via sensors/actuators feedbacks.*
Thanks to technological advances of the last few years, detecting and electrically eliciting trains of action potential from large *in vivo* and *in vitro* neuronal networks makes it possible to generalize and extend the closed-loop paradigm sketched in Figure 1. (A) The use of *in vitro* preparations, such as brain slices or cortical cell cultures coupled to arrays of extracellular electrodes, represented the first attempt at feeding a reduced nervous system with electrical stimuli determined by its own spontaneous and evoked spiking activity. Exploiting substrate multi electrode arrays (MEAs) as a bidirectional interface between spike-detection and stimulation control electronics, simple forms of artificially recreated sensory-motor behavior and elementary learning/conditioning were demonstrated and explored. (B) A similar strategy has been successfully attempted in *in vivo* primate cortical electrophysiology: the voluntary motor commands of a computer-game cursor or of a robotic arm, could be replaced by the information extracted from cortical multiunit activity, without degrading the monkey performance of reaching the tasks. These attempts are still in their infancy for both technical aspects and outcomes.

might be emulated, by appropriately translating the measured spike-train into a stimulating current $i_{k+1}(t)$ whose amplitude is proportional to the mean spike frequency evoked at the previous step $V_k(t)$.

In the very same context, the experiments by S. Marom on cultured networks of cortical neurons, growth on MEAs, represent another example. In fact, a similar iterative/on-the-fly approach has been employed to interrupt the electrical stimulation delivered through a few MEA electrode, as some global fit criteria was matched (30).

The active reduction of spontaneous population bursting, in the same cultured networks, by Potter and collaborators (35) is another successful example of distributed stimulation with an amplitude depending on the network global activity just preceding the stimulus delivery.

RESULTS

In this section, we review the results obtained by the closed-loop approaches previously introduced, in the light of the scientific questions that drove these attempts, resulting into substantial advances in our understanding of the dynamics and plasticity of single-cell and neuronal networks.

Information transfer and synchrony in iteratively-recreated cortical networks

There are two competing theories on the role of synchrony in the encoding of information in the CNS. The first proposes that synchrony is responsible for neural coding, in such a way that global neuronal activity becomes highly correlated during behavioral tasks or during sensation since it carries an intrinsic functional meaning. The other theory proposes that the neuronal code is represented just by firing rates, namely the number of action potential emitted across a large population instantaneously. Clearly, according to the second proposal, spike synchrony plays only a minor role and could, in principle, even be detrimental to information coding and propagation.

In the last years, extensive theoretical investigations demonstrated that networks of interacting neurons can perform under both hypotheses, depending on the conditions. But regardless of the neural coding strategy, what kind of transfer reliability is anyway achieved by a large feed forward network of cortical neurons? In (27) an interesting *offline* closed-loop approach has been explored to answer this question. While recording from just a single neuron in an acute slice of the rat somatosensory cortex, Reyes replicated iteratively the consequence of signal propagation in an abstract network of m layers, each assumed to contain w cells, and characterized by sparse connectivity.

After collecting the spiking response, elicited by noisy current injection into the neuronal somatic compartment, he built an effective postsynaptic current waveform from each of the action potential elicited, equivalent to the net synaptic inputs which would be fed to the following layer in real feed forward network architecture, scaled so that the voltage deflections were comparable to postsynaptic potentials (PSP) measured experimentally in previous pair recordings. This process was iterated for w times, once for each neuron of each layer. To emulate the signal propagation across layer, while monitoring the degree of spikes synchronization, at every suc-

cessive iteration the activity was summed up from the previously evoked spike train and injected again into the neuron.

Interestingly, in all the cases investigated the neuronal firing remained asynchronous for the first 2-3 iterations, while becoming more and more synchronous in the following layers. This strongly suggest that under the same condition, repeating the procedure in parallel, across several neurons of a feed forward network, instead of serially in a population emulated by a single-neuron, synchronous activity could build up and propagate across layers. To further understand whether the noisy barrage of background of excitatory and inhibitory inputs could disrupt and influence the synchrony, the author could simply emulate them by direct *dynamic-clamp* injection. Finally, by recording and stimulating a few cells at the same time, while repeating the same iterative procedure, it was possible to investigate the role of neuronal heterogeneity, in terms of distinct whole-cell input resistance, excitability threshold as well as frequency-current response. This did not prevent synchrony to build up after 2-3 simulated layers.

Information transfer in hybrid thalamic circuits

The fact that the thalamus acts as a gate for the cerebral cortex, modulating the flow of information depending on the state of arousal, is well established. However, the underlying mechanisms of the gating of sensory information, depending on the level of inhibition of the thalamocortical relay neurons by the reticular nucleus remain largely unclear. Le Masson and coworkers developed a unique *hybrid* approach to reconstruct and fully control the thalamocortical circuit loop in thalamic acute slices (7, 17, 37). In details, they recreated *in vitro* the thalamic circuits of the dorsal lateral geniculate nucleus. It consisted of a (real) thalamocortical neuron, impaled by sharp glass pipette, and of a synthetic reticular model interneuron that was connected bidirectionally under *dynamic-clamp* through the amplifier and the current-injection control (Fig. 1C).

Replicating the occurrence of visual inputs by somatic current injection, and mimicking *in silico* the spiking response of GABAergic neurons, the hybrid circuit design allowed them to fully control the conductances modulating the inhibitory synaptic coupling. A similar control of the experimental conditions would have never been possible by any other means. Similarly to the physiological intrathalamic recurrent microcircuit, these authors showed that if the gain of the recreated thalamic inhibitory feedback-loop is strong enough, the emergent dynamics of the hybrid circuit were dominated by oscillations, in the form of spindle waves. Such a stereotypical pattern recurred periodically in a manner very similar to the activity occurring in sleep-related states, regarded as a signature of early stages of sleep. This was a consequence of the rebound burst generation in the thalamocortical neuron, which in real-time affected the activity of the model neuron, changing its own level of inhibition.

Such a network state imposes a strong temporal decorrelation of the visual inputs and thalamic relay output. On the other hand, a lower gain coupling the feedback inhibition together with modulators of arousal (mimicked *in vitro* by bathing the

slice with noradrenaline), helps to increase input-output correlation in relay neurons. Thus, these authors quantitatively demonstrated how the combined use of feedback inhibition and neuromodulation of excitability allow the thalamic circuitry to gate the flow of sensory information to the cortex, in the form of trains of action potentials. The combination of low reliability and low efficiency of spike transmission explains how the thalamic circuit, during sleep, may disrupt the flow of sensory input. On the contrary, during arousal, reliable synaptic transmission and short-latency responses to incoming stimuli is a necessary condition for information relying. As a whole, their results showed that hybrid thalamic circuits act as a filter for input spikes with a temporal precision that is set by both the strength of intrathalamic inhibitory synapses and the state of membrane properties of TC neurons. It is however quite likely that under *in vivo* conditions, such combined features constitute a versatile regulatory system allowing fine up- and down regulation of signal transfer, over a continuum from sleep to arousal.

Injecting and ablating voltage-dependent ion channels

Similar investigations have been carried out by the group of E. Marder in invertebrate neurons (26). Together with other groups, these authors specifically focused on the role of the synaptic coupling and of the intrinsic voltage-gated conductances on the single-neuron pace-making and on the generation of rhythmic activity in small neuronal invertebrate networks (31, 32). With regards to the study of the impact of intrinsic voltage-gated conductances on the neuronal excitability, it is interesting to note that by the very same approach sketched in Fig. 1C to emulate and "inject" artificial ion-channels into excitable cells, a functional ablation can be obtained (18). In this case, when no appropriate selective pharmacological tools are available to silence one specific population of ion channels, the injection of a *negative* conductance, whose activation is accurately matching the current to ablate, has the effect of removing in a isopotential compartment the global impact of the ionic flow through those channels.

Understanding single-neuron input integration and gain modulation

The major difference between *in vivo* and *in vitro* experiments investigating the integrative properties of the neocortex is represented by the substantial lack, in the case of acute tissue slices, of the ongoing spontaneous activity. This largely results from the slicing procedure, where an extremely large number of afferents from other brain areas are severed and where only a subpopulation of the cells survives the acute slicing procedure. An extremely important implication of such a weakness is represented by the different *in vitro* "synaptic environment". Therefore, while neurons lack of the majority of their natural afferent inputs irreversibly lost, it may be possible to recreate the conditions of their normal operation by massively replicating the barrage of excitatory and inhibitory synaptic potentials in the form of conductance fluctuation. Obviously, the *dynamic-clamp* is the ideal tool to do this (8,9) and to study the response properties of individual neurons.

In a recent paper, Reyes and collaborators (5) could dissect for the first time how

the neuronal response was modulated by the level of background synaptic activity. The slope of the firing rate versus input current decreased when the total amount of synaptic background, realized by *dynamic-clamp*, is increased. This result implies that background synaptic input *in vivo* can be seen as a gain control mechanism.

Controlling global network activity by distributed stimulation

Among the most interesting features of long-term cultures of dissociated neurons, is the emergence of spontaneous coordinated electrical activity, lasting for the entire lifetime of the culture (i.e. several months). This consists in the low-rate emission of asynchronous action potentials by individual neurons as well as by episodes of bursting, synchronized throughout large portions of the cultured network. In the case of cultures of neurons dissociated from neocortex, individual cells have been shown not to possess intrinsic bursting or pace-making properties. The collective bursting is thus a population phenomenon requiring recurrent glutamatergic synaptic transmission as a necessary condition. Furthermore, the suppression of the population bursts by pharmacological agents substantially alters the spontaneous activity as a whole, eventually blocking it completely. On the other hand *in vivo* bursts are probably enrolled in synaptogenesis and neuronal development, resulting into mature cultured networks with stable properties across days and weeks *in vitro*. Bursting activity in mature cultures has been considered as a symptom for developmental standstill and compared to a nervous system deprived from thalamic inputs. Indeed, such an activity pattern has often been related to the stereotypical physiological patterns of *in vivo* activity occurring in cortical networks during sleep or ketamine-xylazine anesthesia, where the gating of the thalamocortical loop is largely suppressed. Alternatively, the coordinated character of such activity has been compared to the pathological occurrence of massive synchronized cortical spiking during epileptic seizures.

Considering the last interpretation, it is obvious that devising a technique aimed at reducing burst activity might indicate the directions for the treatment of drug-resistant epileptic patients. It is precisely in the present context that Potter and his coworkers hypothesized that the persistence of global bursts in dissociated cortical cultures may be due to a lack of input from other brain areas and that it could be suppressed by providing a surrogate of such inputs (24, 35). To test this hypothesis, they grew monolayer cultures of cortical neurons and glia from rat embryos on multi-electrode arrays (MEAs), and used electrical stimulation to substitute the missing inputs. Quantifying the “burstiness” of the cultures during spontaneous activity and during different stimulation protocols, they observed that slow stimulation through individual electrodes increased “burstiness”. However rapid stimulation reduced bursting. Interestingly, only when they made the stimulation intensity dependent on the actual level of spontaneous asynchronous global spiking, therefore closing the loop, they observed a dramatic and significant elimination of bursts for any culture considered. In other words, irrespectively on the optimal condition for the coupling between MEA electrodes and cultured neurons and on the effectiveness of individual electrodes to elicit global spiking activity, an active (feed back) stimulation pro-

tocol greatly enhanced burst control without disrupting completely the underlying asynchronous spontaneous activity.

Learning and conditioning in in vitro networks of cortical neurons

As discussed in the Methods, several authors reported success in studying *ex vivo* cultured networks by extensive, long-term and non-invasive MEA monitoring and manipulation of the distributed electrical activity (11, 20, 30). On the functional side, plasticity of the global and local electrical activity has been demonstrated on several time scales, as one of the most striking properties, which are experimentally controllable. What is more interesting has been the attempt at translating the concepts of training and learning to simplified closed loop paradigms, first proposed by the group of S. Marom at the Techion Israel Institute of Technology (20, 30). In fact, by employing extensive computational resources for the real-time electrophysiological multi-site signal acquisition, processing and interpretation, it is possible to define a fast experimental manipulation of the environmental conditions of the cultures (e.g. distributed electrical stimulation), whose parameters are determined on-line by the response of the system.

The idea behind these experiments was simple and effective: cultures were stimulated electrically by means of 2 substrate electrodes selected from those available in the MEAs. Subsequently, the evoked response was quantified in terms of a series of reverberating action potentials detected at the remaining electrodes. Then, a training session was designed in such a way the electrical stimulation was delivered repeatedly at low frequencies (0.3 - 1 Hz), until a defined pattern of activity appeared as evoked by stimulation in any 10 consecutive trials. As soon as this happened, electrical stimulation was suspended. The extremely surprising outcome of such a closed-loop procedure, which explores the role of a kind of external reward/punishment signal (or its absence), is that once the desired response criteria are satisfied, the system is able to retain its performances. As opposed to other paradigms of learning, such as associative learning, Marom thus clearly demonstrated the existence of a form of "selective learning" in a nervous system that is extremely simplified and lacks any organization.

As expected no learning was expressed in control experiments, where the control loop was purposely opened and the stimulus delivered irrespectively of the system response. This proved that indeed the removal of the repeated electrical stimulation was the key signal to learn.

DISCUSSION

As a consequence of a shift of paradigm, predicted by several philosopher of science in the last decades, an increasingly larger number of physicists and engineers are becoming interested in Biology and, in particular, in the Neurosciences. Not only the contributions from the interactions among different cultural backgrounds and languages are undoubtedly fruitful, but they are pushing the use and advances of

new sophisticated techniques in the Neurosciences. Although "technology for the sake of technology" is clearly bound to failure, we are convinced that this is not the case for the approach reviewed here. We believe that the technical contributions discussed here indeed constituted a successful balance between technical advances and the driving scientific questions.

It is our belief that the methodological trend discussed here will continue to expand as new and clever applications are being designed, and that these applications will continue to reveal fundamental aspects of the working of the neurons and networks, under realistic *in vivo*-like conditions. Below, we shortly address some of the most important future directions and implications and we also discuss their relevance for prospective clinical applications, in the field of brain-machine interfaces.

The bridge between experimental and theoretical approaches

In the discussion of the relevant experimental approaches outlined in the present paper, we hope to convey the idea of designing a realistic perturbation to the biological system of interest, for the sake of investigating its structure and function. Recording the *in vitro* response to stereotyped stimulations and protocols might not be the most effective way to gather a conclusive understanding on the working of ion channels, synapses, single-neurons and neuronal microcircuits.

By closing the loop, it is possible to literally *program* an artificial functional feedback with defined rules and constraints, limited only by the computational resources available. This obviously opens the barriers between mathematical modeling and experiments, leading to fruitful direct interactions between the two.

Future directions: optical methods for monitoring and activation

In the coming years, we expect a substantial involvement of optical methods, representing an alternative to electrophysiological techniques in providing a direct feedback measure. Today, both voltage- and calcium-sensitive-dyes make it possible to access single-cell dynamics over a time scale that is comparable to those of electrical phenomena. In addition, it has been demonstrated effectively that imaging can be used to detect the (spiking) electrical activity in extended neuronal compartments as well as in whole neuronal populations (6, 23). In addition, conventional, fast confocal and 2-photon laser-scanning microscopy has been successfully employed, greatly reducing light scattering and photo-bleaching, enabling independent quantitative detection of the spatial distribution of the spontaneous/evoked electrical activity of the *in vitro* microcircuits. In some experiments, high numerical-aperture objectives were already employed, leading to image fields of up to 400 - 700 μm.

As an approach complementary to distributed electrical stimulation, photo-activation of caged neuroactive compounds made impressive progresses (2), promising to become a tool to close the loop in acute tissue slices and cell cultures. Indeed, we anticipate the important contributions of the recent advances in temporally and spatially patterning of laser-beam stimulation, representing a key element for an advanced photo-stimulation at the level of a network (33).

Dynamic linearization using state feedback

As future theoretical perspectives, we expect that results derived from classic non-linear Control Theory may provide important inputs to the field of real-time feed back systems. In the very same context of closed-loop feedback protocols, one of the most likely candidates is the *dynamical linearization* of a non-linear dynamical system via a state feedback signal (4). In general terms, this is equivalent to the problem of synthesizing a control input *u(t)*, for instance by means of some adaptive algorithm implemented on a computer (as in Fig. 2A), which will cause the dynamical system

$$\dot{x} = g\big(x(t), u(t), t\big)$$

to have a response *y(t)* which matches some specified "template" system. While such a framework is normally employed in such diverse fields as industrial process control and avionics, it is easy to anticipate its applicability from single ion-channels, neurons and network of interacting neurons, up to sensory maps and large-scale systems (e.g. the motor cortex). Indeed, when the "template" is a linear relationship, the original system will become *dynamically linearized* by means of an appropriate input, instantaneously depending on the actual output of the system. It is therefore apparent that an in-depth understanding of the structure of the non-linear dynamical system will be available by direct inspection of the particular *control law*, optimally selected by the adaptive algorithm while dynamically compensating for the non-linearities of the input-output relationship of the original system.

Relevance for neuroprosthetics and clinical applications

Millions of people worldwide are every year affected by injuries and severe pathologies of the CNS. The most common are spinal injuries, resulting in paraplegia and quadriplegia. Restoring even the smallest independence to perform a motor action is thus perceived as an immense benefit. Despite the attempts made by clinical research so far, few therapeutic options are available and most of them not yet routinely reliable. While a great effort has been made so far to guide and drive the growth of severed fibers, other studies are attempting to bypass the lesion by means of neuroprosthetic devices. Such an approach is greatly relying on physiological plasticity of the CNS and it is precisely based on the closed-loop concepts reviewed so far. Voluntary motor commands might be accessed by decoding electrical activity recorded from cortical and subcortical neurons, which remained intact above the spinal damage. Such signals might then be used to reactivate biological or artificial actuators. Such a neuroprosthetic device would make use of chronic intracranial recordings in motor cortical areas. Similarly to the experiments sketched in Figure 2B, a mathematical model would take care of extracting the motor commands from the multichannel extracellular activity. In the case of an artificial actuator, the output of the real-time model would be used to reproduce the outcome of the patient's desired movement.

Today, a few clinical applications, employing live EEG recordings, have already been implemented and increasing interests are expected to boost the success of such

approaches in the future, in analogy to very successful examples of neuroprostheses, such as the cochlear implants and the deep brain stimulators for pain treatment, autonomic motor disorders, Parkinson's tremors and for treatment of drug-resistance chronic epilepsy. It is interesting to note that the closed-loop strategy outlined in Figure 2A, and explored in the work of Potter (35), is precisely focused at controlling the emergence of episodes of synchronous firing activity, upon fine tuning of the intensity of distributed electrical stimulation. Finally, we believe that clinical applications will only arise through an in-depth preliminary characterization of the scope of feedback control protocols in *in vitro* and *in vivo* models of the CNS.

SUMMARY

Reflected at any level of organization of the central nervous system, most of the processes ranging from ion channels to neuronal networks occur in a closed loop, where the input to the system depends on its output. In contrast, most *in vitro* preparations and experimental protocols operate autonomously, and do not depend on the output of the studied system. Thanks to the progress in digital signal processing and real-time computing, it is now possible to artificially close the loop and investigate biophysical processes and mechanisms under increased realism. In this contribution, we review some of the most relevant examples of a new trend in *in vitro* electrophysiology, ranging from the use of *dynamic-clamp* to multi-electrode distributed feedback stimulation. We are convinced these represents the beginning of new frontiers for the *in vitro* investigation of the brain, promising to open the still existing borders between theoretical and experimental approaches while taking advantage of cutting edge technologies.

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