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Development, learning and memory in large random networks of cortical neurons: lessons beyond anatomy

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1. Introduction

1.1 Outline

The phenomena of learning and memory are inherent to neural systems that differ from each other markedly. The differences, at the molecular, cellular and anatomical levels, reflect the wealth of possible instantiations of two neural learning and memory universals: (i) an

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extensive functional connectivity that enables a large repertoire of possible responses to stimuli; and (ii) sensitivity of the functional connectivity to activity, allowing for selection of adaptive responses. These universals can now be fully realized in *ex-vivo* developing neuronal networks due to advances in multi-electrode recording techniques and desktop computing. Applied to the study of *ex-vivo* networks of neurons, these approaches provide a unique view into learning and memory in networks, over a wide range of spatio-temporal scales. In this review, we summarize experimental data obtained from large random developing *ex-vivo* cortical networks. We describe how these networks are prepared, their structure, stages of functional development, and the forms of spontaneous activity they exhibit (Sections 2–4). In Section 5 we describe studies that seek to characterize the rules of activity-dependent changes in neural ensembles and their relation to monosynaptic rules. In Section 6, we demonstrate that it is possible to embed functionality into *ex-vivo* networks, that is, to teach them to perform desired firing patterns in both time and space. This requires 'closing a loop' between the network and the environment. Section 7 emphasizes the potential of *ex-vivo* developing cortical networks in the study of neural learning and memory universals. This may be achieved by combining closed loop experiments and ensemble-defined rules of activity-dependent change.

1.2 Universals *versus* realizations in the study of learning and memory

Learning and memory are behavioral concepts. Studying the physiological substrates of learning and memory requires a proper transformation of these behavioral concepts into the language of physiology. It is generally believed that behaviors are not mapped to single spikes generated by any one neuron, but rather to groups of spikes. These functional neural activity groups may originate from a single neuron or from populations of neurons firing in synchronic or diachronic manners (e.g. Edelman, 1987; Abeles, 1991). The structure of the vast majority of behaviorally relevant neural activity groups is not predetermined by genetics, nor dictated by some sort of an 'all-knowing teacher', *homunculus*. Rather, neural activity groups are formed and modulated throughout life in a dynamic, activity-dependent manner (reviewed in Quartz & Sejnowski, 1997), conforming to evolution and environmental constraints. The *formation* of neural activity groups is *learning*; their *conservation* is *memory*.

The variance in the structures of neural systems within and between species, on the one hand, and the constancy of basic behavioral phenomena of learning and memory, across individuals and species on the other, implies that the formation and conservation of neural activity groups is governed by a set of underlying universals. These universals may be realized in many different ways. At present, the neurobiological experimental approach to the study of formation and conservation of neural activity groups emphasizes specific realizations, such as particular forms of molecular machineries [e.g. receptor-mediated intracellular signaling cascades (Sanes & Lichtman, 1999)], particular forms of neuromodulatory effects [e.g. dopamine as a reward signal (Schultz, 1998)] or specially arranged structures [e.g. the role of the hippocampus in learning (Eichenbaum, 2000)]. Descriptions of specific realizations, both microscopic and macroscopic, are invaluable, especially for diagnostic and treatment-oriented purposes. This is true even when the action of underlying universals is unknown. Yet, as far as comprehensive understanding is concerned, collecting facts about specific realizations is by itself insufficient. The complexity of neural systems suggests that accumulation of such facts may lead the field astray rather than offering a coherent large picture. We argue that to

understand how neural substrates give rise to behavior one must understand the underlying universals. Thereafter, hypotheses regarding specific realizations become tenable.

In neural systems, two universals of learning and memory exist. These are: (i) an extensive functional connectivity that enables a large repertoire of possible responses to stimuli, and (ii) sensitivity of the functional connectivity to activity, allowing for selection of adaptive responses. In order to study the action of universals, it is desirable to have an experimental system that allows separation of these universals from their specific realizations. However, this is impossible since all experimental systems, *in vivo* and *ex vivo*, are constrained by specific realizations. Thus, we are left with a problem: understanding the universals is a prerequisite for an appropriate description of the role played by unique realizations but any attempt to set up, i.e. realize, a 'general' experimental model system may interfere with our ability to observe the action of the universals involved. This problem is inherent to the experimental scientific approach but can be overcome, at least partially, through selection of appropriate experimental strategies.

2. Large random cortical networks developing *ex vivo*

Of the various alternatives, large random cortical networks developing *ex vivo* are probably the most appropriate experimental model systems for studying the universals governing formation and conservation of neural activity groups. These networks demonstrate extensive functional connectivity and sensitivity of that connectivity to activity. Moreover, the networks are relatively free of predefined constraints and intervening variables. Alternative models, such as acute cortical slices and cultured slices allow one to explore 'what-is-there', but not 'how-it-got-to-be-there'. The latter question is tightly related to development, and slices have only a limited capacity to develop.

The *ex-vivo* developing model system enables extensive sampling and manipulating of the relevant variable, i.e. electrical activity. While many things can be measured in a neural system, electrical activity is most relevant to the organization and function of networks: processes that do not express themselves in propagation of electrical activity through the system must be deemed secondary, even irrelevant, from the point of view of brain function and behavioral science.

The *ex-vivo* developing cortical network system enables measurement procedures that interfere little with the action of universal factors. Moreover, it allows for study over wide range of timescales.

All these advantages led experimentalists to evaluate *ex-vivo* developing large random networks as biophysical models for study of the universals that act to form and conserve neural activity groups.

2.1 Preparation

Ex-vivo developing cortical networks are composed of cells obtained by means of mechanical and enzymic treatment from cortices of embryonic or early postnatal animals, usually rats. The preference for early stage cells is both mechanical and biochemical (Banker & Goslin, 1991; Higgins & Banker, 1998). In general, the later in development that cells are harvested, the less probable it is that they will survive and adapt to a new environment. At the time of

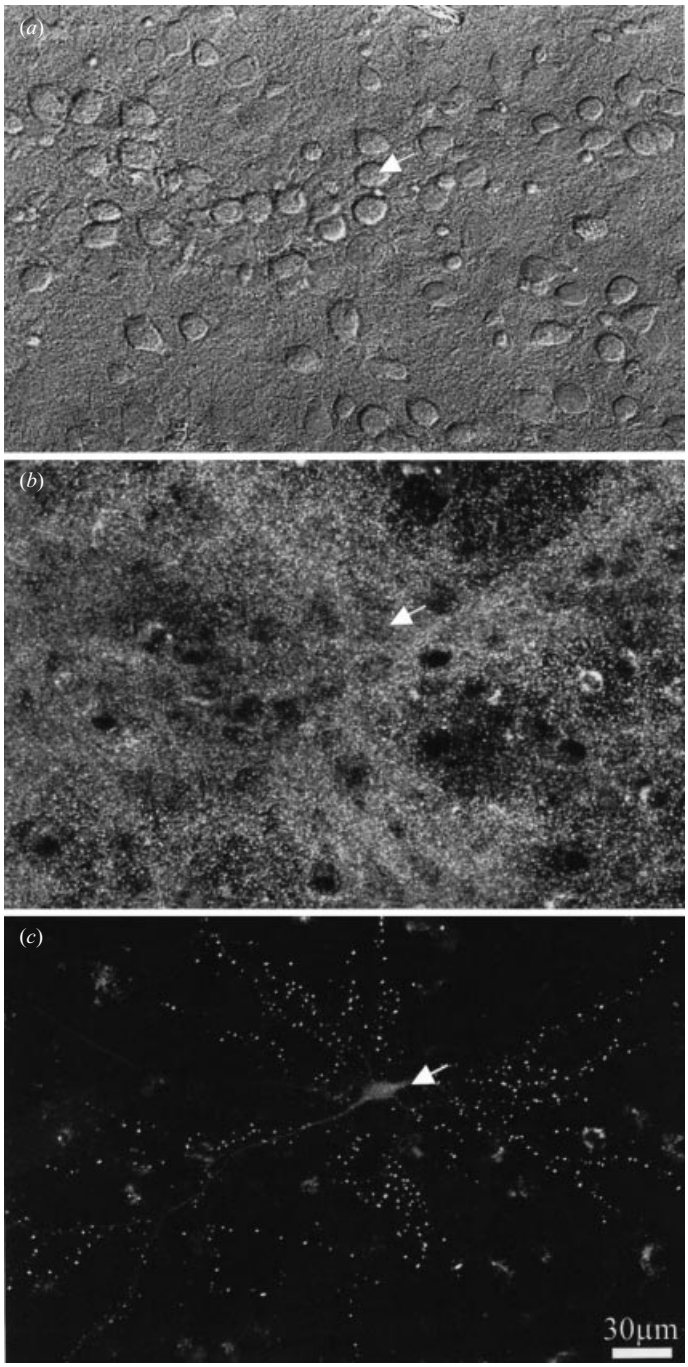

Fig. 1. Immense number of functional synapses connecting between neurons in the mature phase (N. Ziv and Y. Ramati; unpublished results, with permission). (*a*) A DIC image of cortical neurons in culture. (*b*) Functional presynaptic boutons in the same field visualized by FM 4-64 labeling of synaptic vesicles. Each punctum represents at least one presynaptic bouton. Note the enormous density of synaptic connections formed in these preparations. (*c*) Glutamatergic synapses belonging to a single cortical neuron in the same field visualized by expression of a fluorescent variant of the post-synaptic density molecule PSD-95. Individual synapses are clearly discernible. Arrows point to the cell body of the labeled neuron in all three panels.

plating, after their extraction from the cortex, most of the neurons are round or oval, having no axo-dentritic extensions. A typical cortical network, developing in a 20-mm diameter culture dish, may contain up to ~150000 neurons. The neurons begin to extend processes within hours after plating (Huettner & Baughman, 1986), and proceed in culture from a population of unconnected individual neurons, which are completely independent from each other structurally, to a densely connected mature phase. Once mature, the network forms a monolayer, with axo-dentritic branches that extend over 1 mm, and an immense number of functional synapses (Fig. 1).

While some degree of cell purification can be accomplished (Higgins & Banker, 1998), such procedures are not common in the preparation of cortical networks. Thus, the preparation contains all the types of cells that are present in the cortex at the time of extraction, including glial cells. Using antibody staining, it was found that the distribution of types of cells in *ex-vivo* networks is similar to that found *in vivo* (Neale *et al*. 1983; Huettner & Baughman, 1986; Nakanishi & Kukita, 2000). Thus, 10–25 % are inhibitory GABAergic cells (similar to the cerebral cortex *in vivo* where roughly 10–20 % of neurons exhibit GAD activity, that is, the conversion of glutamate to GABA); 2–3 % are acetylcholine-synthesizing cells (similar to the fraction of cerebral cortex cells that exhibit ChAT enzyme activity, the synthesis of ACh, *in vivo*; see Eckenstein & Thoenen, 1983; Huettner & Baughman, 1986). A detailed study by Huettner & Baughman (1986) offers further comparisons of *in-vivo* and *ex-vivo* morphological and physiological cellular properties, including the distribution of subpopulation of neurons in culture.

The survival of networks depends on plating density. A typical network containing ~ 150000 neurons in ~ 300 mm^2 can survive many months (Huettner & Baughman, 1986; Gopal & Gross, 1996). Potter & DeMarse (2001) developed a technique that allows networks to survive for over a year (see below). Comprehensive discussions about maintenance, growth media and growth factors, may be found in Higgins & Banker (1998) and Baughman *et al*. (1991), and references therein. The main phenomena described in the present review are, by and large, independent of the exact growth conditions (including nutrient composition of bathing medium, supplemental growth factors, or type of substrate and container) as long as the cells under study are healthy.

2.2 Measuring electrical activity

Practically all the standard electrophysiological approaches may be exercised in studies of large random cortical networks developing *ex vivo*. Studies involving single electrode techniques serve for gathering high-resolution data about the activity of single cells and pairs of cells. For the study of ensemble activity and its development, multi-electrode stimulation and recording techniques are applied (Stenger & McKenna, 1994). In this case, cortical cells, obtained as described above, are plated directly onto substrate-integrated multi-electrode array (MEA) dishes (Gross, 1979; Gross *et al*. 1982; Meister *et al*. 1994; Stenger & McKenna, 1994). These dishes are commercially available from various sources with approximately 60 electrodes, 10–50 μm diameter each, spaced 100–500 μm from each other to allow a variety of experiments (Fig. 2). Alternatively, the techniques involved in preparing MEA dishes are basic, and may be applied in a standard microelectronic facility. Typical electrode-solution impedance is < 100 kΩ. Transparent MEA dishes allow optical access to the preparation. The various types of insulation layers, pretreated with adhesive substrate [such as poly-L-

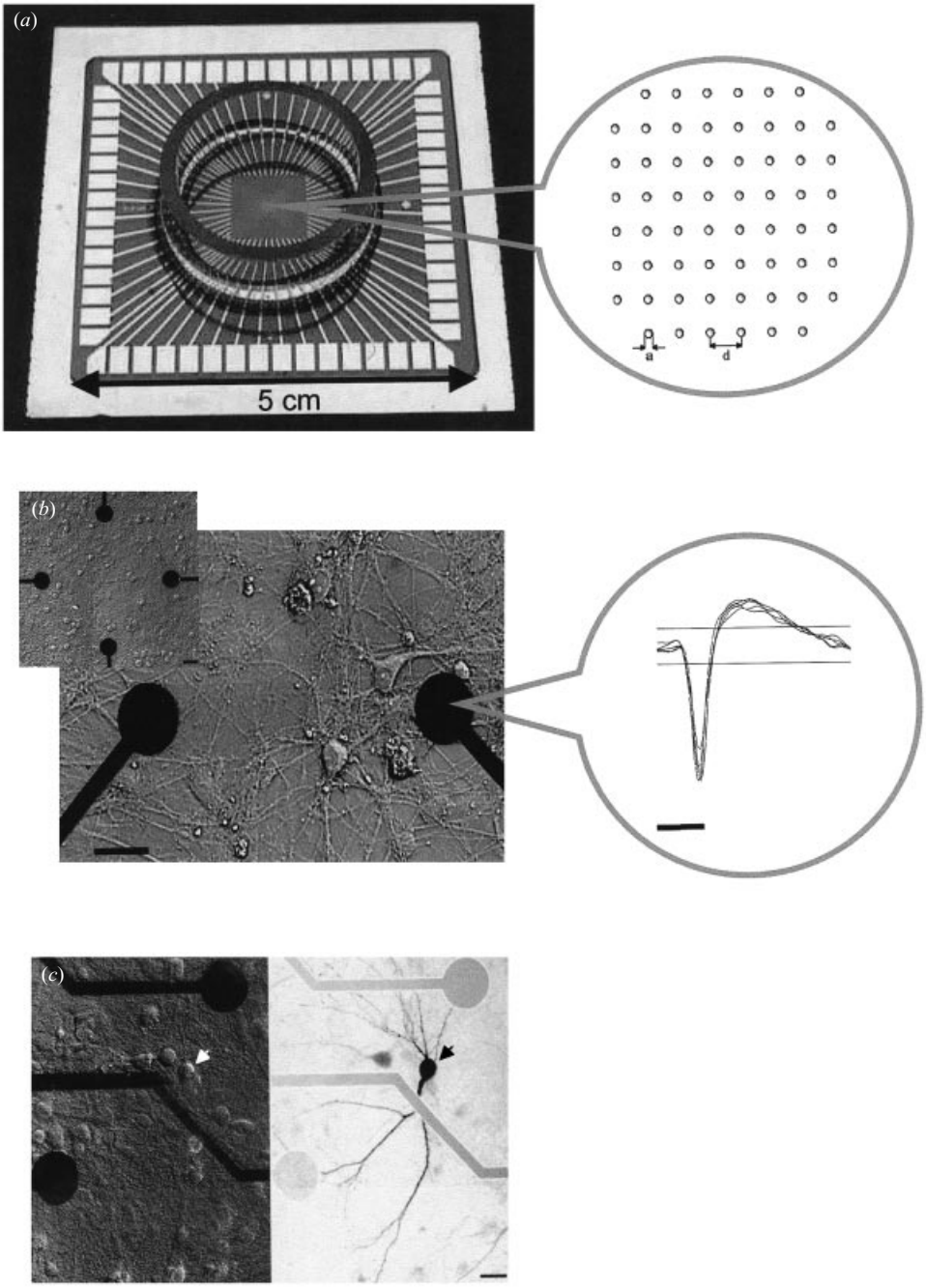

Fig. 2. (*a*) A commercially available substrate integrated multi-electrode array (MEA) containing 60 electrodes (Multi Channel Systems, Reutlingen, Germany, with permission). The diameter of a single electrode (a) and the distance between electrodes (d) range from 10–50 μm and 100–500 μm, respectively. (*b*) *Ex-vivo* developing cortical network growing on a substrate integrated MEA (only the four center electrodes are visible, two of which are enlarged). The exposed electrode tip occupies only part of the circular terminal (bar = 30 μm). Aligned action potentials recorded from one electrode are shown at the right. The distance between the horizontal lines depicts ±8 root mean square units, which, for this particular electrode amounts to approximately ±7 μV (time bar = 1 ms). (*c*) (Kindly provided

lysine, collagen or laminin (Robinson *et al*. 1993; Jimbo *et al*. 1999)], all form good surfaces for the development of neural networks. An authoritative description of the MEA techniques and their various uses may be found in Stenger & McKenna (1994).

In order to support long-term recordings from MEA dishes, the micro-incubation environment must be arranged. This is achieved by, for example, a filtered, heated and humidified air/CO_2 (95/5%) gas mixture, and electrically heated MEA platform at 37 °C. Recently, Potter & DeMarse (In Press) developed a method for keeping cortical networks on MEA dishes alive and stably active for over 1 year. They seal their culture chambers with a membrane that is permeable to CO_2 and O_2, and relatively impermeable to water vapor. By keeping these cultures in a non-humidified incubator, they are able to greatly reduce or eliminate problems with infection and increase in bath osmolality, while maintaining pH and O_2 homeostasis.

A typical electrical setup includes two stages of amplification to achieve a final gain of $\times 10\,000$–$40\,000$, with frequency limits from near 200–10 000 Hz (sufficient for proper spike detection and identification). Recording electrodes may be used for passing local stimulating currents. Data is digitized and stored for later analysis by A/D boards at a sampling frequency of $> 24\,000$ samples per second per channel. Data can be visualized, and preliminary analysis and reduction obtained in real-time using standard desktop computers and appropriate software interfaces. Since the recording electrodes are surrounded by several cell bodies, electrical activity is often picked up from several sources (typically 2–3 neurons). For some analyses, it is necessary to separate these sources; i.e. to assign recorded spikes to identified single neurons. Over the past 30 years, several types of algorithms and techniques have been optimized to allow identification of individual cells based on spike shape (Lewicki, 1998; see also Hulata *et al*. 2000).

Multi-electrode recordings may be used in conjunction with other, more conventional, means for measuring electrical activity, including patch-clamp related methods and optical monitoring. Thus, combining the *ex-vivo* network preparation with a computer-based, long-term, multi-electrode stimulation and recording techniques allows collection of a large bank of activity and stimulation data spanning from milliseconds to weeks, over a lengthscale of micrometers to millimeters.

3. Spontaneous development

3.1 Activity

Without exception, every *ex-vivo* developing cortical network shows spontaneous activity that is first detected in the form of uncorrelated firing towards the end of the first week in culture (Kamioka *et al*. 1996). At later developmental stages, single cells show activity typically composed of sporadic action potentials and clusters of action potential, superimposed on spontaneous voltage fluctuations around a resting potential of approximately -60 mV (Nakanishi & Kukita, 1998). These phenomena are observed in all networks, regardless of

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by N. Ziv, unpublished results). A cortical neuron expressing EGFP growing on an MEA substrate (arrowheads). Fluorescence image on the right is displayed using an inverted grayscale for purposes of clarity (bar = 20 μm).

the exact source from which cortical cells are extracted, or the means of measurement. Indeed, these behaviors appear in *ex-vivo* developing networks originating from almost all brain regions, not only the cortex (Van den Pol *et al*. 1996).

The attributes and frequency of spontaneous sporadic single spikes and synchronized clustered activity are dependent upon the age of the network. Synchronous regular bursting activity is mostly evident at earlier stages of network development (9–12 days in culture). A richer pattern emerges later (22–33 days), when the network exhibits a complicated non-periodic, synchronized, clustered activity with minute-to-minute fluctuations in the probability of firing (Habets *et al*. 1987; Kamioka *et al*. 1996) (Fig. 3). This behavior does not change for more than 2 months and thus represents the mature state of the network.

Maeda *et al*. (1995) noted that as the network matures (from 3 to 40 days), the frequency and propagation velocity of synchronized clustered activity increases markedly (0.01–0.5 Hz and 5–100 mm s^{-1}, respectively). There seems to be no uniquely defined 'pacemaker' that drives the network to burst; rather, Maeda *et al*. (1995) show that the origin of spontaneous bursts varies randomly with each burst, and that physical separation of a network into several parts using a laser yields synchronous bursting activity with different frequencies and phases in each part.

3.2 Connectivity

The spontaneous activity of the network, whether sporadic or synchronized, is correlated with the development of synaptic connections (Van Huizen *et al*. 1985; Habets *et al*. 1987; Muramoto *et al*. 1993). Thus, a rapid increase in the number of synaptic structures with a mature appearance was observed from approximately 5–25 days using electron microscopy. This change in physical structure coincides with the development of spontaneous electrical activity. Indeed, increase in the frequency of synchronous clustered activity directly follows the number of the synapses in the network (Muramoto *et al*. 1993). Subsequently, the process of functional network maturation (marked by complicated non-periodic, synchronized clustered activity with minute-to-minute fluctuations in the probability of firing) is accompanied by an overall decline in the number of synapses that start in the fourth week and continue for approximately 40 days (Van Huizen *et al*. 1985).

Synaptic potentials are detectable within 2 days *in vitro* (Baughman *et al*. 1991). Nakanishi and colleagues have used two electrodes in the whole-cell recording mode to observe connectivity between randomly chosen pairs of neurons in mature networks (Nakanishi & Kukita, 1998, 2000; Nakanishi *et al*. 1999). They found that in $\sim 40\%$ of the randomly chosen pairs an action potential evoked in one cell yields an EPSP (excitatory post-synaptic potential) in the other cell with a mean response latency of ~ 4 ms and a mean amplitude of ~ 4 mV (Nakanishi & Kukita, 1998). Eleven out of 22 such pairs had a response latency of 1–3 ms (corrected for conduction time). These observations led Nakanishi & Kukita (1998) to estimate the single synaptic time delay to be ~ 2 ms. Taken together with estimates from others (e.g. Jimbo *et al*. 1999) it is safe to conclude that in these networks at their mature phase each neuron is mono-synaptically connected to 10–30% of all other neurons.

Nakanishi & Kukita (1998) also show that although the delay between an action potential in a given neuron and the appearance of an EPSP in another, chosen at random, results from 1 to 10 synaptic transmission delays, no obvious relationships are apparent in distances between pairs of cells and the synaptic delay.

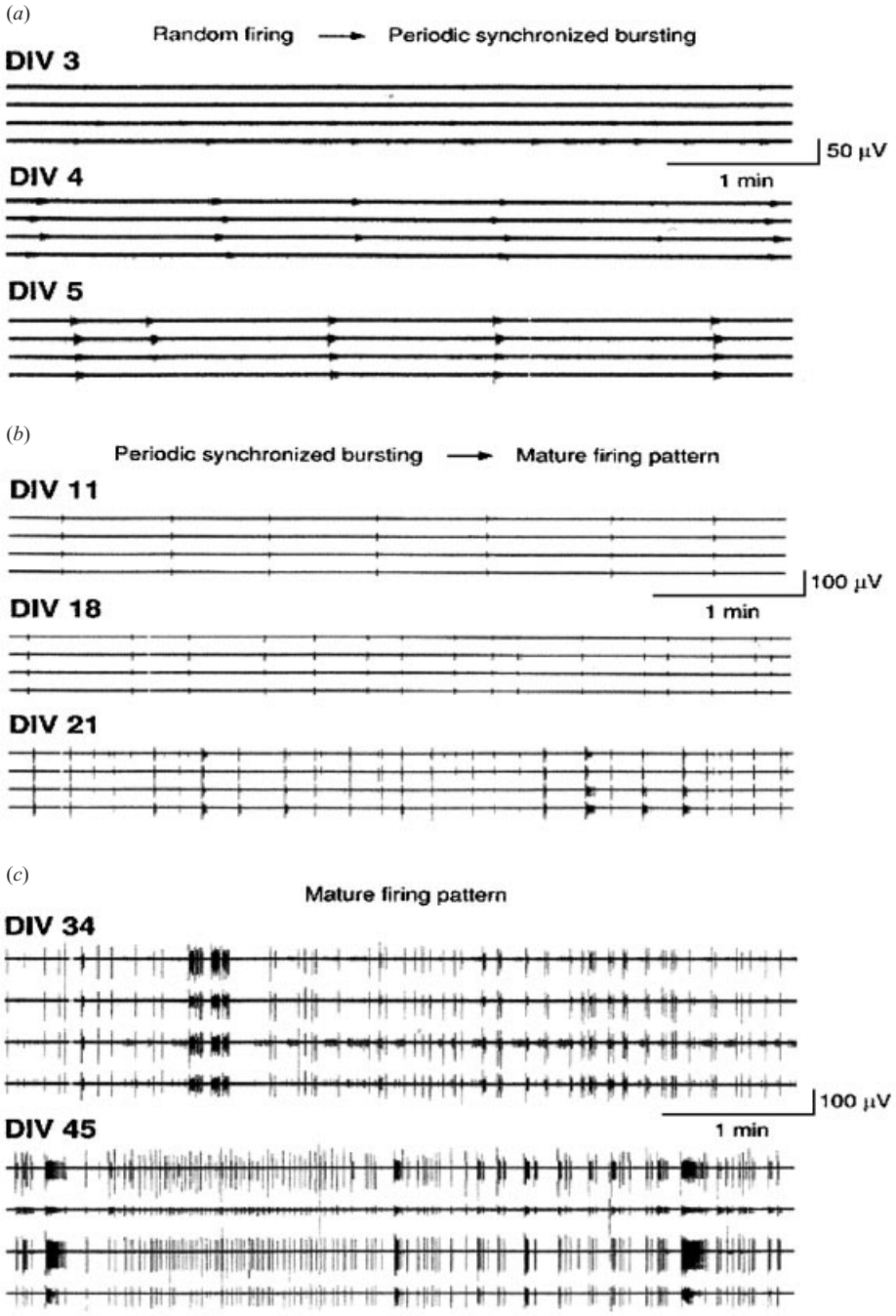

Fig. 3. Developmental changes in neuronal activity (from Kamioka *et al.* 1996); the story of four simultaneously recorded sites. (*a*) Random firing observed at div 3 and transformed into bursts separated by long intervals in the following day. The activity at div 4 indicates that the bursting does not initiate at the same site in a 'pacemaker' fashion. (*b*) Tightly synchronized activity appears at div 11 and changes to complicated bursting after div 21. (*c*) Mature firing pattern; complex high-order patterns of spike and bursts.

To estimate the stability of these spontaneously formed connections, we looked at the number of times significantly occurring activity pairs, defined in terms of an action potential that entails another action potential with a precise time delay between the two, appear in consecutive time frames. We found that the counts of $\sim 70\%$ of the pairs remain unchanged after 5 h of spontaneous activity (Shahaf & Marom, 2001). Furthermore, the stability is not sensitive to the time delay between pair elements (G. Shahaf & S. Marom, unpublished results), indicating the existence of mechanisms that support chains of precise propagation (Abeles, 1991).

Experiments using both electrical and dye coupling tests support the conclusion that neurons are not coupled through gap junctions (Nakanishi & Kukita, 1998). Coupling via astrocytes has also been excluded. Although astrocytes are extensively coupled, they are not coupled to adjacent neurons; and while they do respond electrically to synchronous neural activity, this response seems to be mediated by local neurotransmitter release from nearby neurons (Murphy *et al*. 1993; however see Haydon, 2001).

4. Consequences of spontaneous activity: pharmacological manipulations

An important aspect of studies that seek to relate brain function and behavior has been the search for principles that relate neural activity to development and modification of neural structure to function at psychologically relevant timescales. It is generally assumed that neural electrical activity can leave behind 'structural traces', thereby changing the functional properties of the system. The history of this idea goes back more then a century to William James (1890) who imagined 'traces' left by 'previous currents … deepening old paths or making new ones'. The theories of James, together with Thorndike's general Law of Effect (Thorndike, 1931) and its later rendition at the level of a single synaptic connection (Hebb, 1949), have served as the basis for an extensive and successful ongoing experimental program for more than 50 years. Indeed, experiments performed in large random cortical networks are found to be illuminating in this context (see below).

4.1 Structural consequences

Tetrodotoxin abolishes all electric activity in cortical networks. Picrotoxin is a GABAergic (inhibitory) synapse blocker that disinhibits network activity. A systematic series of studies, aimed at uncovering the structural consequences of pharmacological manipulation of activity was conducted using tetrodotoxin and picrotoxin (Van Huizen *et al*. 1985, 1987a, b; Van Huizen & Romijn, 1987; Ramakers *et al*. 1990; Corner & Ramakers, 1991, 1992). These studies show that inhibition of activity by tetrodotoxin prevents network maturation: neural outgrowth and branching is enhanced, and the large-scale elimination of synapses, that is a hallmark of network maturation during the fourth week *in vitro*, is prevented (Van Huizen *et al*. 1987b). In contrast, accelerated maturation occurs in networks that are chronically disinhibited by picrotoxin (Van Huizen *et al*. 1987a). In other words, reducing activity increases neural outgrowth and prevents normal synapse elimination, while enhanced activity has just the opposite effect (Van Huizen *et al*. 1987a). The conclusion is that network maturation requires spontaneous, possibly synchronous, electrical activity. If electrical activity is inhibited, the network keeps on 'exploring' by outgrowth and branching.

Van Huizen *et al*. (1987b) also found that persistent high synapse density maintained during long-term inhibition of network activity by tetrodotoxin, did not progress to control mature levels after removal of the toxin. They interpreted this finding to indicate the presence of a 'critical period' after which 'bioelectrically controlled elimination of redundant connections no longer occurs'.

4.2 Functional consequences

The functional consequences of pharmacological manipulation of spontaneous activity were studied by Ramakers and colleagues (Ramakers *et al*. 1990, 1991; Corner & Ramakers, 1991, 1992), and Kamioka *et al*. (1996). Corner & Ramakers (1992) considered the physiological consequences of chronic exposure of networks to either tetrodotoxin or picrotoxin. They reported that after removal of tetrodotoxin from the medium, the neurons fired spontaneously in stereotyped regular clusters of action potentials. This behavior is similar to firing patterns seen in early stages of network development and in the presence of picrotoxin. In contrast, picrotoxin-treated, chronically disinhibited networks that were returned to normal growth medium demonstrated a rich repertoire of firing patterns, even more extensive than age-matched controls. Based on these and related findings Corner & Ramakers (1992) suggest that regular neuronal activities 'accelerate the maturation of excitatory connections … [and are] crucial for the development of adequate inhibitory synaptic transmission'.

The dramatic changes in numbers of synapses with development raises a question addressed by Turrigiano and colleagues (Desai *et al*. 1999). How do neurons, given the limited range of possible firing rates, maintain responsiveness to both small and large synaptic inputs? Turrigiano and colleagues show that in response to changes in overall level of activity cortical neurons in cultured networks regulate intrinsic excitability to promote stability in firing. Thus, neurons deprived of spontaneous activity for two days increase their sensitivity to current input by selectively regulating voltage-dependent conductances.

Turrigiano *et al*. (1998) also suggest a mechanism to ensure that firing rates do not become saturated during developmental changes in synaptic input number and strength. They show that the cumulative strength of all of an individual neuron's synaptic inputs increases or decreases as a function of activity. Chronic block of activity results in increased post-synaptic current amplitude, while drug-induced activity enhancement leads to decreased post-synaptic current amplitude. Related to this finding, Van Huizen & Romijn (1987) report that the 'mean size of synaptic structures depends … on the functional state of the tissue at the moment of fixation, being larger in tetrodotoxin-silenced cultures than in bioelectrically active ones'. These observations are in agreement with results obtained in other neuronal *ex-vivo* preparations (Rao & Craig, 1997; O'Brien *et al*. 1998).

In a thoughtful study, Van Ooyen *et al*. (1995) provide a model of activity-dependent development of structure and function for cultured cortical networks. They show that many of the experimental observations considered above can be imitated by a surprisingly simple model in which neurons organize themselves into a network under the influence of their intrinsic activity. Their model includes excitatory and inhibitory neurons, and posits that neuritic field growth depends on individual levels of activity, and that connection between neurons occurs when fields overlap. The model successfully reconstructs a host of seemingly unrelated phenomena: the transition of the network from quiescence to active mode; the transient overproduction of synapses; enhanced outgrowth of neurites and prevention of

synapse elimination after chronic activity block; different growth kinetics for synapses in the shaft and in spines; delayed onset of pruning relative to onset of activity; advancement of synapse elimination after chronic block of inhibitory transmission; a critical period for synapse elimination, but not for synapse formation; and, size difference between the fields of inhibitory (small) and excitatory (large) neurons. The importance of this analysis (Van Ooyen *et al.* 1995) is in the emergence of all these phenomena without assuming predetermined, dedicated, time-scheduled mechanisms.

5. Effects of stimulation

5.1 Response to focal stimulation

When focal stimulation is applied to a network, for example, by passing current between two adjacent electrodes, or between an electrode and a distant reference point, the network responds by producing a propagating wave of activity (Fig. 4). The response is built of three clear components (Jimbo *et al.* 2000; Shahaf & Marom, 2001): an early component seen immediately following the stimulus, a refractory period, and a late component. The early component terminates within ~ 20 ms, and reflects direct activation of neurons by the stimulation. Accordingly, it is precise and reliable; that is, spikes in this early component appear with the same time delay relative to stimulus onset with sub-millisecond precision, and the probability of their appearance is relatively high (Fig. 5). The early component is followed by a period with low spike probability. While the cause for the transient quiescence is not completely understood, it is probably related to activity-dependence of processes involved in excitability (refractoriness) and synaptic transmission machinery (Tsodyks *et al.* 2000). Then comes the late component, a 'reverberating wave' that can last for hundreds of milliseconds, and looks very similar to the synchronized spontaneous clustered activity described earlier in the text (Section 3). The preciseness and robustness of spikes in this late component is dramatically decreased relative to the early component (Fig. 5), indicating the underlying participation of a multitude of alternate pathways.

5.2 Stimulation-induced changes in connectivity

The ability to drive the network to respond provides a means for studying changes in functional connectivity induced by external stimuli. A series of recent studies by Jimbo and colleagues (Jimbo *et al.* 1998, 1999; Maeda *et al.* 1998; Tateno & Jimbo, 1999), revealed interesting aspects of stimuli-induced, large-scale (ensemble) changes in connectivity. Maeda *et al.* (1998) used MEA recording and stimulation to observe modification of synchronized activity in cortical cultures in response to electrical stimulation. They found that the response of the network to a weak localized test stimulus could be potentiated by a transient strong stimulation. This is manifested as an increased probability of eliciting synchronized bursts by a weak stimulation, an increased frequency of spontaneous bursts and number of spikes per burst, and increased speed of burst propagation. These effects last for at least 20 min.

Recently, Jimbo *et al.* (1999) reported an intriguing result that concerns stimuli-induced changes in connectivity. First, as in the above-mentioned studies (Maeda *et al.* 1998; Tateno & Jimbo, 1999), they demonstrate that local tetanic stimulation induces long-lasting (> 30 min) changes in the responsiveness (number of spikes) of many neurons in the

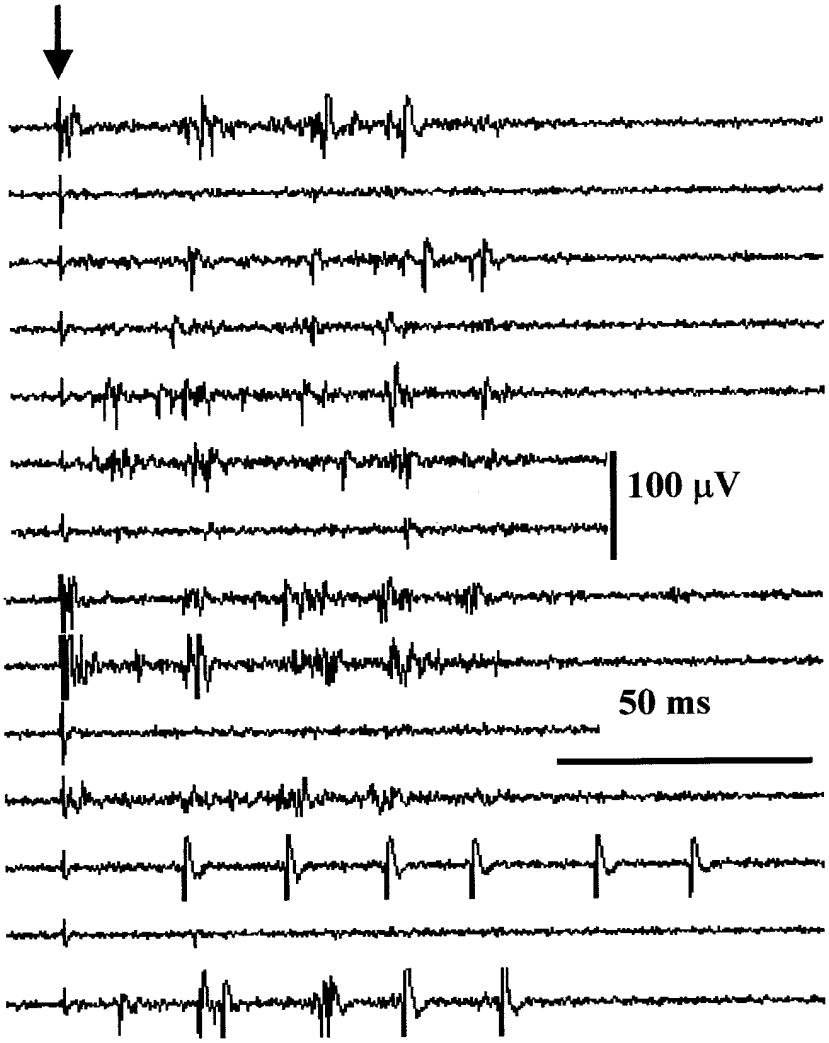

Fig. 4. A ±50 μA stimulus pulse that lasts 420 μs, delivered at the time shown by the arrow. Responses recorded simultaneously from 14 different sites in the network. (From Shahaf & Marom, 2001.)

network. What is so interesting is that for a given site of tetanic stimulation, activated neurons show similar changes in activity level (Fig. 6). That is, all the activated neurons either increase their responsiveness or decrease their responsiveness to the stimulus (Jimbo *et al.* 1999). The result is surprising in its simplicity, especially in light of the large number of neurons and synapses involved. Jimbo *et al.* (1999) conclude that 'potentiation or depression is pathway specific, not neuron-specific'. They found that the initial correlation between activity of the neurons dictated whether potentiation or depression occurred: tightly correlated pathways became potentiated when activated; loosely correlated pathways became depressed. The results of Jimbo *et al.* (1999) are a wonderful demonstration of the upgrading of simple monosynaptic rules (e.g. Markram *et al.* 1997) to neural ensembles, where they still appear to operate simply.

In another study, Tateno & Jimbo (1999) looked more closely at the temporal structure of spike trains evoked by stimuli, and concluded that changes (potentiation or inhibition)

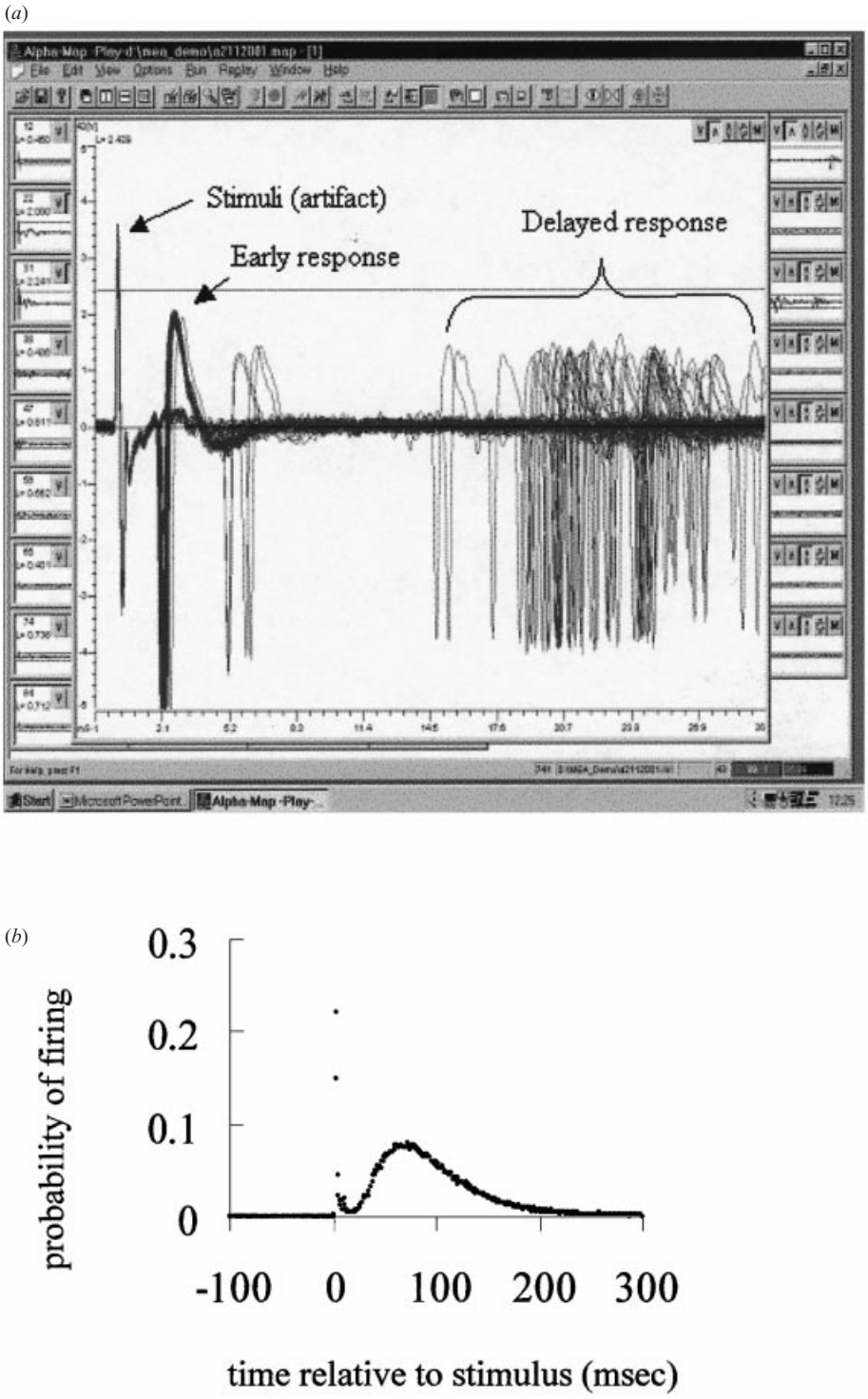

Fig. 5. (*a*) One hundred aligned responses (recorded repeatedly from one site), to focal current stimuli delivered in another site. Note the accuracy and reliability of the early response, probably representing direct activation by the stimulus, compared to the 'noisy' nature of the late response. (*b*) Peri-stimulus time histogram (PSTH). The first peak represents direct activation of neurons by the stimulus; the

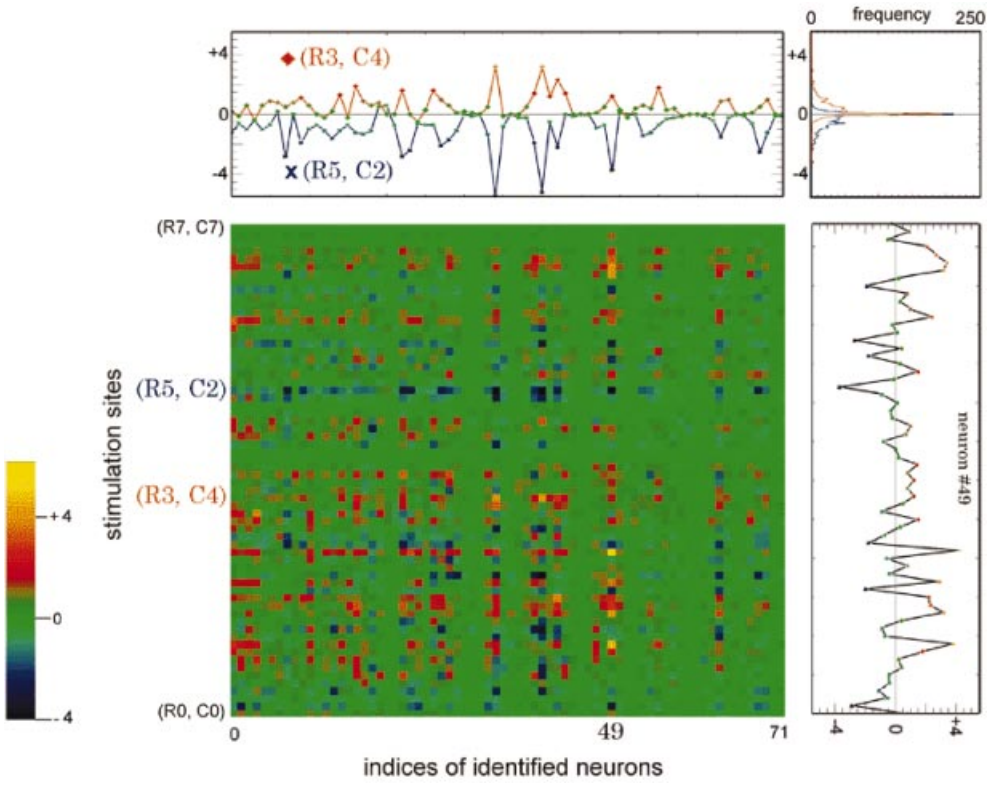

Fig. 6. Network activity changes induced by tetanus (from Jimbo *et al*. 1999). Stimulus pulse was applied through each of the 64 sites sequentially, and the total number of spike generated in each of 72 detected neurons was counted. This procedure was repeated 10 times before and after tetanus, and the average was computed for each case. The difference in the averages is displayed using a color map in a 72×64 matrix, with green indicating no change, and red–yellow and blue–black corresponding to increased and decreased activity, respectively (color scale on left). The profiles of two example responses to stimulation at sites (R3, C4) and (R5, C2) are plotted in the upper panel, showing that the population of neurons responds homogeneously with increased or decreased activity, respectively. The upper right inset shows the distribution of changes in two groups (*red* and *blue*) of 10 selected pathways, showing that almost all stimulus pathways fall into one of two one-sided distributions: increased activity or decreased activity. In contrast, the responses of a single cell (cell 49) to the 64 stimulus pathways show a mixture of enhancement and depression.

were often manifested in the fine structure of spike trains and correlations between activities that are not necessarily obvious when one looks at global statistics.

6. Embedding functionality in real neural networks

We have seen that *ex-vivo* developing large random networks of cortical neurons are extensively connected. Connections may be modified by externally generated focal stimuli as the networks develop but achieve a stable configuration. These properties (extensive

second peak represents a reverberating response. A series of 1200 stimuli is delivered through a pair of electrodes, and the responses in 10 randomly chosen active electrodes are recorded. The total number of responses (counted in 1 ms time bins) divided by 12000 is presented, time-locked to the stimulus event. (From Shahaf and Marom, 2001.)

connectivity, activity-dependence of connections, and stability) fulfill much of what is needed in order to embed functionality into a neural system; that is, to 'teach' the network to 'do things' defined in terms of spatio-temporal firing patterns. Potter and his group recently stated their intention to meet this challenge (DeMarse *et al*. 2000, 2001). They interface *ex-vivo* developing cortical networks grown on MEAs in a closed-loop with a computer. Spatio-temporal patterns of activity in the cultures are used to control the behavior of a simulated body ('Animat') that could move in a computer-generated virtual world. The effect of the interaction between Animat's movements and the environment are fed back into the MEA in the form of spatio-temporal electrical stimulus patterns, in real time. While at its preliminary phase, the hope of Potter and his group is to use this system to map changes in network activity patterns onto different Animat behaviors.

While feedback is an important component for learning, teaching the network to perform a task requires something more. In what follows, we wish to address an issue that every attempt to embed functionality in real neural networks must consider: the physiological realization of the behavioral concept of a 'reward', that is, the mechanism for selection of an 'appropriate' stimulus-response association over other 'inappropriate' ones.

6.1 Facing the physiological definition of 'reward': two classes of theories

In the study of learning, it is important to distinguish between two different questions: (1) What are the neural mechanisms that underlie the formation and modulation of associations? (2) What are the principles that underlie the selection of 'appropriate' associations over 'inappropriate' ones? The mechanisms underlying the formation and modulation of associations has been a topic of intense research, some of which has been described above in the context of *ex-vivo* developing cortical networks. Although much is yet to be discovered, there is a wealth of data at various levels of network organization regarding the function of ion channels, receptors, synapses, axonal/dendritic architecture, and higher order structures, that can, in principle, serve to explain activity-dependent modification of associations between stimuli and responses. Here we focus on the second question, the principles underlying the selection of an 'appropriate' association. Psychologists handle this issue by invoking the concept of reward, a cornerstone for many general theories of learning. We present here our thoughts and studies that seek to realize the behavioral concept of reward in neural systems in general, and *ex-vivo* developing neural networks in particular.

In recent years, considerable experimental and theoretical effort has been directed towards identification of neural structures and mechanisms that are responsible for rewarding adaptive behaviors (e.g. Schultz, 1998; Kalivas & Nakamura, 1999; Spanagel & Weiss, 1999; Gisiger *et al*. 2000; Schultz & Dickinson, 2000). Underlying these endeavors is the notion of mapping the behavioral concept of reward to a neural entity that strengthens a subset of synapses based on past performance of the neural system. Another approach to the concept of reward is related to general learning theories that were advocated by behaviorists such as Clark Hull and Edwin R. Guthrie over 50 years ago (Hull, 1943; Guthrie, 1946). These psychological theories, which we collectively refer to as Stimulus Regulation Principle, stress the effect of the reward on the driving stimulus. Specifically, the reward acts to reduce the driving stimulus, precluding the acquisition of any new stimulus–response (SR) associations. No separate neural rewarding entity is postulated or needed for shaping behavior.

Consider, for instance, a thirsty monkey undergoing a training procedure. In order to be rewarded by juice, the monkey is required to touch a defined area within a grid that is projected on a touch screen. Initially, the monkey explores, performing many 'wrong' touches. As long as this is the situation, the monkey is not rewarded by juice. Once the monkey performs as required, the rewarding juice is given. Now, according to the 'reward as a strengthening entity' class of theories, the juice causes some uniquely defined neural entity to affect a subset of synapses in the brain thus increasing the chance of a similar behavior when the same circumstances are encountered in the future. According to the Stimulus Regulation Principle, the reward abolishes the stimulus (thirst) and therefore the exploratory behavior of the monkey ceases; the last associations that were made between thirst and the experimental environment are left unaltered. No neural reward entity is required and no active strengthening of synapses occurs after performance of the appropriate movement.

While not mutually exclusive, the two classes of theories, in relation to reward, imply different cellular and synaptic mechanisms. The 'reward as a strengthening entity' theories imply that there is a substance (neuromodulator) released by some neural reward entity that is capable of modulating the efficacy of a given synapse after it has been activated. Accordingly, recently active synapses should be in some fashion selectively available for modulation by the rewarding neuromodulator. In contrast, the Stimulus Regulation Principle advocates that neural connectivity changes are due to the persistence of a driving stimulus and proceed under the direction of activity-dependent rules: an exploration process. If the output of the system changes the driving stimulus by its removal, there is no longer a drive for further connectivity change and the system is 'frozen' in its last conformation; no specific cellular and synaptic reward mechanism needs be postulated. In that respect, the notion of reward under the Stimulus Regulation Principle is more primitive, whereas the 'reward as a strengthening entity' might be considered an evolved, or unique version.

6.2 Closing the loop

Timed exposure of an *ex-vivo* cortical network to a neuromodulator, such as dopamine, may be a feasible means for selection of 'appropriate' responses to a stimuli; although we are not aware of reported attempts in this direction, the 'reward as a strengthening entity' class of theories calls for such an approach. We find it attractive (and relatively easy) to test the more primitive realization of the reward concept, the Stimulus Regulation Principle, in *ex-vivo* networks. We have asked: can one drive a network by repeated stimulation to explore the space of possible connectivity, and, then, simply by removing the stimuli, stably maintain a particular predefined configuration? The answer to that enquiry has been 'yes' (Shahaf & Marom, 2001). Thus, we perform closed-loop experiments in which cultured cortical networks interact with a computer-controlled environment and find that eliminating a driving stimulus when a desired predefined response occurs is sufficient for selective learning and memorizing of arbitrarily chosen tasks defined in terms of neuronal firing patterns.

This realization of the principle is straightforward: Each experiment starts by stimulating a network through a pair of electrodes and observing the responsiveness of all other (i.e. the non-stimulated) electrodes. An electrode that responds 50 ($\pm$10) ms after a stimulus with an average response to stimulus (R/S) ratio of 1/10 or less is selected. In other words, before training, it takes at least 10 stimuli in order to evoke one action potential in the selected

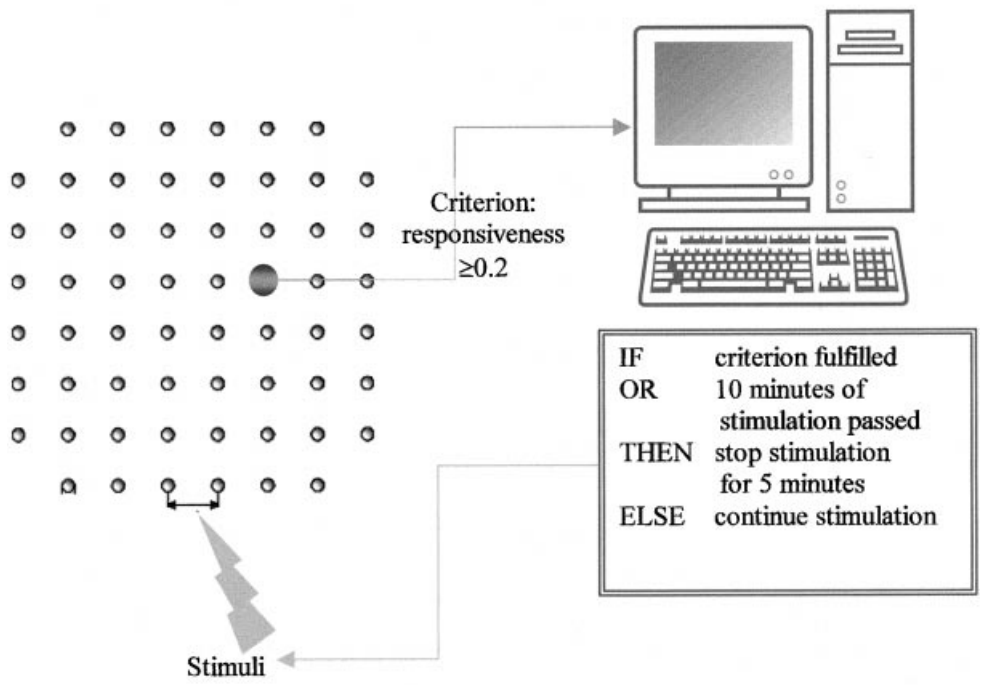

Fig. 7. Realization of the Stimulus Regulation Principle in a MEA system.

electrode within the designated time frame of 50 (± 10) ms after a stimulus. During the training phase, the learning task is to increase the R/S ratio of the selected electrode to 2/10 or greater in the designated time window of 50 (± 10) ms after a stimulus. The network is continuously stimulated at a low constant frequency (typically 1 to $\frac{1}{3}$ Hz). A computer constantly monitors the R/S ratio of the selected electrode, and once the criterion of R/S $\geqslant 2/10$ is fulfilled the computer automatically stops the stimulation. After 5 min, the network is stimulated again (at the same low frequency) until the criterion R/S $\geqslant 2/10$ in the same selected electrode is fulfilled again. This stimulation cycle, which is composed of 5 min without stimulation followed by low-frequency stimulation until the R/S criterion in the selected electrode is fulfilled, is repeated many times. As a rule, if the criterion is not fulfilled within 10 min of stimulation, the stimulation is stopped for 5 min. Hence, the maximal duration of one stimulation cycle is 15 min (i.e. 10 min of stimulation and 5 min of quiescence). The latency for reaching the predetermined criterion (referred to as response time) in each stimulation cycle is used as a measure for the strength of SR connection, and may be viewed as a measure of the degree to which the task was learned. The closed-loop design is schematized in Fig. 7.

An example of a result of this learning procedure is shown in Fig. 8. It includes the responses of a selected electrode before (left column) and after (right column) training. The 11 traces of each panel show the responses to 11 consecutive stimulation pulses. Note that the activity within the 50 (± 10) ms window (depicted) is markedly increased after the training phase.

In order to ensure selectivity of the R/S ratio increase in the selected electrode, we amend the procedure by concomitantly monitoring a second electrode in the array, which serves as

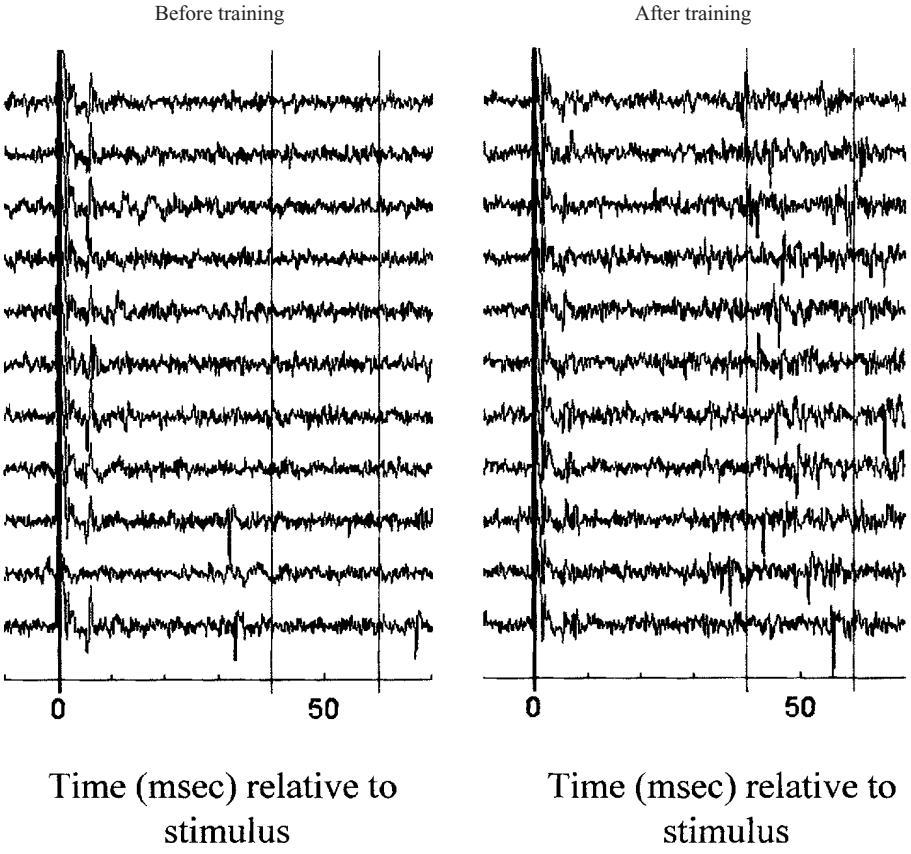

Fig. 8. Example of learning in a cultured network of cortical neurons. Each trace within a panel shows recordings obtained 10 ms before the stimulus to 70 ms after the stimulus, before (left) and after (right) the training procedure. Note that the responsiveness of the electrode within the designated time window increased appreciably. (From Shahaf & Marom, 2001.)

a measure for global network responsiveness. The idea is to ensure selectivity by removing the stimulus only if the fulfillment of the R/S criterion in the selected electrode is not accompanied by similar increase in the responsiveness of the second electrode. Figure 9 (first eight columns) summarizes the selective learning data. Changes in R/S ratio of the selected electrodes (filled circles) and 10 control electrodes (stars) are depicted for eight experiments from eight different networks. For each network, the 10 control electrodes were chosen by analyzing the data, after the completion of the experiment, based on their similarity to the R/S ratio of the selected electrode before the training. The change, depicted by f, is defined as the ratio between the responsiveness before training, and responsiveness after training, normalized to the change in R/S ratio of the selected electrode. Thus, $f = 1$ means a change in R/S ratio that is identical to the change measured in the selected electrode. $f > 1$ and $f < 1$ mean that the relevant response of a control electrode increased or decreased, respectively, relative to the selected electrode. Note that the strengthening in the R/S ratio of the selected electrode is generally higher relative to the responsiveness change in the control electrodes. Also note that since the selected and control electrodes demonstrate low responsiveness before the training, a bias towards an average increase of R/S ratio during training is introduced. The reported effect is selective inasmuch as the increase in R/S ratio of the

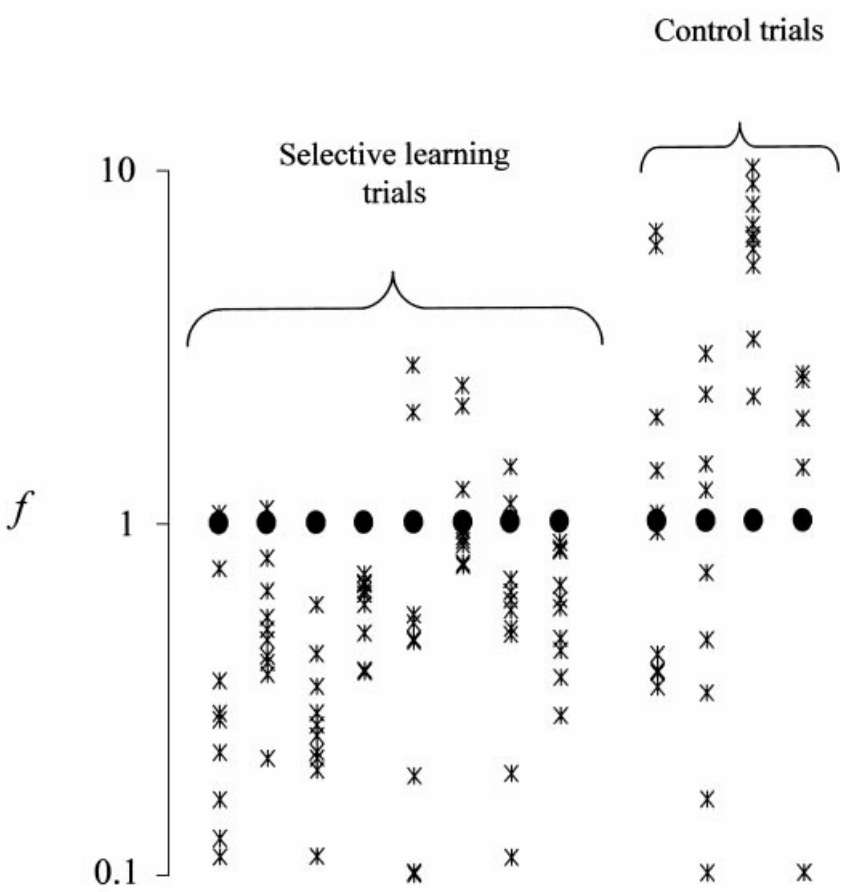

Fig. 9. Changes in the R/S ratio, depicted by *f*, of the selected electrode (filled circles) and 10 control electrodes (stars) for eight learning experiments (left) and four control experiments (right). Control data is from protocols in which each cycle consisted of 10 min of stimulation and 5 min of quiescence, regardless of response (see main text). *f* is normalized to the R/S change of the selected electrode. (From Shahaf & Marom, 2001.)

selected electrode is more than the average increase for the control electrodes. The probability of the selected electrode to be ranked fourth or higher (out of 11), as is the case in the eight experiments shown, is $< (4/11)^8$.

Figure 10 shows three learning curves [(i), (ii), (iii)], differing in learning kinetics. In these curves, the response time (i.e. time required for the selected electrode to fulfill the R/S $\geqslant 2/10$ criterion) is plotted against the number of stimulation cycles. (Recall that each stimulation cycle is composed of 5 min without stimulation followed by low-frequency stimulation until R/S $\geqslant 2/10$ criterion is fulfilled.) The figure demonstrates that the time required to instruct a network to perform the task varies, reflecting the arbitrariness of the procedure by which the tasks are chosen and the idiosyncrasies of the networks. This variability faithfully represents the spectrum of learning curves observed in our networks. Figure 10(iv) shows the average of 16 learning curves. Each point depicts the average time (in seconds) to accomplish the task in one cycle within a series of cycles.

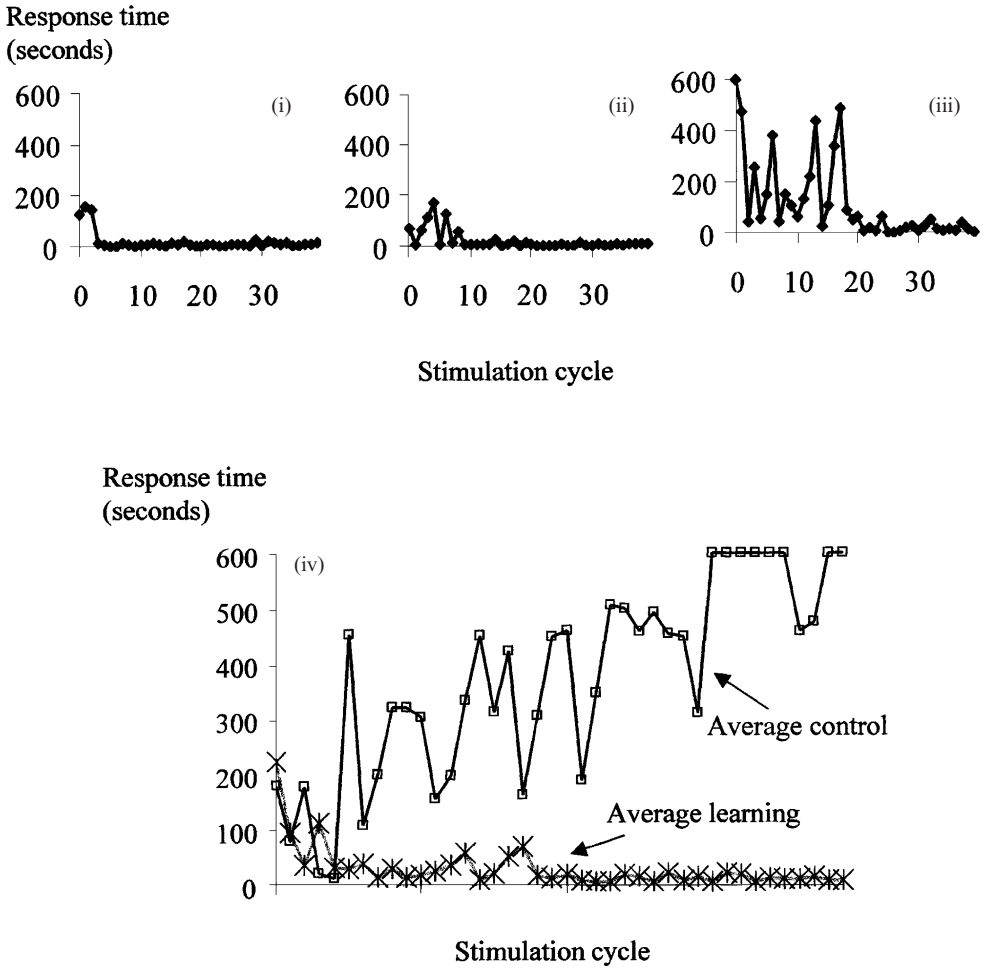

Fig. 10. (Modified, from Shahof & Marom, 2001.) Three learning curves [(i), (ii), (iii)], differing in their learning kinetics. The response time (i.e. the duration of stimuli series until criterion is fulfilled) is plotted against the number of stimulation cycles. Panel (iv) shows the averaged learning curve (stars; $n = 16$) and control curve (squares; $n = 4$). Each point depicts the average time (s) to accomplish the task in one cycle within a series of cycles.

The notion that 'driving' stimulus removal is necessary for selecting 'appropriate' network responses, is further supported by a control experiment that is also presented in Fig. 10: in this experiment, the fulfillment of the R/S criterion in the selected electrode did not lead to stimulus removal (i.e. the attainment of the criterion was ignored). The stimulation was delivered for 10 min interrupted by 5 min of quiescence, regardless of the responses recorded from the selected electrode. Figure 9 (last four columns) shows the change in the R/S ratio of the selected and control electrodes in four such experiments. Without exception, learning does not occur if the 'appropriate' response does not remove the stimulus. Moreover, the response time (i.e. the time required for first appearance of R/S $\geqslant 2/10$ within each stimulation cycle) plotted against the stimulation cycle number shows large fluctuations. Figure 10(iv) shows the averaged control curve ($n = 4$). Taken together with the average learning curve, the graphs of Fig. 10(iv) provide an indication for the robustness of the

phenomena: when the loop is closed and the response is allowed to remove the stimulus, learning is obtained; when the loop is open, i.e. the computer is instructed not to remove the stimulus when the selected electrode criterion is fulfilled, the curves 'explore away'.

The neural substrates for learning in these networks are synchronous bursts of activity evoked by driving stimuli. As shown above (Fig. 5), the timing at which spikes appear within an evoked burst are precise and robust at first, but become compromised at the late phase of the burst. We suggest that the 'noisy' nature of spike timing in the later, influences the synchronization between neurons to produce changes in the efficacy of synapses and serves as a substrate for functional changes in the network. Regardless of the exact mechanism, such experiments show that conditions sufficient for learning by a selection process can be realized without the involvement of a neural rewarding entity and are embodied in large random networks of neurons developed and maintained *ex vivo*. From the theoretical point of view, such results convey an important message, supported by behavioral studies and psychological theories advocated over 50 years ago by psychologists such as Hull and Guthrie: it is not necessary to assume a separate mechanism for the biological realization of a reward and the process of exploration for solutions; the behavioral concept of reward might well be considered as a change in (or removal of) the drive underlying the exploration of possible modes of response. Stimulus removal is an intentionless natural principle to allow adaptation to a rich and unrestricted environment.

7. Concluding remarks

Here is a dream: imagine thousands of cortical neurons on a plate, developing *ex vivo* to produce a large, highly connected network that survives for months on the experimental setup. The network continuously and seamlessly interacts with the world via electronics and computers that allow timed local electrical stimulation and application of neuromodulators. Using these timed stimuli and drug applications we attempt to teach the network to respond to defined electrical stimuli with defined sets of action potentials distributed in time and space. We then interrogate the system, asking: what is required to teach the network to 'do things'? How many 'things' can the network learn to do? At what level of complexity? What does one gain by increasing the number of cells in the network? The number of connections? The types of connections? Can the 'knowledge' of such network be represented? Can one identify rules of change and development? Can one use such rules, together with the representation of present 'knowledge', in order to drive the network from a present 'undesirable' state to another 'desirable' one at will? Is there a critical period for teaching the network to do 'things'? How does 'forgetting' occur? Can one use methods of training, or drugs, to enhance performance, to make the network learn faster, and to delay forgetting? What does one gain by separating the network to morphologically distinct modules, by restricting its morphology to well-defined structures, in short, by introducing anatomy?

This review suggests that we are not *that* far from realizing the dream: *ex-vivo* developing large random networks can survive for many months. Bio-electronic interfaces exist, allowing real-time bi-directional interaction between the network and the world (e.g. DeMarse *et al.* 2001). A basic protocol for teaching the networks to express desired responses is available (Shahaf & Marom, 2001) and, most importantly, the ability to characterize rules of changes in connectivity, at the level of neural ensembles, has been demonstrated (e.g. Jimbo *et al.* 1999).

However, the most interesting part of the work is still ahead. Our conviction is that, at this point in time, efforts to understand the action of general neural factors underlying behavior should be addressed towards: (1) establishing a useful characterization of network 'state' at the ensemble level; (2) extending the characterization of activity-dependent rules of connectivity change at the level of neural *ensembles*; and, (3) harnessing such ensemble-level rules and characterization of state, together with various physiological realizations of the reward concept, endeavor to teach networks to perform more complex tasks. Thereafter we may be ready to address questions that relate to complex features found in native neural systems. These questions are of a general nature, and they are most relevant to the entire brain-behavior endeavor. Answering these questions should be the business of biophysicists, and not just neuroscientists and psychologists, as biophysics is concerned with describing general principles of organization in the biological world, and understanding the universals underlying formation and conservation of neural activity groups in networks is such a challenge. We hope that this review will encourage biophysicists to direct their attention and resources to this subject.

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