Can patterned external input carve out patterned functional neuronal networks in vitro?

Daniel Wagenaar, May 2002

Introduction

Many sensory areas in the brain have long been known to be patterned by inputs from the senses. That is, while the gross morphology of these areas forms irrespective of input, the development of fine structure requires sensory input. A famous example is the dependence of the formation of ocular dominance columns on binocular viewing in cats (Wiesel and Hubel 1963; Wiesel 1982). It was suggested that differences between correlations in intra-ocular and inter-ocular firing patterns are responsible, and indeed Stryker and Strickland (1984) showed that artificially induced correlations between binocular inputs disrupt column formation. The development of barrels in rat somatosensory cortex similarly depends critically on experience (Levitt et al. 1997; Buonomano and Merzenich 1998; Stern et al. 2001). Recent research suggests that experience dependent patterning of barrel cortex is mediated directly through electrical activity: barrels form only when thalamico-cortical signalling pathways are intact. Hannan et al. (2001) show that a knockout mouse with a signalling deficit downstream of cortical mGluRs exhibits drastic reduction in barrel patterning, despite unchanged innervation by thalamic projections, indicating that synaptic activity is paramount.

Although this initial patterning can only take place during a developmentally critical period, forms of large scale experience-dependent cortical plasticity can occur even in adults, as witnessed by the remapping of somatosensory cortex after loss of body parts. Merzenich et al. (1984) showed that the area in owl monkey somatosensory cortex normally representing the middle finger, is overtaken within several months after amputation by neighboring areas representing the second and fourth finger. Similarly, in human amputees, hyperacuity has been observed for touch localization on the stump of the amputated limb (Haber 1955), indicating that the cortical real-estate previously occupied by representation of the now lost limb has been overtaken by its neighbors. On a more positive note, extensive training of particular bodily function extends cortical representations in healthy animals (Wang et al. 1995; Siucinska and Kossut 1996; Xerri et al. 1999) and humans (e.g. Pantev and Lutkenhoner (2000)).

The study of plasticity in vitro so far can be broadly divided in two categories. The first type of investigation focuses in detail on interactions between a very small number of cells. Repeated paired-pulse stimulation with inter-pulse intervals in the tens of ms range has been shown to reliably elicit plasticity in neocortical slice (Markram et al. 1997; Egger et al. 1999). In dissociated hippocampal culture, the direction and amount of changes in synaptic strength was found to depend on precise timing between pre- and post-synaptic induced action potentials (Bi and Poo 1998). The second type probes plasticity at a larger scale: at network level. In cultures very similar to the ones I will use, various forms of tetanic stimulation have been shown to induce plasticity within minutes (Maeda et al. 1998; Jimbo et al. 1998; 1999). Changes have been shown to last several hours, but there is no data indicating whether they decay on timescales longer than that.

Proposal

I propose to study whether input dependent developmental and adult connectional plasticity are innate to cortical neuronal networks, by studying them in dissociated culture, separated from thalamus and distant cortical areas. This separation will also isolate the formation of functional networks from proto-patterning in earlier developmental stages in vivo. I will study the time course of pattern formation, and determine whether young and mature cultures react differently to potentially structure-inducing stimuli.

Specific Aims

I will use the following questions to guide my research project:

1. To what extent can cortical cultures of a few thousand neurons form functionally separated sub-networks, analogous to columns or barrels in vivo, solely in response to patterned stimulation? Are these sub-networks spatially delineated as well as synaptically defined?

2. Is there a critical period for sub-network formation, or can it be initiated at any stage of development? Can an established network be modified or overridden by subsequent re-patterning? What are the timescales for formation and decay of sub-networks?

I will study these questions by performing the following steps. Each step will be explained in more detail in subsequent sections.

- *Suppression of global bursts*
  Such bursts form a large part of the spontaneous activity of cortical cultures, and hamper controlled induction of plasticity. Several methods for bringing them under control are outlined under *Quieting the cultures*.

- *Determining parameters for induction of plasticity*
  If some form of organized plasticity can be induced using protocols lasting hours or days, this will help choosing initial parameters for the long-term experiments in a much reduced time-frame. For more details, see *Rapid induction of plasticity*.

- *Carving out functional sub-networks*
  The experiments that address Specific Aim 1 are described in the sections *Long term stimulation paradigm* and *Diagnostics*.

- *Testing for critical periods*
  Specific Aim 2 will be addressed by the methods described under *Time course and critical periods*.

Before going into details, let me give a brief overview of general methodology and of the results obtained so far.

General methods

Neurons and glia will be dissociated from embryonal (E18) rat cortex and plated on dishes with embedded micro-electrodes, in small colonies of 3 mm diameter consisting of about 10,000 neurons.

Cultures will be maintained for up to 2 months in an incubator at 35°C, 5% CO_2, 9% O_2 and ambient humidity, with protective teflon membranes covering the culture dishes to prevent evaporation and infection (Potter and DeMarse 2001). Culture medium will be DMEM with 10% horse serum (Jimbo et al. 1999), and will be completely replaced once weekly.

Stimulation will take place through the surface electrodes, using a custom built stimulus generation circuit that can deliver voltage controlled biphasic stimuli with 2 μs timing precision to any number of electrodes. Stimulation will take place with the culture dish mounted on a multichannel pre-amplifier, inside the incubator[1]. Stimulation will be continuous from the first day after plating until at most four weeks in vitro. This should be a sufficient time period, since the above mentioned forms of experience dependent development take place within that time-frame in vivo. Cortical cultures form interconnections and exhibit spontaneous activity after a few days, and reach a steady state after about one month. Several cultures will be exposed to the experimental stimulation paradigm in parallel.

Extracellular recordings will be made through 59 electrodes, in a rectangular configuration with 200 μm spacing and an area of 1.4×1.4 mm. Data acquired at 25 kHz and 14 bits resolution will be streamed to disk for off-line analysis in most experiments, although on-line spike detection may be used to reduce disk space requirements for very long experiments. The software used in these experiments (Wagenaar et al. 2001) also allows real-time visualization of a culture's activity, an essential check on the experiment's progress.

Stimulation artifacts will be removed from the recording using a local regression filter, and spikes will be extracted based on threshold crossing and waveform validation (Wagenaar and Potter 2002).

For details on specific experiments, see *Research plan* below.

Previous work

The culture methods described above have been used in our lab to maintain cultures for well over a year[2]. In a typical culture, over 90% of electrodes record single-unit activity. Stimulation using several electrodes is routine.

I have found that in cultures reared in the absence of stimulation, neuronal connections appear to pervade the entire culture without preference for connecting locally, or to any particularly defined area. This has been shown both by observing spontaneous activity, where studying cross-correlations between firing patterns on pairs of electrodes revealed culture-wide connectivity (Dudik 2001), and in recorded responses to local stimulation, which span the entire electrode array. Departures from global, undifferentiated, connectivity caused by patterned stimulation will be systematically investigated as detailed below.

I have established that biphasic voltage pulses (anodic first) of a few hundred millivolts amplitude and less than a millisecond total width delivered to any single electrode are sufficient to elicit responses on a substantial fraction of recording electrodes.

[1] This is feasible, because the teflon membrane prevents evaporation of the media in our culture dishes, allowing us to maintain the incubator at electronics-friendly ambient humidity levels.

[2] We believe we may have the world-record by maintaining a culture healthy and active for over 23 months in vitro (Potter, DeMarse, personal communication).

Research plan

Quieting the cultures

If patterned stimulation is to have a significant effect on network development, it is essential that other causes of plasticity be brought under control, lest they should mask the effect under investigation. An important concern in this context is the impact of culture-wide synchronized bursts lasting several hundred milliseconds, which are the dominant form of spontaneous activity in developing neocortical cultures in the absence of stimulation. These bursts resemble epileptiform activity, and are considered unnatural. They are likely to induce background plasticity or reset any induced changes, since essentially the whole culture fires many closely timed action potentials during bursts, and they occur frequently (every 2–10 s). Indeed, Maeda et al. (1998) have shown that culture-wide bursts lead to changes in subsequent responses to electrical stimulation.

I will atempt to reduce global bursting in several ways. Firstly, I will test the effect of using elevated extracellular Mg^{2+} concentrations, which partially blocks NMDA channels, thus increasing the threshold for bursting. Maeda et al. (1995; 1998) have shown that this way of burst suppression does not hamper the induction of plasticity. A similar approach is to lower the extracellular Ca^{2+} concentration, which also limits NMDA activity. Finally, I will test whether my (high frequency) stimulation paradigm in itself reduces the predominance of bursting, following up on the discovery by Latham et al. (2000) that whether cultures burst or fire tonically at low rates depends on the fraction of endogenously active cells; the hypothesis being that tonic stimulation can play the same role as a population of endogenously firing cells.

Long term stimulation paradigm

I will attempt to induce the formation of sub-networks by choosing two sets of four electrodes in opposite corners of the array, and applying patterned stimulation to each group independently. In the following, I will refer to the electrodes used for such patterned stimulation as ‘patterning electrodes’. Four electrodes in each of the other two corners of the array will be designated controls.

The details of the stimulation patterns will be modified based on the results obtained in the short term experiments described below, but one plausible starting point is the following pattern. Every 10 ms, a stimulus will be delivered to one electrode, and the electrodes within a group will be stimulated sequentially, resulting in a per-site frequency of 25 Hz, comparable to physiological firing rates. One hundred pulses will thus be delivered to one group within a second, after which stimulation will switch to the other group. After another second, the first group will again be targeted. Thus, precisely timed correlated activity is induced in axons responding to electrodes within each group, but not between groups. This, I propose, may coerce their postsynaptic targets to form two distinct sub-networks.

I will start with small, dense cultures that only just cover the electrode array, because I expect that small cultures in which many cells are directly fired by my stimuli will be most influenceable by this stimulation paradigm. However, this is an open question, and I will investigate effects of culture size explicitly. It has been suggested that even cultures of only a thousand cells are large enough to consist of several function units (Gross and Kowalski 1999).

Diagnostics

Stimulation will be interrupted once or twice a day to open a one hour window for probing connectivity, as well as for necessary manipulations such as replacing medium and visual inspection

of culture health. Interrupting the stimulation paradigm for probing is necessary, since otherwise only a few milliseconds of response could be recorded before the next stimulus arrives. Recording responses for longer intervals is important, because stimuli elicit precisely timed responses in downstream neurons over a time course of tens of milliseconds, and the dishwide activity remains elevated for hundreds of milliseconds post stimulus.

I will monitor the formation of patterned functional networks through several measures. Before listing these, it should be pointed out that splitting into sub-networks can occur in two ways: geometrically, and non-geometrically. By geometric splitting I mean that cells in one corner of the culture form strong interconnections among themselves, as do cells in the other corner, but the cells form weaker long distance connections. Non-geometric splitting means that cells do form two ensembles with stronger internal than external connections, but these ensembles are not spatially separated.

- *Spatial spread*
  I will compare the spatial spread of responses to stimuli on patterning electrodes with responses to stimuli on control electrodes, as well as responses in non-stimulated control cultures. The response can be divided into three generations of spikes: first, action potentials recorded from axons that are directly stimulated; second, action potentials from neurons one or a few synapses downstream, which respond with reliabilities up to 50% and temporal jitter between 1 and 5 ms; and third, spikes from cells with elevated firing rate after stimulation, but no precise timing. Considering each of these three generations of responses, I will investigate whether patterned stimulation selectively enhances local responses over long-distance responses. Additionally, I will test whether stimulation preferentially elicits responses on other patterning electrodes in the same group. These measures only probe geometric splitting.

- *Similarity of response patterns*
  Consider the array-wide responses to stimuli on one particular patterning electrode. Are these more simular to the responses to stimuli on other patterning electrodes in the same group than in the other group? I will test this hypothesis by comparing the sizes and relative latencies of peaks observed in the post-stimulus firing histograms of individual electrode channels. Array-wide responses to single electrode stimulation include spikes with 1–2 ms timing precision and moderate reliability, which form clearly distinguishable peaks in such histograms. Thus, I will establish whether populations of neurons emerge that responds in one characteristic way to stimuli from any of the electrodes in one group, but not, or differently, to stimuli from the other group of electrodes. In this way, non-geometric splitting is probed as well. The same techniques will allow me to determine whether responses to stimuli within a group of patterning electrodes are more stereotypical than within a group of control electrodes.

- *Clustering of responses elicited from other electrodes*
  I will test whether responses to stimuli on electrodes neighboring the patterning electrodes resemble the response to the patterning electrode more closely than responses to stimulation farther away. This is another test for geometric separation of sub-networks. I will study whether the responses to stimulation on other electrodes can be classified into two clusters, one for each group of patterning electrodes. This would indicate to what extent the entire culture is patterned by the experience.

– *Cross-correlograms for probing connectivity*
I will use cross-correlation analysis on spontaneous activity as well as on evoked activity (Aertsen et al. 1989) to test the hypothesis that the culture divides into two separate functional networks, spatially separated or not. This would show by the emergence of a subdivision of electrodes into two sets, with strong connections between the cells recorded on electrodes within one set, but weaker connections between cells recorded on electrodes in different sets.

Rapid induction of plasticity

Knowing a class of stimuli that rapidly induce plasticity will be valuable when studying the effects of long term stimulation. Before embarking on a series of month-long experiments, I will optimize the stimulation parameters in hour-long and day-long experiments. Stimulus patterns that induce plasticity within an hour, even if they do not induce strong regionalization will be a starting point for finding stimulus patterns that induce regionalization in longer experiments. If stimuli can be found that induce some regionalization within a day, they will be the starting point for the exploratory phase of the long-term experiments. Parameters that will be tuned include strength of individual stimuli, and the timing between them. If necessary, I will increase the number of patterning electrodes. I may also try to promote plasticity using acetylcholine receptor agonists (Patil et al. 1998).

Timecourse and critical periods

Within the first month in vitro, I will investigate whether there exists a shorter window during which stimulation has the most profound impact on the formation of connections.

Additionally, I will take mature cultures (one month or older) that have been reared without patterning stimulation, as well as mature cultures that have been exposed to patterning stimulation. Stimulation as described above will be applied to these cultures for one week, except that I will pick patterning electrodes in the other two corners. During this week the same questions of development will be studied as before. Thus I will be able to quantify to what extent mature cultures remain plastic, and whether initial patterning can be overridden. If plasticity is found to be significantly reduced in mature cultures, as seems likely, I will establish the time course of this reduction.

Finally, I will establish whether patterning influences are permanent, or whether they decay over time. If they do decay, I will determine the timecourse of decay. To this end, I will maintain cultures for a further four weeks after initial patterning, without constant stimulation in the second phase of the experiment. I will use the same diagnostics to test for the disappearance of patterns that were described for monitoring the development of patterns.

Follow up

If functional connections indeed become spatially organized as a result of patterned stimulation, it will be interesting to study whether this has an anatomical basis. There are at least two possible ways in which a culture can split into functional units: synapses within the units can be strengthened relative to synapses between the units, or processes between the units can be eliminated. These two possibilities can be distinguished by staining a subset of neurons and determining whether their processes span the entire culture, or just the functional sub-network. The techniques will require further exploration, as there are several possibilities: a subset of neurons can be stained with DiI before plating, which will allow tracing their morphology for up to two weeks. Alternatively,

dye injection can be used at any age to stain a small subset of neurons, but it would be much more attractive to study the time-course of pattern formation using non-terminal imaging methods. Preliminary experiments to mix a small fraction of GFP-expressing neurons with ordinary neurons are underway (Potter, personal communication).

An attractive option is to determine where neurons that are stimulated through the patterning electrodes project. One could use an endocytosis marker such as FM1-43 to label pre-synaptic terminals involved in each stimulation pathway. In a first step, excitatory synapses would be blocked by AP5 and CNQX, so that only pre-synaptic terminals on directly stimulated axons are stained. After washing out the synaptic blockers, a second application of the fluorescent marker and a further set of stimuli also stains downstream synapses. Only one stimulus pathway can be investigated at a time, but it may be possible to approach a second one the next day, depending on the toxicity and permanence of the dye, which will need to be determined.

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