Sensitivity of cortical cells to precisely timed paired pulse stimulation in culture

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Introduction

Recurring firing patterns with high temporal precision involving two or more neurons have been observed in many neural systems, including cortex. Precisely timed spike trains, especially if synchronized between several neurons, carry much more information than can be transmitted through a simple rate code (Rieke et al. 1997). The question remains whether animals actually exploit this source of information: Do neurons respond specifically to correlated inputs (Alonso et al. 1996; Roy and Alloway 2001)? After all, it is hardly surprising that correlations in firing patterns occur in cells that have partially overlapping receptive fields; if no downstream neuron responds specifically to correlations, they are mere epiphenomena. Despite years of theoretical argumentation and observational experiments, the question whether millisecond scale timing is used broadly[1] in cortical coding remains unresolved, with increasing polarization between camps.

Proponents have argued that precisely timed activity does occur in many cortical areas including visual cortex (Brecht et al. 1998) and pre-motor cortex (Prut et al. 1998), and why wouldn't these correlations be used? Correlations between firing patterns in pairs of cells in LGN can be used by experimenters to reconstruct more information about visual stimuli than can be obtained from reading the two spike trains independently (Dan et al. 1998). Thus, LGN encodes stimulus information in correlations between pairs of firing cells, and Alonso et al. (1996) have demonstrated a correlation between the level of synchrony in LGN neurons and the activity of their targets in V1.

Moreover, cells were found in motor cortex that increased their synchrony without changing their firing rate while the animal performed certain behaviors (Riehle et al. 1997), and in pre-motor cortex recurring sequences of three or more precisely timed spikes were observed, some of which were linked to particular overt behaviors (Prut et al. 1998). Similar patterns have been reported in hippocampus (Nádasdy et al. 1999; Nádasdy 2000). All these examples indicate that there might be a link between the degree of synchronization between sets of neurons and the impact on their target cells.

Opponents have pointed out that in none of these cases is there direct proof that the precise timing itself is responsible for the response: For example in the case of synchrony in motor cortex, it remains possible that this synchrony is a side effect of increased firing in a non-observed cell that is directly responsible for the behavior (Shadlen and Movshon 1999). Furthermore, models have been used to argue that relatively small networks of neurons can decode a pure rate code within one inter-spike interval (Shadlen and Newsome 1998), weakening the strength of the information-theoretical argument.

However, there exists a large literature of modelling studies that indicate that synchrony in synaptic inputs matters to neurons. Both detailed single cell models (Mel 1993; Azouz and Gray 2000) and large scale network models (Lumer et al. 1997) have been used to argue that spike output depends on precise timing of inputs. Electrophysiology on single cells has provided further

[1] In certain specialized areas, the use of precise timing is well established. For example, barn owls have neural circuitry in the laminar nucleus of their brain stems that uses interaural time differences to locate sound sources. Neurons in this area are sensitive to synchrony at timescales down to 100 μs (Carr and Konishi 1990).

evidence: cells that respond with highly variable spike trains to current steps, respond with reproducible spike trains to current injections with richer spectral content (Mainen and Sejnowski 1995). More recently, conductance clamp studies have been used to argue that reliable output can result from natural inputs (Harsch and Robinson 2000), and photo-uncaging of glutamate has been used to show the sensitivity of hippocampal XX cells to precise timing in their synaptic inputs (Margulis and Tang 1998).

Although plasticity will not be investigated in this study, it deserves mention that precise timing has also also been implicated in long term plasticty in hippocampus (Bi and Poo 1998) as well as in short and long term plasticity in cortex (Steriade 1999; Egger et al. 1999; Sjöström et al. 2001). Finally, and also outside the scope of this study, synchronous firing has been proposed as a solution for the binding problem (reviewed in Singer (1999)), although much skepticism remains (Shadlen and Movshon 1999). More detailed surveys of the state of the art can be found in recent reviews (Usrey and Reid 1999; Salinas and Sejnowski 2001; Bi and Poo 2001).

Despite this considerable body of work, the only system in which causality between precisely timed activity and downstream responses has been conclusively proven is audition, and insect olfaction. MacLeod et al. (1998) have demonstrated that odor discrimination in a population of mushroom body cells is specifically disrupted if synchronicity in antennal lobe [sp] is disrupted. Outside of auditory pathways, I am not aware of any study in the mammalian CNS that directly proves causation between synchrony or precise timing and behavior. In fact, to the best of my knowledge, no direct causal link between precisely timed spikes in a pair of cortical cells and the response in a third cell has been established.

Proposal

I will study the responses of cortical cells in culture to precisely timed evoked spikes on a pair of other cells in the culture. Specifically, I will determine the smallest difference in ISIs that postsynaptic cells are sensitive to, and compare this timescale to the membrane time constant of cortical cells.

This study will add to previous studies (Mainen and Sejnowski 1995; Margulis and Tang 1998; Harsch and Robinson 2000), because the cell whose sensitivity to timing of inputs will be measured, will receive stimulation through its own synapses, a better approximation of natural circumstances than conductance clamp. Another helpful aspect of this study is that several cells in the same network will be monitored simultaneously, so the extent of timing sensitivity will be probed. This study will not elucidate whether the position of synapses along a dendrite are a determining factor of sensitivity, nor will it determine whether sensitivity varies according to subpopulations of cortical cells. It will, however, help determine whether inhibition or facilitation has the greater sensitivity.

Specific Aims

I will study the following questions:

– What is the maximum sensitivity of cortical neurons in culture to timing between their afferents?

– What is the relative role of the two major classes of receptors of excitatory synaptic transmission?

– Can precisely timed inhibition play a role in cortical information flow?

– What is the maximum sensitivity of cortical neurons in culture to variations in the frequency of periodic inputs?

Methods

Neurons and glia will be dissociated from 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 cultures to prevent evaporation and infection. Culture medium will be DMEM with 10% horse serum (Potter and DeMarse 2001), and will be completely replaced once weekly. Cultures will be left to mature for three weeks before experiments start.

Extracellular recordings will be made through 59 electrodes with 10 μm diameter and 200 kΩ impedence at 1 kHz, in a rectangular configuration with 200 μm spacing. 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.

Stimulation will take place through two of 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 channels (up to 16). Stimuli of 200–700 mV amplitude, 400 μs per phase, anodic phase first will be used.

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

Methods for subsequent analysis will be detailed below.

Preliminary results

I have demonstrated that stimulation on single electrodes directly evokes action potentials in several axons. Varying stimulation voltage changes the number of affected axons, but does not affect the latency or height of recorded action potentials. These 'first generation' spikes are timed with 100 μs precision with respect to the sharp cathodic transient current at the positive-to-negative edge in the voltage controlled stimulus pulse. For supra-threshold stimuli, reliability of directly evoked activity ranges up to 90%, and is stable for many hours.

The first generation of action potentials evokes a second generation of responses in post-synaptic cells. Timing of these post-synaptic action potentials is less precise, between 1 and 5 ms, correlated weakly with latency, and reliability rarely exceeds 25%. Second generation events are observed at latencies between 5 and 50 ms, beyond which the temporal precision is generally so low that individual events can no longer be extracted even from histograms constructed from several hundred trials. Individual stimuli result in an enhancement of firing rates throughout the culture that decay to background levels after 100–500 ms. Occasionally, stimuli induce dishwide bursts that closely resemble a form of spontaneous bursting commonly observed in these cultures. Such bursts may start up to 150 ms after the stimulus, and occur in a small fraction of trials, if at all. Trials in which bursts are induced, or which coincide with spontaneous bursts will be explicitly discarded in the proposed experiments.

Experiments

Sensitivity to inter-spike intervals

The first series of experiments for this project will establish how widespread sensitivity to precise timing is, and the maximal sensitivity of cultured cells to timing patterns in their inputs.

I will record the responses to stimulation of a pair of electrodes A and B, varying the inter-pulse-interval (IPI). Both electrodes will evoke action potentials in axons of several cells, each timed with about 100 μs precision. Thus, pairs of spikes on different cells will be evoked, each with their own precisely defined inter-spike-interval (ISI). When changing the IPI, each of these ISIs will either remain fixed (if both cells are excited by the same electrode), or exactly follow the change in IPI (if the cells are excited by different electrodes). Since the network is densely interconnected, there will be many neurons in the culture that receive inputs from more than one directly stimulated axon. Most likely, at very short IPIs the responses to paired stimulation will differ from the linear sum of responses to individual stimuli, because an unknown combination of facilitation and refractoriness. That in itself will not be particularly enlightening. However, if neurons are indeed sensitive to precise timing in their afferents, one expects to find specific enhancement or suppression of responses from some cells at particular IPIs. The sensitivity of these downstream neurons to changes in ISI between their inputs will be established by comparing the response to the paired stimulus with the (properly aligned) sum of responses to individual stimulation of electrode A and B, and systematically searching for facilitation (or depression) at particular IPI values. The width of facilation peaks (or depression dips) will be taken as a measure of the sensitivity of postsynaptic neurons to precise timing in their inputs.

A first experiment

I will now describe in some detail the first experiment I plan to perform, as well as the analysis I will apply to the results.

I will take a three week old culture of about 10,000 cells and choose two electrodes (A and B) through both of which I can excite a number of axons directly and evoke postsynaptic responses on at least 10 electrodes each. Stimulation voltage will be tuned so as not to induce a long-lasting dishwide burst. I will obtain the responses on 57 recording electrode to 100 trials each of paired stimulation between electrodes A and B with IPIs of 5, 6, 7, ..., 30 ms. Recording will start 10 ms before the pulse on electrode A, and last until 100 ms after the pulse on electrode B. Additionally, I will record the responses to 1,000 trials of stimulating electrode A and B individually, recording from 10 ms before stimulation to 100 ms after. The pre-stimulus part of the recording will be used to monitor changes in spontaneous activity through the course of the experiment. The trials at different IPIs as well as the single-electrode trials will be randomly interspersed, and performed at a frequency of 0.5 Hz. The entire experiment consists of $26 \times 100 + 2 \times 1000 = 4600$ trials, and will take 2:30 h and occupy 1.2 GB of disk space. To reduce drift in experimental conditions, the culture dish will remain inside the incubator during the entire session.

Spikes will be extracted from the raw data, and histograms of spike times on each of the recording channels will be constructed for each of the stimulation conditions. Bin size will be 1 ms. Denote the firing rate in response to paired-pulse stimulation by $f_c(t_A, t_B)$, where c labels the recording electrode, and t_A and t_B are the latencies with respect to stimulation on electrode A and B respectively (so $t_A - t_B$ is the IPI). Further, denote the firing rate in response to single-electrode stimulation by $f_c^A(t)$ and $f_c^B(t)$. The difference between the observed paired-pulse response and the linear expectation based on single-electrode stimulation can then be calculated as

$$d_c(t_A, t_B) = f_c(t_A, t_B) - f_c^A(t_A) - f_c^B(t_B).$$

The following diagrams show responses in a variety of scenarios.

[A excites, no effect of B; B excites, no effect of A; A and B excite, no interaction; A and B excite, positive interaction; A excites, B inhibits: synchdia.m]

As seen in the figure, responses to paired pulse stimulation that are sensitive to the exact timing can be isolated by extracting the peaks in the graphs of d_c.

The experiment will be replicated on 12 pairs of stimulating electrodes in four cultures. I will collect the parameters of all ISI sensitive responses and answer the following questions:

- Out of all well-defined responses to stimulation at B, how many are modulated by prior stimulation at A? (And how many responses to stimulation at A are modulated by subsequent stimulation at B?)
- Out of these modulated responses, how many depend on the IPI?
- For those that depend on IPI, what is the typical width of facilitation? Of inihibition? Can IPI sensitivity be better than the membrane time constant?

Subsequent experiments

The above experiment will help determine the parameters for subsequent experiments aimed at the same questions. Additionally, I will repeat the experiment with NMDA receptors blocked using AP5, as well as with AMPA receptors blocked using CNQX. Thus I will determine the roles of the two major cortical receptors of excitatory synaptic transmission.

Extending the assay to interactions between three or more inputs is attractive, but the exponential increase in search space of IPIs is a limiting factor. The first such experiments should focus on triplets of stimuli composed of pairs for which timing sensitivity has been demonstrated.

Sensitivity to frequency of periodic inputs

A related experiment probes the sensitivity of cortical neurons to difference in input frequency. For this experiment, I will periodically stimulate a single electrode, at frequencies between 1 Hz and 100 Hz. Stimulation will be applied for 5 seconds, followed by 5 seconds silence, after which another frequency will be picked. Fifty frequencies in the range 1 to 100 Hz will be tried in random order, and responses will be recorded. At the low end of the spectrum, it is clear that increasing the frequency will increase the firing rate of responsive cells commensurately. At the high end, saturation may occur. Both extremes are of limited interest; it will be more interesting whether some cells respond most strongly to specific frequencies. If so, the width of this frequency optimum will be determined. This will serve as another estimate of the sensitivity of cortical cells to precise timing of their inputs.

Choice of model

It will definitely be desirable to extend this study to acute cortical slice, and subsequently even to the intact brain. There are, however, several compelling reasons to start in dissociated culture:

- Working with dissociated culture allows many experiments on the same culture, reducing the time spent on dissection, and the number of animals sacrificed.
- Using multi-electrode array (MEA) technology allows one to record single unit activity from a large number of cells in culture. This will allow a broad search for neurons that respond to precise timing on a given pair of stimulated cells. Recording from large numbers of single units in cortical slices is still problematic (Potter 2001).

– Dissociated cultures have been reported to exhibit higher connectivity than the natural cortex, further increasing the probability of finding a cell that responds to both members of a pair of stimulated cells. For this very same reason, of course, the work should be extended to slice where network topology more closely resembles the natural cortex.

– Cultures have relatively low spontaneous activity (except during culture-wide bursts, which will be explicitly excluded from any recordings). Thus, the signal-to-background ratio of stimulus-evoked activity is high.

– An advantage of culture over intact brains, though not so much over slice, is that it is easy to apply and subsequently wash out pharmaceuticals, to pinpoint the role of different receptors in the observed responses.

– My experience in culturing dissociated cortex and stimulating such cultures will allow me to start the proposed experiments straight away. All required preliminary experiments have been performed already.

Outlook

The expected result of the experiments proposed here, is that some neurons in culture indeed are sensitive to precise timing in their inputs. In that case, this study will determine the extent of this sensitivity. It will be of particular interest to find whether this sensitivity is significantly better than the timescale set by cortical cells' membrane constants. In this scenario, the next question will be whether the density of connections is instrumental in determining sensitivity, and whether the results extend to cortical slices.

If after extended experiments no evidence is found for sensitivity to precise timing, this suggests that if such sensitivity exists in the cortex, it is due to highly specified connectivity in the brain, and is not an emerging property of randomly connected networks of cortical neurons. Another possible cause for negative results could be that synchrony between a larger number of inputs is required before significant impact is registered. I will study this possibility by varying the strength of the stimuli.

If sensitivity to precise timing is indeed observed, this will be significant further evidence that synchronization between different neurons is indeed used as a mechanism of communication in cortex, and an explicit estimate of the timescale of this communication channel will have been obtained.

References

J. M. Alonso, W. M. Usrey, and R. C. Reid. Precisely correlated firing in cells of the lateral geniculate nucleus. *Nature*, 383(6603):815–819, 1996.

R. Azouz and C. M. Gray. Dynamic spike threshold reveals a mechanism for synaptic coincidence detection in cortical neurons in vivo. *Proc. Natl. Acad. Sci. U. S. A.*, 97(14):8110–8115, 2000.

G.-Q. Bi and M.-M. Poo. Synaptic modifications in cultured hippocampal neurons: Dependence on spike timing, synaptic strength, and postsynaptic cell type. *J. Neurosci.*, 18(24):10464–10472, 1998.

G. Q. Bi and M. M. Poo. Synaptic modification by correlated activity: Hebb's postulate revisited. *Annu. Rev. Neurosci.*, 24:139–166, 2001.

M. Brecht, W. Singer, and A. K. Engel. Correlation analysis of corticotectal interactions in the cat visual system. *J. Neurophysiol.*, 79(5):2394–2407, 1998.

C. E. Carr and M. Konishi. A circuit for detection of interaural time differences in the brain-stem of the barn owl. *J. Neurosci.*, 10(10):3227–3246, 1990.

Y. Dan, J. M. Alonso, W. M. Usrey, and R. C. Reid. Coding of visual information by precisely correlated spikes in the lateral geniculate nucleus. *Nat. Neurosci.*, 1(6):501–507, 1998.

V. Egger, D. Feldmeyer, and B. Sakmann. Coincidence detection and changes of synaptic efficacy in spiny stellate neurons in vat barrel cortex. *Nat. Neurosci.*, 2(12):1098–1105, 1999.

A. Harsch and H. P. C. Robinson. Postsynaptic variability of firing in rat cortical neurons: The roles of input synchronization and synaptic nmda receptor conductance. *J. Neurosci.*, 20(16): 6181–6192, 2000.

E. D. Lumer, G. M. Edelman, and G. Tononi. Neural dynamics in a model of the thalamocortical system .2. the role of neural synchrony tested through perturbations of spike timing. *Cereb. Cortex*, 7(3):228–236, 1997.

K. MacLeod, A. Backer, and G. Laurent. Who reads temporal information contained across synchronized and oscillatory spike trains? *Nature*, 395(6703):693–698, 1998.

Z. F. Mainen and T. J. Sejnowski. Reliability of spike timing in neocortical neurons. *Science*, 268 (5216):1503–1506, 1995.

M. Margulis and C. M. Tang. Temporal integration can readily switch between sublinear and supralinear summation. *J. Neurophysiol.*, 79(5):2809–2813, 1998.

B. W. Mel. Synaptic integration in an excitable dendritic tree. *J. Neurophysiol.*, 70(3):1086–1101, 1993.

Z. Nádasdy. Spike sequences and their consequences. *J. Physiol.-Paris*, 94(5-6):505–524, 2000.

Z. Nádasdy, H. Hirase, A. Czurkó, J. Csicsvari, and G. Buzsáki. Replay and time compression of recurring spike sequences in the hippocampus. *J. Neurosci.*, 19(21):9497–9507, 1999.

S. M. Potter. Distributed processing in cultured neuronal networks. In M A L Nicolelis, editor, *Progress in Brain Research*, volume 130. Elsevier Science, 2001.

S. M. Potter and T. B. DeMarse. A new approach to neural cell culture for long-term studies. *J. Neurosci. Meth.*, 110(1-2):17–24, 2001.

Y. Prut, E. Vaadia, H. Bergman, I. Haalman, H. Slovin, and M. Abeles. Spatiotemporal structure of cortical activity: Properties and behavioral relevance. *J. Neurophysiol.*, 79(6):2857–2874, 1998.

A. Riehle, S. Grun, M. Diesmann, and A. Aertsen. Spike synchronization and rate modulation differentially involved in motor cortical function. *Science*, 278(5345):1950–1953, 1997.

F. Rieke, D. Warland, R. de Ruyter van Steveninck, and W. Bialek. *Spikes: Exploring the neural code*. MIT Press, 1997.

S. A. Roy and K. D. Alloway. Coincidence detection or temporal integration? what the neurons in somatosensory cortex are doing. *J. Neurosci.*, 21(7):2462–2473, 2001.

E. Salinas and T. J. Sejnowski. Correlated neuronal activity and the flow of neural information. *Nat. Rev. Neurosci.*, 2(8):539–550, 2001.

M. N. Shadlen and J. A. Movshon. Synchrony unbound: A critical evaluation of the temporal binding hypothesis. *Neuron*, 24(1):67–77, 1999.

M. N. Shadlen and W. T. Newsome. The variable discharge of cortical neurons: Implications for connectivity, computation, and information coding. *J. Neurosci.*, 18(10):3870–3896, 1998.

W. Singer. Neuronal synchrony: A versatile code for the definition of relations? *Neuron*, 24(1): 49–65, 1999.

J. Sjöström, G. G. Turrigiano, and S. B. Nelson. Rate, timing and cooperativity jointly determine cortical synaptic plasticity. *Neuron*, 32:1149–1164, 2001.

M. Steriade. Coherent oscillations and short-term plasticity in corticothalamic networks. *Trends Neurosci.*, 22(8):337–345, 1999.

W. M. Usrey and R. C. Reid. Synchronous activity in the visual system. *Annu. Rev. Physiol.*, 61: 435–456, 1999.

D. A. Wagenaar and S. M. Potter. Real-time multi-channel stimulus artifact suppression by local curve fitting. *J. Neurosci. Meth.*, 120(2):113–120, 2002.