Unique features of action potential initiation in cortical neurons

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Online supplementary information (Part 1 of 3):

Experimental methods and data analysis

In this part of the Supplementary Information we describe in detail all experimental and data analysis methods used in this study. This includes a description of the stationarity criteria for MP recordings and the estimation of AP (action potential) onset potentials and rapidness. We characterize our data sample, and show that rapid AP onset and substantial variability of AP onset potential are found in all cortical cell classes and argue that they are genuine characteristics of cortical neurons.

Contents

Experimental methods

All experimental procedures used in this study were in accordance with the guidelines published in the European Communities Council Directive (86/609/EEC, 1986) and were approved by a local animal welfare committee (Bezirksregierung Arnsberg, Germany).

In vivo intracellular recordings were made in adult cats (3.0-4.5 kg). Surgery and animal maintenance were similar to those used in our previous studies (Volgushev et al., 2000, 2002). Anaesthesia was induced with a mixture of ketamine hydrochloride (Ketanest, Parke-Davis GmbH, Germany, 0.3 ml/kg, i.m.) and Rompun (Bayer, Germany, 0.08 ml/kg, i.m.). Surgery was started after stable anaesthesia with complete analgesia was achieved. Sometimes this required additional doses of the anaesthetic. After tracheal and arterial cannulations, the animal was placed in a stereotaxic frame, the skull was exposed and a craniotomy (about 5 mm diameter) was done over area 17 of the visual cortex centred at P4/L3 (Horsley-Clark). A brass-cylinder (diameter 20 mm) was cemented over the opening. The hydraulically driven microelectrode holder (Narishige Instruments, Japan) was mounted directly onto the skull with screws and dental cement. All wound edges and pressure points were treated with a local anaesthetic (Xylocaine, Astra GmbH, Germany) every 5-8 hours. Muscle relaxation with alcuronium chloride (Alloferin, ICN Pharmaceuticals, Germany) and artificial respiration were started either at this point, or earlier during the surgery, to avoid respiratory depression due to additional doses of the anaesthetic. Thereafter adequate anaesthesia was maintained throughout the experiment by a gas mixture of N_2O:O_2 (70:30) and 0.2-0.4% halothane (Eurim-Pharm, Germany). Artificial respiration was performed with a cat/rabbit ventilator

(Model 6025, Ugo Basile, Biological Research Apparatus, Comaria-Varese Italy). The volume (20-40 cm^3) and the rate of stroke (7-15 per minute) were adjusted to maintain end-tidal CO_2 between 3.5 and 4.0%. End-tidal CO_2, body temperature, heart rate, blood pressure and EEG were continuously monitored. Body temperature was maintained around 37-38°C. Fluid replacement was achieved by the intraarterial administration of 6 ml of Ringer solution containing 1.25% glucose, per hour. Paralysis was maintained by i.a. infusion of alcuronium chloride (0.15 mg/kg/h) in Ringer's solution. The experiments lasted usually 2-4 days. At the end of the experiment, animals were sacrificed with an overdose of anaesthetics.

In vivo intracellular recordings from visual cortical neurons were made with sharp electrodes filled with 2.5M potassium acetate, or 1M potassium acetate and 1% biocytin (Sigma-Aldrich GmbH, Germany). Electrode resistance was 70-120 MΩ. After amplification (Axoclamp 2B, Axon Instruments, USA, and additional DC-amplifier, total gain x20 to x100) and low-pass filtering at 3-5 kHz, the data were digitized at 10-40 kHz and stored on a computer (PC-586; Spike-2, Cambridge Electronic Design, Great Britain). Visual stimuli (moving gratings of different orientation and direction of movement) were generated on the screen of a second computer using subroutines of the Vision Works stimulation system (Cambridge Research Systems, New Hamshire, USA). Parameters of visual stimulation, sequence of stimuli (randomized) and communication to the data acquisition computer were controlled by custom-written software. The screen was positioned 57 cm in front of the animal and was focussed on the retina using appropriate lenses. Background illumination was 2.37 cd/m^2. Luminance of the dark and light stimuli was 0.02 and 12.8 cd/m^2, respectively. Stimuli were presented monocularly to the dominant eye. Cells were classified as simple or complex according to standard criteria (Orban, 1984) and by using the spike response modulation index, defined as a half of the

peak-to-peak modulation divided by the mean increase of the spiking frequency during presentation of an optimal moving grating (Dean & Tolhurst, 1983; Skottun et al., 1991).

In vitro intracellular recordings were made in slices of rat or cat visual cortex and rat and mice hippocampus. The details of slice preparation and recording were similar to those previously used (Volgushev et al., 2000). The Wistar rats or mice (P21-P35 Charles River GmbH, Suzfeld, Germany) were anaesthetized with ether, decapitated and the brain was rapidly removed. One hemisphere was mounted onto an agar block and 350-400 μm thick sagittal slices containing the visual cortex and/or hippocampus were cut with a vibrotome (TSE, Kronberg, Germany or Leica, Bensheim, Germany) in ice cooled oxygenated solution. Slices of the cat visual cortex were prepared from brains obtained at the end of acute *in vivo* experiments in which one of the hemispheres remained intact. The animal was deeply anaesthetised by increasing halothane concentration in a N_2O:O_2 (70:30) gas mixture to 3-3.5% and perfused with an ice-cooled oxygenated solution of the same ionic composition as that used for slice preparation. The visual cortex of the intact hemisphere was exposed, and a block of tissue containing the visual cortex was cut, removed from the cat, and slices (400-500 μm) were prepared as above. After cutting, the slices were placed into an incubator where they recovered for at least one hour at room temperature before moving on of them in the recording chamber. The solution used during the preparation of the slices had the same ionic composition as the perfusion/ extracellular solution. It contained (in mM) 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 D-glucose and bubbled with 95% O_2 and 5% CO_2. Recordings were made with the slices in submerged conditions at 32-35°C or at room temperature. Recordings at room temperature were performed to check if this factor

was critical for the observed fast dynamics of action potential initiation. Temperature in the recording chamber was monitored with a thermocouple positioned close to the slice, 2-3 mm from the recording site. In experiments with tetrodotoxin (TTX), it was added to the extracellular solution at concentrations 10-300 nM. Whole-cell recordings using patch electrodes were made from neurons of different morphology (pyramidal, non-pyramidal) and located in different layers, selected under visual control using Nomarski optics and infrared videomicroscopy (Dodt & Zieglgänsberger, 1990; Stuart, Dodt & Sakmann, 1993). The patch electrodes were filled with K-gluconate based solution (in mM: 127 K-Gluconate, 20 KCl, 2 $MgCl_2$, 2 Na_2ATP, 10 HEPES, 0.1 EGTA and 0.3-1.0% biocytin) and had a resistance of 3-6 MΩ. Action potentials were evoked by depolarising current steps or by synaptic stimulation. Synaptic responses were evoked by electric shocks applied through bipolar stimulation electrodes located 0.5-1.5 mm below or lateral to the recording site. After amplification using Axoclamp-2A (Axon Instruments), and low-pass filtering at 3-5 kHz, data were digitised at 10-40 kHz and fed into a computer (PC-486; Digidata 1200 interface and pCLAMP software, Axon Instruments). The EGTA, HEPES, potassium gluconate, Na_2ATP and TTX were obtained from Sigma, and the remaining chemicals were obtained from JT Baker BV (Deventer, Holland).

Data sample

The sample of neurons analysed contained different types of cells. In the *in vivo* data from cats, we classified some of the recorded cells electrophysiologically, according to their intrinsic membrane properties, which were assessed from responses to injection of depolarising current steps. All electrophysiological types of cells described so far in the neocortex were represented in our sample, including regular

spiking (RS), fast spiking (FS), intrinsically bursting (IB) and chattering or fast rhythmic bursting (FRB) neurons (Fig. 4 in the paper). Some of the neurons were labelled with biocytin during the recording. Among these neurons, cells of different morphological types and different location within cortical layers were encountered. Microphotographs in Fig. 1SI give examples of a layer V pyramidal cell, a layer IV spiny stellate cell and a layer III pyramidal cell from our sample.

In vitro recordings were made in slices of rat and cat neocortex and rat and mouse hippocampus. We made whole cell recordings with patch electrodes under visual control. This allowed visual pre-selection of cells of different morphological types and layer location. According to the classification based on the intrinsic membrane properties, three types of neocortical neurons were encountered *in vitro*: regular spiking (RS), fast spiking (FS) and intrinsically bursting (IB) cells.

The diversity of our *in vivo* and *in vitro* data, which include cortical neurons of different morphology, layer location and intrinsic membrane properties, makes the sample representative.

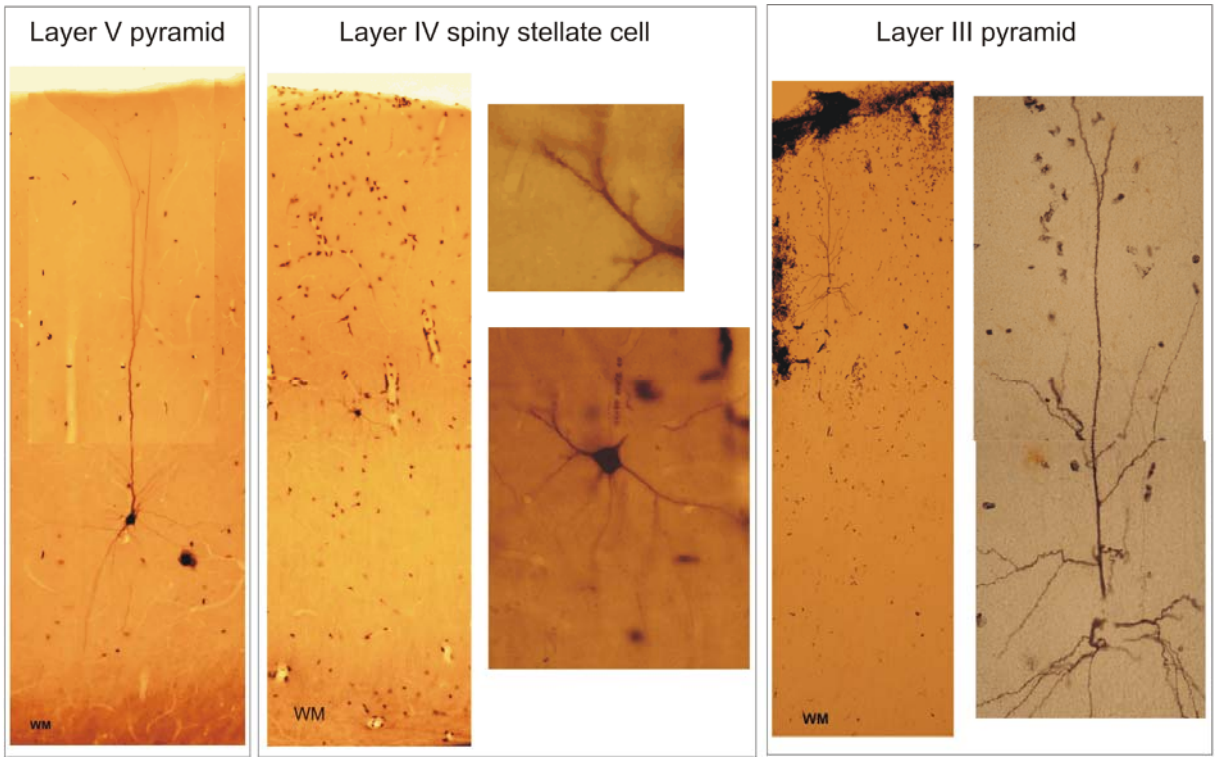

Fig 1SI: Microphotographs of examples of a Layer V pyramidal cell, a layer IV spiny stellate cell and a layer III pyramidal cell from our sample. In each panel, the images to the right were taken at larger magnifications.

Data analysis

We analyzed data from 47 cells recorded *in vivo*. In each cell, we recorded responses to the presentation of moving gratings of different orientations (duration 5-7s), and periods of spontaneous activity (10-120s). Altogether, we analyzed 409 *in vivo* recordings. For comparison, we also examined data from 22 cells recorded in slices (17 in rat, 3 in mouse, 2 in cat), resulting in 70 *in vitro* recordings.

Each recording contained at least 5 action potentials (APs), and the AP onset potentials and AP peak amplitudes fulfilled the following stationarity criterion. The stationarity of each recording was tested by computing the standard deviation of the values of the MP, the AP peak potentials and the onset potentials for increasing window sizes ranging from 100ms to 7s. A recording was classed as stationary if

the average standard deviation in peak amplitude and spike onset potential differed by less than 10% for window sizes between 100ms and 7s.

All of our recordings fulfilled this criterion and did not exhibit slow drifts of the MP, of the AP onset or peak potential.

All recordings were interpolated to a resolution of $\Delta t = 10\mu s$ using the Matlab pchip interpolation filter (Matlab V6.5 (R13)). For each interpolated recording, we computed the temporal derivative *dV/dt*:

(1) $$\frac{dV}{dt}(t_n) = (V_{n+1} - V_{n-1})/(2\Delta t)$$

In Fig 1-3, this temporal derivative is graphed against the instantaneous MP, yielding a "phase plot" representation (see main text).

In each recording, we first detected APs using a voltage threshold set at –30mV. For each AP, we then determined the onset potential using as a criterion the point where the velocity of the AP exceeded 10mV/ms. For all analyses, we took only APs into account that were separated from preceding APs by more than 30ms.

The AP onset rapidness shown in Fig. 4 was determined as follows: For each AP a line was fitted to the AP curve in the phase plane (*V vs. dV/dt)* representation at *dV/dt*=10 mV/ms. The slope of this line is defined as the onset rapidness of a single AP. The onset rapidness for a recording is then given as the mean onset rapidness of all APs in the recording. The onset rapidness *in vitro* was measured at temperatures 32-35°C and at room temperature. The AP onset span of a recording is defined as the difference between maximum and minimum AP onset potential. In *in vitro* experiments, APs were mostly elicited by constant current injections and the

membrane potential exhibited no fluctuations. Thus the onset span was assessed only *in vivo*.

Action potential onset rapidness and onset variability are genuine properties of cortical neurons

In the paper, we show that the large AP onset rapidness together with the large AP onset potential variability observed in *in vivo* intracellular recordings from cortical neurons is outside the range of behaviours that can be described by Hodgkin-Huxley type models. We are not the first to observe these features of cortical APs per se. Sharp, step-like onsets of action potentials recorded *in vivo* can be seen in previous publications from several laboratories, as soon as APs are shown at fine temporal resolution (see for example, cat visual cortex: Fig. 2 in Gray, McCormick 1996 Science 274:109-113; Fig. 5 D,E in Bringuier et al., J.Physiology 1997, 500:751-774; Fig. 2A in Azouz, Gray 1999; cat somatosensory cortex: Fig. 6D, 8B in Yamamoto et al 1990, Exp. Brain Res. 80:12-22; rat somatosensory cortex: Fig.11 in Brecht et al., J. Physiology 2003 553:243-265; cat area 7: Fig. 4 in Timofeev et al., Neuroscience 2002, 114:1115-1132). Also the large variability of AP onset potentials has been reported before (Azouz, Gray 1999, 2000; Volgushev et al. 2002) and can be seen in many of the examples cited above. Nevertheless, it is important to assure that these features represent genuine properties of cortical neurons and are not caused by experimental manipulations or other artefacts.

In is hard to imagine that the large AP onset rapidness represents a measurement artefact. First, the recording circuit in electrophysiological experiments exhibits properties of a low pass filter due to resistance and capacitance of the recording electrode. It is thus expected to rather wash out rapid acceleration of membrane

potential changes. Second, rapid AP onsets are observed using both sharp and patch electrodes, that differ considerably in their electrical properties. Third, direct evidence that rapid AP onsets are not induced by measurement apparatus comes from the *in vitro* experiments with TTX described in the paper (Fig.5). There, the AP onset dynamics was observed to change from *in vivo*-like rapid onsets in control conditions to gradual Hodgkin-Huxley type onsets during application of low concentrations of TTX and back to rapid onsets upon washout of the drug. These reversible modifications of AP onsets were observed in every cell tested with TTX, in an unmodified setup and under continuous recoding. Together these observations indicate that large AP onset rapidness is a genuine property of cortical neurons.

Various lines of evidence from our experiments as well as published results of other labs indicate that the variability of AP onset potentials is also not of an artefactual nature. In principle, it is conceivable that a non-stationary potential drop between the reference electrode and the extracellular space of the measured cell or a non-stationary state of the measurement electrode might bias the variance of AP onset potentials towards increased values. However, these factors would manifest themselves as systematic trends. Further, such general changes of electrode offset potential would shift all measured potential values, including mean membrane potential and AP peak. In our analyses, we therefore applied strict stationarity criteria such that all quantifications of AP onset dynamics were based on recordings that were very stable (c.f. above). Examples of widely different AP onset potentials obtained under stationary recording conditions are apparent in Figure 2 (inset). There three APs were superimposed such that the times of peak potentials were aligned. While the peak potentials of the APs are virtually identical, the onset potentials of the three APs differ by up to 8mV For our data, this is a representative example. In

general, we find that AP peak and onset potentials are often uncorrelated (data not shown). Additional evidence that AP onset variability is not due to recording non-stationarity, but is a genuine property of neocortical neurons is provided by the fact that onset potentials of two APs directly succeeding one another and separated by short inter-spike-intervals (<25 ms) are substantially different, as predicted by incomplete deinactivation of sodium channels. We note here, that to exclude the influence of this type of variability we did not include in our analyses the APs appearing less than 30 ms after the preceding spike. Furthermore, similar AP onset potential variability was recently observed *in vitro* in dynamic clamp experiments (de Polavieja et al., 2005) that were designed to minimize sources of non-stationarity. De Polavieja stimulated cortical neurons with temporally fluctuating inputs and observed onset potential variability indistinguishable from *in vivo* recordings. In such *in vitro* experiments the stability of the potential measurement is well controlled and AP onset potential variability is observed even if the stability of the recording is maximized by using double-electrode impalement of single neurons, where one electrode is used for membrane potential measurement and another one for dynamical electrical stimulation.

In summary, these facts show that rapid AP onsets and large variability of AP onset potentials are robust phenomena reproducible under a wide variety of recording conditions. Most importantly, AP onset rapidness can be artificially and reversibly turned down in cells recorded *in vitro*. Large AP onset potential variability is observed even if the stationarity of the recording is controlled to the highest possible degree. We are thus confident that large AP onset rapidness and onset potential variability are genuine features of AP initiation in cortical neurons.

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