Nanostimulation: manipulation of single neuron activity by juxtacellular current injection

by

Arthur R. Houweling\textsuperscript{1,2*}, Guy Doron\textsuperscript{1*}, Birgit C. Voigt\textsuperscript{1}, Lucas J. Herfst\textsuperscript{1}

& Michael Brecht\textsuperscript{1}

* These authors contributed equally

\textsuperscript{1} Bernstein Center for Computational Neuroscience
Humboldt University of Berlin
Philippstr. 13 Haus 6
10115 Berlin
Germany

\textsuperscript{2} Department of Neuroscience
Erasmus Medical Center
PO Box 2040
3000 CA, Rotterdam
The Netherlands

Corresponding author:
A.H. (a.houweling@erasmusmc.nl), M.B. (michael.brecht@becn-berlin.de)

Submitted to Journal of Neurophysiology
Abstract

In the mammalian brain many thousands of single neuron recording studies have been performed but less than ten single-cell stimulation studies. This paucity of single-cell stimulation data reflects a lack of easily applicable single-cell stimulation techniques. We provide a detailed description of the procedures involved in nanostimulation, a single-cell stimulation method derived from the juxtacellular labeling technique. Nanostimulation is easy to apply and can be directed to a wide variety of identifiable neurons in anesthetized and awake animals. We describe the recording approach and the parameters of the electric configuration underlying nanostimulation. We use glass pipettes with a DC resistance of 4-7 MΩ. Obtaining the juxtacellular configuration requires a close contact between pipette tip and neuron and is associated with a several-fold increase in resistance to values ≥ 20 MΩ. The recorded action potential (AP) amplitude grows to ≥ 2 mV and neurons can be activated with currents in the nanoampere range – hence the term nanostimulation. While exact AP timing has not been achieved, AP frequency and AP number can be parametrically controlled. We demonstrate that nanostimulation can also be used to selectively inhibit sensory responses in identifiable neurons. Nanostimulation is biophysically similar to electroporation, and based on this assumption we argue that nanostimulation operates on membranes in the micrometer area directly below the pipette tip, where membrane pores are induced by high transmembrane voltage. There is strong evidence to suggest that nanostimulation selectively activates single neurons and that the evoked effects are cell-specific. Nanostimulation therefore holds great potential for elucidating how single neurons contribute to behavior.

Key words: single-neuron stimulation, reverse physiology, microstimulation, juxtacellular labelling
**Introduction**

To establish causal relationships between neuronal activity and behavior it is necessary to manipulate the occurrence of action potentials (APs) in neurons. The first neural stimulation tools consisted of electrodes placed onto the surface of the brain (Fritsch and Hitzig 1870; Penfield and Boldery 1937), which allowed currents in the milliampere range to activate cortical tissue and thereby initiate movements or elicit sensory percepts depending on the location of the electrode. The subsequent development of the microstimulation technique (Asanuma and Sakata 1967), in which a fine metal wire with a small diameter tip is inserted into the brain, enabled a more precise spatial control of neural activation with currents in the microampere range. This technique has been particularly successful in establishing that activation of local neuronal populations leads to movements (Asanuma 1989) or artificial sensory percepts (Bartlett and Doty 1980; Butovas and Schwarz 2007; Murphey and Maunsell 2007; Romo et al. 1998; Schmidt et al. 1996; Tehovnik et al. 2003) that can even influence sensory decisions (Afraz et al. 2006; Salzman et al. 1990). However, these findings have been difficult to interpret at the level of individual neurons because microstimulation does not allow identification and quantification of the stimulated cellular elements (Tehovnik 1996). These shortcomings can be overcome in single-cell stimulation experiments (Brecht et al. 2004).

Though single-cell stimulation is a conceptually and experimentally attractive approach, single-cell stimulation has so far been applied only in a relatively small number of studies. The reasons for this are probably related to the mass action views of neural processing (Shadlen and Newsome 1998) which predict a negligible effect of single neuron activity. Two novel developments, however, have provided evidence for the idea that – at least in some circumstances – the brain computes with small but powerful sets of neurons. First, it has become clear that neural activity is more sparse than previously anticipated (Brecht et al. 2005; Greenberg et al. 2008; Olshausen and Field 2004). Second, recent single-cell stimulation studies have demonstrated an effect of single neuron activity on movement (Brecht et al. 2004; Herfst and Brecht 2008), sensations (Houweling and Brecht 2008) and brain state (Li et al. 2009). Stimulation of identified single neurons has only become possible with the application of intracellular and whole-cell recording techniques to the anesthetized animal, but such recordings are difficult to obtain in the awake animal (Crochet and Petersen...
2006; Lee et al. 2006; Margrie et al. 2002; Steriade et al. 2001). In vivo whole-cell recordings are also typically limited to relatively few recording sessions for a given cortical area due to the deterioration of the exposure after dura removal. As an alternative to intracellular stimulation approaches, juxtacellular stimulation has been used in a variety of studies (Andrew and Fagan 1990; Brons et al. 1982; Cruikshank and Weinberger 1996; Fregnac et al. 1992; 1988; Lavallee and Deschenes 2004). Here we characterize this stimulation technique, which is also termed nanostimulation because of the nanoampere-range stimulation currents. Nanostimulation allows addition and subtraction of APs in single identifiable neurons of awake (Houweling and Brecht 2008; Voigt et al. 2008) and anesthetized animals (Herfst and Brecht 2008).
Experimental Procedures

We used standard surgical and electrophysiological techniques to prepare animals (Wistar rats, ~ P35 at the day of surgery) for chronic, head-fixed recording of the barrel cortex (P3 mm, L5 mm relative to bregma) (Margrie et al. 2002). For acute experiments animals (n = 16) were anesthetized with urethane (1.5-2.0 g/kg, intraperitoneal). Glass pipettes for nanostimulation were filled either with intracellular solution containing (in mM) K-gluconate 135, HEPES 10, Na2-phosphocreatine 10, KCl 4, MgATP 4, and Na3GTP 0.3 (pH 7.2), or Ringer solution containing NaCl 135, KCl 5.4, HEPES 5, CaCl2 1.8, and MgCl2 1 (pH 7.2). The juxtacellular signal was amplified and low-pass filtered at 3 kHz by a patch-clamp amplifier (Dagan, Minneapolis, MN) and sampled at 10 or 25 kHz by a Power1401 data acquisition interface under the control of Spike2 software (CED, Cambridge, UK). Since nanostimulation elicits large DC shifts in the potential recorded during current injections, a digital DC remove filter was applied online (which subtracts at each point in time the average potential within Δt = ±1 ms) to allow monitoring of evoked APs. Series resistance and capacitance were not compensated. All experimental procedures were performed according to Dutch and German guidelines on animal welfare under the supervision of local ethics committees.
Results

Recording approach

Nanostimulation is based on a commonly used procedure to stain individual neurons recorded extracellularly in vivo (Pinault 1996). Using glass pipettes, Pinault showed that dyes such as biocytin enter a neuron if the pipette is close enough to fire the cell during alternating on/off 200 ms current injections of a few nA. To establish the juxtaglottic configuration required for nanostimulation we use the following procedure. Neurons are searched for blindly using a glass whole-cell recording pipette (typical resistance 4-7 MΩ) while monitoring the pipette resistance using 1 nA current pulses (Fig. 1a, ‘Search’). Once the resistance reaches a certain level (typically 20 MΩ or above), the search pulse is switched off to check for the presence of APs indicating contact with a neuron. If APs remain smaller than 2 mV over a period of a few minutes, we move the pipette in small steps (2.5 μm) towards the neuron until the amplitude reaches 2 mV or larger (Fig. 1a, ‘Approach’). An attempt is then made to make the neuron fire short trains of APs by brief (200 ms) positive current injections of increasing strengths (Fig. 1a, ‘Entrainment’). Special care is taken to avoid signs of damage to the neuron (hyperpolarization shifts, AP broadening, strong reduction of AP amplitude during the current injection, or an increase in spontaneous activity), which may occur with current injections beyond those that elicit a maximum firing rate. Since nanostimulation elicits large DC shifts in the juxtaglottic potential during current injections, a high-pass filter is applied to the recording to monitor the modulation of AP firing. If the neuron cannot be entrained, the pipette is advanced a few steps and the entrainment procedure is repeated. If entrainment fails or the neuron is lost the same pipette can be used to search for a new cell. Typical nanostimulation currents needed to modulate neuronal AP firing range between 3-30 nA. On average, 8.8 ± 5.6 (s.d.) nA is required to fire 8-12 APs in barrel cortical neurons (Fig. 1b). The large DC potential shifts (and their equivalent transients in the high-pass filtered traces, indicated by triangles in Fig. 1a) complicate the detection of spikes for a brief period of 1-2 ms at the onset and offset of current injections, and may result in a small underestimate of the total number of elicited APs (∼1-2% for 200 ms current injections). There is an inverse relationship between the juxtaglottic circuit resistance (total resistance measured by current injections $R_{\text{total}}$ – pipette resistance in the bath...
This inverse relationship is predicted by the juxtacellular circuit diagram (Perkins 2006), from which it follows that the fraction of injected current that enters the cell equals \((R_{\text{total}} - R_{\text{pipette}})/(R_{\text{patch}} + R_{\text{cell}})\), where \(R_{\text{cell}}\) is the input resistance of the cell and \(R_{\text{patch}}\) the resistance of the small membrane patch directly underlying the tip of the pipette. There is also a negative correlation of spike height with the amount of current needed to elicit APs (Fig. 1c). This latter dependence is surprising as there was no significant relationship between spike height and circuit resistance \((r = 0.26, p = 0.31, t\text{-test})\).

In our view, perhaps the most important advantage of nanostimulation is that this technique is very easy to apply to a variety of preparations and neurons. Fig. 2a illustrates our experimental setup for nanostimulation in the brain of chronically prepared animals. Much like tungsten microelectrodes, nanostimulation pipettes can be advanced through the intact dura (Fig. 2b). The robustness of nanostimulation pipettes against minor mechanical obstacles like the dura and brain surface contaminations (as they typically occur in chronic preparations) is very different from in vivo whole-cell recordings or sharp microelectrode recordings, which are greatly affected by such obstacles. As a consequence it is easy to target deep brain structures with nanostimulation pipettes – we never had a situation in which pipettes would break or irreversibly occlude in such experiments. Deep penetrations will result in damage to the overlying brain structures and for these applications it is useful to pull pipettes with long thin shanks. We have been able to apply nanostimulation to hundreds of neurons in widely different brain regions including the barrel cortex (Fig. 2c, in this case a spiny stellate cell, one of the smallest cortical neurons), the thalamus (Fig. 2d), and the facial nucleus (Fig. 2e), which contains some of the largest neurons in the mammalian brain.

We assessed the stability of the nanostimulation configuration by quantifying the durations of nanostimulation sessions on 79 cells recorded in the barrel cortex of awake animals involved in a detection task \((n = 8, \text{unpublished data})\). For all these sessions a minimal number of each of several stimulation trial types had been presented satisfying an inclusion criterion for behavioral analysis (Houweling and Brecht 2008). In this data set recording durations (i.e. elapsed time between first and
last effective nanostimulation trial) ranged between 5-86 minutes, with an average duration of 22 ± 17 (s.d.) minutes and a median duration of 15 minutes. We did not collect detailed statistics on ‘bad’ recordings that did not satisfy our inclusion criterion. In these 8 animals, 79 successful recordings were obtained in 63 daily sessions (each lasting 2-3 hours), yielding an average success rate of 1.25 included cells per experiment.

To assess the health of neurons during the course of a nanostimulation experiment, we quantified spontaneous activity during experiments on neurons recorded in the barrel cortex (n = 70) and visual cortex (n = 20) of awake animals involved in a detection task (Houweling and Brecht 2008). Spontaneous firing rates were quantified in 1 s blocks preceding nanostimulation trials (see Fig. 1d, Houweling and Brecht 2008). In 13 out of 48 neurons (27%) for which recording durations exceeded 10 minutes, mean spontaneous activity during the 5-10 minute period following the first nanostimulation trial was significantly altered compared to the initial 0-5 minute period (shuffle test, α = 0.05). In 2 out of 48 neurons (4%) spontaneous rates decreased (on average 1.3 spikes/s), and in 11 neurons (23%) spontaneous rates increased (on average 2.4 spikes/s). For the remaining cells that displayed stable spontaneous firing rates during the first 10 minutes and for which recording durations exceeded 20 minutes, mean spontaneous activity during the 15-20 minute period following the first nanostimulation trial was significantly altered in 5 out of 17 neurons (29%) compared to the initial 0-5 minute period. In 1 out of 17 neurons (6%) spontaneous rates decreased (2.5 spikes/s), and in 4 neurons (24%) spontaneous rates increased (on average 3.6 spikes/s). Thus, in a fraction of neurons small changes in spontaneous firing rate may accompany nanostimulation experiments. It must be noted however that many factors may have contributed to the observed changes in spontaneous firing rates over the course of these behavioral experiments, including changes in arousal state and possible long-lasting effects of nearby microstimulation on circuit organization.

In our experiments we used sustained DC current injections and consistently found that such prolonged current steps do not allow for a precise control of spike timing (Fig. 3). Typically, AP firing is uniformly distributed over the nanostimulation interval (Fig. 3a). Current injections that elicit an equal number of APs produce a
large trial-by-trial variation in the timing of individual spikes (Fig. 3b), even when the evoked spike trains are aligned on their first APs (data not shown). A variety of discharge patterns have been observed, including adapting spike trains reminiscent of regular spiking pyramid cells. Some control of spike timing may be obtained by restricting current injections to short pulses, although elicited APs are difficult to detect because of the large stimulation artifacts that last 1-2 ms (data not shown). In some cases AP firing is temporarily increased for a few hundred milliseconds following the end of the current injections (Fig. 3a). These after-discharges (the origin of which we will address in the discussion) can be prevented by a same size negative current injection directly following the positive current injection (data not shown).

Parametric control of spike frequency and number

To explore whether nanostimulation can be used to control AP frequency, we varied current intensity in barrel cortex neurons of anesthetized rats (n = 4). We first determined the maximal amount of current well tolerated by the cell by increasing the current to a point where further increases would jeopardize the viability of the recording. This current intensity (range 4-19 nA, mean 9.5 nA, n = 10 cells) elicited on average 13.3 ± 5.6 APs during 200 ms injections and we then applied 25, 50, 75 or 100 percent of this maximal current. In an experiment on a layer 6 inverted pyramidal neuron (Fig. 4a), nanostimulation evoked on average 15 APs (i.e. 75 Hz) in response to the 100% current (8 nA) and proportionally fewer APs with 75%, 50% and 25% of the maximal current (Fig. 4b,c). Average AP frequency varied linearly with nanostimulation current in the studied range for this cell (Fig. 4c, R^2 = 0.80), as well as for our population of cells (Fig. 4d). Although there was considerable variability in the evoked number of APs for a given current intensity, linear regression indicates that the control of AP frequency by varying nanostimulation intensity was good in all cells (R^2 = 0.48-0.97, median 0.73).

Next we determined whether nanostimulation can be used to control AP number. To this end we varied the duration of nanostimulation current applied to neurons in the barrel cortex of anesthetized rats (n = 6). Again, we determined a maximal stimulation current for each cell (range 3-30 nA, mean 9.7 nA, n = 12 cells). We then applied 50% of this current intensity for 100, 200, 400 and 800 ms durations. In a layer 4 pyramidal neuron (Fig. 5a), 50% current intensity evoked on average 4 APs during a
100 ms current injection, and systematically more APs with longer stimulus durations (Fig. 5b,c). The average AP number varied linearly with nanostimulation duration in the studied range for this cell (Fig. 5c, $R^2 = 0.95$), as well as for our population of cells (Fig. 5d). Although there was considerable variability in the evoked number of APs for a given current duration, linear regression indicates that the control of AP number by varying stimulus duration was good in all cells ($R^2 = 0.49-0.98$, median 0.90).

Inhibition of spiking activity

Finally we determined if nanostimulation could also be used for inhibiting AP activity. We obtained sensory responses in 7 barrel cortex neurons of anesthetized rats (n = 6) by applying a 100 ms air puff to the whiskers. To inhibit AP firing we first determined a maximal stimulation current for each cell as before (range 5-16 nA, mean 9.1 nA) and applied the same intensity but as a negative current of 200 ms duration. We then presented air puff stimulation together with negative current injection and interleaved with air puff-only stimuli. As illustrated in Figure 6 for a layer 6 pyramidal neuron (Fig. 6a), sensory AP responses to the air puff (Fig. 6b, top) were abolished when paired with a negative nanostimulation current (Fig. 6b, bottom). Inhibition of sensory evoked activity was also clearly evident in the population averages (Fig. 6c).
Discussion

In this study we provide a detailed description of nanostimulation procedures. We demonstrate that nanostimulation can be directed to a variety of identifiable neurons in anesthetized and awake animals. Furthermore, nanostimulation allows control of AP frequency and AP number. Finally we demonstrate that this technique can be used to selectively inhibit sensory responses in identifiable neurons.

Properties of nanostimulation

Table 1 gives an overview of electric stimulation techniques. As indicated in the table the effects of the juxtacellular current injections used for nanostimulation are in many ways similar to those of intracellular current injections. First, neurons are excited by positive currents and inhibited by negative currents. Second, nanostimulation current-AP-frequency curves are close to linear for cortical neurons (at least for 200 ms current steps), as described for intracellular recordings (Creutzfeldt 1995; Nowak et al. 2003). Third, sustained DC current injections result in AP responses with large temporal jitter, much like AP responses elicited by DC current injections in the whole-cell configuration (Mainen and Sejnowski 1995). Fourth, most cells do not fire instantaneously upon current onset. This may reflect a capacitive charging of the neuronal membrane, which also delays AP initiation upon intracellular current injection. Different from intracellular current injections, nanostimulation may induce in some neurons a small increase in spontaneous AP firing following the injection. This phenomenon might reflect processes related to membrane resealing, as discussed below.

Biophysical foundations: nanostimulation compares to electroporation

An intact patch of lipid membrane underlying the tip (1-2 μm diameter) has a resistance in the GΩ range (Perkins 2006). The intact patch therefore supports only limited juxtacellular current influx, as current will rather flow through the much smaller juxtacellular seal resistance (tens of MΩ). While the exact mechanisms underlying juxtacellular labeling and nanostimulation remain to be established, it seems likely that their biophysical processes compare to electroporation (Chang et al. 1992). Theoretical (Abidor et al. 1979; Freeman et al. 1994) and experimental work
on electroporation suggests that it is based on the reversible electric breakdown of the
lipid membrane (Benz et al. 1979) leading to membrane pore formation (Neumann et
al. 1982). Nanostimulation and juxtacellular labeling share five major characteristics
with electroporation, and recognizing these will help in understanding the biophysics
of nanostimulation:
1. Similar voltage range. In a variety of studies on lipid bilayers and cell membranes a
reversible electric breakdown has been observed at voltages between ~ 0.5 and 1.5 V
(Abidor et al. 1979; Benz et al. 1979; Neumann et al. 1982; Tsong 1987;
Zimmermann 1986). We have no direct measurements of the transmembrane voltage
in our experiments, but as illustrated in Fig. 1b, neuronal stimulation requires ~ 9 nA
in a typical experiment, with a total resistance of ~ 25 MΩ (of which ~ 5 MΩ is due
to the pipette). One might therefore expect a voltage of 180 mV (ΔV = 9 nA · 20 MΩ)
at the electrode tip. This voltage value is somewhat lower than the estimates for
electroporation (0.5 - 1.5 V), but these have been obtained with electric fields applied
to entire cells and short voltage pulses (milliseconds rather than hundreds of
milliseconds as in our study).
2. Irreversible membrane breakdown at higher voltages. Beyond the voltage specified
above, excessive AP firing and cell death have been observed in our experiments and
previous labeling studies (Pinault 1994; 1996). These are presumably a consequence
of an irreversible membrane breakdown as observed in experiments on cell
membranes and lipid bilayers (Chernomordik et al. 1987).
3. Similar time course. Both our findings (see Fig. 3) and studies on membrane
electroporation suggest a fast onset and offset of membrane effects (with a time
constant in the millisecond range) and a second slow membrane recovery process
(with a time constant in the range of hundreds of milliseconds to seconds) which is
thought to reflect membrane resealing (Chernomordik et al. 1987; Weaver 1993).
4. Dramatic increase in conductivity. A dramatic increase in membrane conductivity
is observed both in electroporation of cells (Chernomordik et al. 1987) and lipid
bilayers (Benz et al. 1979) as well as in nanostimulation, where currents in the nA
range can activate cells. These currents would be entirely ineffective in a conventional
extracellular configuration (as discussed below the microstimulation currents used for
conventional extracellular stimulation are roughly three orders of magnitude larger).
5. Formation of aqueous pores. The formation of aqueous pores in the lipid bilayer is the key event in the reversible electric breakdown of membranes (Abidor et al. 1979; Weaver 1993). It seems highly likely that this is also the mechanism underlying juxtacellular labeling and nanostimulation, as the biocytin that enters neurons in juxtacellular labeling (Pinault 1994; 1996) and nanostimulation (Houweling and Brecht 2008) is normally not membrane-permeable.

Nanostimulation is biophysically different from iontophoresis and microstimulation
While there are strong similarities of juxtacellular labeling and nanostimulation to electroporation, it seems unlikely that these methods are similar to iontophoresis (Hicks 1984) as originally proposed (Fregnac et al. 1992; Pinault 1994). Iontophoresis is not based on the electric breakdown of membranes and has different functional characteristics from electroporation (Hui 1998). From Table 1 it is also obvious that nanostimulation is biophysically different from microstimulation, which relies on currents in the microampere range rather than the nanoampere range (Tehovnik 1996) reflecting the fact that microstimulation activates cells along extracellular pathways. Microstimulation operates in the tens to hundreds of micrometer scale (Ranck 1975; Stoney et al. 1968; Tehovnik 1996). This is very different from nanostimulation, which presumably activates cells in the micrometer area directly below the pipette tip, where membrane pores are induced by high transmembrane voltage.

Nanostimulation stimulates a single neuron and induces cell-specific effects
A key aspect of both juxtacellular labelling and nanostimulation is that these procedures target single neurons. The evidence for this idea has been reviewed in depth before (Herfst and Brecht 2008; Houweling and Brecht 2008; Pinault 1994; 1996; Voigt et al. 2008). Briefly, the argument is as follows: (i) Nanostimulation currents (3-30 nA) are about 2-3 orders of magnitude smaller than those thought to be required for activating neurons with microstimulation (> 1 µA) (Stoney et al. 1968). In line with these small currents the biophysical considerations above suggest that nanostimulation acts in the micrometer range below the electrode tip. (ii) Inadvertent stimulation of a second nearby neuron, detected by the presence of large additional AP waveforms, is a rare event (< 1% of APs) (Houweling and Brecht 2008; Voigt et al. 2008). This is consistent with the low probability that two neurons are in close contact with a 1-2 µm tip. (iii) Cells more distant from the pipette than the primary
neuron targeted for nanostimulation, detected by the presence of small additional AP waveforms (< 0.5 mV) in the recording, are not affected in their activity (Houweling and Brecht 2008; Voigt et al. 2008). (iv) Histological data confirm a one-to-one correspondence between single juxtacellulrly activated and labelled neurons (Pinault 1994; 1996). Furthermore, electric destruction of single juxtacellulrly labelled neurons results in the histological recovery of single cells with disintegrated morphologies (Pinault 1994).

In line with these methodological considerations we found that nanostimulation effects were highly cell-specific. For example, whisker movements evoked by single-cell stimulation in the facial nucleus were completely contingent on spiking of the respective neurons (without any movement failures) (Herfst and Brecht 2008). Furthermore, whisker movements varied with respect to direction, amplitude and speed as a function of the identity of the stimulated neuron. In the somatosensory cortex we detected differences in evoked sensory effects between the stimulation of putative interneurons and principal cells (Houweling and Brecht 2008).

Comparison of nanostimulation to other brain stimulation methods

The development of optogenetic methods for neural stimulation (Boyden et al. 2005) and inhibition (Zhang et al. 2007) holds great promise for the creation of a novel set of tools for controlled manipulation of multi-neuron activity. These methods will be particularly attractive when genetically targeted to selected sets of neurons. To allow quantification of elicited AP trains and identification of the stimulated cells these methods will have to be combined with some form of population imaging. An upper bound on the number of stimulated neurons can be obtained by histology (Huber et al. 2008). It remains to be seen if the current limits on the frequency of light-elicited APs (10-30 Hz with channelrhodopsin-2) can be alleviated.

There are four major limitations of conventional electric stimulation techniques (Table 1) such as brain surface stimulation (Fritsch and Hitzig 1870; Penfield and Boldery 1937) or microstimulation (Asanuma and Sakata 1967): (i) the number of activated cells is unknown in these techniques (Tehovnik 1996), as well as (ii) their identity and (iii) the pattern of AP firing, and (iv) these techniques do not allow inhibiting neurons. Both nanostimulation and intracellular stimulation approaches overcome these shortcomings, but nanostimulation is much easier to apply than intracellular recordings. We reason that the application of nanostimulation,
particularly in awake animals, holds great potential for elucidating the cellular mechanisms of brain function.

Acknowledgments

We thank Brigitte Geue and Undine Schneeweiss for technical support, Edith Chorev, Moritz von Heimendahl and Jason Wolfe for comments on the manuscript.

Grants

This work was supported by Erasmus MC, the Bernstein Center for Computational Neuroscience, Humboldt University of Berlin, a VIDI grant (NWO) to A.H., a DAAD stipend to G.D., the FP7 Biotact EU grant and the ERC Neuro-behavior grant to M.B.
Table 1: Overview of electric stimulation techniques

<table>
<thead>
<tr>
<th>Technique</th>
<th>Stimulation current (order of magnitude)</th>
<th>Number of stimulated cells</th>
<th>Spike train control</th>
<th>Cell identification</th>
<th>Application in behaving animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sharp microelectrode recording</td>
<td>~1 nA</td>
<td>1</td>
<td>excellent</td>
<td>possible</td>
<td>difficult</td>
</tr>
<tr>
<td>Whole-cell recording</td>
<td>~1 nA</td>
<td>1</td>
<td>excellent</td>
<td>possible</td>
<td>difficult</td>
</tr>
<tr>
<td>Nanostimulation</td>
<td>~10 nA</td>
<td>1 (occasionally 2)</td>
<td>good</td>
<td>possible</td>
<td>easy</td>
</tr>
<tr>
<td>Microstimulation</td>
<td>~10 µA</td>
<td>unknown</td>
<td>unknown</td>
<td>impossible</td>
<td>easy</td>
</tr>
<tr>
<td>Surface stimulation</td>
<td>~1 mA</td>
<td>unknown</td>
<td>unknown</td>
<td>impossible</td>
<td>easy</td>
</tr>
<tr>
<td>Transcranial magnetic stimulation</td>
<td>unknown</td>
<td>unknown</td>
<td>unknown</td>
<td>impossible</td>
<td>very easy</td>
</tr>
</tbody>
</table>
Figure legends

Fig. 1. Nanostimulation procedure. (a) Nanostimulation procedure illustrated in a barrel cortical neuron recorded in the awake headfixed animal. Upper traces display the juxtacellular potential (‘search’ phase) and which are high-pass filtered during ‘approach’ and ‘entrainment’ to monitor AP firing, while lower traces indicate current injection. During entrainment 200 ms current injections are repeated once every 5 s. Triangles indicate stimulation artifacts. (b) Relationship between total resistance of the juxtacellular circuit ($R_{\text{total}} - R_{\text{pipette}}$) and current intensity required to fire 8-12 APs in a series of experiments in the anesthetized animal ($n = 17$ cells). The black superimposed curve represents the theoretically expected relationship $y = c/x$, where $c$ is a constant which was fitted to the data. (c) Relationship between AP height and average current intensity applied in barrel cortical neurons ($n = 59$) recorded in the awake behaving animal during a single-cell stimulation detection task (Houweling and Brecht 2008).
Fig. 2. Nanostimulation setup and examples from different brain regions. (a) Schematic (top view) of rat head with recording cylinder and head fixation post. (b) Schematic (side view) of the brain exposure and nanostimulation pipette in a chronically prepared animal. Nanostimulation examples from (c) barrel cortex, (d) thalamus and (e) facial nucleus. Reconstructed neurons are shown below the respective voltage traces.
Fig. 3. Spikes elicited by nanostimulation occur randomly during the injection interval. (a) Spike raster plot (top) and post-stimulus time histogram (bottom) of a barrel cortex neuron recorded in the awake behaving animal. (b) Raster plot of the subset of trials in which exactly 13 APs were evoked during current pulses of 10 nA.
Fig. 4. Effect of current intensity on spike frequency in barrel cortex neurons. (a) Reconstruction of a stimulated layer 6 inverted pyramidal neuron with dendritic tree (red); axon was not filled and not reconstructed. Barrels are indicated in brown. L, layer. (b) Example AP discharges of the neuron for nanostimulation at different current intensities. Triangles indicate stimulation onset and offset artifacts. (c) Average AP frequency varied linearly with stimulus intensity in this neuron. (d) Population averages obtained by normalizing evoked AP numbers at 100% nanostimulation current. Error bars in c and d indicate standard deviations.
Fig. 5. Effect of stimulus duration on spike number in barrel cortex neurons. (a) Reconstruction of a stimulated layer 4 pyramidal neuron with dendritic tree (red) and axon (blue). Conventions as in Fig. 4. (b) Example AP discharges of the neuron for nanostimulation at different durations. (c) Average AP number varied linearly with stimulus duration in this neuron. (d) Population averages obtained by normalizing evoked AP numbers at 800 ms stimulus duration. Error bars in c and d indicate standard deviations.
Fig. 6. Negative current nanostimulation can prevent sensory responses in neurons of barrel cortex. (a) Reconstruction of a stimulated layer 6 pyramidal neuron with dendritic tree (red) and axon (blue). Conventions as in Fig. 4. (b) Spike raster plot and post-stimulus time histogram of the neuron while presenting air puff stimuli to the whiskers (top) and when paired with negative juxtacellular current injection (bottom). (c) Population averaged responses for air puff stimuli alone (solid line) and air puff stimuli paired with negative current injection (dotted line) (n = 7). Firing rates for each neuron were normalized using the baseline AP firing rate.
References


Chernomordik LV, Sukharev SI, Popov SV, Pastushenko VF, Sokirko AV, Abidor IG, and Chizmadzhev YA. The electrical breakdown of cell and lipid


Figure 1

(a) Search  Approach  Entrainment

<table>
<thead>
<tr>
<th>Time (s)</th>
<th>Spike Pattern</th>
<th>Current (nA)</th>
<th>Voltage (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>t = 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 115</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 340</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 550</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 584</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 594</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 600</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 611</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 618</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 719</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 742</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t = 762</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(b) Current (nA) vs. $R_{\text{total}} - R_{\text{pipette}}$ (MΩ)

(c) Current (nA) vs. Spike height (mV)
Figure 3
Figure 4


(b) Graph showing the relationship between nanostimulation current (%: 25%, 50%, 75%, 100%) and action potential (AP) frequency (Hz). The graphs display the effect of various current intensities on AP frequency.

(c) Graph showing the normalized AP frequency in relation to nanostimulation current (%: 25%, 50%, 75%, 100%). The normalized frequency is plotted against the nanostimulation current.

(d) Schematic representation of the current application, showing a 100 ms duration with a 2.5 mV Vm and 8 nA Istim.
Figure 6

(a) Diagram showing brain regions.

(b) Graphs showing firing rate over time with air puffs and current.

(c) Graph showing normalized firing rate over time.