LETTERS

Behavioural report of single neuron stimulation in somatosensory cortex

Arthur R. Houweling[1,2] & Michael Brecht[1,2]

Understanding how neural activity in sensory cortices relates to perception is a central theme of neuroscience. Action potentials of sensory cortical neurons can be strongly correlated to properties of sensory stimuli[1] and reflect the subjective judgements of an individual about stimuli[2]. Microstimulation experiments have established a direct link from sensory activity to behaviour[3,4], suggesting that small neuronal populations can influence sensory decisions[5]. However, microstimulation does not allow identification and quantification of the stimulated cellular elements[6]. The sensory impact of individual cortical neurons therefore remains unknown. Here we show that stimulation of single neurons in somatosensory cortex affects behavioural responses in a detection task. We trained rats to respond to microstimulation of barrel cortex at low current intensities. We then initiated short trains of action potentials in single neurons by juxtacellular stimulation. Animals responded significantly more often in single-cell stimulation trials than in catch trials without stimulation. Stimulation effects varied greatly between cells, and on average in 5% of trials a response was induced. Whereas stimulation of putative excitatory neurons led to weak biases towards responding, stimulation of putative inhibitory neurons led to more variable and stronger sensory effects. Reaction times for single-cell stimulation were long and variable. Our results demonstrate that single neuron activity can cause a change in the animal's detection behaviour, suggesting a much sparser cortical code for sensations than previously anticipated.

Based on its volume[7] and density of neurons[8], rat somatosensory cortex contains an estimated two million neurons. The detection of single-cell stimulation might therefore be a difficult task, and we adopted a behavioural paradigm designed for observing single-cell effects (Fig. 1a). We first trained animals to report short (200 ms) trains of microstimulation pulses. Stimulation of somatosensory cortex evokes tactile sensations in humans[9], and animal studies have demonstrated an interchangeability of tactile stimuli and cortical microstimulation[10]. We mainly targeted deep cortical layers, where microstimulation detection thresholds are lowest in rat barrel cortex[11]. Tongue lick responses were rewarded with a drop of water and counted as a hit if a lick occurred within 100–1200 ms from stimulus onset (Fig. 1b). Animals typically learned this microstimulation report task in a single session and detection thresholds decreased to 2–5 μA within days (Supplementary Fig. 1). These values are comparable to the lowest cortical microstimulation detection thresholds reported in humans[12] and animals[11,13]. To be able to detect potentially weak effects of single-cell stimulation, we encouraged guessing (a non-conservative response criterion) by introducing only mild negative reinforcement (a 1.5 s time-out) for false-positive responses (licks without preceding stimulation).

Once animals responded consistently to low microstimulation currents, we approached a cortical neuron closely with a glass pipette and evoked short (200 ms) trains of action potentials by juxtacellular stimulation, a technique developed to label individual neurons[14]. Juxtacellular stimulation currents (3–43 nA, mean 12.6 nA) strongly modulated action potential firing in barrel cortex neurons (Fig. 1c, d). On average, we evoked 14.2 ± 6.5 (s.d.) action potentials during current injection, a 25-fold increase over the average spontaneous firing rate. The close apposition of neuron and pipette in the juxtacellular configuration in behaving animals typically resulted in short experimental sessions per cell. Microstimulation in the vicinity of the neuron (at an average distance of about 75 μm) was adjusted such

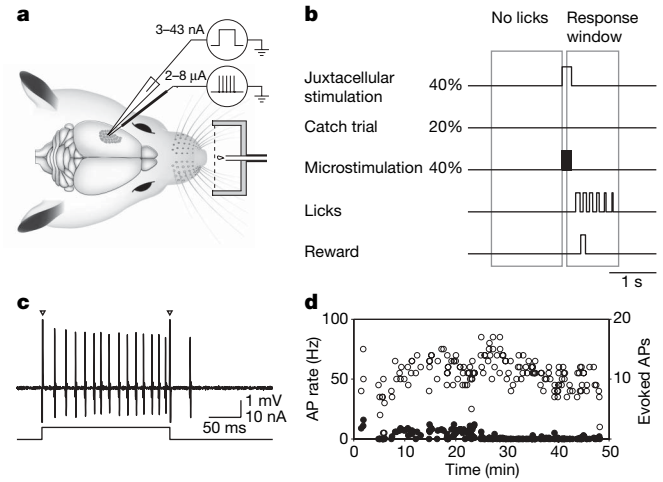

Figure 1 | Behavioural setup and single-cell stimulation. **a**, Stimulation experiments were performed in the barrel cortex of awake rats. Animals responded to stimulation by interrupting a light beam (dashed line) with multiple tongue licks. The time of the first lick was taken as the reaction time and reward was delivered for correct responses (right). Top, single-cell stimulation pipette with stimulation current wave form (upper) and tungsten microelectrode with stimulation pulse train (lower). **b**, Three types of stimulus were presented at random intervals (Poisson process, mean 3 s): microstimulation (2–8 μA) (40% probability), juxtacellular single-cell stimulation (40%) and no (or subthreshold) current injection 'catch' trials (20%). Licks within the interstimulus interval led to an additional 1.5 s delay to presentation of the next stimulus (left box) and were rewarded after a stimulus (right box) for all three trial types. **c**, Single-cell stimulation trial by juxtacellular current injection. Triangles indicate stimulation onset and offset artefacts. **d**, Evoked action potentials (open circles) in a series of stimulation trials. Spontaneous action potentials (solid circles) were quantified for 1 s before each stimulation. The left *y* axis label applies to both spontaneous and evoked action potentials; the right *y* axis label applies to evoked action potentials.

[1]Bernstein Center for Computational Neuroscience and Humboldt University Berlin, Philippstrasse 13, House 6, 10115 Berlin, Germany. [2]Department of Neuroscience, Erasmus Medical Center, PO Box 2040, 3000 CA, Rotterdam, The Netherlands.

that current intensities were close to the animal's detection threshold, resulting in an average hit (detection) rate of 75%. The action potential firing of most cells was affected during and after microstimulation (Supplementary Fig. 2).

Microstimulation and single-cell stimulation trials were randomly interleaved with 'catch' trials (with no or subthreshold current injection) (Fig. 1b). In paradigms with random stimulus presentation times, catch trials can be used to estimate chance performance and to guard against inadvertent cues[15]. To assess single-cell effects when the animal was attentive, we confined our analysis to those single-cell stimulation and catch trials flanked by correct microstimulation responses.

Figure 2 shows an experiment on a regular spiking layer 5b pyramidal neuron with a slender apical dendrite (Fig. 2a). Juxtacellular stimulation evoked on average 9.1 action potentials during the current injection (Fig. 2b top). Lick responses (red squares) occurred mainly after single-cell stimulation (Fig. 2b top) and microstimulation (Fig. 2b bottom), but only once after no stimulation catch trials (Fig. 2b middle). Quantification of responses (Fig. 2c) suggests that the animal reported single pyramidal cell activity. Even though this neuron was one of the cells with the strongest behavioural effects, this effect was not significant on the single neuron level ($P = 0.099$, Fisher's exact test). This is not unexpected given the limited number of trials (see Methods).

A population analysis revealed, however, that single-cell stimulation biased animals towards responding. Figure 3a shows, for 51 neurons, that animals responded significantly more often in single-cell stimulation trials (mean hit rate 22.0%) than in no-current-injection catch trials (mean false-positive rate 17.9%; $P = 0.022$). To test if single-cell detection was dependent on the firing of the stimulated neuron rather than on inadvertent cues associated with the current injection, we stimulated a further set of 19 neurons. We applied single-cell stimulation as usual, but instead of the no-current catch trials we presented subthreshold (10% of the single-cell stimulation current) catch trials. Subthreshold current injections activated neurons only weakly or not at all. Animals also responded significantly more often in single-cell stimulation trials than in subthreshold catch trials (Fig. 3b; mean hit rate 27.4%; mean false-positive rate 20.6%; $P = 0.019$). Stimulation effects were distributed evenly across animals (Supplementary Fig. 3). Having verified that single-cell stimulation led to significant biases in two independent sets of neurons (Fig. 3a, b), we wanted to confirm that this effect did not result from the inadvertent stimulation of neighbouring neurons or other nonspecific effects. Thus, we injected current (25 nA, twice the average current applied with juxtacellular stimulation) through the pipette into extracellular space (instead of applying it to a neuron). These control experiments showed that animals did not report current injection into extracellular space (Fig. 3c; $n = 90$; mean hit rate 18.7%; mean false-positive rate 19.0%; $P = 0.598$). To test if single-cell stimulation effects (Fig. 3a, b) were different from those of extracellular current injection (Fig. 3c), we compared effect size (hit rate − catch trial response rate) across those two data sets and observed a significant difference ($P = 0.008$). Finally, we tested if single-cell stimulation effects were specific to the attended (and trained) cortical area. As before, microstimulation was applied to barrel cortex, but we now stimulated single neurons in visual cortex. Animals did not report single-cell stimulation in visual cortex (Fig. 3d; $n = 21$; mean hit rate 21.2%; mean false-positive rate 20.1%; $P = 0.319$), suggesting that stimulation effects are specific to the attended cortical area.

Further observations show that the animals' responses were caused by the stimulation of single and not multiple neurons. (1) Juxtacellular stimulation currents were approximately three orders of magnitude lower (3–43 nA) than those required for evoking motor or sensory responses with microstimulation (2–200 μA). (2) Although we occasionally observed the inadvertent stimulation of a second neuron by the appearance of a second large action potential waveform in our recordings, such inadvertent stimulation was rare (accounting for only about 1% of evoked action potentials across experiments; Supplementary Fig. 4). All results presented here were also significant when single-cell stimulation trials with secondary action potentials were excluded. (3) Firing rates of more distant cells (with action potentials less than 0.5 mV) were not modulated (Supplementary Fig. 5). (4) Juxtacellular labelling typically fills single neurons[14].

Because microstimulation in barrel cortex can evoke whisker movements, we combined stimulation experiments with whisker tracking to assess if rats sense single-cell stimulation indirectly by detecting movements. Our data argue against such an indirect mechanism: near detection threshold microstimulation did not evoke movements even though it was reported (Supplementary Fig. 6a) and single-cell stimulation did not evoke whisker movements (Supplementary Fig. 6b).

The bias towards responding evoked by single-cell stimulation was weak on average (approximate 5% effect size: single-cell stimulation hit rate – catch trial response rate). As illustrated in Supplementary Fig. 7, the strength of the effect depended greatly on the animal's overall response rate. When animals were conservative (low response

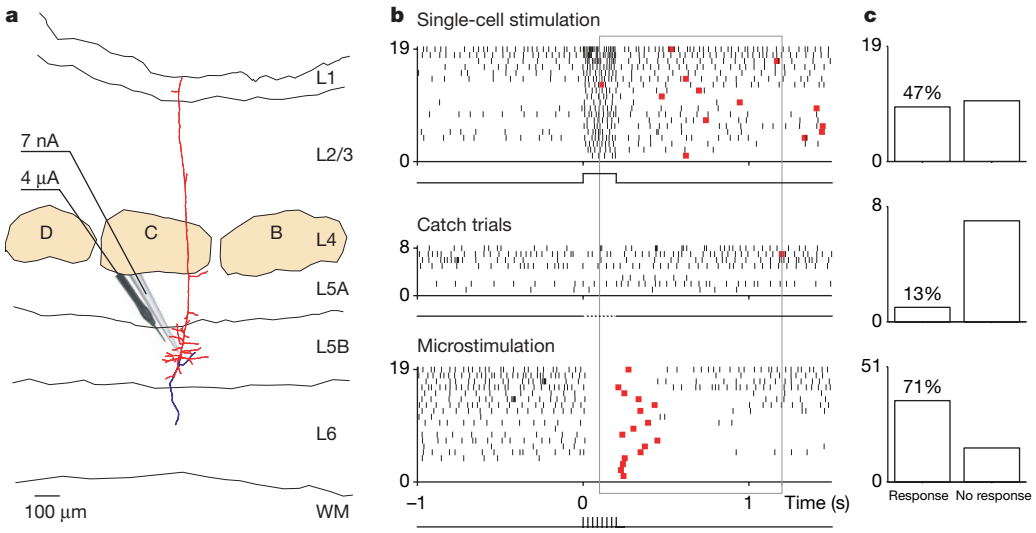

Figure 2 | Behavioural responses to stimulation of a single layer 5b pyramidal neuron. a, Reconstruction of the stimulated neuron with dendritic tree (red) and axon (blue, incompletely filled). Superimposed is a micrograph of a stimulation pipette and a tungsten microstimulation electrode aligned along the electrode track. Barrel rows (brown) are labelled with letters. L, layer; WM, white matter. **b**, Action potential (ticks) raster plots and first lick responses (red squares) during juxtacellular single-cell stimulation trials (top), no-current-injection catch trials (middle) and 19 randomly selected microstimulation trials (bottom). The neuron was inhibited during and after microstimulation (stimulation current, 4 μA). **c**, Quantification of responses to single-cell stimulation, catch trials and microstimulation.

rate) the effect was small (and not significant); at high response rates, however, the effect size was about 9% (and significant). Most interestingly, the variance of stimulation-evoked sensory effects was greater for putative interneurons than putative excitatory cells ($P = 0.0023$, see Methods), which we distinguished based on spike width and firing pattern (Supplementary Fig. 8). Whereas stimulation of putative excitatory neurons led to weak but significant biases towards responding, stimulation of putative inhibitory neurons led to stronger and more variable sensory effects (Fig. 3e). In particular, in 3 out of 11 putative interneurons the effect was stronger than in any of the 59 putative excitatory cells or 90 control experiments. In two of these three putative interneurons most hits were observed for trials in which the evoked firing rates were not higher than the population average, suggesting that interneuron action potentials are in some cases more readily detected than action potentials of excitatory cells.

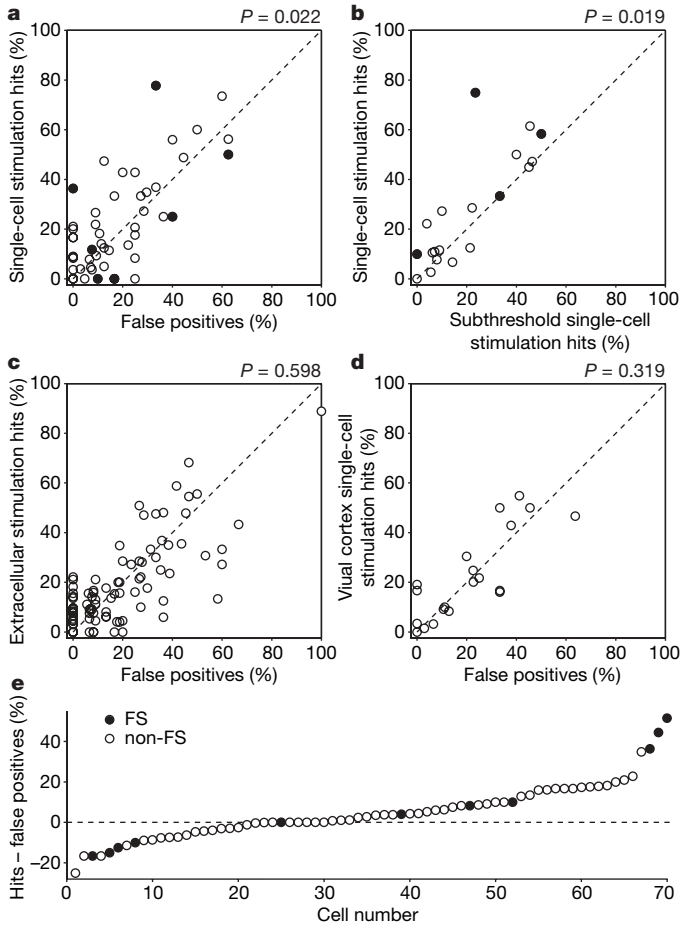

Figure 3 | Initiation of action potentials in single barrel cortex neurons causes biases towards responding. **a**, Response rates for single-cell stimulation trials (hits) versus no-current-injection catch trials (false positives) ($n = 51$ neurons; note several points coincide). Fast spiking, putative interneurons (filled circles); non-fast spiking, putative excitatory neurons (empty circles). **b**, Response rates for single-cell stimulation trials (hits) versus subthreshold current injection catch trials ($n = 19$ neurons). Conventions as in **a**. **c**, Response rates for trials in which we applied 25 nA (twice the average juxtacellular stimulation current) into extracellular space (hits) versus no-current-injection catch trials (false positives) ($n = 90$ stimulation sites). **d**, Response rates for single-cell stimulation trials (hits) versus no-current-injection catch trials (false positives) for visual cortex neurons ($n = 21$), while microstimulation was applied in barrel cortex. Animals had been trained to report microstimulation in barrel cortex. **e**, Distribution of sensory effects (single-cell stimulation hit rate − catch trial response rate) across putative interneurons and putative excitatory neurons in barrel cortex single-cell stimulation experiments (conventions as in **a**).

Reaction times for single-cell stimulation were long and variable (Figs 2 and 4a, b) compared with microstimulation responses (Fig. 4c). Although effect size could be considerable in individual cells (Fig. 2), we did not observe single-cell responses with close to 100% hit rates and short reaction times as observed often in microstimulation trials. Thus, single cells never led to a strong, perceptually saturating signal. Microstimulation at 2–8 μA presumably activates multiple neurons, which may account for the difference between single-cell stimulation and microstimulation. Even smaller currents (less than 2 μA) are known to activate cortical neurons[16]. It remains to be seen if such currents lead to a microstimulation performance comparable to that of single-cell stimulation.

The combination of single-cell stimulation and control experiments shows that the activity of single sensory cortical neurons can lead to a behaviourally reportable effect. It has been estimated that a single barrel cortical column contains approximately 8,500 excitatory cells that generate about 1,550 spontaneous action potentials in a 200 ms period and about 4,000 action potentials in response to a small (3.3°) whisker deflection[17] that is close (about 60% hit rate) to the animal's detection threshold[18]. Given these numbers it is surprising that adding approximately 14 action potentials over 200 ms in a neuron is detectable. Other measurements suggest lower rates of

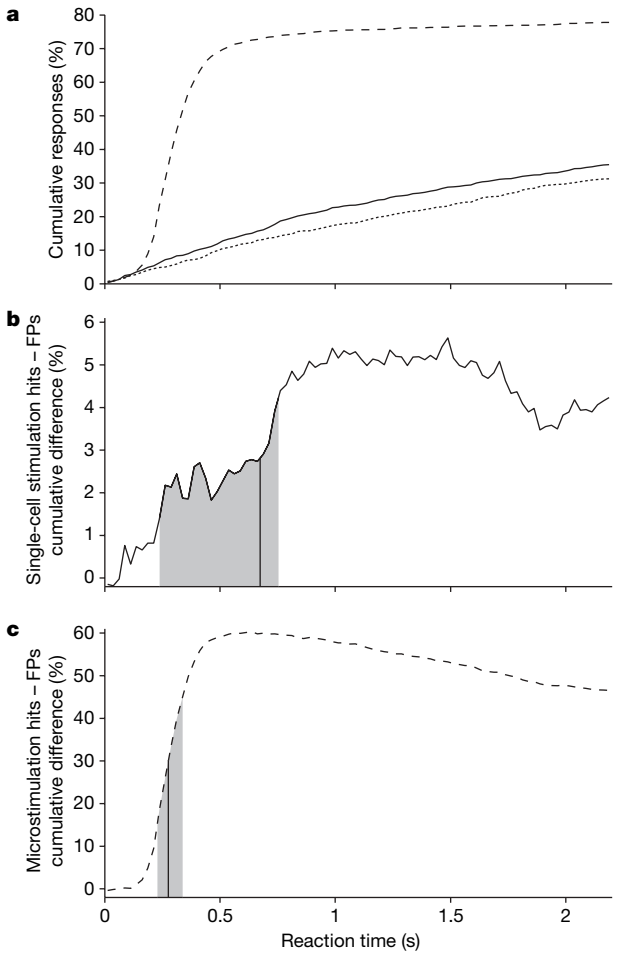

Figure 4 | Reaction times for single-cell stimulation are long and variable compared with microstimulation responses. **a**, Cumulative distribution of reaction times for microstimulation (dashed), single-cell stimulation (solid) and catch trials (dotted). **b**, Difference of the cumulative distributions of reaction times for single-cell stimulation and catch trials. This isolates the contribution of single-cell stimulation from false-positive responses. The vertical line marks the time where 50% of the peak difference is reached; the grey area marks the time from 25% to 75% of the peak difference. **c**, Difference of the cumulative distributions of reaction times for microstimulation and catch trials. Conventions as in **b**.

ongoing[19–21] and sensory-evoked[22–25] cortical action potential activity. The detectability of single-cell stimulation might therefore be related to the sparseness of cortical activity. A single cortical pyramidal cell connects to several thousand postsynaptic neurons[26], but most of these connections are weak[27]. Likewise, a single inhibitory neuron connects to thousands of local neurons. Depending on ongoing membrane potential fluctuations, variable sets of postsynaptic cells may become activated or suppressed, which might contribute to the variable reaction times. Some models of sensory decision making contain a temporal integration step during which sensory evidence is accumulated[28]. It is conceivable that the weak single-cell signals require longer temporal integration, thereby contributing to the long reaction times. The mechanisms that hold the sensory information between single-cell stimulation and reaction remain to be determined.

Our finding that stimulation of putative interneurons (which project locally) can lead to strong sensory effects suggests that (1) local circuits are involved in the read-out of single-cell activity and that (2) read-out mechanisms are sensitive to suppression of action potentials. Cortical microstimulation evokes pronounced and long-lasting inhibition (Fig. 2 and Supplementary Fig. 2). Thus, our animals might have been trained to detect the suppression of activity. The present results, with the classic single afferent stimulation experiments by Vallbo and colleagues[29] and single-cell stimulation experiments in rat motor cortex[30], demonstrate the behavioural relevance of single neuron activity. These studies establish a reverse physiology approach in which one analyses responses to cellular activity rather than cellular responses as in classical physiology. In further studies it should be possible to establish how the frequency and number of action potentials are related to the evoked sensations.

METHODS SUMMARY

Animals were trained to report microstimulation (40 cathodal pulses at 200 Hz, 0.3 ms pulse duration) applied to the barrel cortex through a tungsten microelectrode at a depth of 1,500 μm. In the first training session, current intensities no greater than 200 μA were applied; subsequently current intensity was decreased according to detection performance. During training animals were put on a water restriction schedule with daily access to water *ad libitum* for one hour after the experiment. Once the animal performed at detection thresholds no greater than 5 μA in at least one block of trials on two consecutive days, we switched to the single-cell stimulation report task; here microstimulation currents were on average 5.0 ± 1.6 μA. To stimulate single neurons close to the microstimulation site, we glued a tungsten microelectrode close to the tip of a glass pipette (average tip separation approximately 75 μm). The construct was inserted through the intact dura to a mean depth of $1{,}400 \pm 271$ μm, whereby the actual depth from the cortical surface was less because of dimpling and oblique penetrations. From the histologically identified neurons it appears that most single-cell stimulation experiments were performed in cortical layers 4, 5A and 5B. Cells were classified as fast spiking neurons (putative interneurons) if the action potential width was no greater than 0.4 ms (peak to trough) and/or if they responded with at least 50 action potentials (that is, at least 250 Hz) during at least one 200 ms current injection (see Supplementary Fig. 8). The remainder of cells were classified as non-fast spiking (putative excitatory) cells.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to A.R.H. (arthur.houweling@bccn-berlin.de) or M.B. (michael.brecht@bccn-berlin.de).

METHODS

Experimental procedures. We used standard surgical and electrophysiological techniques to prepare animals ($n = 15$ Wistar rats, about P35 at the day of surgery) for chronic, head-fixed recording of the barrel cortex (P3, L5 relative to bregma)[20]. Glass pipettes for single-cell stimulation were filled either with intracellular solution containing (in mM): K-gluconate 135, HEPES 10, Na_2-phosphocreatine 10, KCl 4, MgATP 4, and Na_3GTP 0.3 (pH 7.2), or Ringer's solution containing NaCl 135, KCl 5.4, HEPES 5, $CaCl_2$ 1.8, and $MgCl_2$ 1 (pH 7.2). Penetrations were targeted to the E, D and C whisker row representation of barrel cortex. All experimental procedures were performed according to Dutch and German guidelines on animal welfare under the supervision of local ethics committees.

Analysis. Cells were included if at least five catch trials and five single-cell stimulation trials had been presented that satisfied our inclusion criterion. All single-cell stimulation and catch trials were included for which the animal responded to both the preceding and the succeeding microstimulation. We also included all trials where the animal responded only to either the immediately preceding or immediately succeeding microstimulation. All numbers on single-cell stimulation experiments refer to these included trials. For our barrel cortex experiments an average of 30.2 single-cell stimulation trials and 17.7 catch trials were included per cell. Applying the inclusion criterion led to the exclusion of many trials with low response rate (on average 14.0 single-cell stimulation trials and 7.7 catch trials). When all trials were considered, animals still responded significantly more often in single-cell stimulation trials than in catch trials (data not shown). From these trial numbers it becomes clear that it is difficult to obtain a significant result on the single neuron level. Monte Carlo simulations show that approximately 480 single-cell stimulation trials (about 16 times more than our average) are required to obtain a significant outcome with 90% probability (one-sided binomial test by normal approximation, $\alpha = 0.05$) assuming a 10% effect size (30% hits, 20% false positives).

As we trained animals to report stimulation of the barrel cortex, we tested the prediction that single-cell stimulation led to responses (hits). Thus, differences between hit rates and false-positive rates were evaluated by using a one-sided, paired *t*-test, and differences between single-cell stimulation experiments and control experiments by using a one-sided, unpaired *t*-test. However, all results presented here were also significant when applying two-sided *t*-tests or when using Monte Carlo simulations of the statistical distributions. To test non-parametrically if the variance of sensory effects was greater for putative interneurons than excitatory neurons (see Fig. 3e), we ranked all neurons on effect size and computed the variance of interneuron ranks and compared this with a Monte Carlo distribution based on random permutations of the ranks.

It is our impression that the interneuron inclusion criteria (action potential width no greater than 0.4 ms and/or a response of at least 50 action potentials during at least one 200 ms current injection) were conservative and may have led to the false classification of potential interneurons as excitatory cells. The difference in sensory effects between putative interneurons and excitatory cells persisted when more inclusive interneuron inclusion criteria were chosen (data not shown). Further observations support our classification scheme. First, morphologically identified excitatory neurons were correctly classified as putative excitatory cells (Supplementary Fig. 8). Second, maximum discharge patterns of putative excitatory cells showed significantly more irregular spike intervals than those of putative interneurons ($P = 0.037$, two-sided *t*-test on the coefficient of variation), which showed very little or no accommodation (Supplementary Fig. 8) in agreement with *in vitro* studies[31]. Spontaneous firing rates (measured in a 30 s period at the onset of each experiment before any current injections) were similar between putative interneurons (mean 2.1 Hz) and putative excitatory neurons (1.6 Hz).

Histology. On the last day of experiments with an animal, we included biocytin (1.5%) in the stimulation pipette and processed brains as described previously[22]. Even though combining cell recovery and behavioural measurements was difficult, we recovered ten neurons or fragments of neurons. Three of these entered our data set (satisfied the trial number inclusion criterion) and were identified as excitatory neurons (see Supplementary Fig. 8). Combining cell recovery and behavioural measurements is difficult because one is limited to a single session per animal, and even aborted single-cell stimulation attempts can stain neurons, which complicates neuron identification. We evaluated the tissue damage by electrode constructs in 8 out of 15 animal brains. In these brains the damage to the barrel cortex target area was rated as either minimal or weak.

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