COOLING IN CAT VISUAL CORTEX: STABILITY OF ORIENTATION SELECTIVITY DESPITE CHANGES IN RESPONSIVENESS AND SPIKE WIDTH

C. C. GIRARDIN*[1] AND K. A. C. MARTIN

Institute of Neuroinformatics, ETH/University of Zurich, Winterthurerstraße 190, 8057 Zurich, Switzerland

Abstract—Cooling is one of several reversible methods used to inactivate local regions of the brain. Here the effect of cooling was studied in the primary visual cortex (area 17) of anaesthetized and paralyzed cats. When the cortical surface temperature was cooled to about 0 °C, the temperature 2 mm below the surface was 20 °C. The lateral spread of cold was uniform over a distance of at least ~700 μm from the cooling loop. When the cortex was cooled the visually evoked responses to drifting sine wave gratings were strongly reduced in proportion to the cooling temperature, but the mean spontaneous activity of cells decreased only slightly. During cooling the strongest effect on the orientation tuning curve was on the peak response and the orientation bandwidth did not change, suggesting a divisive mechanism. Our results show that the cortical circuit is robust in the face of cooling and retains its essential functionality, albeit with reduced responsiveness. The width of the extracellular spike waveform measured at half height increased by 50% on average during cooling in almost all cases and recovered after re-warming. The increase in spike width was inversely correlated with the change in response amplitude to the optimal stimulus. The extracellular spike shape can thus be used as a reliable and fast method to assess whether changes in the responses of a neuron are due to direct cooling or distant effects on a source of its afferents. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: area 17, orientation tuning, cortical circuitry, reversible inactivation, cooling loop, spike broadening.

For reversible inactivation of a large area of brain, cooling is much preferred to drugs as a method (e.g. Schiller and Malpeli, 1977; Sherk, 1978; Girard and Bullier, 1989; Ferster et al., 1996; Lomber et al., 1999; Huang et al., 2007). The cold affects cell responses by reducing transmitter release and the spike conductance's (Brooks, 1983). *In vitro* studies in rat visual cortex have shown that even moderate cooling to 22–25 °C lowers the transmitter release probability of synapses (Volgushev et al., 2004) and increases the transmission failure rate (Hardingham and Larkman, 1998). In addition to synaptic effects, cooling also changes the biophysical properties of the cells. Vol-

gushev and colleagues have shown that voltage-gated sodium channels are less sensitive to cold than are voltage-gated potassium channels (Volgushev et al., 2000b). This results in a slower repolarisation and a broader action potential. Other changes in the basic cell properties such as higher input resistance and higher excitability were reported at lower temperatures (Volgushev et al., 2000a,b). Very low temperature can also influence the conduction of action potentials along axons (Brooks, 1983).

Because of the location of the cooling device it is difficult to inactivate completely the deeper neural tissue by surface cooling. To avoid damaging the tissue in contact with the cooling device by freezing, the cooling temperature must be kept above 0 °C. Thus a surface temperature of 3 °C leaves the tissue 2 mm deeper at around 20 °C (Girard and Bullier, 1989; Lomber et al., 1999) and although they are less responsive, cells can still spike at 20 °C (Brooks, 1983). With the cooling probe at 3 °C, the temperature of the cortex 2 mm lateral from the probe rises to 20 °C (Lomber et al., 1996) and thus normal brain temperature (35–37 °C) is only reached beyond 2 mm lateral from the cooling coil. For this reason, it is important to ensure that any changes thought to be due to deactivation of afferent input (e.g. Sherk, 1978; Michalski et al., 1993; Casanova et al., 1997; Huang et al., 2007) are not simply a direct effect of the spread of cold to the recorded neurons.

The robustness of orientation selectivity is a general property of visual cortical cells maintained through changes in temporal frequency (Moore et al., 2005), speed (Hammond and Smith, 1983), absolute contrast (Sclar and Freeman, 1982; Skottun et al., 1987), or level of contrast adaptation (Ohzawa et al., 1982, 1985) (but not spatial frequency, see Hammond and Pomfrett, 1990). Even the dramatic effect of cooling on the cell's biophysical properties does not alter orientation selectivity (Michalski et al., 1993; Casanova et al., 1997). The main effect is a proportional decrease of the response amplitude.

One goal of the present study was to examine in more detail the changes in the responsiveness of individual neurons when the activities of large portions of the local circuits in which they are embedded are reduced by cooling. A second aim was to identify a simple means of distinguishing direct effects of cooling from indirect effects. As a control we first measured the temperature within the cortex while the surface was cooled in order to estimate the spread of the cold within the tissue from an omega-shaped cooling coil placed on the surface. In separate experiments we recorded extracellularly from single cells located within

[1] Present address: University of Konstanz, Biologie-Neurobiologie (M11), 78457 Konstanz, Germany.
*Corresponding author. Tel: +49-7531-88-2117; fax: +49-7531-88-3894.
E-mail address: Cyrille.Girardin@uni-konstanz.de (C. C. Girardin).
Abbreviations: HWHH, half width at half height; RPR, response peak ratio; TWR, tuning width ratio.

the cooled region of area 17 and measured their orientation tuning with moving bars and drifting sine wave gratings. We compared both the spontaneous activity and the visually-evoked responses to the stimuli under cooling and control temperatures. Finally, we measured the width of spikes recorded extracellularly, to determine whether changes in the extracellular spike width could be used online as a fast and reliable method to assess whether changes in the response properties of a cell are due to direct cooling or due to inactivation of circuits that provide its input.

EXPERIMENTAL PROCEDURES

Animal preparation

Five adult cats were used for these experiments and prepared for *in vivo* recordings. The experiments were carried out under authorization of the Cantonal Veterinary Authority of Zurich to KACM. The number of animals used and their suffering was minimized. The detailed procedure has been described earlier (Girardin et al., 2002). Briefly, anaesthesia was induced with a mixture of xylazine (0.5 mg/kg, Rompun; Bayer, Leverkusen, Germany) and ketamine (10 mg/kg, Narketan, Chassot, Bern, Switzerland) and maintained with saffan (through a venous cannula, ~0.1–0.2 ml/kg/h, Schering Plough Animal Health, Welwyn Garden City, UK). Animals were paralyzed (mixture of gallamine triethiodide (5 mg/kg/h, May & Baker, UK) and d-tubocurarine chloride (0.5 mg/kg/h, Sigma Aldrich, Buchs, Switzerland)) and ventilated with 30/70% mixture of O_2/N_2O through a tracheal cannula. Halothane (0.5%, Arovet AG, Zollikon, Switzerland) was used for potentially painful procedures (e.g. durotomy) and during initial surgery (1–2%). Electrocardiogram (EEG), blood pressure, rectal temperature, and expired CO_2 were monitored continuously and kept in physiological ranges. Neutral power lenses and atropine were applied on the eyes. Eyes were refracted and focused at a distance of 57 cm. A craniotomy was made at Horsley–Clarke sterotaxic coordinates of zero to −6 mm anterior-posterior and from the midline to the lateral sulcus. This exposed the central representation of the visual field in area 17. The cooling coil was shaped to fit snugly within the craniotomy over the lateral gyrus. All single unit recordings were made within the diameter of the loop. A plastic recording chamber was glued on the skull with dental cement and filled with agar to stabilize the cortex. Recordings were made with high impedance (5–10 MΩ) micropipettes (Hugo Sachs Elektronik, March-Hugstetten, Germany) filled with biocytin (1.5% (Sigma Aldrich) in 0.5 M CH_3KSO_4 and 0.01 M phosphate buffer) or Pontamine Sky Blue (2% (Sigma Aldrich) in 0.5 M sodium acetate and 0.5 M sodium chloride).

Visual stimulation and data acquisition

The receptive fields of the recorded cells were plotted by hand on a tangent screen and then computer controlled bars, and sine wave gratings were displayed to determine the orientation tuning. The orientation of the bar or gratings varied randomly. The temporal and spatial frequencies of the stimuli were adjusted to optimize the cell response. The stimuli were displayed on a screen (Tektronix 608 monitor, phosphor P31, USA). Data were collected via the software Spike2 (CED, Cambridge, UK). The analogue recorded trace was passed to an electronic spike trigger (Neurolog System of Digitimer, NL200) to record only the spike events. These were used to construct the orientation tuning curves. The raw analogue signal was anti-alias filtered and sampled at 5 kHz. These data were used to measure the extracellular spike width.

Cortical cooling

Cortical cooling was performed with a cooling loop described by Lomber et al. (1999). The loop consisted of a stainless steel tube (inner diameter: 0.2–0.35 mm, outer diameter 0.4–0.65 mm, Robert Helwig GmbH, Berlin, Germany) through which chilled ethanol was pumped by variable speed peristaltic pump. The ethanol was cooled within an ethanol/dry ice bath at −76 °C. The loop, which had an outside diameter of about 2 mm, was shaped by hand to fit the curved surface of the cortex. The temperature of the loop was measured using a thermocouple (Omega, Deckenpfronn, Germany) soldered on the loop and connected to a digital thermometer (Model HH-25 TC from Omega). Because of differences in thermal conduction for metal versus the pia and brain, the temperature measure on the loop was a few degrees lower that the actual brain surface. The temperature could be accurately controlled (to 0.5 °C) by changing the flow rate of ethanol through the loop. During single cell recording the temperature was always kept above 0 °C to avoid damaging the surface of the brain. All tubes from the cooling setup (except the cooling loop itself) were flexible silicon tubes. In one animal additional temperature measurements were made within the brain with a thermocouple, constructed using 25 µm thick copper and constantan wires (Omega). A copper wire was inserted in one barrel of 2-barreled pipette and a constantan wire was inserted in the other barrel. The constantan wire was pulled out of the pipette tip and inserted in the other barrel so that it touched the copper wire; temperature was measured at the point of contact between both wires. The total tip size of this construction was about 200 µm.

Data analysis

The mean response was used to calculate the orientation tuning curves during control, cooled, and recovery epochs. The orientation tuning curves were fitted with a Gaussian curve and the half width at half height (HWHH) was measured from the fitted curve. For untuned cells (or when cell response was almost zero for all orientations due to cooling) the HWHH was defined as half of the range of the stimuli used to measure the orientation tuning curve. For every recording 20–300 spikes were randomly selected from a period of about 5 min, at a stable temperature. For each spike, 20 sampled data points (4 ms) were fit with a spline (MATLAB built-in function). The full width at half height was measured on this smoothed version of the spike. Recordings with less than 20 spikes were discarded.

RESULTS

Temperature measurements

In one animal the temperature was recorded with a thermocouple advanced into the primary visual cortex up to a depth of 3 mm (Fig. 1A). To study the short range lateral spread of the cold the temperature was measured at two locations relative to the cooling loop: in the centre of the loop (lateral distance to the loop was about 700 µm) and immediately next to the loop (Fig. 1B). The surface temperature was kept constant (Fig. 1C) while the measurement probe was lowered into the brain in 150 µm steps. The measurements were repeated at two different surface temperatures (0 °C and 8 °C, grey and black symbols, respectively). Since the temperature varied linearly with depth a linear regression was used to calculate the gradient. At a surface temperature of 0 °C the gradient was 8.5 °C/mm and at a surface temperature of 8 °C the gradient was 6 °C/mm. The temperature recorded in the cortex was the same for both positions of the probe (centre
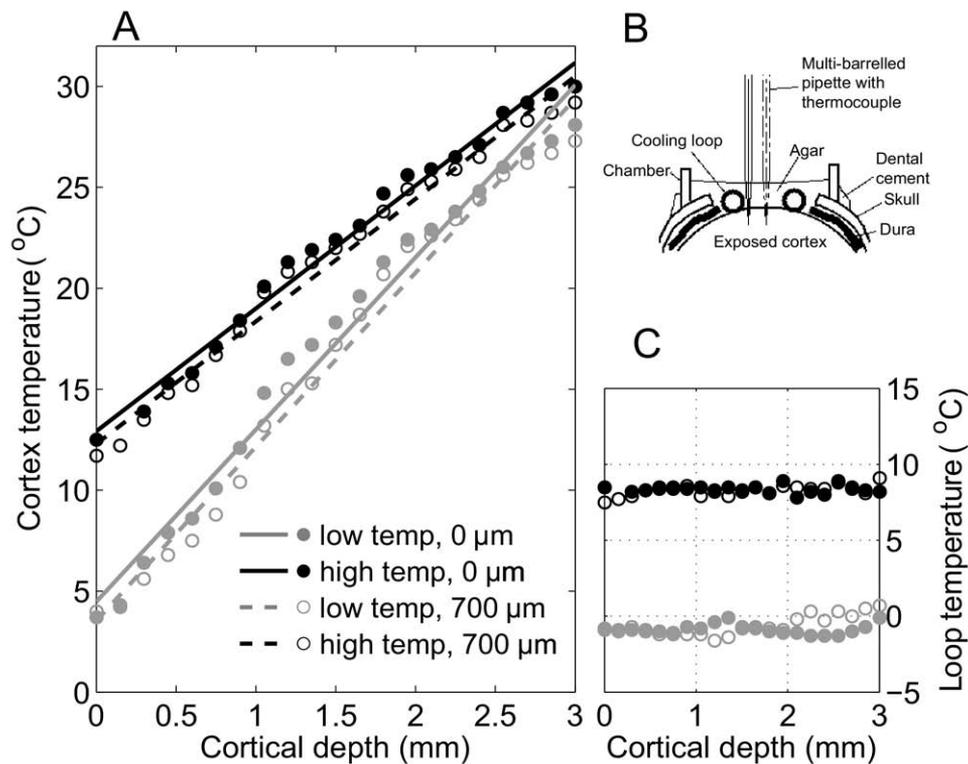
Fig. 1. Temperature measurements in the cortex. (A) Temperature in the cortex relative to depth and distance to cooling loop. Open circles show measurements performed in the centre of the loop (700 μm see panel (B) and closed circles show measurements performed next to the loop. Grey symbols are for measurements performed at low temperature (0 °C, see panel C) and black symbols are for measurements performed at high temperature (8 °C). Full lines are linear regressions through closed circles and dashed lines are linear regressions through open circles. (B) Cross section through the temperature measurement setup. The thermocouple was either inserted in the centre of the loop (dashed thermocouple, corresponding to open circles and dashed lines in (A) or near the loop (full line thermocouple, corresponding to the closed circles and the full lines in (A)). (C) Temperature of the cooling loop (surface temperature) during the measurements shown in (A). Grey and black symbols for low (0 °C) and high (8 °C) temperature, respectively. Open and closed circles for measurements at the centre (700 μm) of the loop and next to the loop, respectively. Symbols overlap in some cases.

of the loop or next to the loop), showing that the lateral and the radial spread of the cold is the same over the 700 μm tested here. Moreover the data show that a surface temperature of 0 °C is just sufficient to reduce the tissue temperature to 20 °C at 2 mm below surface. This means that the deep layers are unlikely to be completely inactivated, since we recorded visually evoked responses in neurons near the surface whose temperatures were probably below 20 °C (Fig. 3).

Spontaneous response and orientation selectivity

Figure 2 shows an example of a cell that was recorded at control temperature and at two different surface temperatures (15 °C and 5 °C). The cell was also recorded after the cortex had rewarmed ('recovery'). The stimulus was a drifting sine wave grating presented for 3.3 s. Only the response to the preferred orientation is shown in Fig. 2. The cell response was still modulated by the stimulus even when the number of spikes was very low due to cooling (see panel C).

The spontaneous activity was analyzed for 59 control/cooled pairs (37 cells because some cells were tested with two different cooling temperatures). During cooling the spontaneous activity decreased significantly in 33 cases, increased in two cases and did not change significantly in 24 cases (2-sample t-test, $P<0.01$, data not shown). All cells for which there was no significant change already had a very low spontaneous activity (<1 spike/sec). Across all control/cooled pairs the mean spontaneous response during the control period (3.35±0.80 spikes/sec, mean±SEM) was significantly higher than during cooling (1.16±0.26 spikes/sec) periods (2-sample t-test, $P=0.01$, Fig. 3). A recovery recording was obtained in 29 cells. The spontaneous activity recovered in 15 cells (no significant difference with control), was larger in nine cells, and smaller in five cells. At the population level the mean spontaneous activity was not significantly different for control and recovery (2.4±0.8 spikes/sec for control and 2.14±0.7 spikes/sec for recovery, 2-sample t-test, $P=0.8$). The difference in spontaneous activity was also significant between control (2.39±0.66 spikes/sec) and cooling (0.88±0.25 spikes/sec, 2-sample t-test, $P=0.03$) if only the 29 cells with a recovery recording were considered.

The orientation tuning curves of four cells recorded in the same penetration are presented in Fig. 4. In each case the cell was first recorded at control temperature. The
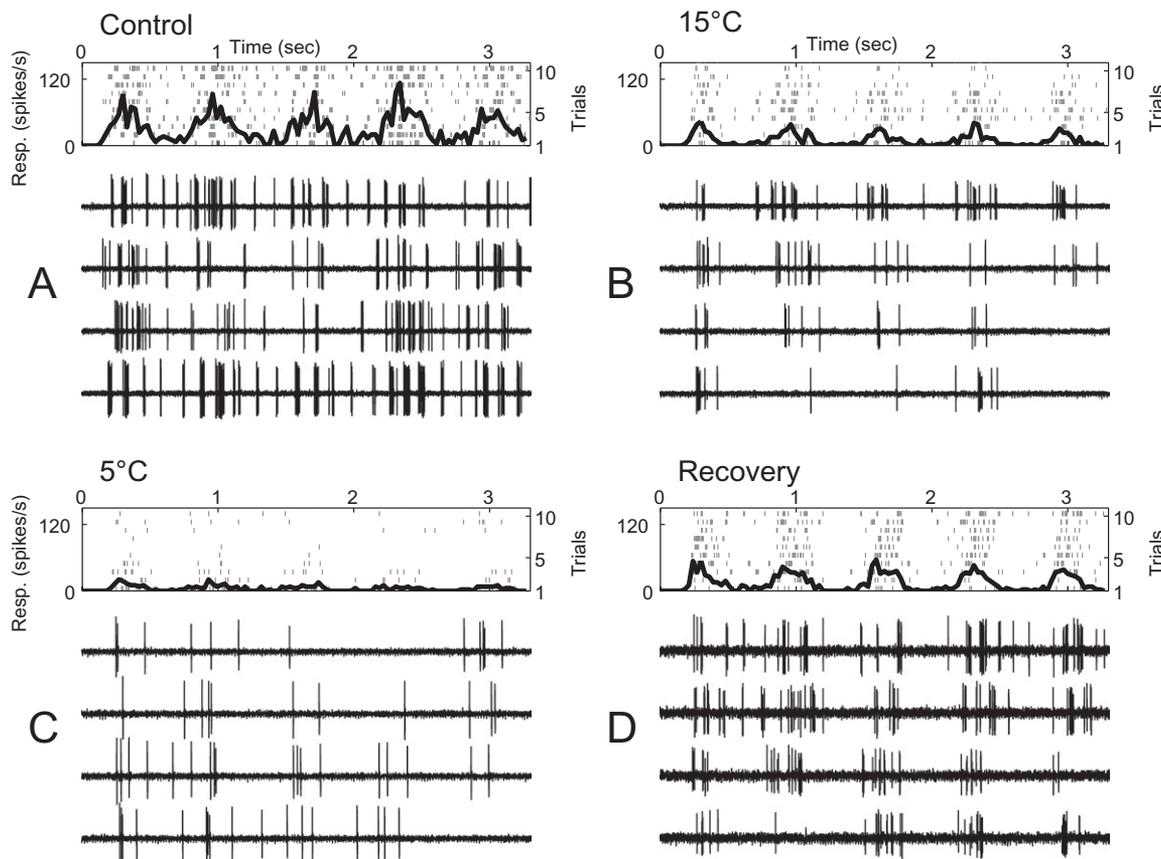
Fig. 2. Example of cell responses recorded at different surface temperatures. (A) Control conditions. (B, C) Surface temperature was 15 °C and 5 °C, respectively. (D) Recovery conditions. In each case the response to the preferred orientation is shown (same cell in all panels). In each panel the poststimulus time histogram (thick line, bin size is 30 ms, left y-axis) and the raster plot are shown on the top (each grey line is a spike, each trial ($n = 10$) is shown, right y-axis). The four traces on the bottom of each panel are selected examples of analogue recorded spikes taken from the 10 trials that were recorded. The stimulus was a five cycles sine wave grating presented for 3.3 s ($=1.5$ Hz). Note that the cell response was still modulated by the grating stimulus even at the lowest temperature as shown in the poststimulus time histogram in (C).

measure was then repeated at two different cooling temperatures (11–12 °C and 2–6 °C in the examples shown on Fig. 4). Finally, the orientation tuning curve was recorded after the cortex had rewarmed. The pipette was then advanced deeper into the cortex to record the next cell. The effect of cooling was always to decrease the evoked response. In general lowering further the temperature decreased further the response (compare dark and light grey curves). Nevertheless, the effect was variable. For example, the responses of cell 3 were not very different at 12 °C and at 2 °C. For cell 4 the response at 3 °C was again lower than at 11 °C. This may indicate that the temperature sensitivity of each cell might be different, or that some local variation in temperature independent of the depth can occur (see Discussion), or that the circuit elements that give rise to the response are being differentially affected. Sometimes the cells responded more during the recovery measurements (cell 1 and cell 3, see Discussion).

To see whether the cooling inactivation changed the orientation selectivity, we measured the HWHH of the tuning curve from the Gaussian fit (Henry et al., 1974; Sclar and Freeman, 1982; Orban, 1984; Sompolinsky and

Shapley, 1997; Anderson et al., 2000; Moore et al., 2005). If the HWHH increases, the selectivity decreases, because the cell can respond to a broader range of orientations, whereas a decrease of the HWHH means the orientation selectivity sharpens. In the single examples presented in Fig. 4 the tuning width did not change under cooled conditions. To verify that this was true for all cells we plotted the HWHH under cooled conditions versus the HWHH for control for every control/cooled pair (Fig. 5). In general cells clustered around the identity line and thus their HWHH did not change significantly when cooled. But for some cells the HWHH did change and example orientation tuning curves of such cells are shown in the insets of Fig. 5. Here the tuning increase (broadening) was likely due to the large response decrease and not to a modification of the basic circuit function during cooling. These examples also revealed that when the response was almost completely suppressed during cooling (as in the insets of Fig. 5) the fit curve was flat, increasing the HWHH (see Experimental Procedures for estimation of HWHH in cases of small response). To examine the effect of response suppression on the tuning width we calculated the tuning width
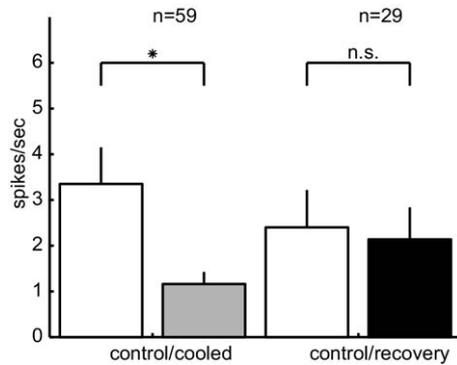
Fig. 3. Effect of cooling on spontaneous response. Mean spontaneous activity during control (white), cooling (grey), and recovery (black). During cooling the spontaneous activity was significantly smaller than during control (star, 2-sample t-test, $P=0.01$). The spontaneous activity was not significantly different between control and recovery (n.s.). The number of control/cooled pairs is larger ($n=59$) than the number of control/recovery pairs ($n=29$) because some cells were tested with two cooling temperatures (see text for details).

ratio (TWR) by dividing the HWHH during cooling by the HWHH during control (TWR=HWHH$_{cooling}$/HWHH$_{control}$). Then the response peak ratio (RPR) was computed by dividing the peak response (maximum of the fit) during

cooling by the peak response during control (RPR= Peak$_{cooling}$/Peak$_{control}$). When TWR was plotted against RPR the results show that a large tuning increase (for example TWR$>$2, dashed line) was correlated with a large response suppression (Fig. 6). On the other hand some cells having large response suppression kept the same tuning width showing that, generally, no increase in HWHH occurred. The same trend, although weaker, was visible for tuning narrowing showing that there was no 'iceberg' effect on the orientation tuning curve during cooling (see triangles in Fig. 6). In other words the response suppression was divisive and not subtractive.

Spike width

We next analyzed the spike width from extracellular recordings like those shown in Fig. 2. The changes in spike width with cooling could be easily heard by playing the recording through an audio amplifier and speaker. During cooling the sharp "click" of a spike became progressively dulled, but returned after rewarming. This change in the sound of the spike was due to the progressive increase of the spike duration during cooling. To quantify these changes, each spike was reconstructed with a spline function and the width was measured from this curve (see

Fig. 4. Orientation tuning curve during cooling. Orientation tuning curves of four cells recorded in the same penetration and showing the cell response versus the stimulus orientation. The cells were found in the cortex one after the other with Cell 1 recorded first and Cell 4 last. Each cell was tested under control conditions (full black line), two cooling surface temperatures (dark and light grey lines) and recovery conditions (dashed line). The error bars are the SEM. The cells were recorded at the following depths from the top of the gyrus: 1.3 mm (cell 1), 1.5 mm (cell 2), 2.6 mm (cell 3) and 3.1 mm (cell 4).
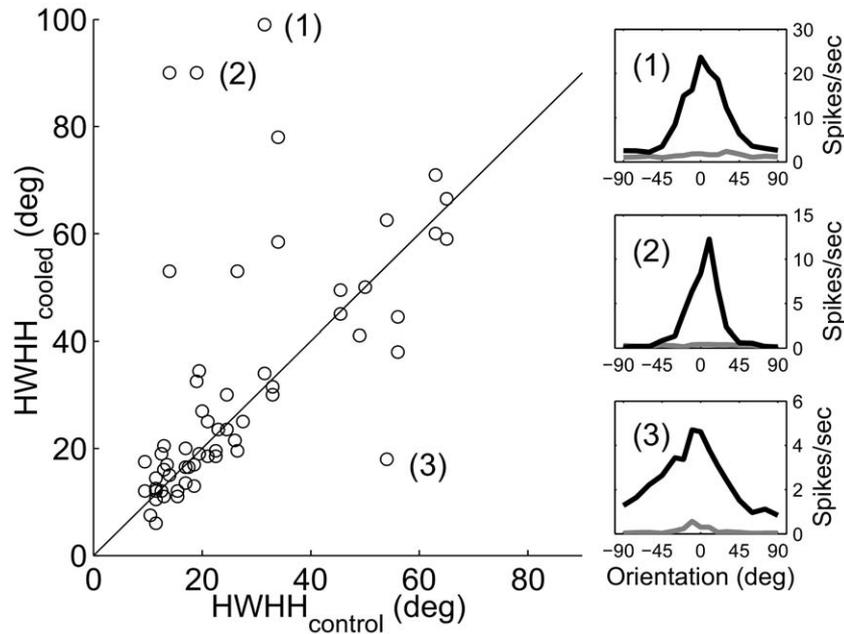
Fig. 5. Orientation tuning width does not change during cooling. Scatter plot showing the half-width at half-height (HWHH) in degrees under control (x-axis) and cooled (y-axis) conditions. The diagonal is the identity line. The insets show the orientation tuning curves for three examples with large increases (1 and 2) or large decreases (3) of the tuning width. Control and cooled values are plotted in black and grey, respectively. Note that these cells almost stopped responding during cooling. Most cells still responded during cooling and kept their HWHH constant since they are close to the identity line.

Experimental Procedures). Between 20 and 300 spikes were randomly selected from the stable temperature period. Two control/cooled pairs out of 59 were discarded because less than 20 spikes could be detected on the analogue signal. The mean number of spikes tested was 247 (range: 46–300) for control and 205 (range: 23–300) for cooled. The comparison for each pair is important here, since the absolute extracellular spike width might depend on the distance to the cell, or the signal could have been filtered differently by different recording pipettes.
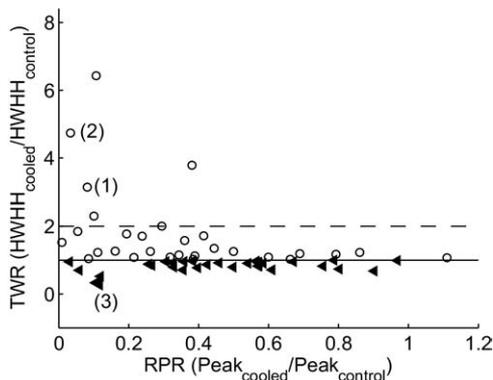

Fig. 6. Large tuning changes are correlated with large response suppression. Scatter plot of the TWR plotted against the RPR. Open circles and black triangles show TWR larger (broadening) and smaller (narrowing) than 1, respectively. The dashed line at 2 shows that only a few cells had a 100% broadening. The data points marked with (1), (2), and (3) correspond to those shown in Fig. 5 (symbol of data point (3) larger for clarity). Note that pairs with large tuning increase or decrease are also showing a large response suppression (small RPR).

The scatter plot of Fig. 7A shows the results for 57 control/cooled pairs. During cooling the spike width increased significantly (2-sample t-test, two-tailed, $P<0.05$) in 53 cases (dots above identity line), decreased in one case (dot below identity line) and did not change in three cases (open circles, two of which being the same cell tested at two temperatures). At the population level this corresponds to a mean increase of 50% (compare white and grey bar in Fig. 7C). This increase was significant (2-sample t-test, one-tailed, $P<0.01$). The data for control/recovery pairs are shown on Fig. 7B ($n=29$). At the population level there was no difference between control and recovery (compare white and black bar in Fig. 7C). However, at the single cell level the spike width remained the same between control and recovery in only 12 recordings (41% of the pairs). For the remaining cells ($n=17$) the spike width was either slightly larger ($n=10$) or slightly smaller ($n=7$) during recovery compared to control. Nevertheless, the spike width differed significantly between cooling and recovery in 15 out of these 17 cases (2-sample t-test, $P<0.01$). Fig. 8 shows that, for those recordings, the spike width increased during the cooled phase and then decreased again during the recovery (but without reaching the same width as under control conditions).

To compare across conditions, a mean spike was first calculated for each recording under control, cooling and recovery conditions using the spline fit. Then, an average spike for each condition was calculated (Fig. 7D). The average cooled spike was clearly wider than the average control and recovery spikes. To test whether the spike width broadening was related to the response reduction
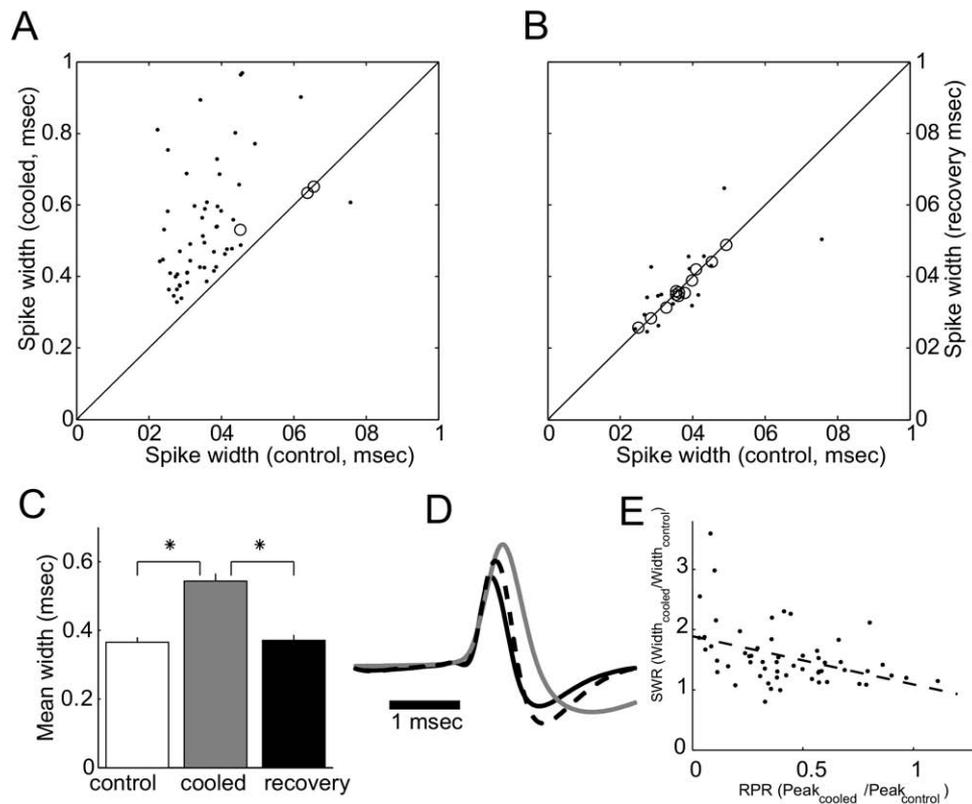
Fig. 7. Spikes become broader under cooling conditions. (A) Scatter plot comparing the spike width during control (x-axis) and cooling periods (y-axis) for 57 pairs. Pairs for which spike width was significantly different between both measures are shown with black dots (n=54) and pairs with no difference are shown with open circles (n=3). Note that a significant decrease during control only occurred in one case. The diagonal is the identity line. (B) Scatter plot comparing control and recovery recordings. Symbols as in (A). (C) Mean spike width for control (white), cooled (grey), and recovery (black). The differences between control and cooled and between cooled and recovery are significant (stars) while the difference between control and recovery is not (2-sample t-test, one-tailed, P<0.01). The SEM are shown. (D) Mean spikes for control (black), cooled (grey) and recovery (dashed). (E) Changes in spike width were correlated with response reduction. Each data point from panel A is plotted as SWR (spike width ratio) against its corresponding RPR. The dashed line shows the linear regression.

under cooling conditions, the spike width ratio (SWR= $Width_{cooling}/Wdth_{control}$) was plotted against the RPR (see above and Fig. 6). As shown in Fig. 7E the correlation coefficient (Pearson) was negative ($r=-0.41$) and the correlation was significant ($P=0.0014$). This indicates that, for large response reductions (i.e. small RPR), the spike widening was also large (large SWR).

DISCUSSION

Cortical cooling was used here to study the properties of single cells in the cat primary visual cortex when the activity of the local circuit was greatly reduced. We found that the preferred orientation and the tuning width of most cells were not affected by cooling phase, despite evident modification of the biophysical properties of the cells (spike width increase). Moreover we also showed that the spike width is a reliable method of assessing whether a cell has been directly cooled.

Technical considerations

The cooling method developed by Lomber et al. (1999) and used here enables accurate control of the cortical surface temperature. Precise control of the temperature is important in order to avoid damage of the cortical surface and superficial layers by cryogenic lesions. For example a temperature of −6 °C during only 2–3 min produces lesions in the supragranular layers (Schwark et al., 1986). The inactivation method is attractive because it is easily reversible, and we found that most cells recovered from cooling. Studies using chronic implants of the cooling loop have shown that repeated cooling does not alter neuronal responsiveness or animal behaviour and does not produce anatomical damage (Keating and Gooley, 1988; Lomber et al., 1996, 1999). Light microscopic examination of the tissue from our cooling experiments showed no damage.

The temperature measurements inside the cooled cortex, performed with a small purpose-built thermocouple, gave a cooling gradient of 6−8 °C/mm. This is within the range (2–10 °C/mm) reported in earlier studies (Kalil and Chase, 1970; Schiller and Malpeli, 1977; Girard and Bullier, 1989; Michalski et al., 1993; Lomber et al., 1996; Casanova, 2002). The gradient depends on the cooling device and on its cooling efficacy (Girard and Bullier, 1989). For example Girard and Bullier (1989) measured the temperature inside the brain using a cooling plate applied to the dura whereas we used a cooling loop in direct
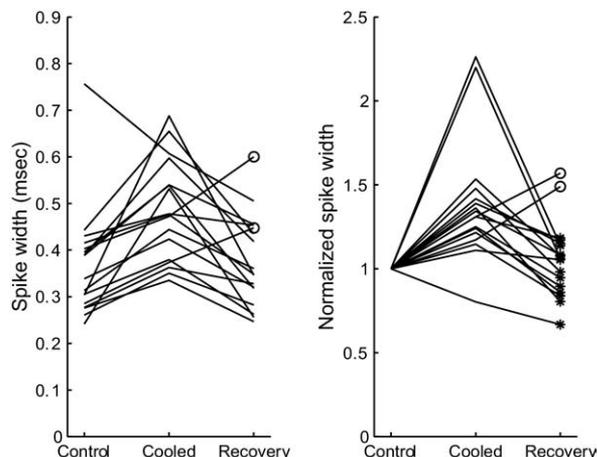
Fig. 8. Spike width recovered after termination of cooling. Seventeen recordings for which the spike width for control and recovery were significantly different (dots in Fig. 7B). The left panel shows the spike width for control, cooled, and recovery. Note that even in these 17 cases the spike width increased from control to cooled and then decreased again during recovery. The right panel shows the relative spike width (data were normalized by dividing by the width under control conditions). The spike width decreased significantly in 15 out of 17 cases (stars) from cooled to recovery. In two pairs the spike width increased from cooled to recovery (open circles).

contact with the brain. Lomber et al. (1999) report that the temperature at a depth of 900 μm was between 20 and 24 °C when the cooling loop was at 8 °C (see their Fig. 12B). We used a similar loop and with a surface temperature of 8 °C our measurement of 20 °C at 1000 μm is in good agreement with the measurements of Lomber et al. (1999).

The brain surface may well be cooler than the core body temperature (see Zauner et al., 1995) and additional heat loss could occur through the open skull and dura. This is consistent with the observations of Michalski et al. (1993) who found that the brain temperature was between 34 and 36 °C about 1 mm below the surface and that this depends on the size of the craniotomy. In our experiments the brain was covered with a 1 cm thick layer of agar, which should provide some insulation, but at the surface the temperature was always slightly lower than the core body temperature measured with a rectal probe. In our experiments the control cooling loop temperature was between 30 and 33 °C, depending on the cat, which was about four degrees lower than the actual brain surface temperature, as is evident in Fig. 1. Likewise, when the cooling loop was around 8 °C (black curves, panel C) the surface temperature was about 12 °C (black curves, panel A). The same difference appeared when the cooling loop temperature was at 0 °C (grey curves, panel C), and in that case the surface temperature was 4 °C (grey curves, panel A). Therefore we concluded that the control cortical temperature was always above 34 °C, a temperature well above that needed to block synaptic activity (Brooks, 1983). Some cells responded more during the recovery than during control. This rebound effect might be due to a higher synaptic release probability during rewarming (Volgushev et al., 2004).

Orientation selectivity during cooling

When the cortex was cooled the selectivity to orientation and direction of motion were preserved, in agreement with previous studies (Girard and Bullier, 1989; Michalski et al., 1993; Ferster et al., 1996; Casanova et al., 1997). Even when partly inactivated by cooling the cortical circuitry continues to provide the necessary input to the recorded cell to maintain its properties. The question of the role of the cortical circuit in orientation selectivity was directly addressed by Ferster et al. (1996). In their study the cortex was deactivated by cooling and using patch clamp recordings the sub-threshold responses of layer 4 simple cells were tested with oriented drifting gratings. They showed that the orientation tuning curves of the sub-threshold response under cooled and normal conditions have the same width. The tuning curves differed in amplitude, because the cells responded much less in a cooled cortex. Their interpretation was that the residual tuning came from the thalamic input (confirming the hypothesis of Hubel and Wiesel (1962) regarding the generation of orientation selectivity in simple cells) since they assumed that the cortical cells from all layers did not spike. Doubt has been cast on this interpretation, because in cortical cells the orientation selectivity of the sub-cortical input is different from the orientation selectivity of the spiking output (Shapley et al., 2003) and because the cortex might not have been completely inactivated (Vidyasagar et al., 1996; Crook et al., 2002). Ferster et al. (1996) themselves reported some residual multiunit activity in layer 6 of the cooled cortex. Since layer 6 provides a large excitatory input to layer 4 (Gilbert and Wiesel, 1979; Martin and Whitteridge, 1984; Ahmed et al., 1994; Grieve and Sillito, 1995) it could be that the activity recorded in layer 4 of the cooled cortex was partly due to input from layer 6. In any case, it seems difficult to completely inactivate the cortex through its entire depth even while holding the surface temperature at 0 °C. For example Girard and Bullier (1989) showed that the temperature of their cooling plate must be as low as −8.5 °C to obtain a temperature of 10 °C 2 mm below surface (see their Fig. 2). With the cooling loop at 3 °C Lomber et al. (1999) claim that the entire grey matter down to layer 6 is below 20 °C and thus completely inactivated, but this is not cold enough to block activity through the entire cortical depth since spiking activity is only blocked below 10 °C (Brooks, 1983). In our study, neurons remained spontaneously active and could be driven by visual stimuli even when the cortical surface was cooled below 10 °C. However, we found in the present study, as have others (Michalski et al., 1993; Casanova et al., 1997), that the cooled, spiking cortical circuit is capable of maintaining orientation selectivity of the output (spikes).

We observed that the form of the orientation tuning curve recorded under normal conditions compared to that recorded under cooled conditions can be estimated by dividing the cell response by a constant number: the response decreased, but the tuning width did not change. Michalski et al. (1993) tested different combinations of tuning widths and amplitudes for excitation and inhibition

(both modelled as Gaussian functions). The sum of the excitatory and inhibitory functions gave the orientation tuning curve. They found that no matter which component of the input orientation was selective, only a divisive reduction reproduced the effect of cooling on the tuning function.

This effect of cooling on the tuning function is very similar to what happens if the mean contrast of the stimulus is lowered (Sclar and Freeman, 1982; Skottun et al., 1987). The tuning width is preserved irrespective of the contrast level. This property is called contrast invariant orientation tuning and its origin is still unclear (see, e.g. Sompolinsky and Shapley, 1997; Troyer et al., 1998; Ferster and Miller, 2000; Alitto and Usrey, 2004; Finn et al., 2007; Banitt et al., 2007). Adaptation to the stimulus contrast also produces a divisive effect (Ohzawa et al., 1982, 1985) called contrast gain control.

However, when the contrast is lowered the thalamic input is smaller, but the cortical processing is normal. On the other hand when the cortex is cooled, not only is the input to the cortex weaker (because the thalamocortical synapses are cooled), but the entire cortical activity is reduced (synaptic and spiking activity) to give a response that is simply scaled in a divisive manner. Thus, cortical cooling changes the gain of the circuit without affecting its basic functionality. The mechanism underlying this "cooling gain" is probably a mixture of effects on spike conductance's (Volgushev et al., 2000a,b) and synaptic transmission (Brooks, 1983; Hardingham and Larkman, 1998; Volgushev et al., 2004). Broader spikes could keep presynaptic voltage-dependent calcium channels open for longer time (more calcium influx in the terminal), leading to higher release probability. Conversely, slower diffusion and vesicle docking in cooled terminals could lower the probability of transmitter release. The time course of the postsynaptic potentials is also slower in cooled cortex and thus dendritic integration may also be more efficient (Hardingham and Larkman, 1998). Nevertheless, that the number of spikes produced decreases with cooling, points to the dominant effect being a decrease in synaptic transmission, which would produce the divisive change in gain observed.

The effect of surface cooling depended not only on the depth (distance to the cooling loop) but also varied from cell to cell. As shown in Fig. 4 the response amplitude of cell 3 was the same at 12 and 2 °C, while cell 4 (deeper and thus further from the loop) was affected differently by similar temperatures. Thus the sensitivity to cold differs from cell to cell, as reported by others (Girard and Bullier, 1989; Casanova et al., 1997). This individual variance makes the measured gradients only a guide to the degree of inactivation of a region, not an absolute measure. The spread of temperature probably depends on the blood vessel pattern (Girard and Bullier, 1989; Lomber et al., 1999) so that a cell close to a blood vessel that is also in contact with the cooling device will be more strongly affected.

Spike width

In the present study a careful analysis of the spike waveform revealed some features that are consistent with intracellular recordings *in vitro* (Moseley et al., 1972; Hardingham and Larkman, 1998; Volgushev et al., 2000b). Since the pipette resistance and capacitance are not carefully controlled during extracellular measurements, the absolute spike width is of less significance than the changes that occur when spikes are collected from the same cell under control and cooling conditions. The spike width increased in 93% (53/57) of the pairs and the mean spike width increased during the cooling. At the population level the mean spike width between control and recovery was not different, although the spike width for some individual cells did differ significantly between control and recovery, probably because the blocks of recordings were performed many minutes apart and so small shifts in the pipette could cause unspecific changes. More importantly, there was a significant decrease in spike width from cooling to recovery (Fig. 8).

Many researchers have used cooling methods to study the influence of one brain area on another (e.g. Sherk, 1978; Girard and Bullier, 1989; Michalski et al., 1993; Casanova et al., 1997; Hupe et al., 1998). A major interest in these studies is to deactivate one region (the input region) while ensuring that the recording region remains at physiological temperature. This may be difficult if the two regions are close-by, such as V1 and V2. Temperature measurements can be made inside the brain with a small thermocouple (Kalil and Chase, 1970; Schiller and Malpeli, 1977; Girard and Bullier, 1989; Michalski et al., 1993; Lomber et al., 1996; Casanova, 2002), but these measures are not easy to perform in combination with recording and most measurements of activity and temperature inside the brain have been done in separate experiments, as here. On the other hand, monitoring the spike width is a reliable and sensitive method of discovering if neurons in a region remain at physiological temperature (Sherk, 1978) when a neighbouring region is cooled (i.e. if their spike width does not alter with changes in temperature). It thus does not depend on calculation of temperature gradients that must be measured after the recordings or after the behavioural test are finished (Lomber et al., 1996) and because it can be seen at the resolution of single cells, the spike width of each neuron can be used as a reference probe to check whether changes in responsiveness are due to direct effects of cooling or not. The advantage of measuring the spike width is that the broadening can easily be detected by ear if the electrode voltage is played on an audio monitor and checked quantitatively online by averaging a few tens or hundreds of spikes. In this manner it can be quickly decided whether the effect comes from the spread of cold to the recorded cell (in which case the spikes will be broader), or is a consequence of an input modification because cells upstream were deactivated.

CONCLUSION

In summary, results from cortical cooling experiments should be interpreted with care when cooling is used to inactivate the same region where the recordings are done, because it is difficult to estimate the level of responsiveness of the cortex in deep layers. Moreover, cooling the cortex decreases the cortical gain rather than inactivating it completely, as indi-

cated by our observation that the orientation bandwidth does not change even in strongly suppressed cells. This means that some essential information about the stimulus properties are still being signalled, albeit weakly. Our results also show that direct effects of cooling on single neurons can be easily determined by monitoring the spike width during cooling. Thus cooling is best used as a deactivation method to decrease the drive in a region upstream to the recording region. Because of the depth gradient, output neurons located in the superficial layers of cortex will be more effectively deactivated than those in the deep layers. However, even partial deactivation can provide valuable data as to the functional relationship of one region with another.

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