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SYNAPTIC TRANSMISSION IN THE NEOCORTEX DURING REVERSIBLE COOLING

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Abstract—We studied the effects of reversible cooling on synaptic transmission in slices of rat visual cortex. Cooling had marked monotonic effects on the temporal properties of synaptic transmission. It increased the latency of excitatory postsynaptic potentials and prolonged their time-course. Effects were non-monotonic on other properties, such as amplitude of excitatory postsynaptic potentials and generation of spikes. The amplitude of excitatory postsynaptic potentials increased, decreased, or remain unchanged while cooling down to about 20°C, but thereafter it declined gradually in all cells studied. The effect of moderate cooling on spike generation was increased excitability, most probably due to the ease with which a depolarized membrane potential could be brought to spike threshold by a sufficiently strong excitatory postsynaptic potential. Stimuli that were subthreshold above 30°C could readily generate spikes at room temperature. Only at well below 10°C could action potentials be completely suppressed. Paired-pulse facilitation was less at lower temperatures, indicating that synaptic dynamics are different at room temperature as compared with physiological temperatures.

These results have important implications for extrapolating *in vitro* data obtained at room temperatures to higher temperatures. The data also emphasize that inactivation by cooling might be a useful tool for studying interactions between brain regions, but the data recorded within the cooled area do not allow reliable conclusions to be drawn about neural operations at normal temperatures.

Key words: visual cortex, cellular electrophysiology, temperature, excitability, action potentials.

Effects of temperature on the membrane properties, propagation of spikes and synaptic transmission have long been appreciated. However, most of these studies were done on preparations from invertebrates or lower vertebrates,[16,18,20,24,26,37,41,55] while there is only limited data from higher vertebrate.[2,5,27,44,51] However, there are indications that temperature dependence of the functioning of mammalian and non-mammalian cells can be quite different[26] (cf. also Refs 18 and 27). These differences are not surprising, since mammalian nerve cells are adapted to perform best around 36–38°C, but the nerve cells of lower vertebrates and invertebrates need to work at lower temperatures and in a much wider range: from slightly above 0°C to about 35°C, but rarely higher. For that reason, the results of experiments on invertebrates and lower vertebrates may have only a limited relevance for mammals.

Recently we have described modifications of the basic membrane properties and spike generation in neocortical cells, associated with changes of the recording temperature.[54] It is reasonable to expect that changes such as cooling-induced depolarization of cells and broadening of the action potential that we have shown should have an impact on synaptic transmission. Therefore, we set out to investigate properties of synaptic transmission in neocortical slices during cooling and re-warming. This study had two main purposes. First, we wanted to assess the possibility of a comparison of results of *in vitro* experiments, which are usually performed either above 30°C or at room temperature, not both. Most often the results obtained at room temperature are simply taken as valid for drawing conclusions about functioning of the nerve cells under physiological conditions. Our present results expose the limitations of such inferences. The second aim was to help in interpreting *in vivo* experiments that use cooling as a tool of inactivation of specific brain regions.[7,15,22,33,36,39,43,45,52] It has often been implicitly assumed that cooling leads to a gradual reduction of nervous activity, particularly the occurrence of spikes, either spontaneous or stimulus induced. However, some studies indicate that this assumption might be not correct, and that gradual cooling leads first to an increased reactivity of nerve cells, and only thereafter to the blockade of activity.[2,4,5] Results of the present study confirmed that excitability of neurons is increased during moderate cooling, and depolarization block of spike generation occurred only at temperatures below 10°C. An understanding of the cellular processes that take place during cooling can provide a framework for adequate interpretation of the experimental results.

Part of these data appeared in abstract form.[8]

EXPERIMENTAL PROCEDURES

Slices

Slices of the visual cortex of 27–63-day-old Wistar rats (Charles River GmbH, Suzfeld, Germany) were prepared by conventional methods.[53] The rats were anaesthetized with ether, decapitated and the brain was rapidly removed and put into an ice-cold oxygenated solution. One hemisphere was mounted on to an agar block and 350–400-μm-thick sagittal slices containing the visual cortex were cut with a Vibrotome (TSE, Kronberg, Germany) in ice-cold oxygenated

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Abbreviations: EGTA, ethyleneglycolbis(aminoethyl ether)tetra-acetate; EPSP, excitatory postsynaptic potential; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; NMDA, *N*-methyl-D-aspartate; PPF, paired-pulse facilitation.; TBS, Tris-buffered saline.

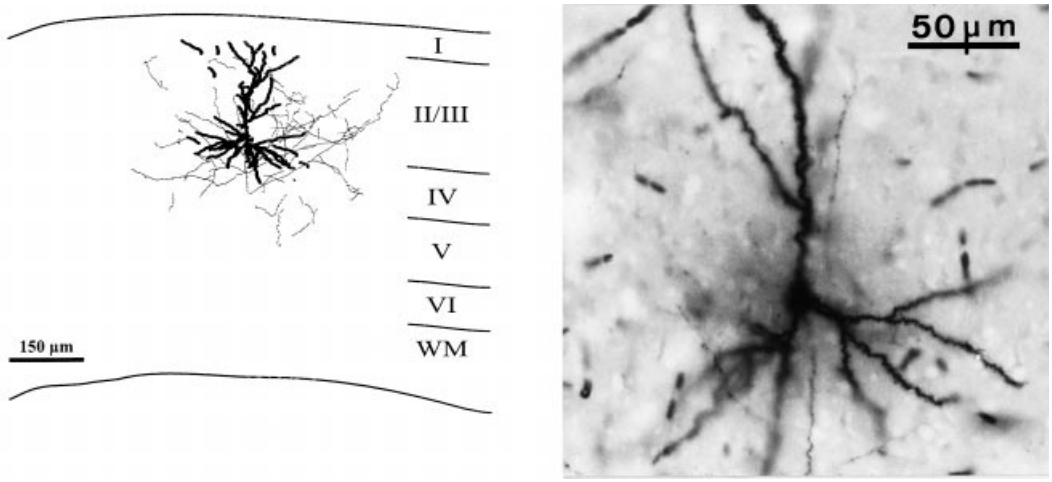

Fig. 1. Morphological verification of a recorded cell as a layer 2/3 pyramid. Reconstruction on the left shows the dendritic tree (thick lines) and axon collaterals (thin lines) drawn from two sections of a slice showing a cell body and its spatial location within the cortex. The microphotograph shows the cell body and spiny apical and basal dendrites as well as the initial part of the axon and its collaterals.

solution. After cutting, the slices were placed into an incubator where they recovered for at least 1 h at room temperature. Then a slice was placed in a recording chamber. The solution used during the preparation of the slices had the same ionic composition as the recording medium (as below), except for L-glutamine. The methods used in this study were in accordance with the guidelines published in the European Communities Council Directive (86/609/EEC, 1986) and were approved by the regional animal welfare committee (Arnsberg, Germany).

Recording and data analysis

Perfusion medium contained (in mM): 125 NaCl, 2.5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1.25 NaH_2PO_4, 25 $NaHCO_3$, 25 D-glucose and 0.5 L-glutamine bubbled with 95% O_2/5% CO_2. Temperature in the recording chamber was varied from 7 to 35°C and monitored with a thermocouple positioned close to the slice, 2–3 mm from the recording site.

Patch-electrodes were filled with a K-gluconate or Cs-methanesulphonate-based solution (in mM: 127 K-gluconate or Cs-methanesulphonate, 20 KCl, 2 $MgCl_2$, 2 Na_2ATP, 10 HEPES, 0.1 EGTA and 0.3–1.0% biocytin) and had a resistance of 2–6 MΩ. Whole-cell recordings from pyramidal and non-pyramidal neurons in layers II–VI in slices of the visual cortex were made under visual control using Nomarski optics and infra-red videomicroscopy.[12,48] Some of these cells were labelled with biocytin to verify their morphological type and position within the cortex. Responses to current or voltage steps and to synaptic stimulation were recorded. Synaptic responses were evoked by electric shocks (50–100 µs, 5–300 µA) applied through bipolar stimulation electrodes located 0.5–1.5 mm below or lateral to the recording site. Electric shocks were applied as paired pulses (50-ms interpulse interval) at 0.017–0.066 Hz.

After amplification using Axoclamp-2A (Axon Instruments), both electrophysiological and temperature data were digitized at 5 or 10 kHz and fed into a computer (PC-486; Digidata 1200 interface and pCLAMP software, Axon Instruments) for off-line processing using programs written by one of us (M.V.). Statistical analysis was based on a two-tailed Student's *t*-test and Wilcoxon–Mann–Whitney test. The differences were considered as significant at $P < 0.05$ unless stated otherwise. The data are presented as mean ± S.E.M.

The excitatory postsynaptic potential (EPSP) amplitudes were measured as the difference between the mean membrane potential within two windows of 1–5 ms width, one positioned immediately before the response and another one around the peak of the averaged EPSP (five to 50 individual responses), or on the last portion of the rising slope. To calculate the latency of the EPSP, its origin was determined as the point from which the potential was rising over the next 1–5 ms with a gradient within a pre-set range. The time between the determined EPSP origin and stimulus artefact was taken as response latency. The spike amplitudes were measured in two ways: from the origin of the regenerative process (spike threshold) and from the resting membrane potential to the peak. We have used both these measurements because of large depolarizing shifts of the resting membrane potential associated with cooling. The spike area was measured as the integral area under the curve from spike origin to the moment when the amplitude after the peak exceeds the threshold by less than 15 mV.

Morphology

The tissue containing biocytin-labelled cells was processed as follows: slices were fixed overnight in 2–4% paraformaldehyde containing 0.5% glutaraldehyde (E. Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (pH 7.4) and cut into 60-µm sections on a freezing microtome. After several rinses in phosphate buffer and Tris-buffered saline (TBS; Tris-buffer, Trizma® Base plus Trizma hydrochloride, pH 7.6, Sigma), the sections were incubated in avidin–biotin complexed with horseradish peroxidase (1:200, ABC, Vector Laboratories, Burlingame, CA, U.S.A.) diluted in TBS containing 0.05% Triton X-100 at 4°C overnight. The sections were then rinsed in TBS, followed by Tris and incubated for the enzymatic reaction in 0.005% 3,3′-diaminobenzidine-4-HCl (Sigma) in TBS supplemented with $CoCl_2$ intensification for 20 min. Hydrogen peroxide (final concentration 0.00125%) was added to complete the enzymatic reaction. The sections were washed in Tris buffer, then in phosphate buffer and they were then postfixed in 1% OsO_4 (Sigma) in phosphate buffer for 15–30 min, dehydrated and embedded in Durcupan ACM resin (Fluka, Neu-Ulm, Germany) on slides. Three-dimensional reconstruction was made using a 100× oil objective and the Neurolucida system (MicroBrightField, U.S.A.).

Chemicals

The following chemicals were obtained from Sigma (Deisenhofen, Germany): biocytin, Cs-methanesulphonate, EGTA, HEPES, K-gluconate, L-glutamine, Na_2ATP, tetrodotoxin. The remaining chemicals were from J.T. Baker B.V., Deventer, Holland, when not indicated otherwise.

RESULTS

Whole-cell recordings were made from different types of neurons in slices of the visual cortex of adult rats. Cells of a certain morphological type and location within the cortex were pre-selected using infra-red videomicroscopy with contrast enhancement.[12] Some of the cells (19 of 32) were injected with biocytin and their morphological type was verified. A typical example of a layer 2–3 pyramidal cell is shown in Fig. 1, with a characteristic pattern of axon collaterals and dendritic tree. Both apical and basal dendrites are densely

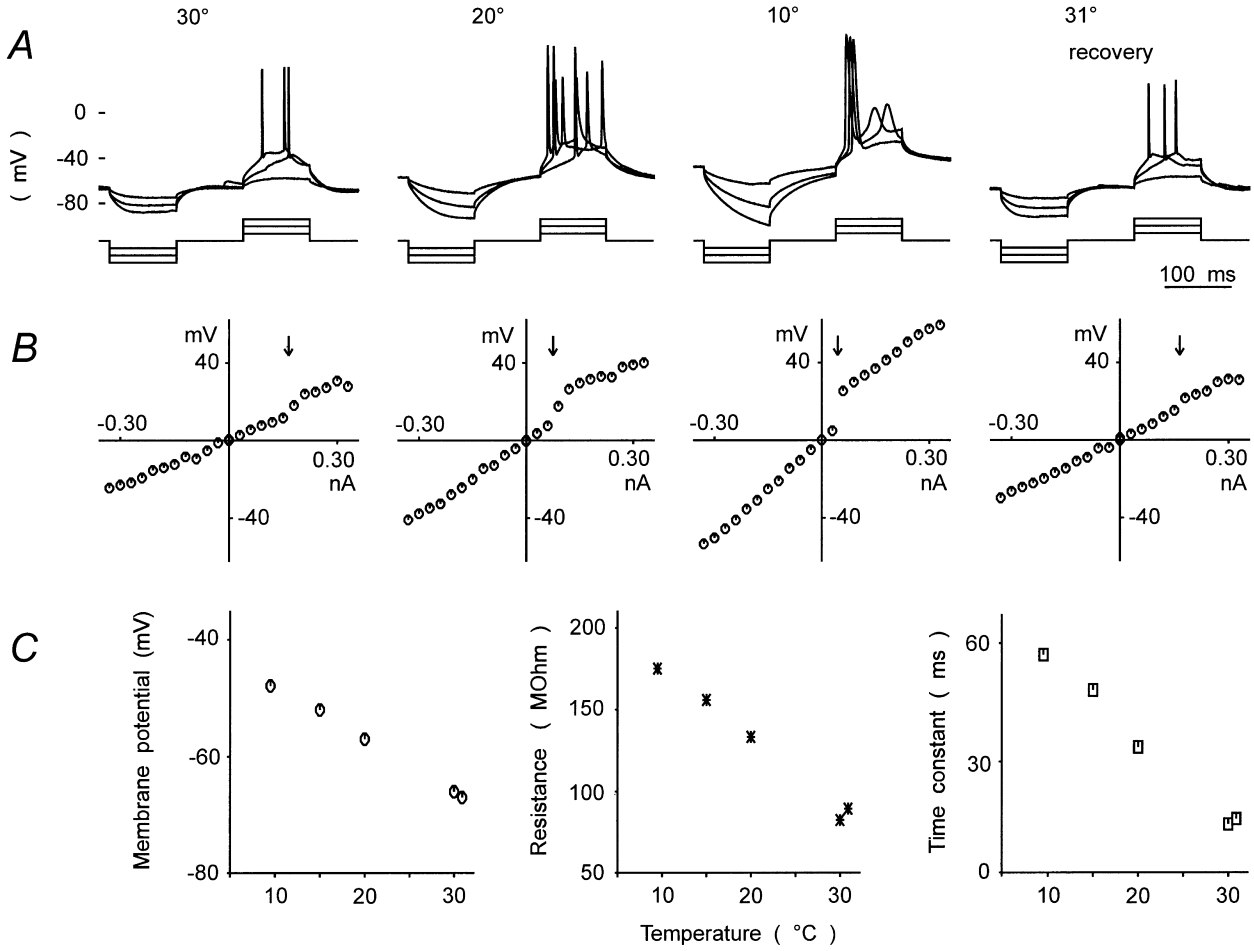

Fig. 2. Temperature effects on the responses of a layer 5 pyramidal cell to current injections. (A) Responses of a cell to negative and positive current steps (0.1, 0.2 and 0.3 nA; 100 ms) recorded at different temperatures (as indicated). (B) Current–voltage relationships plotted using the data in A. Arrows indicate distortion of current–voltage relation due to spike generation. (C) Dependence of membrane potential (left), input resistance (middle) and membrane time-constant (right) on the temperature.

covered with spines. These features allow unambiguous identification of this cell as a spiny pyramidal cell.

Fourteen out of 32 cells recorded for this study were layer 2–3 pyramids, eight were layer 5 pyramids and six were non-pyramidal cells comprising three spiny stellates from layer 4 and three aspiny (putative inhibitory) stellates. The remaining four cells were neither labelled nor could they be identified unambiguously during the recording.

In 18 out of 32 cells stimulation strength was adjusted to evoke weak synaptic responses, which remained subthreshold for action potential generation over the whole range of temperatures tested. In 14 cells, stronger stimuli were applied, which led to spike generation at all temperatures tested (seven cells) or within some range of temperatures (seven cells).

Temperature dependence of basic membrane properties

Temperature has pronounced effects on both passive and active membrane properties. Lowering the temperature leads to depolarization of the resting membrane potential, increase in the cells' input resistance and the membrane time-constant, as well as to an increase in the amplitude and broadening of spikes induced with steps of depolarizing current (Fig. 2). All of these changes were reversible, and there was a complete recovery upon rewarming.

The membrane potential and the input resistance, measured at higher temperatures (25–32°C), were significantly different in different cell types. The membrane potential was most negative in layer 2–3 pyramidal cells (-77.4 ± 0.6 mV), differing significantly ($P < 0.01$) from the membrane potential of non-pyramidal cells (-68.7 ± 0.9 mV) and of the layer 5 pyramids (-62.4 ± 2.9 mV). The non-pyramidal cells had significantly higher input resistance (287 ± 21 MΩ) than the layer 3 pyramids (111 ± 14 MΩ) and the layer 5 pyramids (106 ± 12 MΩ). Despite the differences in the absolute values of the membrane potential and the input resistance, the dependence of these two parameters on temperature was similar in all cell types. With lowering of the temperature, the membrane potential became depolarized, typically by 1–2.2 mV/°C. The gradient of the changes in membrane potential, averaged across the whole sample, was -1.3 ± 0.09 mV/°C. The input resistance increased with cooling, in most cells by more than 2 MΩ/°C (mean -5.2 ± 0.80 MΩ/°C). The increase of the input resistance was accompanied by a proportional increase of the membrane time-constant. In most of the cells (10 out of 16), both the input resistance and the membrane time-constant increased monotonically with cooling, as in the example shown in Fig. 2. However, in some neurons (six out of 16) dependence of the both parameters deviated from the linear at temperatures below 20°C. In the

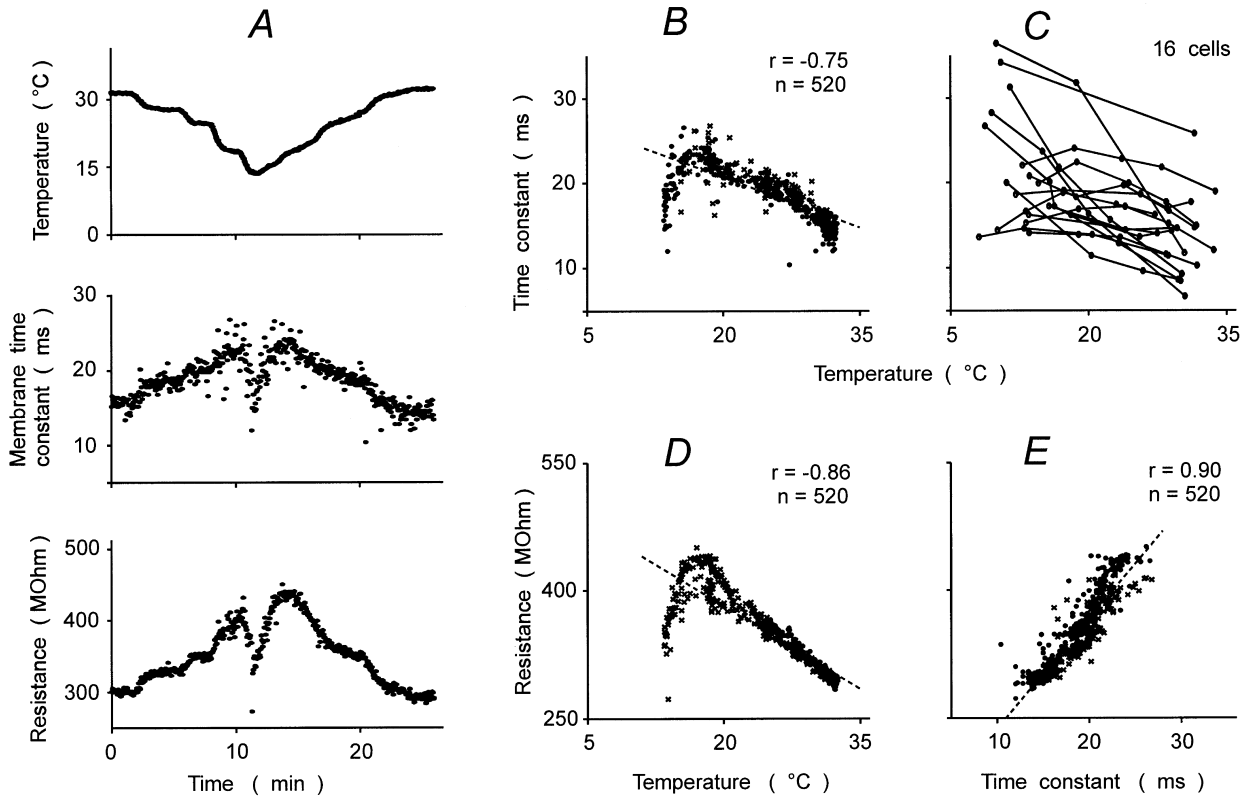

Fig. 3. Dependence of the membrane time-constant and input resistance on temperature in a layer 2 non-pyramidal cell (A, B, D, E) and group data for 16 cells (C). (A) Time-course of changes of temperature in the recording chamber, of the membrane time-constant and of the input resistance in the cell. Membrane time-constant and input resistance were assessed from responses of the cell to negative current steps (−0.3 nA, 100 ms) recorded at different temperatures during cooling and re-warming. (B) Scatter diagram showing dependence of the membrane time-constant on temperature, plotted using the data in A. Values obtained during cooling are shown as crosses; those during re-warming are shown as solid circles. (C) Group data for temperature dependence of the membrane time-constant in 16 cells. For each cell, temperature and membrane time-constant values were averaged within 5°C intervals (small circles) and connected with a line. (D) Scatter diagram showing dependence of the input resistance on temperature, plotted using the data in A. Values obtained during cooling are shown as crosses; those during re-warming are shown as solid circles. (E) Correlation between the input resistance and the membrane time-constant changes. Coordinates of each data point represent values of the input resistance (ordinate) and the membrane time-constant (abscissa) obtained from one response of a cell to current step (same data as in A).

cell shown in Fig. 3, after an almost linear increase of the input resistance and the membrane time-constant during cooling from above 30°C to about 14–17°C, both these parameters decreased upon further cooling (Fig. 3A, B, D). Most probably, this occurred because the cooling-induced depolarization of the cell brought the membrane potential closer to the threshold level for activation of voltage-dependent conductances. Even in these cases, highly significant linear correlation between the input resistance and the membrane time-constant persisted over the whole temperature range (Fig. 3E). Summary data demonstrate that lowering the temperature leads, with minor exceptions, to an overall increase of the membrane time-constant of the cortical cells (Fig. 3C). The membrane time-constant below 20°C was significantly longer than above 25°C (19.4 ± 0.37 ms vs 13.5 ± 0.28 ms, $P < 0.001$).

Alterations in the basic membrane properties may influence the efficiency of synaptic transmission apart from any direct effect of cooling on synaptic transmission itself. To characterize the temperature dependence of synaptic transmission, we recorded EPSPs evoked in neocortical neurons at different temperatures.

Subthreshold synaptic responses

Latency of excitatory postsynaptic potentials. Lowering the temperature of the recording chamber led to an increase of the latency of synaptic responses. In the example in Fig. 4, cooling from 29 to 20°C resulted in an increase of the response latency from 3.4 to 8.5 ms (Fig. 4A, interrupted and dotted vertical lines). At temperatures above 20°C, the latency increase with cooling was essentially linear with a gradient of −0.58 ms/°C (Fig. 4B–D). With further cooling, the latency of EPSPs not only increased more rapidly, but also became less stable, showing considerable jitter (Fig. 4C, D). With cooling below 15°C, the time-course of synaptic responses became very slow, and reliable estimation of the latency was no longer possible.

These changes of temporal characteristics of the synaptic responses were reversible. Both the latency and its jitter returned to their initial values upon re-warming (Fig. 4C, D). This example was typical of our sample. The group data (Fig. 4E) show a clear and strong tendency of response latency to increase with cooling. In different cells, the gradient of the latency increase was between −0.32 and −1.33 ms/°C (mean −0.76 ± 0.07 ms/°C) to 20°C and this gradient became higher at lower temperatures (Fig. 4E).

Amplitude and shape of synaptic responses. Moderate cooling had little effect on the amplitude of the synaptically evoked EPSP as shown in Fig. 5. Despite lowering the bath

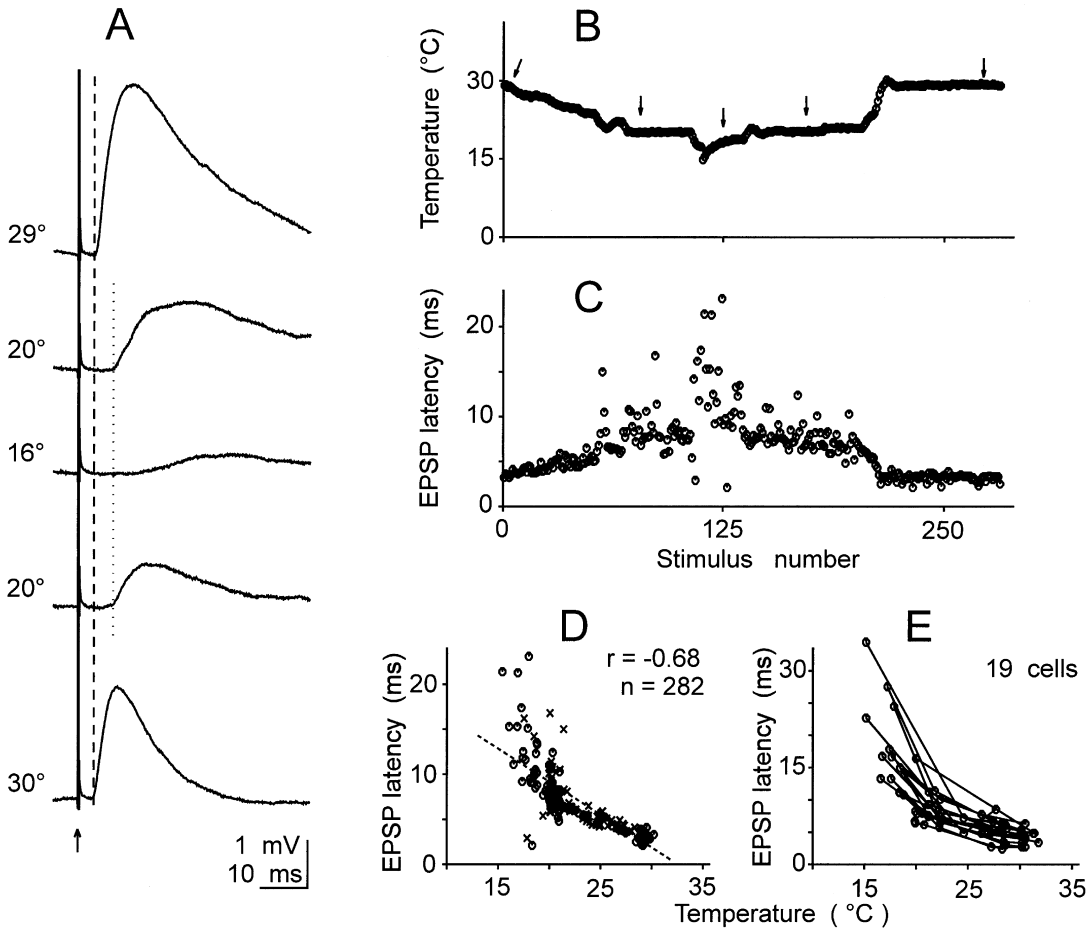

Fig. 4. Effect of cooling on EPSP latency. (A) EPSPs evoked in a layer 5 pyramidal cell (different from the one in Fig. 2) during cooling and re-warming, as indicated in B. The arrow marks the stimulus artefact. Interrupted and dotted lines show latency of responses at different temperatures. (B, C) Time-course of changes of temperature in recording chamber (B) and of EPSP latency (C). Arrows in B indicate the temperature values corresponding to the responses shown in A. (D) Scatter diagram showing dependence of the EPSP latency on temperature; same data as in B and C. (E) Group data for 19 cells. For each cell, temperature and corresponding EPSP latency values were averaged within 5°C intervals (circles) and connected with a line.

temperature from 29 to 20°C, the EPSP amplitude remained unchanged, as becomes evident from comparison of individual responses recorded at 29 and 20°C (Fig. 5A). The initial parts of the time-courses of changes in bath temperature and response amplitude (Fig. 5B, C) also clearly demonstrate that the response amplitude did not decrease during cooling to 20°C. At the beginning of the initial cooling, there was even a small but significant increase of the response from 11.6 ± 0.05 mV at 29°C to 13.0 ± 0.04 mV at 28–25°C ($P < 0.001$) (Fig. 5B, C). A tendency for the PSP amplitudes to be highest at temperatures around 25–27°C is also evident in the respective part of the correlation field of these two parameters (Fig. 5D, crosses at temperatures above 20°C). With cooling, the time-course of the EPSP became slower, thus facilitating temporal summation of afferent inputs. Further, because of the depolarizing shift of the resting membrane potential associated with cooling, the response peak reached progressively higher potential values, and at a temperature around 20°C even reached the threshold for spike generation (Fig. 5A, F). Lowering the bath temperature to 17–16°C led to a gradual decline of the EPSP amplitude, but because of the increased depolarization of the cell, occasional action potentials were evoked even at 16°C (Fig. 5A, C, F). Upon re-warming, the amplitude of the EPSP slowly increased up to the control level, but the membrane potential returned to the initial hyperpolarized state, thus shifting the whole response well below the spike threshold (Fig. 5A, C, F). During re-warming the EPSP amplitudes were lower than at the same temperatures during cooling. This hysteresis in the scatter diagram (crosses and circles in Fig. 5D) might have been due to slow recovery of some intracellular metabolic processes after the cooling, and/or to markedly different temperature gradients during cooling and re-warming used in this experiment.

The highly significant positive correlation between EPSP amplitude and temperature for all the data obtained from this cell between 15 and 31°C (Fig. 5D, $P < 0.001$) shows that the overall effect was a decrease of response amplitude with lowering of the temperature. However, because of an even stronger temperature dependence of the membrane potential, the EPSPs at lower temperatures peaked at a more depolarized potential, and a strong and highly significant negative correlation between the EPSP peak and the temperature was observed (Fig. 5G, $P < 0.001$). Summary data (Fig. 5E, H) show that both these tendencies, namely, decrease of the EPSP amplitude and a higher potential reached at peak response, were evident in most cells in our sample. A mild increase of the EPSP amplitude during the initial cooling, similar to that described above, was observed in seven out of 19 cells.

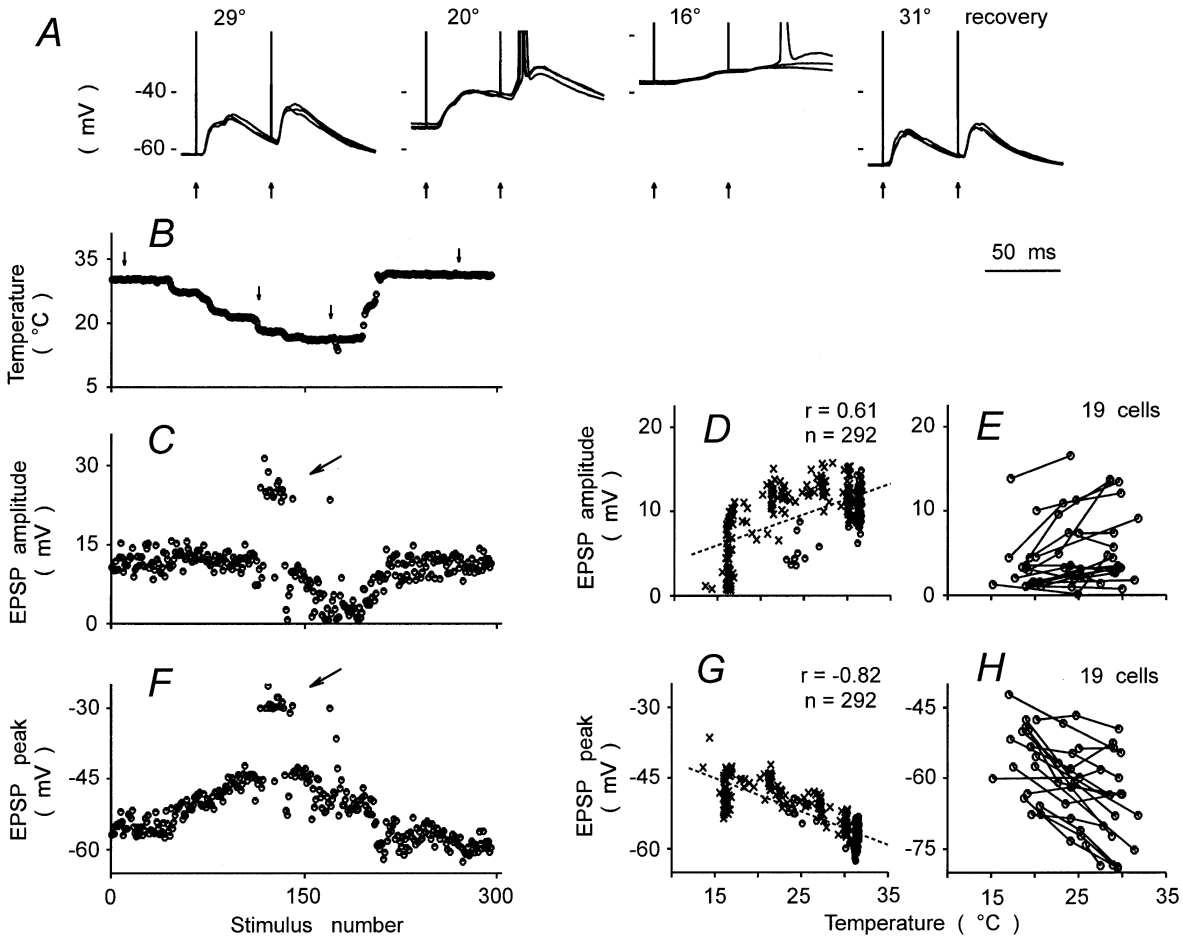

Fig. 5. Changes of the EPSP amplitude and of the membrane potential reached at the peak of the synaptic response with temperature. (A) EPSPs evoked in a layer 5 pyramidal cell (same cell as in Fig. 2) at different temperatures with paired-pulse stimulation. Superposition of three consecutive responses; spikes got truncated by the AD-converter. Arrows mark the stimulus artefact. (B) Time-course of changes of temperature in recording chamber. Arrows indicate values corresponding to the responses shown in A. (C, D) Time-course of changes of the response amplitude (C) and a scatter diagram showing dependence of the amplitude on temperature (D). For this plot, the response amplitude was measured as the peak amplitude of the EPSP evoked by the second stimulus in a paired-pulse paradigm relative to the membrane potential just before the stimulation. Oblique arrow in C points to the values corresponding to truncated spikes. These values were discarded when calculating correlation in D. (E) Group data for 19 cells. For each cell, temperature and corresponding EPSP amplitude values were averaged within 5°C intervals (circles) and connected by a line. (F, G) Time-course of changes of membrane potential reached at response peak (F) and a scatter diagram showing dependence of the EPSP peak on temperature (G). Since response peak was reached during the second EPSP, this measure was used for this plot. (H) Group data for 19 cells. For each cell, temperature and membrane potential at EPSP peak were averaged within 5°C intervals (circles) and connected by a line. A–D, F, G: Data from the same cell. For calculation of correlations in D and G, and of summary data in E and H, responses with spikes were discarded.

Action potential generation

Since cooling-induced depolarization shift of the membrane potential brings a cell closer to its firing threshold, cooling could facilitate spike generation. Indeed, in the above example (Fig. 5) synaptic stimulation reliably evoked action potentials when applied at temperatures around 20°C, but not at higher or lower temperatures. To address the question, whether stimulus intensity which is the threshold for spike generation, changes with temperature, we studied stimulus–response relationships at different temperatures.

Stimulus–response relationships. Synaptic responses evoked in a layer 2/3 pyramidal cell by stimuli of different intensities, applied at three different temperatures, are shown in Fig. 6A. In that cell, changing the temperature from 32 to 20°C had little effect on the stimulus–response relationship. Dependence of the amplitude of responses evoked by the first pulse in the paired-pulse paradigm (EPSP1) on stimulus intensity remained essentially the same (Fig. 6A, B). Amplitudes of responses evoked by the second pulse in a pair (EPSP2) were always slightly lower at 20°C (Fig. 6A, B). This indicates that paired-pulse facilitation (PPF) ratio was lower at 20°C than at 32°C over the whole range of tested stimulus intensities (see below).

However at 20°C, the resting membrane potential was 15 mV more positive than at 32°C and consequently, the stimulus–response curves for both EPSP1 and EPSP2 obtained at 20°C were shifted to more positive potentials (Fig. 6C). As a result, the spike generation threshold was reached with a lower stimulus intensity at 20°C (Fig. 6A, 120 μA). At a yet higher intensity (140 μA), action potentials were evoked reliably by both the first and the second EPSPs during the paired-pulse stimulation at 20°C, while only occasional spikes appeared on top of the second EPSP at 32°C. At 9°C the resting membrane potential was depolarized by a further 10 mV relative to that at 20°C. No clear EPSPs were evoked with stimuli of intensities below 120 μA, and thus the stimulus–response curves remained essentially flat

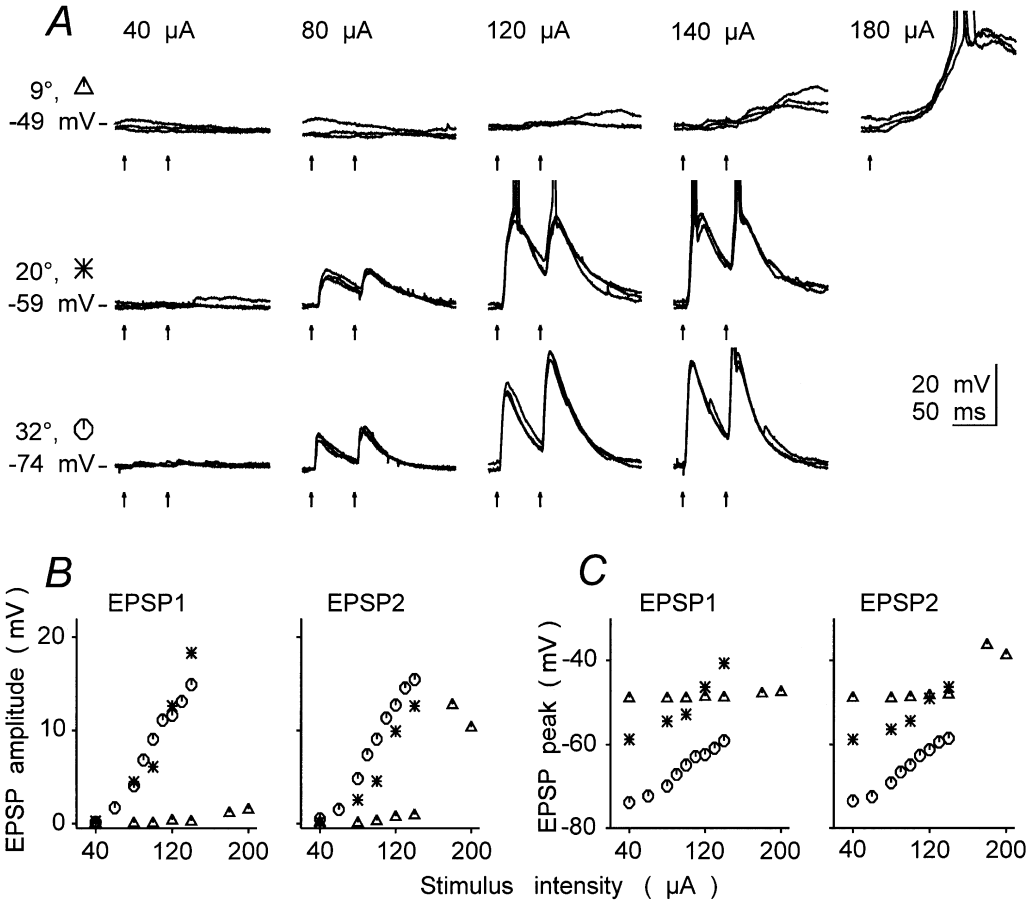

Fig. 6. Stimulus–response relationships in a layer 2/3 pyramidal cell at different temperatures. (A) EPSPs evoked in a layer 3 pyramidal cell at different temperatures by stimuli of increasing intensities. At each intensity and temperature, three consecutive responses are superimposed; spikes are truncated. Arrows indicate stimulus timing. Note changes of the resting membrane potential with temperature. (B) Dependence of the amplitude of EPSPs evoked by the first (EPSP1) and the second (EPSP2) pulse in a paired-pulse paradigm on stimulus intensity at 9°C (triangles), 20°C (asterisks) and 32°C (circles). (C) Dependence of the membrane potential reached at response peak (spikes excluded) for EPSP1 and EPSP2. Data from the same cell.

(Fig. 6A, B). Increasing the stimulus strength to 140 µA led to the appearance of some responses, and stimulation became suprathreshold at 180 µA.

Therefore, the threshold stimulus intensity necessary to evoke spikes was minimal at 20°C, higher at 32°C and even higher at 9°C. Notably, the cell was capable of generating spikes in response to synaptic stimulation even at temperatures as low as 9°C.

Hyperexcitability during moderate cooling. The data on temperature dependence of stimulus–response relationships indicate that moderate cooling to temperatures around 20°C leads to a decrease of the threshold stimulus intensity for spike generation and thus might lead to hyperexcitability of neocortical cells. To evaluate the temperature range at which hyperexcitability occurs, we adjusted the stimulus intensity to evoke strong EPSPs, that were, however, still subthreshold for spike generation and had an amplitude of 15–25 mV at around 30°C. Then test stimuli of that intensity were applied repetitively while changing the temperature in the recording chamber. In the cell illustrated in Fig. 7, the PSP responses remained subthreshold at temperatures above 20°C. Similarly to the data described above, the EPSP amplitude remained unchanged or had a tendency to increase slightly when the temperature was lowered, from 28.0 ± 0.1 mV at 30°C to 28.9 ± 0.09 mV at 26–27°C ($P < 0.1$; Fig. 7C). With further cooling, the response amplitude decreased, and became more variable. Since the membrane potential of the cell strongly depended on temperature and the cell became depolarized with cooling (Fig. 7B), the response peak reached higher values at lower temperatures. At 18–14°C, the depolarization potential at the response peak was maximal, occasionally exceeding spike threshold and thus leading to the generation of action potentials (Fig. 7A, D). With further cooling, the spikes disappeared. However, with stronger synaptic stimulation, the spikes could still be evoked in some of the other cells even at 9 or 10°C (e.g. Fig. 6). The EPSP amplitude recovered upon re-warming, but because of the rapid recovery of the membrane potential, the responses were again well below spike threshold (Fig. 7A). Qualitatively similar results were obtained for the cell recorded in a slice of the cat visual cortex. Synaptically evoked responses were subthreshold at 34°C, but led to action potentials at temperatures of 23–21°C and became subthreshold again upon further cooling (data not shown).

Altogether, we applied this stimulation protocol in 10 cells, and hyperexcitability during moderate cooling was observed in seven cells, including one non-pyramidal cell from layer 4 and one layer 5 pyramid. In the remaining three cells, the responses remained subthreshold at any temperature tested. We note here that the fact that the stimulation with a given intensity did not lead to spike generation

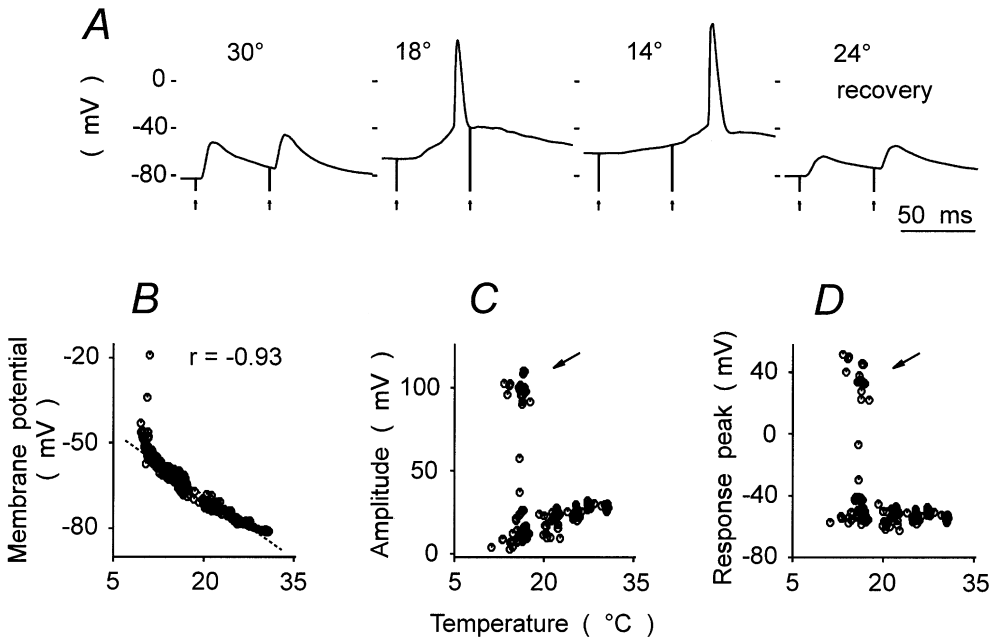

Fig. 7. Subthreshold synaptic stimulation of a rat layer 2/3 pyramidal cell becoming suprathreshold during moderate cooling. (A) Responses evoked in the cell by stimuli of a constant intensity at different temperatures. Stimuli were applied as paired pulses with a 50-ms interpulse interval; arrows indicate stimulus artefacts. (B–D) Dependence of membrane potential (B), response amplitude (C) and response peak (D) on temperature. Note that action potentials (responses with amplitudes of about 100 mV) were generated only at temperatures around 14–18°C. Data from the same cell.

at any temperature does not exclude the possibility that these cells were hyperexcitable at some temperatures, if somewhat stronger stimuli would have been applied. In fact, our stimulation protocol was designed for evaluation of the temperature range in which cells are hyperexcitable, but not for demonstrating hyperexcitability as such, which is a very consistent phenomenon. In Fig. 8 the temperature ranges at which action potentials occurred in the seven cells in response to synaptic stimulation are shown as grey bars. Typically, hyperexcitability was observed at temperatures between 18 and 24°C, although in different cells it could occur from 14 to 26°C (Fig. 8). In all cases, synaptic stimulation of a given amplitude was suprathreshold only within a certain temperature range, but remained subthreshold at lower and higher temperatures.

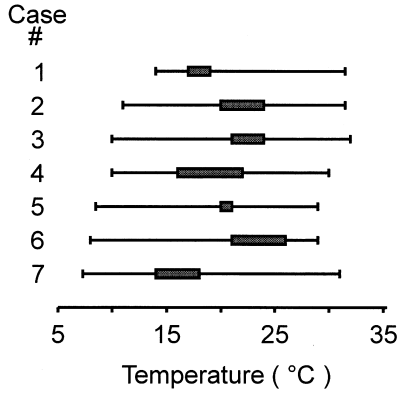

Fig. 8. Temperature range of hyperexcitability of cortical cells. For each of the seven cells, the temperature interval in which spikes were generated in response to synaptic stimulation is indicated as a grey bar superimposed on the whole range of temperatures tested. Stimulus intensity used was: cell 1, 21.2 μA; 2, 275 μA; 3, 222 μA; 4, 150 μA; 5, 200 μA; 6, 210 μA; 7, 300 μA; and was kept constant throughout an experiment.

Action potential threshold and shape. To further investigate mechanisms of hyperexcitability during moderate cooling we studied temperature dependence of the spike generation threshold. It was assessed from responses to strong synaptic stimulation, which was capable of inducing action potentials over a wide temperature range. Threshold for spike generation was estimated as the potential from which the regenerative process started. In the cell shown in Fig. 9, the threshold for action potential generation was less negative (higher) at a lower bath temperature. This is evident from comparison of response traces at different temperatures (Fig. 9A) and from superposition of the spikes evoked by synaptic stimulation at different temperatures (Fig. 9B, left). However, since cooling-induced depolarization of the cell membrane occurred with an even higher gradient than the shift of the spike threshold, the distance from the actual resting membrane potential to the threshold decreased upon cooling (Fig. 9C). For each cell, we performed multiple (17–173) measurements of the spike threshold relative to the actual resting membrane potential at different temperatures, and calculated correlation between these two values. In the majority of cells tested (five out of seven) strong and highly significant positive correlation was found between the relative threshold and temperature ($r > 0.55$, $P < 0.001$). In these cells, cooling brought the membrane potential clearly closer to the spike generation threshold (Fig. 9D). In another cell the relative spike threshold was not correlated with temperature, and the distance from the resting membrane potential to spiking threshold remained unchanged. In one more cell, the relative threshold slightly increased at lower temperatures (Fig. 9D).

Changing the bath temperature also had dramatic effects on the shape and the amplitude of the action potentials. The most pronounced effect of lowering the temperature was broadening of the spikes, which is clearly visible in

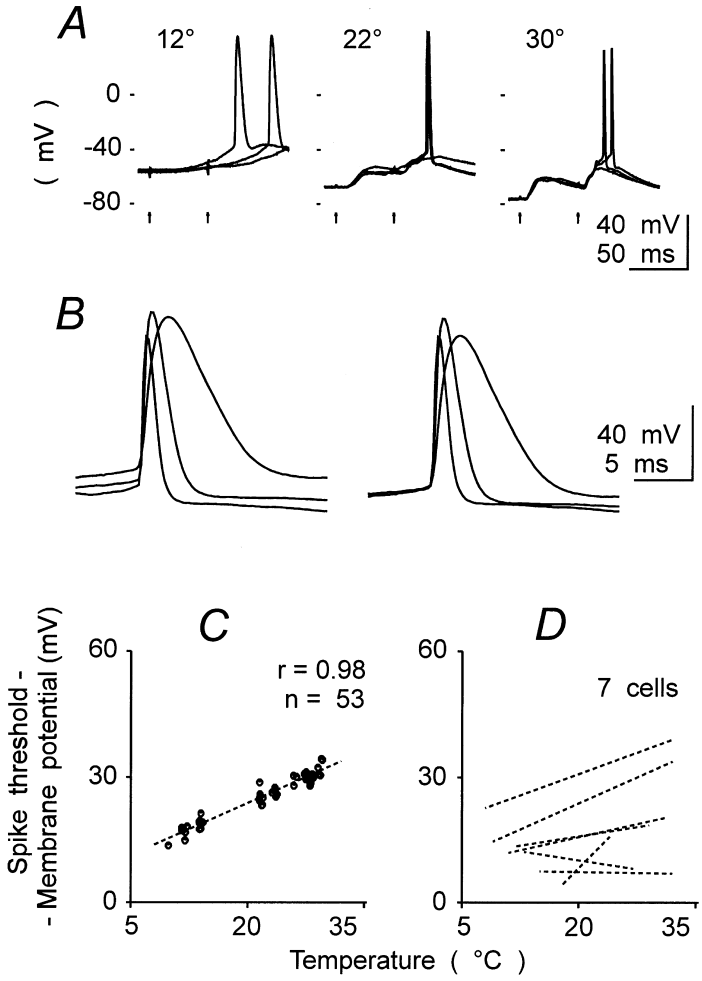

Fig. 9. Effect of cooling on the threshold and shape of synaptically evoked spikes in a layer 2/3 pyramidal cell (A–C) and group data for seven cells (D). (A) Responses evoked in the cell by a suprathreshold stimulus at different temperatures. Stimuli were applied as paired pulses with a 50-ms interpulse interval; arrows indicate stimulus artefacts. Three responses at each temperature are superimposed. (B) Superposition of action potentials evoked at different temperatures. Spikes from A are shown at high temporal resolution. On the left, the origins of the spikes are aligned on the time-scale, and on the right on both time and membrane potential scales to facilitate comparison of their shapes. (C, D) Dependence of the difference between the threshold for spike generation and resting membrane potential on temperature in the cell shown in A (C) and group data for seven cells (D). Each regression line was calculated on the basis of 17–173 data points, obtained for an individual cell. Regression lines are drawn for the temperature intervals in which measurements were made.

Fig. 9B where action potentials generated at different temperatures are superimposed at high temporal resolution. Spike broadening occurred monotonically, over the whole temperature range tested. The amplitude of the action potentials increased with moderate cooling due to a greater overshoot. With further cooling below 20°C, the spike amplitude started to decrease due to both a slightly decreased overshoot and a depolarizing shift of the resting membrane potential and spike threshold (Fig. 9B). In this and in the other cells tested, the amplitude of the action potential was maximal at temperatures around 20–23°C.

Paired-pulse interaction

To assess temperature effects on dynamic properties of synaptic transmission we measured paired-pulse interaction at different temperatures. Most of the factors contributing to paired-pulse interaction are believed to have a presynaptic origin.[14,25,38,42,56] The amplitude of response to the second pulse in a pair depends on the portion of transmitter that remains available for release after the first response, on the speed of refilling of the transmitter vesicle pool and on the facilitatory effect of residual calcium. Due to these factors, PPF ratio has a roughly inverse relation to the transmitter release probability. This relation may be partially masked by the contribution of postsynaptic factors to shaping the response to the second stimulus, such as receptor desensitization, rate of transmitter uptake, or involvement of voltage-sensitive mechanisms of the cell membrane.

For the cell whose responses are shown in Fig. 10A, the PPF ratio was lower at 19°C than at 29°C (0.97 and 1.46, respectively). The same tendency for decrease of the PPF at lower temperatures was also evident for other cells in which measurements were made at least at two temperatures. There was a significant positive correlation ($P < 0.01$) between PPF ratio and temperature (Fig. 10B), and for the majority of cells the PPF ratio decreased at lower temperature (Fig. 10C). As a result, PPF ratio at room temperature (18–23°C) was significantly lower than at 28–33°C (0.83 ± 0.06 vs 1.13 ± 0.03, $P < 0.001$, Fig. 9D). Another effect of lowering the temperature was an increase of the variability of PPF ratios between cells, evident in Fig. 9B–D.

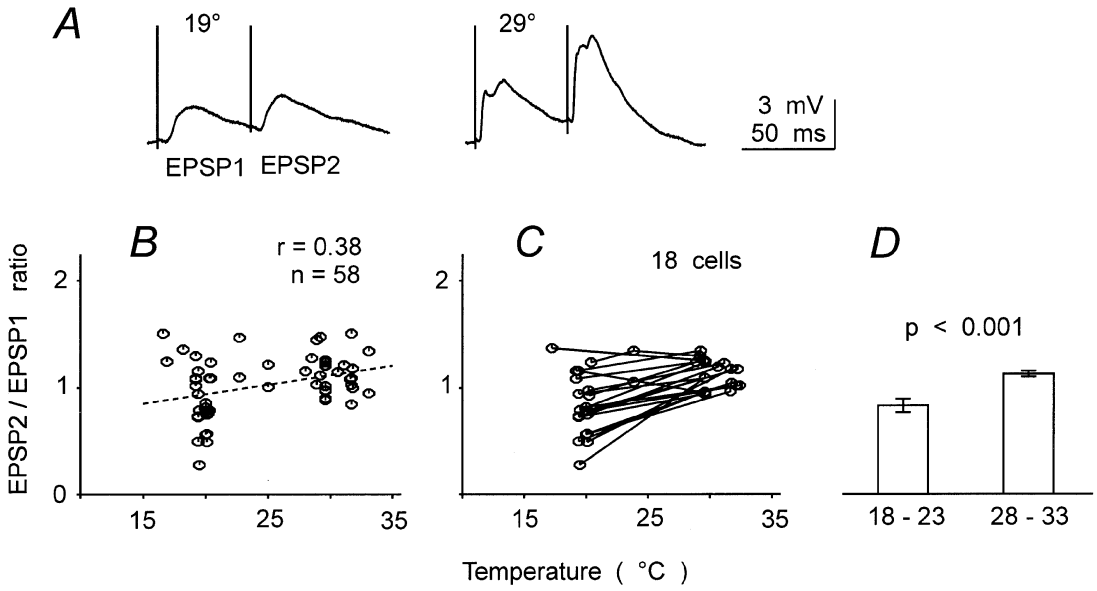

Fig. 10. Effect of temperature on paired-pulse interaction ratio. (A) Averaged EPSPs evoked by paired-pulse stimulation in a layer 5 pyramidal cell at different temperatures. (B–D) Summary data on temperature dependence of the paired-pulse interaction ratio (EPSP2/EPSP1 ratio) in 18 cells. In B, individual data obtained at different temperatures are shown. Any cell contributed only one point at a given temperature. In C, for each cell temperature and paired-pulse interaction, ratios were averaged within 5°C intervals (circles) and connected with a line. In D, paired-pulse interaction ratios for two temperature intervals were averaged across the cells. Vertical scale is the same in B–D.

DISCUSSION

Our results show that basic properties of neuronal membranes, synaptic transmission and generation of action potentials in cortical cells are dramatically influenced by temperature changes. These results have several important implications for both *in vitro* and *in vivo* studies. Firstly, the quantitative description and comparison of parameters of synaptic transmission at different temperatures point out the severe limitations in drawing inferences from results obtained at room temperature regarding the behaviour of nerve cells at physiological temperatures. Secondly, our results show how varying the temperature by several degrees alters the excitability of cortical cells, as well as the timing and duration of their responses. These changes in individual cells' behaviour provide the basis for changes in the population responses associated with even minor alterations of brain temperature.[2,13,34] Thirdly, our results identify cellular mechanisms which modify excitability bidirectionally during gradual cooling, leading first to hyperexcitability and only thereafter to a gradual depolarization block. An important consequence for *in vitro* studies is that cortical cells are hyperexcitable at room temperature. Enhanced excitability of individual cells and increase of the population responses associated with moderate cooling[2,4,34] should also be taken into account in *in vivo* experiments that use cooling as a method of "inactivation" of specific brain regions.

Effect of temperature on basic membrane properties

Basic properties of the cell membrane strongly depend on the temperature.[5,27,44,51,54] Lowering the temperature leads to depolarization of the cell membrane, increase of the input resistance and the membrane time-constant and a broadening of the action potentials. The amplitude of spikes was maximal at temperatures around 20–24°C. The mechanism of these changes rests on the difference in the temperature dependence of the potassium and sodium channels. Potassium conductance, both passive and active, dramatically decreases with cooling, while sodium conductance changes little (see Ref. 54 for details). Decreasing the ratio between potassium and sodium conductance at lower temperatures leads to depolarization of the cell membrane.[27,44,51,54] Absolute decrease of the passive potassium conductance results in an increase of the input resistance and of the membrane time-constant.[19,27,44,51,54] Our finding that a linear relation between the input resistance and the membrane time-constant remained over a wide temperature range suggests that the increase of the membrane time-constant was due mainly to the increased input resistance, and that the membrane capacitance did not change significantly. Cooling-induced decrease of active potassium conductance leads to a broadening of spikes and a marked increase of their area.[19,27,44,51,54] Temperature dependence of passive and active membrane properties and underlying mechanisms are discussed in detail elsewhere.[54]

Temperature dependence of synaptic transmission

Latency and time-course of synaptic responses. Cooling led to a monotonic change of the temporal characteristics of the synaptic responses, namely, increase of latency and its jitter and prolongation of the EPSP time-course. Increases of the response latency and of the dispersion of time of transmitter release are consistent with classical observations at the neuromuscular junction[24] and with field potential recordings from the spinal cord[4] and neocortex.[2] Similar effects were reported recently for excitatory synaptic transmission in the neocortex[19] and cerebellum[46] and inhibitory transmission in the hippocampus.[1] Several mechanisms could be responsible for these changes. A general slowing down of the biochemical processes, diffusion, and channel kinetics may lead to an increase in both EPSP latency and duration. An increase in the membrane time-constant might also prolong duration of synaptic responses. Finally, depolarization of the cell

membrane, associated with lowering of the temperature, may enhance activation of *N*-methyl-D-aspartate (NMDA) receptor-mediated[23,35,49,50] and other voltage-dependent conductances[10,21,31,47] which could further prolong the EPSP duration.

Release probability and reliability of synaptic transmission. Our experimental data suggest that the release probability can either increase or decrease upon moderate cooling. Our finding that PPF ratio is higher at temperatures above 30°C than at room temperature can be interpreted as indicative of increased release probability at room temperature.[14,25,38,42,56] Consistent with that are observations that in some cases the EPSP amplitude increase with moderate cooling[19] and our own data presented here. In contrast, other results of Hardingham and Larkman[19] are indicative of decreased release probability at lower temperatures. They found that in the neocortex, lowering the temperature led to an increase in the failure rate and the coefficient of variation of synaptic responses and a decrease in the frequency of spontaneous PSPs. However, these apparently conflicting data are consistent with the assumption that the strength and duration of short-term depression increase at lower temperatures. Short-term depression is attributed to a depletion of the "ready to release" pool of synaptic vesicles and its incomplete replenishment during short interstimulus intervals.[56] Slower biochemical processes at lower temperatures should result in a slower refilling of transmitter vesicle pool, and thus in an increased duration of short-term depression. This, in turn, can account for a stronger depression of the response to the second pulse in a paired-pulse paradigm, as we had observed. At the same time, with a stimulation frequency of 0.5 Hz used in study of Hardingham and Larkman,[19] slower replenishment of the "ready to release" vesicle pool may lead to a low-frequency depression of synaptic transmission and thus to an apparent decrease in release probability.

Cooling changes several factors that influence the probability of transmitter release and the reliability of synaptic transmission and these factors may also act in opposite directions. Decrease in the activity of K^+ channels associated with cooling may enhance release by two mechanisms. Firstly, it facilitates propagation of an action potential along the axon and decreases the probability of propagation failures at branch points.[9] This factor could be especially important for transmission between neocortical neurons, which typically have connections consisting of several release sites located at different terminals of the same axon.[6,11,30] In the case of compound PSPs, enhanced excitability associated with moderate cooling (see below) may increase the number of presynaptic fibres activated by a given stimulus, or increase reliability of their activation. Secondly, a decrease of the activity of K^+ channels leads to an increase in the width and area of action potentials, and hence a greater Ca^{2+} surge can be expected. A recent study has indeed reported that more Ca^{2+} enters presynaptic terminals at room temperature than at 35°C.[3] Higher levels of presynaptic calcium reached during a broader action potential might enhance transmitter release.[25,38,56] At the same time, the slower biochemical reactions may result in an increased threshold for transmitter release and thus a lower release probability at lower temperatures.

A balance between these factors could account for both increases and decreases of the release probability upon moderate cooling. With cooling below room temperatures, the factors hindering the release start to prevail and synaptic transmission is suppressed. However, synaptic stimulation is still capable of inducing spikes even at temperatures as low as 9°C.

Response amplitude. Both increases and decreases of the amplitude of EPSPs were observed during cooling from physiological temperatures to 30°C[19] or to 20–22°C (our data), but further cooling always led to a reduction in the response amplitude. Interestingly, even in those cases when response amplitude, measured from the resting membrane potential to the peak, decreased with cooling, the EPSP nevertheless peaked at more depolarized potentials at lower temperatures. This occurred because during cooling the gradient of depolarization of the resting membrane potential was usually higher[54] than the gradient of the EPSP amplitude decrease. One important consequence of this relation is a hyperexcitability of cortical neurons at room temperature (see below).

The amplitude of synaptic responses is influenced by several temperature-dependent factors which can act in opposite directions and thus cause either an increase or a decrease of the response amplitude during cooling. The first factor is release probability, which can change in either direction during moderate cooling (see above). The second group of factors include changes of transmitter spread in the synaptic cleft, alterations in the kinetics of receptor binding and in channel kinetics. These factors can also lead to an increase or decrease of the response amplitude at lower temperatures. Thirdly, lowering the temperature decreases transmitter uptake and increases transmitter spillover.[28] Prolonged presence of the transmitter in the synaptic cleft may lead to an increase in the response amplitude. Fourthly, depolarization of the cell membrane, associated with cooling,[27,44,51,54] decreases the driving force for postsynaptic currents and thus decreases their amplitude. However, due to the increased input resistance, less postsynaptic current will be required to produce a membrane potential shift of a given amplitude. The increase of the input resistance will therefore counteract the effect of decreased driving force on the EPSP amplitude. Finally, the depolarization of the cell enhances activation of NMDA receptor-mediated[23,35,49,50] and other voltage-dependent conductances,[10,21,31,47] which could amplify the EPSP.

The net effect of lowering the temperature on response amplitude will depend on a combination of these factors. The contributions of different postsynaptic factors seem to be balanced to some extent, as indicated by little change in the amplitude of spontaneous PSPs with temperature.[19] However, the overall balance of pre- and postsynaptic factors may change with temperature, leading to a non-monotonic dependence of the response amplitude on temperature.

Hyperexcitability at room temperature

Moderate cooling can lead to an increase in the size of evoked potentials, augmentation of the background cortical activity and an increase of the amplitude of population spikes in the hippocampus.[2,4,5,34] These results suggest an increased excitability of the neurons. Three groups of results of the present study demonstrate that cortical cells are hyperexcitable at room temperature. Firstly, cooling usually brings the cells closer to the threshold for spike generation. At the same time, the amplitude of excitatory postsynaptic responses is

influenced relatively little by temperature changes in the range from about 21 to 36°C. Secondly, stimuli of constant strength, applied at different temperatures, often led to action potential generation at temperatures between 15 and 25°C, but remained subthreshold at both lower and higher temperatures. Thirdly, direct assessment of spiking threshold from stimulus–response relationships demonstrated that the stimulus intensity, necessary to evoke spikes was minimal at temperatures around 20°C.

The ionic mechanism of hyperexcitability at room temperature rests on the strong dependence of potassium channel function on temperature.[54] Decrease of potassium conductance during cooling increases the input resistance and also leads to depolarization of the cell membrane. The absolute value of the activation threshold of voltage-dependent sodium channels does not change when the membrane potential is clamped,[54] or may change only a little as a secondary effect of depolarization. As a result, less excitatory input might be sufficient to evoke spikes. Decrease of excitability, associated with further cooling, could be accounted for by a synergistic action of the following factors. Because of the further depolarization, the membrane potential approaches the region of depolarization block for action potential generation. In addition, the rising slope of the EPSP becomes less steep. Taken together, this hinders simultaneous activation of a sufficient number of voltage-gated sodium channels which is necessary for the initiation of an action potential.

One possible consequence of hyperexcitability *in vitro* could be amplification of the effect of tetanic stimulation at room temperature. Together with several additional synergistic factors, such as larger Ca^{2+} influx due to broader spikes[3] and a more depolarized membrane potential, it could result in lowering the threshold for induction of plastic changes of synaptic transmission at room temperature. A tetanus, ineffective at higher, more physiological temperatures, could be strong enough to induce plasticity at room temperature. This conjecture is compatible with experimental data on hippocampal plasticity.[32]

Implications for in vitro *studies: synaptic transmission above* 30°*C and at room temperature*

In vitro studies are usually performed either at 20–24°C (room temperature) or at temperatures above 30°C. Below we briefly compare properties of synaptic transmission in these temperature ranges and discuss possible caveats to bear in mind while drawing conclusions regarding *in vivo* conditions from data obtained at room temperature.

The experimental data and the above considerations indicate that changing the recording temperature from above 30°C to the room temperature range may lead to bidirectional changes of the release probability and the amplitude of responses evoked by single pulses applied at a sufficiently low frequency. At the same time, this temperature change dramatically alters dynamic aspects of synaptic transmission: response latency increases, time-course becomes slower, and the strength and duration of short-term depression increases.[1,19,24,40,44] These changes may be especially apparent in the responses to trains of stimuli. Increased short-term depression will lead to redistribution of synaptic efficacy within the train, namely, to stressing the response to the first pulse in a train at the expense of the following responses. Interestingly, this effect is further amplified by changes in the repetitive firing of cells. At room temperature, the generation of the first spike in response to an intracellular current step is enhanced, but generation of further spikes is suppressed as compared with higher temperatures. Furthermore, at room temperature the first spike in a train has an increased amplitude, while the remaining spikes are smaller.[51,54] One further form of the short-term plasticity which is enhanced at room temperature is frequency depression. Because of the slower replenishment of the vesicle pool after each release, depression will be more apparent at room temperature than at physiological temperatures at lower stimulus frequencies. Thus, for a given stimulation frequency, low-frequency depression can be salient at room temperature, but may play little part at physiological temperatures.

Therefore, only *in vitro* data obtained at temperatures above 30°C are directly relevant for applying the knowledge gained to questions of synaptic transmission *in vivo*. It appears complicated to use data gathered at room temperature for estimating physiological values of parameters like release probability, reliability, response amplitude, and especially dynamic properties of synaptic transmission. Since temperature changes alter a number of different factors, which affect synaptic transmission in opposite directions, reservations are always necessary when inferences about normal synaptic function are drawn from room temperature data.

Implications for in vivo *studies*

Our results have important implications for *in vivo* studies which use cooling as a method of inactivation of specific brain regions. The mechanism of inactivation by cooling is a depolarization block, and before a complete inactivation is achieved, the cells inevitably pass through a phase of hyperexcitability. This conjecture is supported by *in vivo* data on evoked potentials and unit firing.[2,4,33,34] We note here that hyperexcitability only means that cells are closer to the spike threshold, and that less excitatory drive is required to evoke their discharges. As a result, more cells may be brought to spiking by a given stimulus. But at the same time, since the repetitive firing is usually gradually suppressed even with moderate cooling,[51,54] less spikes are expected to be generated by each single neuron during the response. Indeed, a decrease of the number of single-unit spikes in responses to sensory stimulation during cooling has been consistently observed.[17,22,29,33,36,52]

The other consistent and unambiguous effect of cooling was on the temporal properties of synaptic transmission. Even moderate cooling would lead to severe, if not total, destruction of the temporal structure of spike trains. This factor may allow to assess the possible role which precise coherent temporal structure of the output from localized cortical networks may play in brain processes.

Thus, cooling of a brain region should lead to clear and dramatic alteration of its output: a decrease of the total spike count and modification of the temporal pattern of spiking, whereby the initial response may be relatively strengthened at the expense of the later response phases.

However, an extremely complicated picture of changes is expected in a cooled cortical area because of high temperature gradients measured around the cooled region *in vivo*[17,29] and because of differences between individual cells in the dependence of the basic membrane properties[54] and synaptic

transmission on temperature. A complete block of spike generation is reached only when the temperature of the tissue is well below 10°C. Our data show that the cells are still capable of generating spikes in response to synaptic stimuli at temperatures as low as 7–10°C, and *in vivo* data obtained with a thermocouple glued to the recording microelectrode demonstrated that visually evoked responses disappear usually below 10°C, but in some cells were still present even at 5°C.[17,33] The neurons that are not cooled to that extent could be still in a hyperexcitable state rather than "inactivated". Because of the high temperature gradients,[17,29] moderately cooled, and therefore hyperexcitable, tissue will surround the region under the cooling device. For example, cooling the plate, positioned on the cortical surface, even to the freezing point (0°C) may result in only a local silencing of the region located within about 2 mm from the plate, while mixed effects — silencing of some cells but hyperexcitability of some others might occur in the surrounding regions. When the temperature of the plate is 10°C or higher, no secure inactivation is achieved even close to the plate, and only regions of mixed effects or of hyperexcitability are expected.

In general, our data and the above considerations allow us to conclude that local cooling may be an adequate inactivation technique for studying interactions between brain regions, when recording and cooling sites are well separated and sufficient cooling that is required to completely silence one region, or at least to reduce substantially its output, is achieved without significantly altering the temperature of the region under study. On the other hand, cooling is definitely not the best method for studying mechanisms of neural processing within the cooled region,[15,43] because a complicated pattern of changes in a non-uniformly cooled region does not allow prediction of even changes of the major factors, say of an overall balance between excitation and inhibition. Furthermore, since reliable inactivation of the local networks can be achieved only at temperatures that severely affect basal synaptic transmission, cooling is hardly an adequate method for studying the input to the cooled region.[15]

Our results also help to identify cellular processes which underlie changes in the amplitude of evoked field potentials, accompanying minor changes of the brain temperature *in vivo*,[2,13,34] and provide direct evidence for the mechanisms that were suggested to account for the field potential changes.[2,34,37,40] Our data demonstrate that lowering the temperature by a few degrees led to depolarization of the cells and a corresponding increase of their excitability, to an increase of the EPSP duration, and to an increase in both the amplitude and the width of action potentials in the neocortical cells. With warming, all these factors alter in the reverse direction. A multiplicative effect of these factors would explain the large magnitude of field potential changes associated with minor changes of brain temperature.

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