EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

European Journal of Neuroscience, pp. 1–11, 2010 doi:10.1111/j.1460-9568.2010.07453.x

Increased excitability of cortical neurons induced by associative learning: an *ex vivo* study

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Keywords: barrel cortex, BK channels, classical conditioning, intracellular recordings, mice, vibrissae

Abstract

In adult mice, classical conditioning in which whisker stimulation is paired with an electric shock to the tail results in a decrease in the frequency of head movements, induces expansion of the cortical representation of stimulated vibrissae and enhances inhibitory synaptic interactions within the 'trained' barrels. We investigated whether such a simple associative learning paradigm also induced changes in neuronal excitability. Using whole-cell recordings from *ex vivo* slices of the barrel cortex we found that layer IV excitatory cells located in the cortical representation of the 'trained' row of vibrissae had a higher frequency of spikes recorded at threshold potential than neurons from the 'untrained' row and than cells from control animals. Additionally, excitatory cells within the 'trained' barrels were characterized by increased gain of the input–output function, lower amplitudes of fast after-hyperpolarization and decreased effect of blocking of BK channels by iberiotoxin. These findings provide new insight into the possible mechanism for enhanced intrinsic excitability of layer IV excitatory neurons. In contrast, the fast spiking inhibitory cells recorded in the same barrels did not change their intrinsic excitability after the conditioning procedure. The increased excitability of excitatory neurons within the 'trained' barrels may represent the counterpart of homeostatic plasticity, which parallels enhanced synaptic inhibition described previously. Together, the two mechanisms would contribute to increase the input selectivity within the conditioned cortical network.

Introduction

In the adult neocortex, representational maps can be modified by sensory experience and by learning (Kossut, 1992; Buonomano & Merzenich, 1998; Kilgard *et al.*, 2002; Feldman & Brecht, 2005; Ohl & Scheich, 2005; Weinberger *et al.*, 2009). These processes are guided by neuronal activity and are supported by molecular cues (Fox & Wong, 2005). One of the possible mechanisms of barrel cortex plasticity may involve an increase of neuronal intrinsic excitability. Intrinsic excitability changes after learning were recorded in mammals in the cerebellum, hippocampus, and piriform and motor cortices (Brons & Woody, 1980; Moyer *et al.*, 1996; Saar *et al.*, 1998; Schreurs *et al.*, 1998). However, the changes of membrane properties underlying this phenomenon have yet to be fully explained. We investigated this mechanism in the barrel cortex of mice, where organization and plasticity are reasonably well described (Woolsey & Van der Loos, 1970; Feldman & Brecht, 2005).

It has been shown that even in adult animals a change in sensory input can modify physiological properties of neurons in the barrel cortex (Kossut, 1992; Petersen, 2007; Feldman, 2009). We have investigated a form of associative learning based upon the classical conditioning paradigm in which stimulation of a row of whiskers was paired with a tail shock. This treatment results in enlargement of the cortical functional representation of the 'trained' whiskers (Siucinska & Kossut, 1996). Neither whisker stimulation alone nor pseudoconditioning with unpaired presentation of stimuli produced a similar effect.

The expansion of cortical representation following conditioning was NMDA receptor-dependent (Jablonska *et al.*, 1999) and was accompanied by increased expression of mRNA and the protein of regulatory NMDA receptor subunit NR2A (Skibinska *et al.*, 2005). However, it also enhanced GABA and GAD immunoreactivity (Siucinska *et al.*, 1999; Gierdalski *et al.*, 2001; Siucinska & Kossut, 2006) as well as GABAergic synaptic transmission (Tokarski *et al.*, 2007) within the 'trained' barrels. Recently, inhibitory synaptogenesis was found within the changing cortical representation of the 'trained' whiskers (Jasinska

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Received 2 July 2010, revised 24 August 2010, accepted 26 August 2010

et al., 2010). Given the homeostatic rules governing plasticity (Turrigiano & Nelson, 2000) we expected that the increased inhibitory interactions should be paralleled by enhancement of some local excitatory processes. A situation in which enhanced inhibitory synaptic transmission accompanied intrinsic and excitatory synaptic modifications was recently found in piriform cortex after olfactory discrimination learning (Brosh & Barkai, 2009).

As increased neuronal excitability has been reported in several investigations of learning (Moyer *et al.*, 1996; Schreurs *et al.*, 1998; Oh *et al.*, 2003; Saar & Barkai, 2003; Matthews *et al.*, 2008), we hypothesized that it may also operate in learning-induced cortical plasticity within the modified cortical representation of the 'trained' whiskers. The aim of the present study was to examine whether enhanced excitatory activity can be identified in the barrel cortex of adult mice after classical conditioning. We show that whisker conditioning results in increased excitability of layer IV excitatory neurons and suggest that this effect may be due to altered properties of BK channels.

Materials and methods

Experiments were performed on 6- to 7-week-old C57Bl/6 male mice. All experimental procedures were approved by the Local Ethical Commission and were performed in accordance with the European Communities Council Directive of 24 November 1986.

Animal training

Experimental animals were first accustomed to neck restraint for 10 min a day over 2 weeks. Mice from the conditioning group (CS + UCS group) were then placed in the restraining apparatus and vibrissae of row B on one side of the snout were manually stimulated with a fine brush, without touching neighbouring rows (conditioned stimulus, CS). This stimulation lasted 9 s with the last stroke accompanied by electrical shock applied to the tail (unconditioned stimulus, UCS; DC current, 0.5 s, 0.5 mA) and was followed by a 6-s break. The CS–UCS pairings were repeated four times per minute for 10 min/day for 3 days (see Siucinska & Kossut, 1996). The stimulated control mice (CS group) received only stimulation of row B vibrissae applied identically as in the CS + UCS group. The non-stimulated controls (naive group) comprised animals which were only habituated to the neck restraint.

The training sessions were filmed and head movements during application of the CS were counted. The observed reduction in head movements is akin to freezing observed during fear conditioning in which footshock is used as UCS and can be used as an indicator of learning (Cybulska-Klosowicz *et al.*, 2009).

Slice preparation and electrophysiology

Twenty-four hours after the end of training, mice were decapitated under inhalational anaesthesia with 4% isoflurane (Baxter, Deerfield, IL, USA). Brains were quickly removed and immersed in cold (0°C), artificial cerebrospinal fluid (ACSF) of the following composition (in mM): KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 24, $MgSO_4$ 4, $CaCl_2$ 0.5, D-glucose 10, sucrose 219 (300–308 mOsm). This solution (as well as the ACSF used for incubation and recording) was saturated with 95% O_2/5% CO_2 to pH 7.3–7.4. Slices (350 μm) containing a part of the barrel cortex were cut orthogonally to the rows of barrels in an oblique coronal plane (55° to the sagittal plane). Slices were stored in a submersion-type incubation chamber filled with warm ACSF (32°C) containing (in mM): NaCl 126, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 24, $MgSO_4$ 3, $CaCl_2$ 1, D-glucose 10. A single slice was transferred to a submerged recording chamber mounted on the upright microscope (Olympus BX61WI, Tokyo, Japan) and perfused (2–2.5 mL/min) with warm ACSF (32 ± 0.5°C) of composition similar to that in the incubation chamber but with 2 mM $MgSO_4$ and $CaCl_2$ (hereafter standard ACSF).

Individual barrels were identified within the slice under visual guidance with a low-magnification 4× objective (Fig. 1A). Only slices with five clearly visible barrels were used for electrophysiological recordings. Single neurons were visualized using a long-working-distance water-immersion 20× objective, near-infrared (775 nm) differential interference contract optics (IR-DIC) and a Hamamatsu C7500 video camera (Hamamatsu Photonics, Shizuoka, Japan). Whole-cell recordings were performed from visually identified neurons within layer IV of barrels B and D. Recording pipettes (4–6 MΩ) were prepared from standard-wall (1.2 mm OD) borosilicate glass capillaries, and were filled with (in mM): K-gluconate 120, NaCl 5, HEPES 10, EGTA 5, $CaCl_2$ 0.5, $MgCl_2$ 3, Na_2-ATP 2, Na-GTP 0.3; osmolarity: 280–290 mOsm; pH: 7.2–7.3.

In most experiments, the pipette solution also contained the fluorescent dye Alexa Fluor® 555 (50 μM; Invitrogen, Carlsbad, CA, USA) and neurons were filled by diffusion during the 30- to 60-min recordings.

Signals were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) working in the 'fast current clamp' mode. Signals were digitized at 20 kHz using a Digidata 1322A interface and were analysed in PCLAMP10 software (Molecular Devices). Electrophysiological recordings were performed by an experimenter blind to the treatment groups.

Response characteristics of the recorded neurons (Fig. 1B) were evaluated with intracellular injections of 600-ms rectangular current pulses. To determine the relationship between injected current and firing rate, the numbers of spikes evoked by current steps of increasing amplitude (25- or 50-pA increments) were measured in standard ACSF for individual cells (Fig. 2A and B). The average firing rate for each step was then calculated (total number of action potentials during the current pulse divided by the pulse length). Finally, the gain (slope) and firing threshold (measured as a current extrapolated at zero firing rate) parameters were measured from the straight lines fitted to these measurements (Fig. 2B), and averaged (Fig. 2C and D).

Parameters of action potential waveforms were determined from the first spike evoked by the minimal current pulse that elicited firing of the investigated cell. Spike threshold was calculated as a membrane potential at which $dV/dt = 20$ mV/ms. Action potential amplitude was measured as a difference between the threshold and the peak voltage of the spike, and fast after-hyperpolarization (AHP) amplitude as a difference between the threshold and the most negative value of membrane potential immediately following the spike. Rise and decay slopes were derived from the ascending and descending parts of the action potential, respectively, and were calculated as an average voltage slope between 10 and 90% of spike amplitude.

For 13 cells (representative sample taken from 'naive' and 'trained' animals) responses for injected current were additionally recorded in the presence of blockers of AMPA (CNQX, 20 μM), NMDA (CPP, 20 μM) and $GABA_A$ receptors (bicuculline, 10 μM).

The amplitudes of medium and slow AHPs were calculated from membrane potential changes following the current step which evoked 14–16 spikes. Medium AHP (mAHP) amplitude was measured as the maximal value of local negative deflection within 40–60 ms after the end of the pulse and slow AHP (sAHP) amplitude as a negative

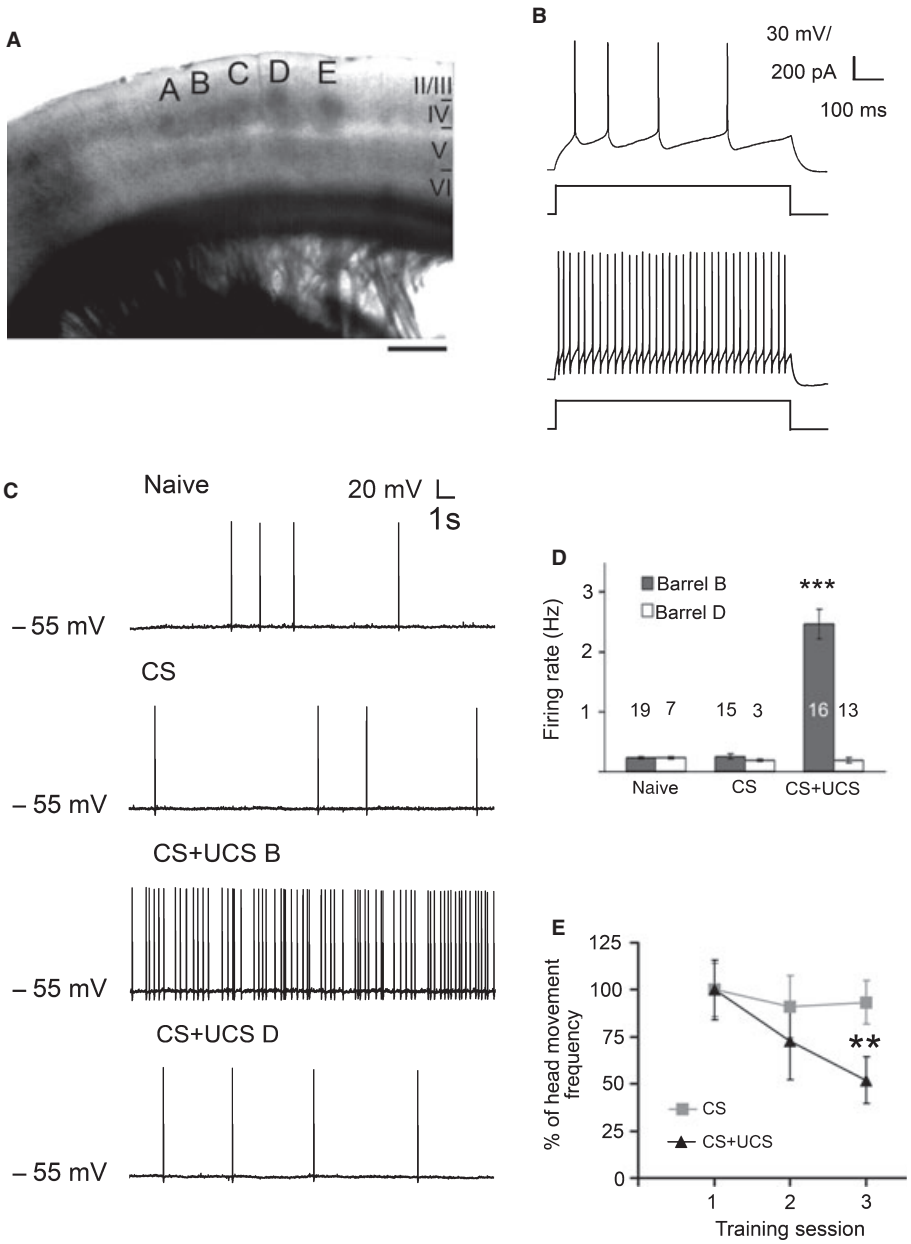

FIG. 1. Conditioning increased threshold firing rate of layer IV excitatory cells. (A) Image of a living slice. Letters denote barrels. Scale bar = 500 μm. (B) Responses to a depolarizing current pulse, characteristic for a regular spiking (RS) excitatory cell (top) and an inhibitory fast spiking neuron (bottom). (C) Representative traces of spiking activity recorded in modified ACSF at the threshold membrane potential from excitatory cells of unstimulated (naive) and stimulated (CS) controls, as well as from stimulated barrel B (CS + UCS B) and control barrel D (CS + UCS D) in trained mice. (D) Mean firing rate for neurons from barrels B and D of the three different groups of animals. The number of investigated cells is indicated on each bar. Asterisks denote significant differences between the mean rate obtained for barrel B in trained mice and each control group ($P < 0.001$). (E) Behavioral effect of conditioning. Reduction of head movement frequency from the first to the third training session was statistically significant for mice from the CS + UCS group ($P = 0.002$, Wilcoxon test) but not the CS group ($P = 0.36$, Wilcoxon). For each experimental group of animals the numbers of head movements were normalized to that during the first training session.

deflection 500 ms after the end of the step. Baseline was obtained from the 5-ms time period preceding the stimulus. Before each current step the membrane potential was adjusted to −70 mV.

Modified ACSF (3.5 mM KCl, 0.5 mM $MgCl_2$, 1.0 mM $CaCl_2$) of the composition close to the interstitial CSF *in situ* (Fishman, 1992; Sanchez-Vives & McCormick, 2000) was used to record spontaneous firing (according to Maffei *et al.*, 2004; Shruti *et al.*, 2008). Finally, iberiotoxin (60–100 nM) was added to the ACSF to block BK channels. In some experiments the non-peptidergic BK-channel blocker paxilline (10 μM) was used instead of iberiotoxin. As no difference between the drugs was found, iberiotoxin was used in most experiments and data were pooled.

After recording, slices were fixed in phosphate buffer saline containing 4% paraformaldehyte. Twelve hours later slices were rinsed three times for 10 min each in phosphate-buffered saline and mounted on glass slides with Vectashield Mounting Medium (Vector Labs., Burlingame, CA, USA). High-resolution images were collected with a Leica SP5 confocal microscope with a 63×, 1.4 numerical aperture oil-immersion objective (see Fig. 4A). Classification of excitatory neurons into pyramidal and spiny stellate cells was based on

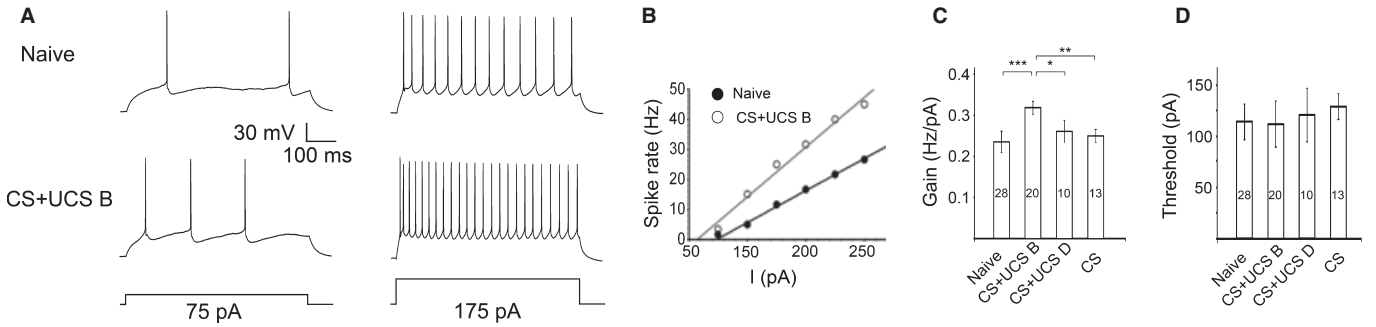

FIG. 2. Conditioning enhanced intrinsic excitability of RS neurons. (A) Firing of cells from barrel B in slices from naive (top) and CS + UCS animals (bottom) during current injections of 75 pA (right) and 175 pA (left). (B) Spike rate vs. injected current for the two cells for which responses are shown in A. (C) Mean gain (slope) of firing rate vs. injected current relationships and mean threshold values (D) of injected current calculated for all groups of mice. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Other details as in Fig. 1.

morphology of their dendritic tree. The presence of an apical dendrite extending out of layer IV into supragranular layers was used as a criterion for pyramidal cells (Feldmeyer *et al.*, 1999; Schubert *et al.*, 2003).

All chemicals were obtained from Sigma (St Louis, MO, USA) except for paxilline (Tocris, Bristol, UK). Throughout the text, the averaged data are presented as means ± SEM. The effect of training on passive and active membrane properties, frequency of spontaneous action potentials and parameters of the input–output relationship was assessed by one-way ANOVA followed by a Tukey–Kramer multiple comparison test. In other instances unpaired Student's *t*-test or paired Wilcoxon tests were used.

Results

The results are based on recordings from a total of 86 layer IV excitatory, regular spiking (RS) neurons, among which 22 belonged to the 'trained' barrel B of CS + UCS mice and 13 to barrel D of conditioned animals. We also recorded activity of 18 excitatory cells of barrels B of mice which received only CS treatment and of 33 cells belonging to barrels B or D of naive mice. Excitatory cells could be distinguished from fast spiking (FS) interneurons based on responses to depolarizing current pulses (Fig. 1B; for a review see Connors & Gutnick, 1990).

A behavioural index of learning was obtained from analysis of head movements during application of CS (Fig. 1E). This revealed that the reduction of head movement frequency from the first to the third training session amounted to 55% ($P = 0.002$, paired Wilcoxon test), indicating an association between CS and UCS (Cybulska-Klosowicz *et al.*, 2009). Note that all animals showed changed behaviour during the first three training sessions (see the learning curve in Fig. 1E). No change in head turning frequency was seen in the CS group.

The average resting membrane potential and input resistance (Table 1) did not differ significantly between the four experimental groups ($F_{3.85} = 0.85$, $P = 0.47$ and $F_{3.85} = 0.009$, $P = 0.99$ respectively, ANOVA). Also most of the parameters characterizing the spike shape were similar for the different groups of cells ($P > 0.28$, ANOVA). However, the amplitude of fast AHPs (fAHPs, Table 1) was significantly smaller for neurons from barrel B in CS + UCS mice than for barrel D in conditioned animals ($P = 0.0004$) and for barrel B from the CS group ($P = 0.008$) and from naive mice ($P = 0.0003$, ANOVA).

To obtain a general measure of neuronal excitability we evaluated the steady-state spike frequency at threshold membrane potential. Cells were depolarized to threshold potential (and began to fire spontaneously) using a manually adjusted DC current injection. In line with previous observations in the visual cortex (Maffei *et al.*, 2004), in standard ACSF the recorded neurons generated no spontaneous spikes at rest, and even with steady depolarization up to −40 mV they fired none or only a few action potentials (data not shown). However, when standard ACSF was replaced with modified ACSF, the composition of which better mimics the brain interstitial fluid *in situ,* the investigated cells reached the threshold for spike generation typically between −54 and −56 mV (mean value −54.7 ± 0.5 was similar for different experimental groups: $F_{3.85} = 1.25$, $P = 0.30$, ANOVA) and at this level they fired consistently for a long period of time. Measurements were taken from the first 30-s period.

The most striking and robust result of the threshold firing frequency test is shown in Fig. 1C. The threshold firing rate measured in layer IV RS cells after simple associative learning increased specifically within

TABLE 1. Passive and active membrane properties of recorded excitatory neurons

	Naive ($n = 33$)	CS ($n = 18$)	CS + UCS B ($n = 22$)	CS + UCS D ($n = 13$)
Resting membrane potential (mV)	−75.2 ± 0.5	−75.4 ± 1.0	−75.8 ± 0.7	−74.5 ± 0.9
Input resistance (MΩ)	144 ± 8	151 ± 11	146 ± 10	146 ± 16
Action potential height (mV)	88.5 ± 1.4	88.9 ± 3.0	87.5 ± 1.8	85.9 ± 1.9
Action potential threshold (mV)	−38.7 ± 0.7	−39.9 ± 1.1	−39.5 ± 0.8	−37.7 ± 1.5
Action potential half-width (ms)	0.69 ± 0.02	0.69 ± 0.02	0.71 ± 0.02	0.68 ± 0.02
Action potential rise slope (mV/ms)	405 ± 15	376 ± 18	382 ± 20	369 ± 24
Action potential decay slope (mV/ms)	−94.8 ± 3.4	−94.6 ± 5.7	−99.2 ± 4.6	−98.0 ± 5.4
Fast afterhyperpolarization amplitude (mV)	−15.5 ± 0.4	−15.9 ± 1.0	−12.3 ± 0.6*	−16.2 ± 0.5

*Differences between fast AHP amplitude measured for barrel B in CS + UCS mice and different controls are significant ($F_{3.85} = 10.26$, $P < 0.001$, ANOVA). Other parameters do not differ between various experimental groups ($P > 0.28$, ANOVA).

the 'trained' barrels which receive information from the stimulated row B of whiskers in CS + UCS mice (CS + UCS B trace). Indeed, these neurons generated action potentials at 2.48 ± 0.2 Hz, about ten times more often (Fig. 1D; $F_{3,69} = 52.16$, $P = 0.00015$, ANOVA) than similar cells from each of the control groups: 'untrained' barrel D in CS + UCS mice (0.25 ± 0.03 Hz), non-stimulated animals (0.24 ± 0.02 Hz) and barrel B in mice which received only CS stimulation (0.23 ± 0.03 Hz). Mean firing rates calculated for different controls were the same ($F_{2,53} = 0.14$, $P = 0.88$, ANOVA).

This elevated firing could be caused by stronger synaptic drive or by an increase of intrinsic excitability. However, using an identical training paradigm we recently found a conditioning-related increase of frequency of spontaneous inhibitory post-synaptic currents and no change in the frequency and amplitude of spontaneous excitatory post-synaptic currents recorded from layer IV neurons in the 'trained' barrel (Tokarski *et al.*, 2007). This would imply a decrease of the excitation/inhibition balance (i.e. weaker driving force) of the synaptic input to the investigated cells. Alternatively, the higher firing rate at threshold could result from the increase in intrinsic excitability (Maffei & Turrigiano, 2008). Thus, we assessed the relationship between injected current and firing rate, the slope of which is referred to as the response gain. We measured the responses to rectangular, 600-ms current steps of increasing amplitude in standard ACSF (Fig. 2A and B). The results presented in Fig. 2C show that the average gain for layer IV RS cells from 'trained' barrels (0.33 ± 0.01 Hz/pA) was significantly ($F_{3,70} = 27.5$, $P = 0.001$, ANOVA) larger than the gain measured for cells from barrel B in CS (0.25 ± 0.01 Hz/pA, $P = 0.003$) and naive mice (0.23 ± 0.02 Hz/pA, $P = 0.0002$) and from 'untrained' barrel D in trained animals (0.26 ± 0.02 Hz/pA, $P = 0.03$; ANOVA). The mean slopes obtained for all control groups were similar ($F_{2,55} = 0.94$, $P = 0.40$, ANOVA). Note also that mean threshold values did not differ between different groups of neurons: those recorded from barrel B in CS + UCS mice (113 ± 23 pA), from control barrel D in trained animals (121 ± 26 pA), as well as from non-stimulated (114 ± 18 pA) and stimulated (129 ± 14 pA) controls ($F_{3,70} = 0.93$, $P = 0.43$, ANOVA; Fig. 2D).

The parameters shown in Fig. 2 were calculated from the relationships between injected current and firing rate measured with active synaptic inputs to the investigated cells. To test whether the active synaptic conductances could affect the responses to current injection we blocked AMPA, NMDA and $GABA_A$ receptors in seven neurons from 'naive' mice and six cells from barrel B of 'trained' animals. The responses to injected current steps for two individual cells before and after blockage of the synaptic conductances are shown in Fig. 3A. The average slope and threshold calculated before and after application of synaptic blockers appeared similar, irrespective of cell group (Fig. 3B and C; $P = 0.42$ for naive and $P = 0.19$ for CS + UCS groups, Wilcoxon test). Note also that after application of synaptic blockers we still observed significantly greater slopes (0.29 ± 0.04 Hz/pA) for cells recorded from 'trained' barrel B, as compared with control neurons from naive animals (0.18 ± 0.02 Hz/pA; $t_{11} = 3.09$, $P = 0.01$, *t*-test). These results indicated that the active synaptic conductances did not significantly affect our results.

Larger response gain for neurons from 'trained' barrels indicated that these cells were more excited by the same depolarizing pulse of current than cells from the control groups. Thus, we concluded that cells from 'trained' barrels had significantly enhanced intrinsic excitability as compared with control neurons.

In order to differentiate morphologically the recorded sample of cells in layer IV we labelled them with Alexa 555 fluorescent dye, which allowed us to distinguish between pyramidal (40%) and stellate (60%) type morphology (Fig. 4A). Interestingly, stellate cells had about 30% greater response gain than pyramidal neurons (Fig. 4B) and this difference was similar for cells recorded from naive mice (0.25 ± 0.01 vs. 0.19 ± 0.01 Hz/pA; $t_{25} = 4.2$, $P = 0.0003$, *t*-test) as well as from barrel B in trained mice (0.36 ± 0.02 vs. 0.28 ± 0.02 Hz/pA; $t_{16} = 3.02$, $P = 0.008$, *t*-test). On the other hand, both cell types had similar threshold current values (Fig. 4C; $t_{25} = -0.28$, $P = 0.78$ for naive and $t_{16} = -0.22$, $P = 0.83$ for CS + UCS group, *t*-tests). Conditioning procedure seems to affect input–output relationships in both morphological classes of cells in a similar way, increasing the gain by about 45% of the corresponding value calculated for naive animals (Fig. 4B, compare appropriate values given above; $t_{24} = 5.46$, $P = 0.0001$ for stellate cells and $t_{17} = 4.45$, $P = 0.0004$ for pyramidal cells). In the following pharmacological parts of the research the results obtained for the two morphological cell groups were pooled, because the relatively small number of cells in each did not allow for quantitative comparisons. It is worth mentioning that hitherto we did not notice any obvious difference in the influence of the drugs on the investigated physiological properties of pyramidal and stellate cells.

An increase in intrinsic excitability might be caused by functional modifications of a specific group of ion channels responsible for the generation of different forms of spike AHPs. The smaller amplitudes of fAHPs in neurons from barrel B in trained mice (Table 1) suggested

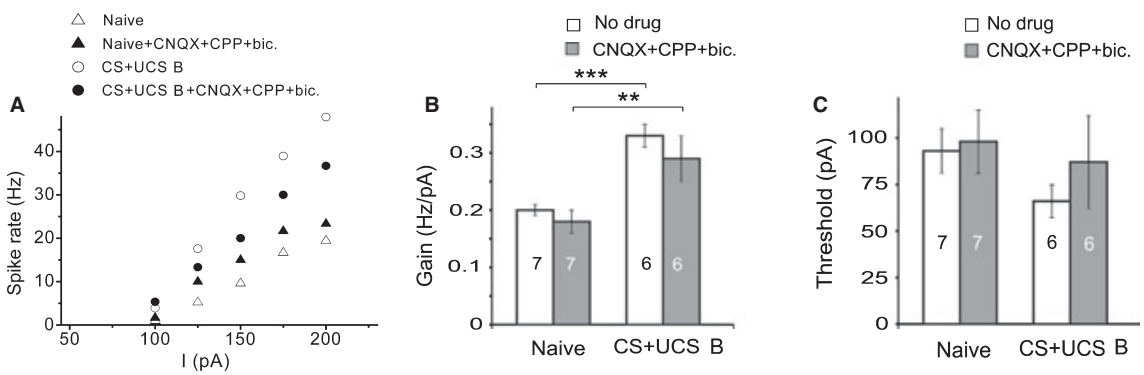

FIG. 3. Active synaptic conductance did not affect the calculated slope and threshold parameters. (A) The firing rate vs. injected current relation measured for the representative neuron from 'naive' mouse and from barrel B in a trained animal. Each cell was investigated in the control condition and then in the presence of synaptic blockers (CNQX, 20 μM; CPP, 20 μM; bicuculline, 10 μM). (B, C) Averaged gain and threshold before (white) and after blocker application (grey), for naive and CS + UCS-B cells. $^{**}P < 0.01$ and $^{***}P < 0.001$.

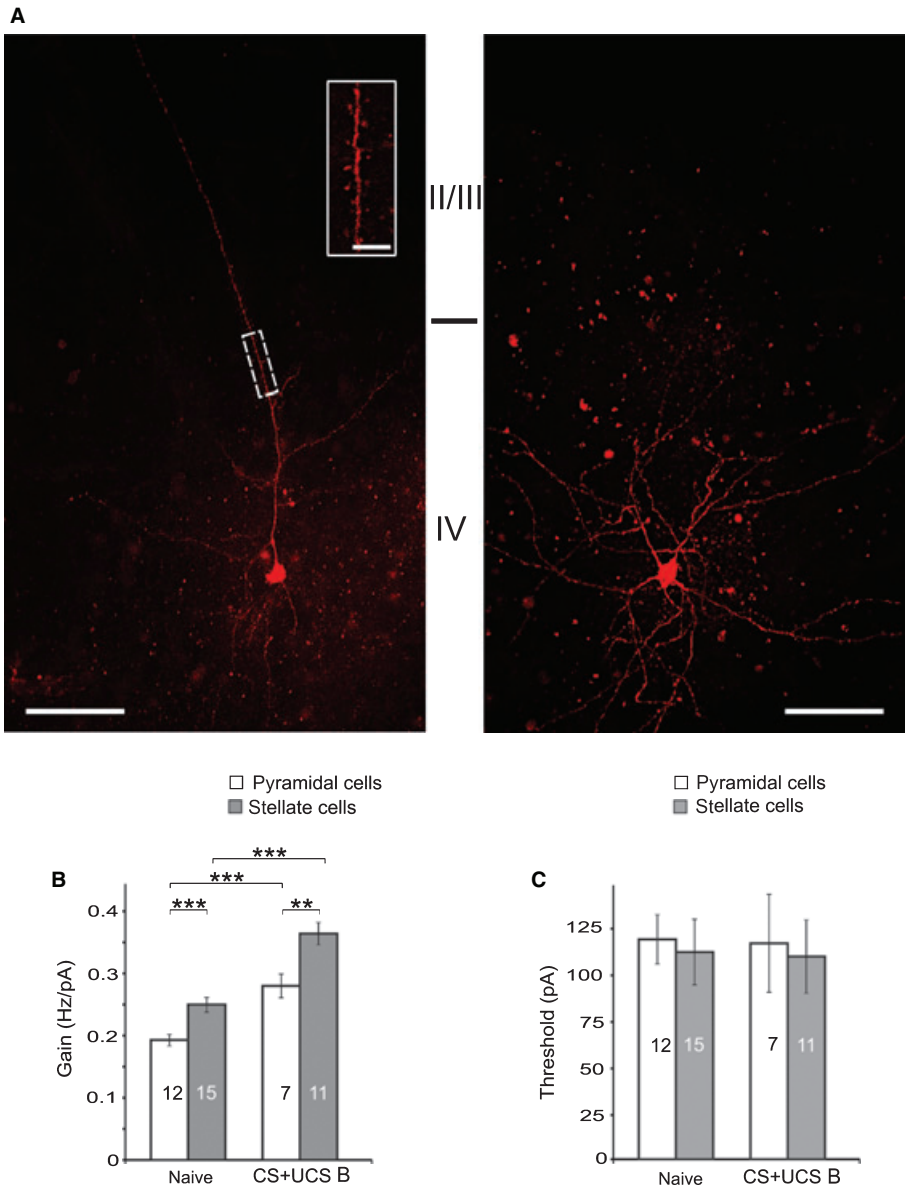

FIG. 4. Conditioning increased intrinsic excitability in both pyramidal and spiny stellate layer IV neurons. (A) Maximum intensity projections of confocal *z*-stacks of the pyramidal (left) and spiny stellate (right) neurons filled with Alexa Fluor 555. Scale bar = 50 μm. Inset: part of apical dendrite with dendritic spines. Scale bar = 5 μm. Averaged gain (B) and threshold (C) for pyramidal (white) and spiny stellate (grey) cells from naive mice and barrel B of 'trained' animals. $^{**}P < 0.01$ and $^{***}P < 0.001$.

a possible role for BK (large conductance) calcium-dependent potassium channels in the observed changes in neuronal excitability. It is known that in many types of neurons these channels are responsible for currents generating fAHPs (e.g. Sah & Faber, 2002). Interestingly, BK channels were previously shown to be involved in inhibition-dependent firing rate potentiation in the medial vestibular nucleus (Nelson *et al.*, 2003) and in increased excitability of hippocampal neurons following eye-blink conditioning (Matthews *et al.*, 2008).

To investigate whether inactivation of BK channels changes the excitability of RS neurons, we added the selective blocker iberiotoxin (60–100 nmol) to the ACSF (Fig. 5). In slices obtained from non-stimulated controls (Fig. 5A, upper row), blocking of BK channels increased the firing rate at threshold membrane potential from 0.27 ± 0.04 to 1.75 ± 0.38 Hz ($P = 0.018$, paired Wilcoxon test), which is close to the mean value obtained for neurons from 'trained' barrel B in CS + UCS animals (2.16 ± 0.18 Hz, Fig. 5B). Simultaneously, consistent with the role of BK channels, the amplitude of the fAHP was on average 14% smaller (−13.4 ± 1.1 mV) than the −15.6 ± 1.1 mV measured without iberiotoxin ($n = 6$, $P = 0.008$, Wilcoxon; Fig. 6C).

A similar increase of firing frequency after application of iberiotoxin was found for samples of neurons from control groups, recorded from non-stimulated barrel D in CS + UCS animals (1.38 ± 0.19 vs. 0.27 ± 0.06 Hz, $P = 0.043$, Wilcoxon test) and from mice which experienced only the CS stimulation (1.42 ± 0.37 vs. 0.18 ± 0.02 Hz, $P = 0.043$, Wilcoxon test; Fig. 5A and B).

In contrast, neurons from 'trained' barrel B in slices obtained from CS + UCS mice showed very little sensitivity to inactivation of BK channels (Fig. 5A, CS + UCS B). Application of iberiotoxin did not

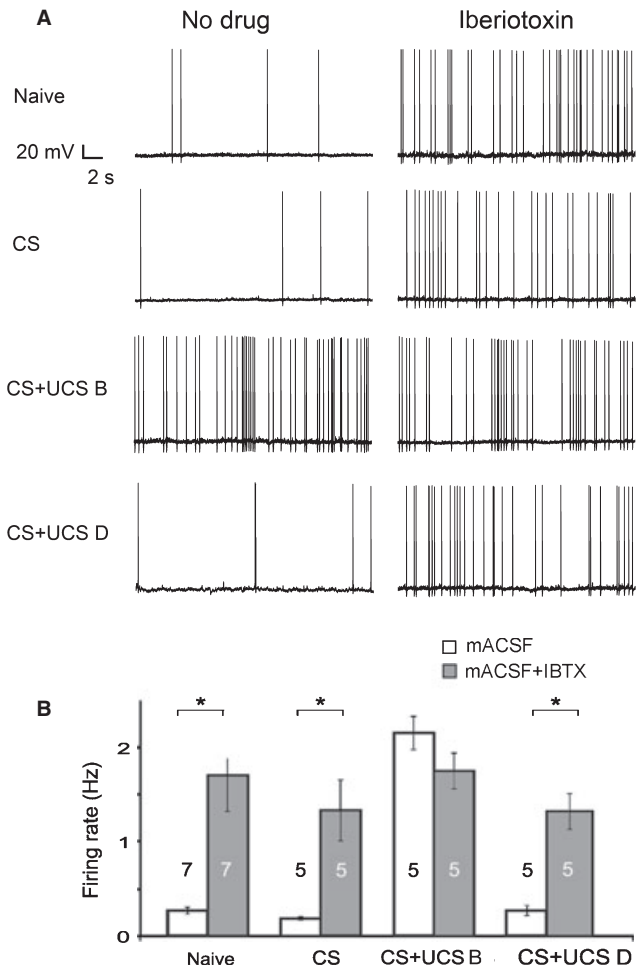

FIG. 5. Pharmacological blockade of BK channels increased threshold firing rate of RS cells in a training-related manner. (A) Representative examples of threshold firing for cells from barrel B in slices from naive, CS, CS + UCS and for control barrel D in trained animals (CS + UCS D), before (left) and after (right) application of iberiotoxin (60 nM). Blockade of the BK channel with iberiotoxin increased firing frequency in controls but not in CS + UCS B animals. (B) Mean firing rates measured for cells from different groups of mice. *$P < 0.05$. Recordings were made in modified ACSF (mACSF).

change the firing rate of these cells (1.76 ± 0.19 Hz with the blocker vs. 2.16 ± 0.18 Hz without; $P = 0.14$, Wilcoxon test, Fig. 5B) and the amplitude of fAHPs (−12.4 ± 0.6 vs. −11.9 ± 0.7 mV; $n = 5$, $P = 0.47$, Wilcoxon; Fig. 6C). Decreased sensitivity to iberiotoxin suggested that in these neurons BK channels were already partially inactivated or that they had altered properties and iberiotoxin could not block them.

To be certain that higher firing rate in control neurons after application of BK channel blocker was due to the increase in intrinsic excitability and not to changes of network influences, we examined the impact of blocking BK channels on input–output relationships between injected current and firing rate. Iberiotoxin was added to standard ACSF containing synaptic blockers (CNQX, CPP and bicuculline). The analysis indicated that inactivation of BK channels increased input–output gain significantly (by 39%) from 0.18 ± 0.02 to 0.25 ± 0.02 Hz/pA for cells from naive mice ($P = 0.013$, Wilcoxon test) and left this parameter unchanged (0.32 ± 0.03 with iberiotoxin vs. 0.30 ± 0.03 Hz/pA) without this drug ($P = 0.72$, Wilcoxon) in neurons from barrel B of trained animals (Fig. 6A). Threshold values showed no difference after application of iberiotoxin ($P = 0.69$ for control and $P = 0.27$ for trained groups, Wilcoxon test, Fig. 6B). These data imply that the observed increase of the neuronal input–output gain after sensory conditioning could be explained by changes in functioning of BK channels. This BK channel-related enhancement of intrinsic excitability seems to be responsible for the higher firing rates in modified ACSF found for neurons from the 'trained' barrel.

To test the possible influence of mAHPs and sAHPs in the observed increase of neuronal excitability after training, we determined their magnitudes. The amplitudes of mAHPs and sAHPs were the same in naive ($n = 19$) and trained ($n = 14$) mice (mAHP: −2.84 ± 0.26 and −2.86 ± 0.47 mV, $t_{31} = 0.11$, $P = 0.96$; sAHP: −1.01 ± 0.10 and −1.04 ± 0.17 mV, $t_{31} = 0.25$, $P = 0.83$, t-tests). This suggested that in our experiment calcium-dependent potassium channels responsible for these two forms of AHPs (see Sah & Faber, 2002) did not change after conditioning.

To determine whether changes of the intrinsic excitability can also be detected in layer IV inhibitory interneurons, we recorded from the population of 31 FS cells. We found that slope (gain) and threshold parameters of the input/output function (see Supporting Information Fig. S1) were similar among the FS neurons of the barrel B investigated in slices obtained from naive ($n = 16$) and trained (CS + UCS; $n = 15$) mice. Average slopes for these two groups were 1.15 ± 0.07 and 1.03 ± 0.07 Hz/pA ($t_{29} = 1.32$, $P = 0.2$, t-test) and mean threshold currents were 301 ± 18 and 273 ± 22 pA ($t_{29} = 0.99$, $P = 0.33$), respectively. This observation indicates that conditioning did not change the intrinsic excitability of fast spiking inhibitory interneurons in layer IV of the barrel cortex, and confirms that previously reported enhanced inhibitory synaptic transmission within the 'trained' barrel results primarily from an increased release of GABA (Tokarski *et al.*, 2007).

Discussion

The results of this study show that classical conditioning, involving vibrissal stimulation paired with a tail shock, specifically enhances intrinsic excitability of those layer IV regular spiking neurons, both pyramidal and stellate, which belong to the 'trained' barrels, i.e. that receive information from the row of whiskers stimulated during conditioning. In contrast, the FS inhibitory cells in the same barrels did not change their excitability after the conditioning procedure. The data from barrels B and D, 'trained ' and 'untrained' in the CS + UCS mice, show that the effect of increased excitability is found only in the 'trained' barrel, and is therefore not due to a non-specific action of any factors induced by tail shock of the somatosensory cortex.

Our data are in line with previous experiments in mammals which reported a learning-induced increase of the intrinsic excitability of neurons in different brain structures, following classical or operant conditioning paradigms (Brons & Woody, 1980; Moyer *et al.*, 1996; Schreurs *et al.*, 1998; Oh *et al.*, 2003; Saar & Barkai, 2003; Matthews *et al.*, 2008). To the best of our knowledge, the present study provides the first demonstration of a learning-induced increase of intrinsic excitability in the thalamorecipient layer IV of the sensory cortex. At variance with previously described decreases in the amplitude of mAHPs and sAHPs (Moyer *et al.*, 1996; Saar & Barkai, 2003) we found no conditioning-related changes of those parameters, which may be due to the different learning procedure and/or the different structure investigated. On the other hand, the learning-induced changes in our experimental paradigm involved a decrease of the fAHP and this effect was similar to results described by Matthews *et al.* (2008) following eye-blink conditioning in the hippocampus.

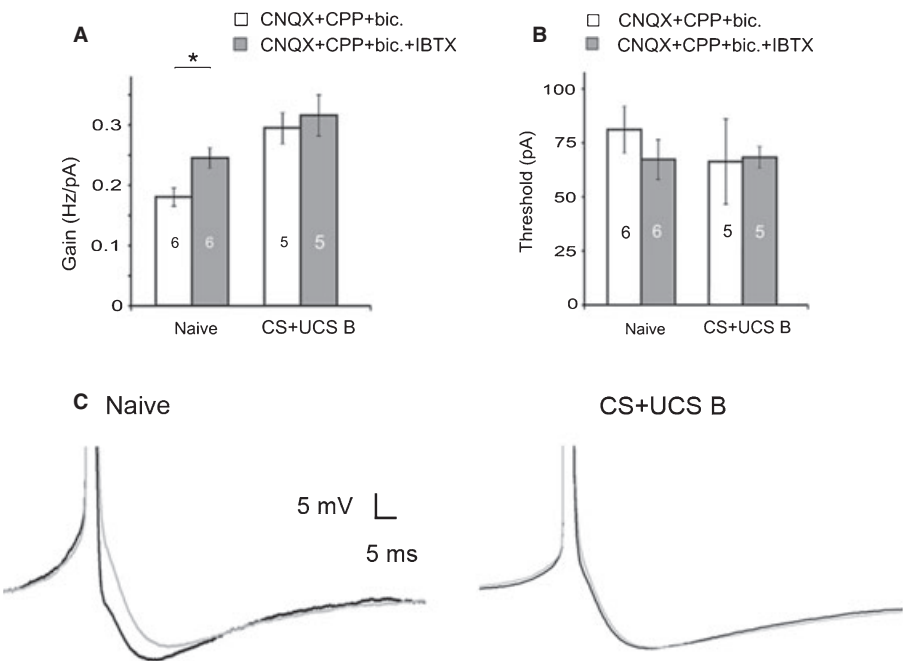

FIG. 6. BK channel blockade increased gain (slope) of firing rate vs. injected current relationships (A), did not affect threshold current values (B) and decreased amplitude of fast AHP (C) in neurons from the naive but not from the CS + UCS groups. (A,B) Mean parameters calculated for the same cells before and after application of iberiotoxin (60 nM). In all cases the standard ACSF contained also CNQX (20 μM), CPP (20 μM) and bicuculline (10 μM) to block synaptic conductances. *$P < 0.05$. (C) Averaged spike shapes from barrel B excitatory neurons before (black lines) and after application of iberiotoxin (grey lines).

The results obtained here suggest decreased activation of BK channels concomitant with a learning-induced increase of intrinsic excitability. This is in line with several other reports, which indicated that a decrease in function of this channel group correlated with higher cellular excitability and firing level. *In vivo*, neurons of the suprachiasmatic nucleus of genetically modified BK channel-null mice (Meredith *et al.*, 2006) as well as pyramidal cells in the CA1 region of the hippocampus after infusion of specific BK channel blockers (Matthews *et al.*, 2008) expressed increased spontaneous firing rates. Similarly, an elevated frequency of spontaneous action potentials was observed in brainstem slices following pharmacological blockade of BK channels (Nelson *et al.*, 2003). Thus, BK channels are important for the regulation of spontaneous neuronal firing rate, even at low frequency (*c.* 2 Hz) (Nelson *et al.*, 2003; Meredith *et al.*, 2006). Moreover, a decrease in activation of BK channels was found to be responsible for the increase of neuronal excitability induced by stronger inhibition in the medial vestibular nucleus (Nelson *et al.*, 2003), for enhanced excitability after learning in the hippocampus (Matthews *et al.*, 2008) and during extinction of fear conditioning in prefrontal infralimbic cortex (Santini *et al.*, 2008). Inactivation of these channels also abolished response adaptation of isolated olfactory receptor cells for repeated odorant stimuli and increased cellular membrane excitability (Kawai, 2002). Finally, blockade of BK channels *in vivo* impeded acquisition of eye-blink conditioning (Matthews & Disterhoft, 2009), suggesting that the proper modulation of intrinsic excitability by this channel group is essential for learning.

It has been proposed that the function of BK channels is regulated by phosphorylation, upstream signalling factors or modulation of the associated calcium channels (Nelson *et al.*, 2003; Matthews *et al.*, 2008). It is also important to note that the function of BK channels can be significantly changed by regulatory β subunits (McManus *et al.*, 1995; Brenner *et al.*, 2005). Although numerous reports (see previous paragraph) have indicated that lower activation of BK channels coincided with higher neuronal excitability, we note that an abnormal up-regulation of these channels can also produce hyperexcitability. A gain-of-function mutation of the α subunit of the BK channel in humans has been found to be linked to a syndrome of generalized epilepsy with paroxysmal movement disorders (Du *et al.*, 2005). In mice, chemoconvulsant-induced seizures can in turn give rise to a BK channel gain-of-function responsible for increased firing (Shruti *et al.*, 2008).

Inconsistent data exist also in the literature regarding the spike frequency range at which BK channels can influence firing evoked by direct current pulse injections. Gu *et al.* (2007) showed that blockade of BK channels had no effect on spike parameters and frequency adaptation of CA1 pyramidal cells at firing frequencies below 40 Hz. On the other hand, investigations performed on similar neurons of the same hippocampal region (Matthews *et al.*, 2008) as well as on different brain structures such as olfactory epithelium (Kawai, 2002), brainstem (Nelson *et al.*, 2003) and cerebral cortex (Shruti *et al.*, 2008) indicated that these frequencies can be much lower, in the range of several hertz.

As BK channels can take part in action potential repolarization, inactivation of these channels can increase spike duration or half-width (Gu *et al.*, 2007; Matthews *et al.*, 2008). However, blockade of BK channels resulted also in a substantial reduction of fAHP but with no decrease of spike width (Womack & Khodakhah, 2002; Nelson *et al.*, 2003). Similarly, Santini *et al.* (2008) observed a reduction of fAHP in prefrontal cortical neurons after extinction of fear conditioning without any changes in spike duration. Therefore, it is possible that the role of BK channels in spike repolarization can vary according to cell type. Thus, further studies are warranted to investigate the detailed mechanism of the involvement of BK channels in the process of regulation of neuronal excitability during learning.

It is worth emphasizing that the changes observed in the present experiments were selective for excitatory neurons of the 'trained' barrel and not in the distant barrel of the same cortical slice, which was unaffected by the stimulation. They were also not observed in the

controls receiving only CS. This shows that the changes were specific to the cortical representation involved in the conditioning, and not due to a non-specific increase in excitability in broad regions of the cortex.

Layer IV in the rodent somatosensory cortex contains two morphological classes of excitatory spiny neurons: stellate and pyramidal (also called star pyramids) cells (Simons & Woolsey, 1984). Higher efficacy of connectivity between cells of the same type (stellate–stellate or pyramidal–pyramidal) together with weaker stellate–pyramidal connectivity (Feldmeyer *et al.*, 1999) and different response dynamics after whisker deflection (Brecht & Sakmann, 2002) indicate that these two morphological groups of excitatory neurons play different roles in signal processing. Pyramidal neurons receive both translaminar synaptic inputs from a home column and transcolumnar inputs from neighbouring barrels, and the axons of these cells show substantial transbarrel targeting (Lübke *et al.*, 2000; Schubert *et al.*, 2003). This enables pyramidal cells to integrate transbarrel signals. Stellate cells are the main target for thalamocortical connections. Their synaptic inputs are mainly limited to their own layer. Presumably these cells perform intracolumnar signal integration and serve to amplify weak thalamic inputs (Schubert *et al.*, 2007).

Higher input–output gain found here for stellate cells as compared with pyramidal neurons appears to be consistent with the hypothesis that these two classes of cells perform different signal processing. There is as yet no clear indication whether stellate or pyramidal neurons in layer IV show differences in excitability (Feldmeyer *et al.*, 1999; Schubert *et al.*, 2003; Cowan & Stricker, 2004). However, the full input–output relationship has not been tested in these reports. Our results for the barrel cortex resemble the differences in electroresponsiveness between stellate and pyramidal cells in layer II of the entorhinal cortex (Klink & Alonso, 1993). Interestingly, modelling studies indicate that neurons with more longitudinally directed dendrites could be less excitable than cells with more spherical dendritic trees (Mainen & Sejnowski, 1996; Helmstaedter *et al.*, 2009).

It is remarkable that layer IV excitatory cells within the 'trained' barrels showed both enhanced intrinsic excitability (this study) and increased inhibitory synaptic input (Tokarski *et al.*, 2007; Jasinska *et al.*, 2010) at the same time (24 h) after the end of associative training. These data demonstrate that at this time-point two, apparently counteractive, mechanisms are at work. In our experiments the intrinsic excitability of FS interneurons, measured on the basis of an input–output relationship, was unchanged by training and stronger inhibition exerted by these cells on excitatory neurons was achieved by changes at the presynaptic site, resulting in increased relase of GABA (Tokarski *et al.*, 2007). However, during experience-dependent plastic changes in layer IV of barrel cortex, FS interneurons can change their intrinsic excitability (Sun, 2009). During normal whisker usage, enhancement of the cortical response to the stimulation of principal vibrissae parallels sharpening of inhibitory tuning of excitatory neurons in relation to non-preferred whiskers (Miller *et al.*, 2001; Sun *et al.*, 2006). It would be interesting to know whether these two mechanisms are also modified in synergy in the 'trained' barrel during the entire course of conditioning-induced plastic changes.

Galindo-Leon *et al.* (2009) similarly showed increased inhibition in the mouse auditory cortex after experience-dependent plastic changes. Studies using other models of learning have also reported learning-induced enhancement of inhibitory synapses (Scelfo *et al.*, 2008; Brosh & Barkai, 2009) accompanying increased excitability.

Moreover, Nelson *et al.* (2003) found that stronger inhibition can cause an enhancement of intrinsic excitability in slices of the mouse brainstem medial vestibular nucleus. The investigated cells were spontaneously active, and their firing rate could be potentiated by a small, transient hyperpolarization resulting from either synaptic inhibitory input or current injection. This potentiation coincided with the increase in intrinsic excitability, and almost completely reduced sensitivity to the BK channel blocker iberiotoxin. These data strongly suggest that increased inhibition may cause, in the same neurons, enhanced intrinsic excitability with inactivation of BK channels. The different sequence of appearance of changes in excitation and inhibition was reported during investigations of plasticity in the primary auditory cortex. Whole-cell recordings in anaesthetized rats (Froemke *et al.*, 2007) showed that during pairing of an auditory stimulus with stimulation of nucleus basalis, the synaptic current responses in the cortical neurons changed dramatically. During the course of such pairing, the inhibitory currents recorded in cortical neurons decreased quickly, followed by a gradual increase of the excitatory currents. After the pairing, the inhibition started to increase slowly, finally rebalancing the persistent enhancement of excitation.

Our results along with data reported from other laboratories suggest that increased inhibition and excitation parallels the plastic changes in neuronal networks. We hypothesize that the observed increase in excitability of layer IV excitatory cells may express one part of a dual process of homeostatic plasticity. In this process the enhanced synaptic inhibition found previously (Tokarski *et al.*, 2007) together with increased excitability (this study) may prevent excitatory neurons within layer IV of the 'trained' barrel from becoming hyper- or hypoactive and maintain their ability to generate action potentials in response to fluctuating excitatory inputs. The mutual enhancement of both mechanisms may also result in increased selectivity of response to contextually 'new' sensory input from 'trained' whiskers sensing the stimuli of novel behavioural significance. To date, evidence for such homeostatic mechanisms comes from experiments on activity- or experience-dependent plastic changes (Desai *et al.*, 1999; Aizenman *et al.*, 2003; Maffei *et al.*, 2004; Maffei & Turrigiano, 2008) and from brain regions critical for learning eye-blink conditioning (Scelfo *et al.*, 2008; cerebellar cortex) and olfactory discrimination (Brosh & Barkai, 2009; piriform cortex). We propose that a similar homeostatic compensatory mechanism accompanies representational plasticity induced by associative learning in the primary somatosensory cortex.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Lack of effects of training on the excitability of barrel B inhibitory interneurons.

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Acknowledgements

This research was supported by Ministry for Science and Education grant no. N303 08131/2682 to M.K. and the Polish MNSW Scientific Network fund. We are grateful to Mark Hunt for language corrections.

Abbreviations

ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid; CNQX, 6-Cyano-7-nitroquinoxaline-2,3-dione; CPP, 3-(2-Carboxypiperazin-4-yl)propyl-1-phosphonic acid; CS, conditioned stimulus; f/m/sAHP, fast/medium/slow after-hyperpolarization; FS, fast spiking; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; NMDA, N-methyl-D-aspartate; RS, regular spiking; UCS, unconditioned stimulus.

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