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Differential down-regulation of voltage-gated calcium channel currents by glutamate and BDNF in embryonic cortical neurons

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Summary

In the embryonic brain, post-mitotic cortical neurons migrate from their place of origin to their final location. Various external factors such as hormones, neurotransmitters or peptides regulate their migration. To date, however, only a few studies have investigated the effects of these external factors on the electrical properties of the newly formed embryonic cortical neurons. The aim of the present study was to determine whether glutamate and brain-derived neurotrophic factor (BDNF), known to regulate neuronal cell migration, could modulate currents through voltage-gated calcium channels (I_{Ca}) in cortical neurons isolated from embryonic day 13 (E13) mouse foetuses. Whole cell recordings of I_{Ca} showed that E13 cortical cells kept 1 day *in vitro* expressed functional low- and high-voltage activated (LVA and HVA) Ca^{2+} channels of T-, L- and N-types. A 1-day glutamate treatment non-specifically inhibited LVA and HVA I_{Ca} whereas BDNF down-regulated HVA with N-type I_{Ca} being more depressed than L-type I_{Ca}. The glutamate-induced I_{Ca} inhibition was mimicked by NMDA. BDNF exerted its action by recruiting trkB receptors and SKF-96365-sensitive channels. BAPTA prevented the glutamate- and the BDNF-dependent inhibition of I_{ca}, indicating a Ca^{2+}-dependent mechanism of action. It is proposed that an influx of Ca^{2+} through NMDA receptors depresses the expression of LVA and HVA Ca^{2+} channels whereas a Ca^{2+} influx through SKF-96365-sensitive TRPC (transient receptor potential protein of C subtype) channels preferentially inhibits the expression of HVA Ca^{2+} channels. Glutamate and BDNF appear as potent modulators of the electrical properties of early post-mitotic neurons. By down-regulating I_{Ca} they could exert a neuroprotective action on embryonic cortical neurons.

Introduction

The normal development of the cortex requires intimately coordinated sequences of cell proliferation and migration, morphological differentiation and synaptogenesis. In mice, most of the neurogenesis that occurs during the formation of the cortex takes place between embryonic day 11 (E11) and E17 (Takahashi *et al*., 1996; Kriegstein & Noctor, 2004). Many external factors such as neurotransmitters, growth factors and hormones are known to regulate not only cell fate and survival but also neuronal migration. For instance, glutamate modulates growth cone motility (Owen & Bird, 1997) and neuronal migration (Komuro & Rakic, 1993). In the embryonic cortex, glutamate, which can be released by E13 cortical cells (Platel *et al*., 2005), is mainly found in the target region for migrating cortical neurons (e.g. the cortical plate) (Behar *et al*., 1999). It has a ten-fold greater chemotropic effect on cortical neuronal migration than GABA. This action is controlled by entry of Ca^{2+} through NMDA receptors (Behar *et al*., 1999).

The brain-derived neurotrophic factor (BDNF) belongs to the neurotrophin family, which comprises several proteins such as nerve growth factor, neurotrophin-3 and neurotrophin 4/5 (Bibel & Barde, 2000). Like glutamate, BDNF is another important external cue regulating the survival, growth and shape of neurons (Bibel & Barde, 2000). This neurotrophin, present in the embryonic cortex (Fukumitsu *et al*., 1998), is essential for the migration of interneurons (Polleux *et al*., 2002) and of cortical neurons to their final destination as well as for the differentiation of cortical neurons and glial cells (Medina *et al*., 2004). However, little is known about the effects of glutamate or BDNF on the electrical properties of embryonic cortical neurons.

Changes in the cytoplasmic concentration of Ca^{2+} ($[Ca^{2+}]_i$) are implicated in pleiotropic cellular processes (Ghosh & Greenberg, 1995; Berridge *et al*., 2000). For instance, many studies have indicated that calcium ions are involved in the control of neuronal growth and differentiation. We initiated this study to verify whether glutamate and BDNF could, in addition to their action on the migration of embryonic cortical neurons, regulate currents through voltage-gated Ca^{2+} channels.

The experiments were performed on dissociated E13 cortical neurons maintained in culture. Whole cell recordings showed that E13 cortical neurons kept for 1 day *in vitro* (DIV) expressed low- and high-voltage activated (LVA, HVA) Ca^{2+} channels (I_{Ca}). A pharmacological analysis revealed the presence of two types of HVA I_{Ca}. One component was sensitive to the L-type Ca^{2+} channel blocker nifedipine whereas the second was inhibited by the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (ω-CTx). Both had similar amplitudes at least when measured after 1 DIV. Chronically applied in the culture medium glutamate

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depressed the expression of LVA and HVA Ca^{2+} channels by recruiting NMDA receptors whereas BDNF preferentially down-regulated HVA Ca^{2+} channels via SKF-96365-sensitive channels. Both modulators exerted their actions via cytoplasmic Ca^{2+} changes. It is proposed that an influx of Ca^{2+} through NMDA channels non-specifically depresses the expression of LVA and HVA Ca^{2+} channels. By contrast, an influx of Ca^{2+} through SKF-96365-sensitive TRPC (transient receptor potential protein of C subtype) preferentially depresses the expression of HVA Ca^{2+} channels.

Materials and methods

Cell cultures

Electrophysiological measurements were performed on cultured cortical neurons dissociated from E13 C57BL6/J mice embryos (vaginal plug was designated E0) (Bouron *et al.*, 2004). Briefly, pregnant mice were killed by cervical dislocation and the embryos were transferred in an ice-cold Ca^{2+}- and Mg^{2+}-free Hank's solution supplemented with gentamycin (10 mg/mL) and D-glucose (6 g/L). The cell suspension was obtained from two to three cerebral hemispheres (without the ganglionic eminences) that were prepared as slabs of neocortex (Owens & Kriegstein, 1998). Cortical cells were mechanically dissociated from these slabs by a series of successive aspirations through a sterile fire-polished Pasteur pipette. The cells were kept in a neurobasal medium supplemented with 2% B27, 500 μM glutamine and 1% penicillin/streptomycin and plated on NUNC Petri dishes (35 mm) coated with poly-L-ornithine. Cultured cells were maintained at 37 °C (95% O_2/5% CO_2) (Bouron *et al.*, 2004). In some experiments, glutamate (30 μM), BDNF (50 ng/mL) or glutamate (30 μM) + BDNF (50 ng/mL) were added to the culture medium immediately after plating of the cells. The procedures were approved by the Veterinary Services of Isère, France.

Electrophysiological measurements

Voltage-gated calcium channel currents (I_{Ca}) were recorded by means of the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). The external recording solution contained (in mM): TEACl 145, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10, D-glucose 10, pH 7.4 (TEAOH). In some experiments, tetrodotoxin (TTX), a specific blocker of voltage-gated sodium channels, was added at a concentration of 0.2–0.4 μM. The patch pipettes, pulled from thick wall borosilicate glass capillaries (1.5 mm o.d. × 0.86 mm i.d., Clark Electromedical Instruments, Phymep, Paris), were filled with the following intracellular solution (in mM): CsCl 120, EGTA 10, $MgCl_2$ 2, Na_2-ATP 3, HEPES 10, pH 7.2 (CsOH). They had a resistance of about 1.2–3.5 M. Electrical signals were stimulated and recorded by means of the pClamp software (version 9.0, Axon Instruments, Dipsi, Chatillon, France). Whole-cell currents, filtered at 1–2 kHz, were sampled at 5–10 kHz on an Axoclamp 200B amplifier (Axon Instruments). Throughout this study cortical neurons were stimulated at a frequency of 0.2 Hz from a holding potential of −80 mV. Capacitive transients were cancelled and the cell capacitance value was read from the amplifier dials. Calcium currents were recorded at room temperature 24 h after plating of the dissociated cells.

Immunocytochemistry

The experiments were conducted according to a protocol described in a previous study (Bouron *et al.*, 2005). Briefly, the cells were fixed with 4% paraformaldehyde in phosphate buffer for 10 min at room temperature. They were then rinsed three times with PBS-Triton 0.2% and incubated for 2 h at room temperature with the neuronal marker anti-β_{III}-tubulin (TuJ1, 1 : 500, Balco). After rinsing with PBS-Triton 0.2%, the cells were incubated with A-488-conjugated mouse anti-IgG2 antibody (1 : 1000, Molecular Probes). The cells were then rinsed twice and incubated with TO-PRO3 (1 : 1000, Molecular Probes) which stains double-stranded nucleic acids.

Calcium imaging experiments

Cytoplasmic Ca^{2+} changes were analysed by means of the Ca^{2+} indicator Fluo-4. The experiments were performed on a Leica TCS SP2 scanning confocal microscope according to a protocol described previously (Bouron *et al.*, 2004).

Drugs

TTX was obtained from Alomone Laboratories (Jerusalem, Israel). K252a and (S)-α-amino-3-hydroxy-5methyl-4-osoxalepropionic acid [(S)-AMPA] were from Calbiochem (VWR, Fontenay sous Bois, France). Nifedipine, ω-CTx, NMDA, CNQX, *trans*-(±)-1-amino-(1S,3R)-cyclopentanedicarboxylic acid (*trans*-±-ACPD) and poly-L-ornithine were from Sigma Aldrich (Saint Quentin Fallavier, France). B27, glutamine and penicillin/streptomycin were from Invitrogen Life technologies (Cergy-Pontoise, France). Fluo-4/AM was from Molecular Probes (Interchim, Montluçon, France). Drugs were bath applied from stock solutions. Data are presented as means ± SEM with *n* being the number of cells tested. Unless indicated otherwise, the significance of the differences between means was determined with the Student's *t*-test.

Results

Determination of the neuronal phenotype of the dissociated cells

We prepared primary cultures of cortical neurons dissociated from E13 mice. When fixed 2–3 h after plating, 65–70% of the cells expressed β_{III}-tubulin, a marker of early post-mitotic neurons (Menezes & Luskin, 1994) which is recognized by the antibody TuJ1. Throughout this study, TuJ1-positive cells were identified as neurons. After 1 DIV the cells extended short and thin neurites and had a cell membrane capacitance of 9.0 ± 0.7 pF ($n = 31$). Immunostaining experiments indicated that ∼85% of the cells were TuJ1-positive (Fig. 1). Thus, in our cell culture system, most of the cells had a neuronal phenotype (Bouron *et al.*, 2005).

Voltage-gated calcium channel currents in E13 cortical neurons

Currents through voltage-gated calcium channels (I_{Ca}) were recorded after 1 DIV. The cells were bathed in an Na^+-free external recording solution where external Na^+ ions were replaced by TEA^+, a blocker of voltage-gated K^+ channels. When stimulated from a holding potential of −80 mV, depolarizing step potentials more positive than −50 mV elicited an inward current (Fig. 2A). Depolarizing step pulses above activation threshold gave rise to an inactivating I_{Ca} whereas a non-inactivating component appeared for step potentials more positive than −20/−10 mV. The fast inactivating current, observed in 60–70% (20/31) of the cells, was never present when the cells were depolarized from −40 mV (Fig. 2B). In most of the

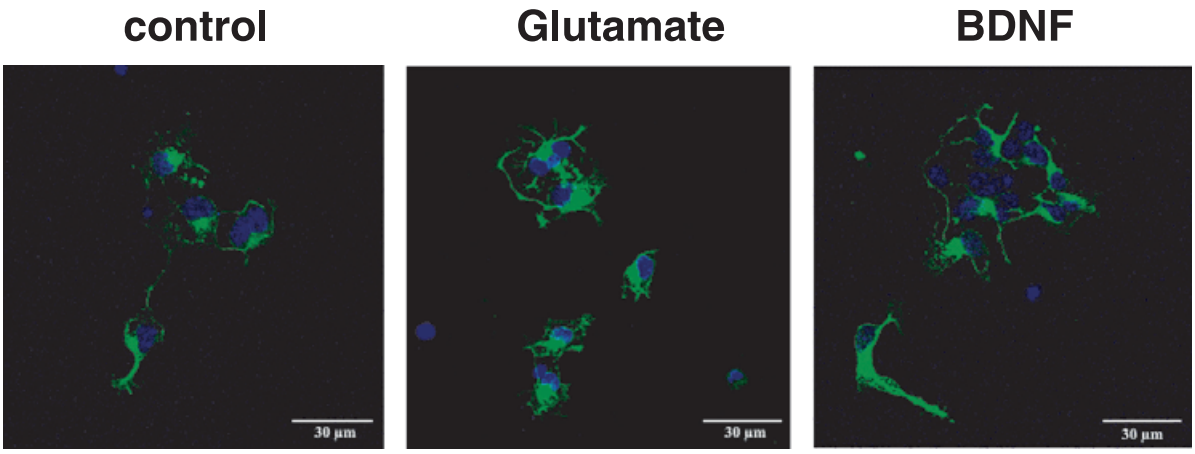

FIG. 1. Determination of the neuronal phenotype of the dissociated embryonic (E13) cortical cells. Cortical cells were mechanically dissociated from slabs of neocortex and kept for 1 day *in vitro* (DIV). TO-PRO3 (blue) was used to identify the nuclei whereas TuJ1 (green) was used to recognize the neuronal protein β_{III}-tubulin. In some experiments, the culture medium was supplemented with 30 µM glutamate or 50 ng/mL BDNF. Most of the cells were β_{III}-tubulin-positive. Scale bars, 30 µm.

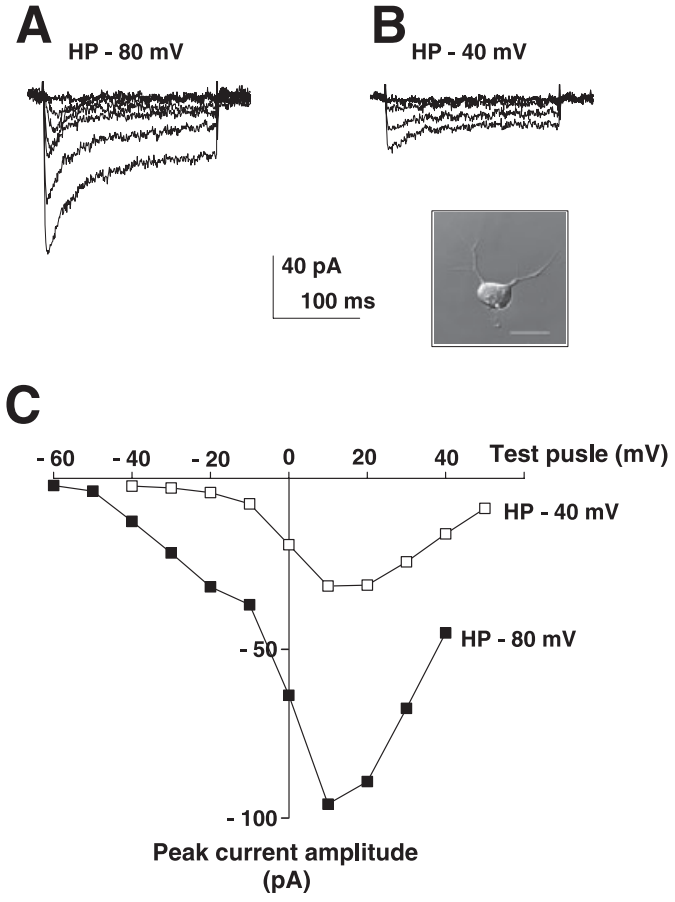

FIG. 2. Evidence of two types of voltage-gated calcium currents in E13 cortical neurons kept for 1 day in culture. Voltage-gated Ca^{2+} channel currents (I_{Ca}) were recorded on dissociated E13 cortical neurons kept for 1 day *in vitro* (DIV). (A) Representative current traces recorded at −50, −40, −30, −20, −10, 0 and +10 mV from a holding potential of −80 mV. (B) Same cell as in A but I_{Ca} were recorded in response to depolarizing pulses applied from −40 mV to −20, −10, 0 and +10 mV. Inset: E13 cortical cell kept for 1 DIV. Scale bar, 7 µm. (C) Current–voltage relationships of the peak current amplitude (in pA) for the cell shown in A and B. Filled squares, holding potential of −80 mV; open squares, holding potential of −40 mV. The cell was stimulated at a frequency of 0.2 Hz.

neurons tested, the maximum current amplitude was observed for a depolarizing step potential of +10 mV (Fig. 2C). The recordings suggest the existence of LVA and HVA calcium channel currents. This was verified by using various blockers of voltage-gated Ca^{2+} channels. As illustrated in Fig. 3A, depolarizing the cells from −80 to −30 mV elicited an inactivating I_{Ca} that could be blocked by the T-type channel blocker $NiCl_2$ (50 µM, $n = 6$ cells tested). Some experiments were performed with TTX (1 µM), a specific inhibitor of voltage-gated Na^+ channels. This toxin did not affect Ni^{2+}-sensitive inward current ($n = 5$ cells tested, data not shown). From these experiments it was concluded that cultured cortical neurons from E13 fetuses express functional LVA or T-type Ca^{2+} channels. The analysis of HVA I_{Ca} was achieved by using nifedipine, an L-type channel blocker, and ω-CTx, an N-type channel blocker. HVA I_{Ca} were elicited by step depolarizations applied from −80 to +10 mV in the presence of 50 µM Ni^{2+} to suppress LVA I_{Ca}. Representative current traces recorded with such a protocol before and after the addition of these Ca^{2+} channel inhibitors are shown in Fig. 3B and C. Nifedipine and ω-CTx partially blocked the HVA I_{Ca}. However, when added together they completely blocked the current (Fig. 3D). Figure 3E is a summary graph showing the percentage of inhibition produced by the HVA Ca^{2+} channel blockers on I_{Ca}. Nifedipine (10 µM) reduced I_{Ca} amplitude by 40–50% ($n = 16$ cells) whereas ω-CTx (1 µM) blocked ∼60% of the I_{Ca} ($n = 10$ cells) (Fig. 3E). These two types of Ca^{2+} channel currents were found in all cells tested. We found no evidence for the presence of a nifedipine + ω-CTx-resistant HVA I_{Ca} ($n = 4$ cells).

Chronic glutamate or BDNF treatment down-regulates I_{Ca}

In some experiments, the culture medium was supplemented with either glutamate (30 µM) or BDNF (50 ng/mL). Under these conditions, 87 and 84% of the cells were TuJ1-positive in the presence of glutamate and BDNF, respectively (Fig. 1). These values were similar to those found in control cultures with ∼80–85% of TuJ1-positive cells. Figure 4A and B show current traces of I_{Ca} recorded in response to step pulses applied from −80 mV after 24 h of glutamate (Fig. 4A) or BDNF (Fig. 4B) treatment. These two neuromodulators did not modify the mean input resistance of the cells. Control, glutamate- and BDNF-treated cells had a mean input resistance of 10.5 ± 3.4 GΩ

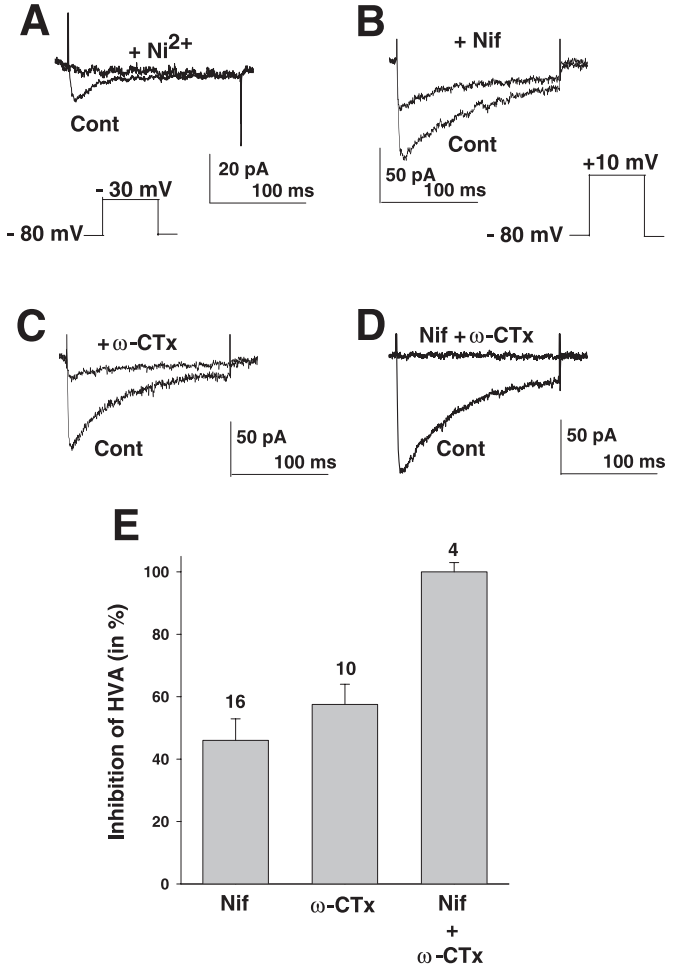

FIG. 3. Cultured E13 cortical neurons express functional T-, L- and N-type calcium channels. (A) Step depolarizations from −80 to −30 mV elicited a transient I_{Ca} that could be blocked by 50 μM Ni^{2+}. The current traces shown were recorded before and after the addition of the T-type I_{Ca} blocker. Some cells, kept in the presence of 50 μM Ni^{2+}, were depolarized from −80 to +10 mV to activate HVA I_{Ca} (B–D). The traces show the inhibition produced by 10 μM nifedipine (B) and 1 μM ω-CTx (C). In some experiments, nifedipine (10 μM) and ω-CTx (1 μM) were added together (D). (E) Summary graph of the inhibitory effect produced by nifedipine (10 μM) and ω-CTx (1 μM) on I_{Ca} recorded in response to depolarizing step potentials applied from −80 mV to +10 mV. I_{Ca} was recorded before and after the addition of the channel blockers. The plot shows the percentage of inhibition produced on HVA I_{Ca}. The recording medium contained Ni^{2+} to block LVA I_{Ca}. The number of cells tested is indicated above each bar. Cont, control (current recorded before the addition of the Ca^{2+} channel blocker); Nif, nifedipine.

($n = 10$), 9.2 ± 2.9 GΩ ($n = 10$) and 11.3 ± 4.1 GΩ ($n = 9$), respectively.

Figure 4C shows current–voltage relations for control cells (filled squares, $n = 31$), glutamate-treated (filled circles, $n = 29$) and BDNF-treated cells (open circles, $n = 20$). Glutamate and BDNF inhibited the peak current amplitude without shifting the voltage-dependence of the channels (Fig. 4C). For instance, the maximum I_{Ca} amplitude, measured at +10 mV, was 47.3 ± 8.6 pA in the glutamate group ($n = 29$) and 46.9 ± 4.1 pA in the BDNF-treated group ($n = 20$) which is significantly smaller than the maximal control I_{Ca} amplitude of 88.2 ± 13.1 pA ($n = 31$, $P < 0.05$). The cell membrane capacitance values were 8.1 ± 0.6 pF for glutamate- ($n = 29$) and 8.3 ± 0.7 pF for BDNF-treated cells ($n = 20$). These values were not different from the cell membrane capacitance values measured in the control group (9.0 ± 0.7 pF, $n = 31$). This shows that none of these external factors, when applied for 1 day, profoundly affected the cell size of E13 cortical neurons at least when assayed with our electrophysiological approach. Consequently, the current density was much lower after glutamate (5.0 ± 0.6 pA/pF) and after BDNF (5.3 ± 0.3 pA/pF), compared with the control group (9.5 ± 1.7 pA/pF, $n = 31$) (Fig. 4D). Some experiments were also carried out with lower concentrations of glutamate (1 and 10 μM). Under these conditions the maximum current densities were 9.2 ± 1.1 pA/pF ($n = 7$, not different from glutamate-untreated cells) and 6.8 ± 0.9 pA/pF ($n = 8$, $P < 0.05$ vs. glutamate-untreated cells) with 1 and 10 μM glutamate, respectively.

Figure 5A, showing normalized density–voltage relations, allows a better comparison of the data. For each group (glutamate, BDNF), the I_{Ca} densities were compared with the normalized maximum I_{Ca} density measured in the control group of cells. Even though glutamate and BDNF down-regulated the maximal I_{Ca} amplitude and density they did not induce similar responses. A clear BDNF-dependent I_{Ca} inhibition was seen for step potentials more positive than −10 mV (Fig. 5A) whereas glutamate down-regulated I_{Ca} regardless of the step potential value (from −40 to +40 mV) (Fig. 5A).

The results presented above suggested that BDNF mainly regulated HVA I_{Ca} whereas glutamate depressed LVA and HVA I_{Ca}. We thus verified the presence of a T-type I_{Ca} in E13 cortical neurons grown for 1 day in culture in the presence of glutamate or BDNF. BDNF-treated cells were stimulated as illustrated in Fig. 3A by applying repetitive depolarizing step potentials to −30 mV from a holding potential of −80 mV. However, because glutamate markedly reduced I_{Ca} amplitude, glutamate-treated cells were stimulated from −80 to −20 mV, instead of −30 mV, in order to elicit larger currents. Ni^{2+} (50 μM) completely blocked the transient I_{Ca} in BDNF-treated cells (Fig. 5B) but not in the glutamate-treated group where the current was Ni^{2+}-insensitive (Fig. 5C) even when higher concentrations (e.g. 150 μM, $n = 3$) were used (data not shown). In conclusion, the LVA I_{Ca}, present in dissociated E13 cortical neurons (Fig. 3A), was strongly attenuated by glutamate (Figs 4C and D, and 5A and C) but was unaffected by BDNF. We next investigated which type of HVA I_{Ca} was down-regulated by glutamate and BDNF. To this end, the same experiments as those described in Fig. 3 were conducted. The cells were grown for 1 day with either glutamate or BDNF. The percentage of I_{Ca} inhibition was determined as in Fig. 3, in the presence of 50 μM Ni^{2+} for the BDNF group of cells but without this T-type Ca^{2+} channel blocker for the glutamate group of cells. The results are summarized in Fig. 6A. After a 1 day of glutamate treatment, both components, namely the nifedipine- and the ω-CTx-sensitive components, were down-regulated. For instance, the nifedipine-sensitive and the ω-CTx-sensitive components represented ∼35% ($n = 10$ cells) and ∼65% ($n = 6$ cells), respectively, of the total HVA I_{Ca} (Fig. 6B). These values were not different from the control group in which these two components represented ∼45% ($n = 16$ cells) and ∼60% ($n = 10$ cells), respectively, of the total I_{Ca} (Fig. 6B). In the BDNF-treated group of cells, the nifedipine-sensitive and the ω-CTx-sensitive components represented ∼75% ($n = 8$ cells) and ∼40% ($n = 5$ cells), respectively, of the total HVA I_{Ca} (Fig. 6B). Thus, glutamate and BDNF down-regulated both types of HVA I_{Ca}. In contrast to what was seen in the other groups (control and glutamate), the nifedipine-sensitive component measured in the BDNF group of cells was more important than the ω-CTx-sensitive I_{Ca} ($P < 0.05$ vs. the ω-CTx-sensitive I_{Ca}). Because BDNF reduced the maximum I_{Ca} amplitude and density, this large nifedipine-sensitive component observed after BDNF indicates that this

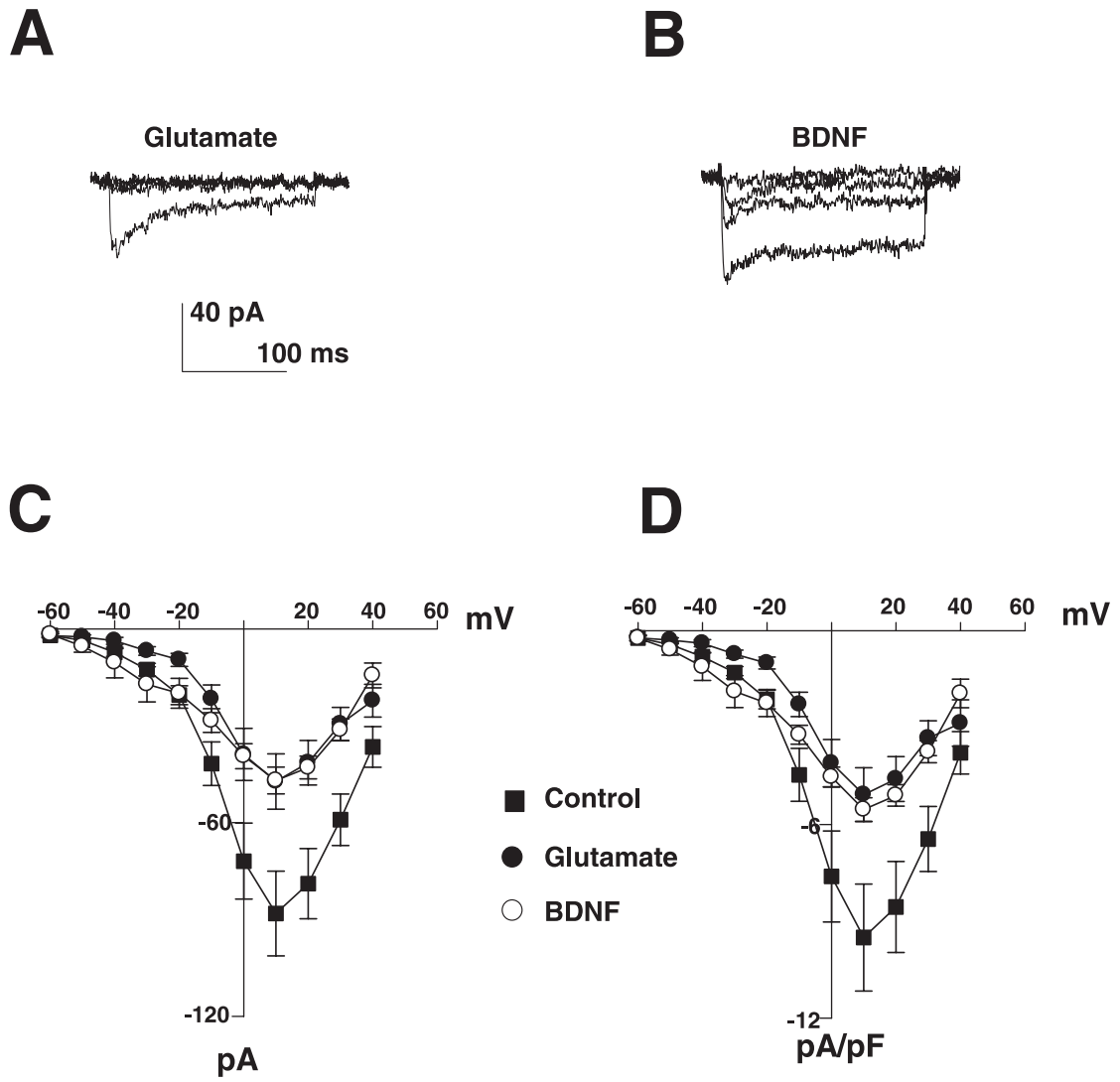

FIG. 4. Effects of a chronic glutamate or BDNF treatment on I_{Ca} amplitude and density. E13 cortical neurons were grown for 1 DIV in the presence of glutamate (30 μM, A) or BDNF (50 ng/mL, B). The current traces were obtained at −50, −30, −10 and +10 mV from a resting potential of −80 mV. Current–voltage (C) and density–voltage (D) relationships are shown for each experimental condition: control cells (filled squares), glutamate-treated cells (filled circles) and BDNF-treated cells (open circles).

neurotrophin repressed more N-type or ω-CTx-sensitive I_{Ca} than L-type or nifedipine-sensitive I_{Ca}.

We determined whether glutamate and BDNF had additive effects on I_{Ca}. Glutamate (30 μM) and BDNF (50 ng/mL) were added together to the culture medium. After 1 DIV, the cell membrane capacitance of the glutamate + BDNF-treated cells was similar to the control group (8.3 ± 0.6 pF, $n = 27$, vs. 9.0 ± 0.7 pF, $n = 31$, $P > 0.05$ vs. control cells), indicating that glutamate + BDNF did not alter cell size. The treatment did not shift the activation threshold of the current but reduced its maximum amplitude by ∼50% (Fig. 7A). The maximum I_{Ca} amplitude and density, measured at +10 mV, were 48.5 ± 14.8 pA and 4.5 ± 1.1 pA/pF ($n = 15$), respectively (Fig. 7A and B). These values were similar to those reported after glutamate or BDNF alone. This shows that the effects of BDNF and glutamate on the HVA I_{Ca} were not additive.

In order to determine whether glutamate and BDNF affect protein expression or the properties of the channels, we investigated whether acutely applied, these substances could modulate I_{Ca}. Some cells were loaded with the Ca^{2+} indicator Fluo-4 and cytoplasmic Ca^{2+} changes were analysed by means of a confocal microscope (Bouron *et al.*, 2004). Cells were stimulated by two successive applications of KCl (50 mM). It caused massive and transient elevations of $[Ca^{2+}]_i$. The analysis of the Fluo-4 signals showed that the two KCl-dependent Ca^{2+} responses had similar amplitudes with a ratio (amplitude of the second signal/amplitude of the first signal) of 1.05 ± 0.05 ($n = 39$ cells) (Fig. 7C, inset). Applied 80 s after the first KCl application, glutamate (30 μM) did not affect the second Fluo-4 response (ratio 0.96 ± 0.04, $n = 20$ cells) (Fig. 7C). Similarly to glutamate, BDNF did not depress the second KCl-dependent Ca^{2+} signal (ratio 0.98 ± 0.06, $n = 19$ cells) (Fig. 7C). In contrast to glutamate, which activated functional NMDA receptors (Platel *et al.*, 2005), BDNF failed to elevate $[Ca^{2+}]_i$. In another set of experiments, whole-cell I_{Ca} were recorded on control cells before and after the addition of glutamate (or BDNF). The results of such experiments are shown in Fig. 7D, and indicate that acutely applied glutamate (or BDNF) did not influence I_{Ca} amplitude, suggesting that neither of them altered the properties of the Ca^{2+} channels but rather their expression.

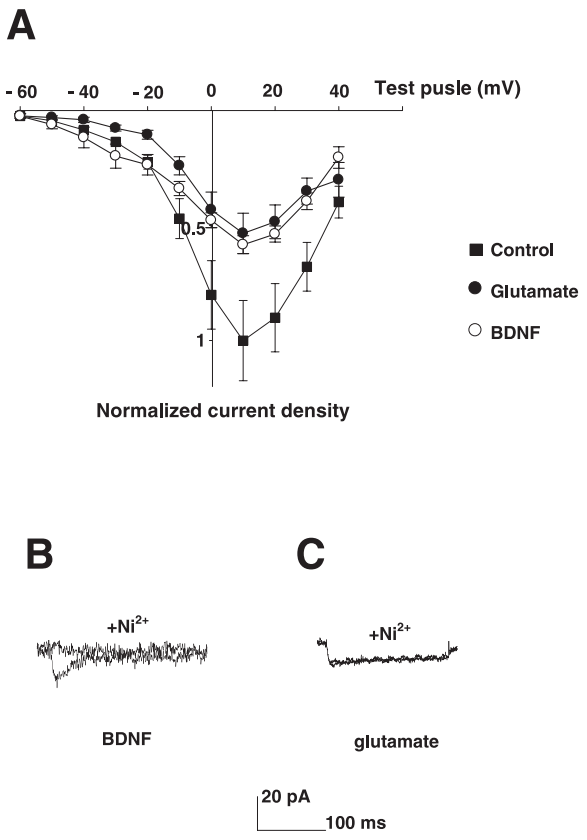

FIG. 5. Glutamate down-regulates LVA and HVA I_{Ca} whereas BDNF inhibits HVA I_{Ca}. (A) The current density–voltage relationships from glutamate- and BDNF-treated cells were compared with the normalized control current density (filled squares). This plot shows that BDNF depressed mainly HVA I_{Ca} whereas glutamate seemed to inhibit LVA and HVA I_{Ca}. Current traces from a BDNF- (B) and a glutamate-treated cell (C). They were stimulated from −80 to −30 mV (BDNF, B) or −20 mV (glutamate, C) without or with Ni^{2+} (50 μM).

Mechanism of glutamate action

We also attempted to gain information regarding the receptors underlying the glutamate-dependent I_{Ca} inhibition. Cells were incubated for 24 h with AMPA (30 μM), NMDA (33 μM) or the mGluR agonist *trans*-±-ACPD (15 μM). A chronic treatment of the cells with AMPA or *trans*-±-ACPD did not change I_{Ca} amplitude (data not shown) or density (Fig. 8). However, and similarly to the glutamate experiments, when the culture medium was supplemented with NMDA, the cells had a lower I_{Ca} than untreated cells (Fig. 8). These results show that the activation of NMDA channels can mimic the glutamate-dependent inhibition of I_{Ca}. In some experiments, the cells were incubated with BAPTA/AM to prevent cytoplasmic Ca^{2+} changes. Control- and BAPTA + glutamate-treated cells had similar I_{Ca} amplitude (data not shown) and density (8.7 ± 1.1 pA/pF, $n = 7$) (Fig. 8). It is suggested that the glutamate-dependent inhibition of I_{Ca} was controlled by an influx of Ca^{2+} through NMDA channels.

Mechanism of BDNF action

In another series of experiments, we investigated the mechanism of BDNF action. Cells were incubated for 24 h with BDNF (50 ng/mL) + K252a (200 nM). The alkaloid-like compound K252a, which potently inhibits the activity of trkB neurotrophin receptors, reversed

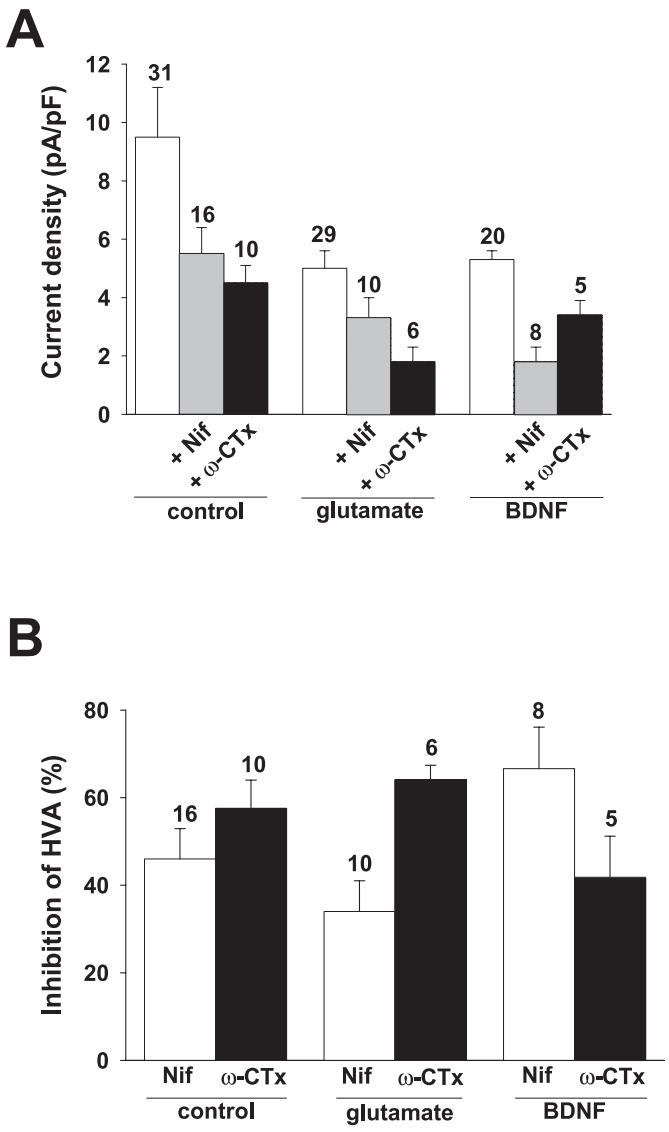

FIG. 6. Inhibition of HVA I_{Ca} by glutamate and BDNF. (A) Summary histogram showing the maximum current density (pA/pF) from control, glutamate- and BDNF-treated cells. In some experiments, nifedipine (Nif, 10 μM) or ω-CTx (1 μM) was added. For the control and BDNF groups, the external medium contained 50 μM Ni^{2+}. (B) Summary graph showing the percentage of inhibition of the HVA I_{Ca} produced by nifedipine (Nif, 10 μM) or ω-CTx (1 μM) in each group of cells (control, glutamate, BDNF). The number of cells tested is indicated above each bar.

the BDNF-dependent I_{Ca} inhibition (Fig. 8). This shows that trkB receptors mediated the BDNF-dependent down-regulation of I_{Ca}. Some cells were also incubated with BDNF + BAPTA/AM (2.5 μM). BAPTA prevented the BDNF-dependent inhibition of I_{Ca} (Fig. 8), suggesting that BDNF controlled a Ca^{2+}-dependent mechanism in response to the activation of trkB receptors. Recent reports have shown that BDNF can exert its cellular action by recruiting SKF-96365-sensitive TRPC Ca^{2+} channels (Li *et al.*, 2005). We therefore verified whether the application of SKF-96365, an inhibitor of TRPC channels (Li *et al.*, 2005; Wang & Poo, 2005), could reverse the BDNF-dependent I_{Ca} modulation. For this, cells were incubated with SKF-96365 (1 or 5 μM) + BDNF (50 ng/mL). After 1 day of treatment, whole-cell recordings of I_{Ca} showed that SKF-96365 reversed in a concentration-dependent manner the BDNF-dependent

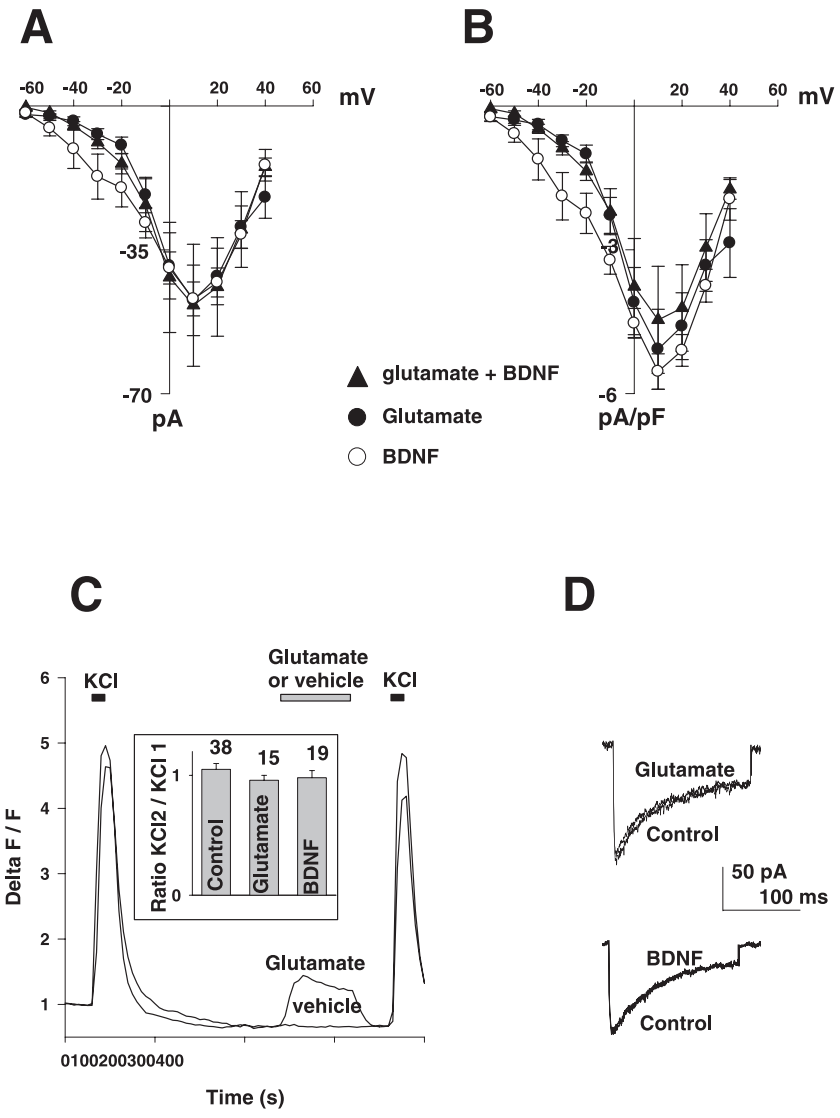

FIG. 7. Effects of a co-application of glutamate + BDNF on I_{Ca}. (A) Current– and (B) density–voltage relationships from cells grown in a culture medium supplemented with 30 μM glutamate + 50 ng/mL BDNF (filled triangles, $n = 15$ cells tested). I_{Ca} was recorded 24 h after the beginning of the glutamate + BDNF treatment. Curves obtained on glutamate- (filled circles) and BDNF-treated cells (open circles) are also shown (same data as in Fig. 3C and D). (C) This figure shows Fluo-4 signals (DeltaF/F) as a function of time (s) in two different cells grown for 1 DIV. Cells were depolarized by two successive applications of 50 mM KCl, which rapidly elevated $[Ca^{2+}]_i$. Glutamate or BDNF (or their vehicle, control cells) were added after the first KCl challenge. In this example, one cell was treated with glutamate (30 μM) and the other one with its vehicle. Inset: summary graph showing the ratio (amplitude of the first KCl-dependent Fluo-4 signal) to (amplitude of the second KCl-dependent Fluo-4 signal). The number of cells tested is shown above each bar. (D) Representative current traces recorded before and after the addition of glutamate and BDNF, the cells were stimulated from −80 to +10 mV. Same protocol as in C.

I_{Ca} inhibition but had no effect on the glutamate-dependent I_{Ca} inhibition (Fig. 8). In response to the activation of trkB receptors and SKF-96365-sensitive channels, BDNF can recruit a Ca^{2+}-dependent signalling pathway leading to a down-regulation of HVA I_{Ca}.

Absence of crosstalk between the glutamatergic and the BDNF systems

Several reports have shown a functional coupling between the glutamatergic and BDNF systems (Marini *et al*., 1998; Xiong *et al*., 2002). For instance, glutamate, via the activation of NMDA receptors, can promote the release of BDNF which, in turn, leads to the activation of trkB receptors (Marini *et al*., 1998). Interestingly, changes in the intracellular concentration of Ca^{2+} can regulate the BDNF/trkB-dependent signalling pathway. Cytoplasmic Ca^{2+} controls the neuronal release of BDNF or the expression of trkB receptors (Hartmann *et al*., 2001; Balkowiec & Katz, 2002; Kingsbury *et al*., 2003). Based on our data showing that glutamate or BDNF induced a Ca^{2+}-dependent inhibition of I_{Ca}, it was therefore possible that glutamate application could regulate I_{Ca} by promoting BDNF release, as shown in cerebellar granule cells (Jiang *et al*., 2003). The hypothesis of a crosstalk between the glutamatergic and the BDNF systems was verified by adding glutamate together with K252a to determine whether trkB receptors were involved in the glutamate-dependent I_{Ca} modulation. After 1 day of treatment, K252a (200 nM) did not reverse the glutamate-dependent reduction of I_{Ca} amplitude (data not shown) and density (Fig. 8). Therefore, glutamate does not act by promoting the release of BDNF.

Discussion

In the developing cortex, neuronal activity influences the formation of neural circuits. It was thus of interest to investigate the effects of glutamate and BDNF, release of which critically depends on neuronal activity, on the electrical properties of early post-mitotic embryonic cortical neurons. The intrinsic neuronal activity of the cells was manipulated by adding glutamate or BDNF to the culture medium and we then analysed their effects on I_{Ca}.

Whole-cell recordings showed that E13 cortical neurons kept for 1 DIV express functional LVA and HVA Ca^{2+} channels. A pharmacological analysis indicates that the HVA I_{Ca} is composed of a nifedipine-sensitive (or L-type) I_{Ca} and of a ω-CTx-sensitive (or N-

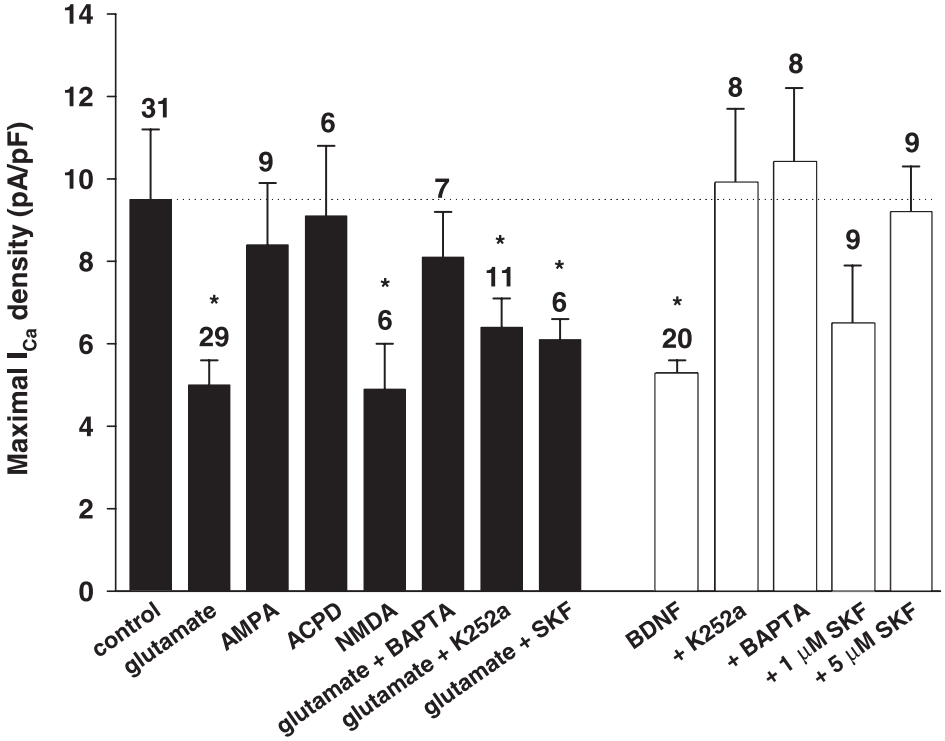

FIG. 8. Glutamate and BDNF act in a Ca2+-dependent manner via NMDA receptors and SKF-96365-sensitive channels, respectively. Histogram showing the influence of the composition of the culture medium on ICa density (in pA/pF). E13 cortical neurons were kept for 1 DIV with a neurobasal medium (control) or kept in a neurobasal medium supplemented with: glutamate (30 μm), AMPA (30 μm), trans-±-ACPD (15 μm, n = 6), NMDA (33 μm), glutamate (30 μm) + BAPTA/AM (2.5 μm), glutamate (30 μm) + K252a (200 nm), glutamate (30 μm) + SKF-96365 (5 μm), BDNF (50 ng/mL), BDNF (50 ng/mL) + K252a (200 nm), BDNF (50 ng/mL) + BAPTA/AM (2.5 μm), BDNF (50 ng/mL) + SKF-96365 (1 and 5 μm). The number of cells tested is indicated above each bar. *$P < 0.05$ vs. control (untreated cells) (one-way anova and Tukey test).

type) I_{Ca}. Each of these HVA I_{Ca} represent nearly 50% of the total HVA current. We find no evidence for the presence of a nifedipine + ω-CTx-resistant HVA I_{Ca}. A 24-h glutamate treatment of E13 cortical neurons leads to a marked reduction of I_{Ca} amplitude and density. Glutamate non-specifically affects T-, L- and N-type Ca^{2+} channel currents. This effect, mimicked by NMDA but not by AMPA or *trans*-±-ACPD, is abolished in the presence of the Ca^{2+} chelator BAPTA. This indicates that glutamate controls a Ca^{2+}-dependent signalling pathway activated in response to the recruitment of NMDA receptors. It is worth noting that E13 cortical neurons express functional NMDA receptors (Platel *et al*., 2005). Glutamate could influence I_{Ca} by altering the number or the opening probability of the Ca^{2+} channels. When acutely applied on cultured E13 cortical neurons, glutamate (30 μM) (and also BDNF) did not regulate I_{Ca} (Fig. 7D). This suggests that, chronically applied, it most likely influences the number of LVA and HVA Ca^{2+} channels and not their biophysical properties. In the embryonic cortex, high levels of glutamate have been reported in proliferative zones of the neuroepithelium. But after E14, there is a gradual decline in glutamate (and also GABA) levels during neurogenesis (Haydar *et al*., 2000). The physiological significance of this decrease as well as the mechanisms controlling it are unknown. Our data suggest that decreasing glutamate levels in the embryonic cortex may facilitate the expression of voltage-gated Ca^{2+} channels. Electrophysiological experiments performed on cortical cells from E13 brain slices could not detect the presence of functional voltage-gated Ca^{2+} channels (Albrieux *et al*., 2004). Because high levels of glutamate are found in E13 murine cortex (Behar *et al*., 1999), the inhibitory action exerted by this neurotransmitter on LVA and HVA Ca^{2+} channels may prevent the recording of I_{Ca} in E13 cortical slices.

The neurotrophin BDNF plays an important role in neural development. For instance, it regulates the formation of cortical networks (Nagano *et al*., 2003). BDNF and the trkB receptors, present in the embryonic cortex, are thought to play a role in cerebral cortex development and formation (Maisonpierre *et al*., 1990; Ernfors *et al*., 1992; Fukumitsu *et al*., 1998). *In vitro*, BDNF up-regulates the migration of embryonic cortical neurons. This effect is mediated by trkB receptors via a Ca^{2+}-dependent mechanism (Behar *et al*., 1997; Medina *et al*., 2004). In addition, exogenously applied BDNF modulates the growth of cortical dendrites (McAllister *et al*., 1997). However, in our experiments, the cell membrane capacitance of BDNF-treated cells was not different from the BDNF-untreated cells, suggesting that the neurotrophin does not profoundly affect the cell size of E13 cortical neurons. Similar results were also obtained on cortical neurons from E18/19 embryonic rats in which control and BDNF-treated cells (for 24 h) had similar cell membrane capacitances (Nagano *et al*., 2003).

A chronic BDNF treatment reduces HVA I_{Ca} amplitude and density without altering LVA I_{Ca}. N-type Ca^{2+} currents were more depressed by BDNF than L-type currents. In neurons, these two types of Ca^{2+} channels fulfil distinct functions. By controlling signalling cascades, Ca^{2+} influx through neuronal L-type Ca^{2+} channels can convey information to the nucleus whereas neuronal N-type Ca^{2+} channels are involved in neurotransmitter release. Interestingly, a chronic BDNF treatment has been shown to change markedly the distribution of embryonic hippocampal Ca^{2+} channels controlling neurotransmitter

release (Baldelli *et al*., 2005). By depressing N-type Ca^{2+} currents BDNF could affect synapse maturation (Baldelli *et al*., 2005) in the immature cortex.

A BDNF treatment has no effect on HVA I_{Ca} amplitude and density in E17 rat basal forebrain neurons (Levine *et al*., 1995) whereas it up-regulates N-, P/Q- and R-type I_{Ca} from embryonic hippocampal neurons (Baldelli *et al*., 2000). It therefore appears that, depending on the cell type, BDNF can affect ion conductances in a specific manner. For instance, a 24-h BDNF treatment up-regulates currents through AMPA channels without affecting NMDA responses (Nagano *et al*., 2003). Exposure of neocortical neurons to BDNF leads to an increased expression of the AMPA receptor subunits GluR1 and GluR2/3 without affecting the expression of NMDA receptor subunits (Narisawa-Saito *et al*., 1999; Xiong *et al*., 2002). Interestingly, acutely applied BDNF also modulates AMPA receptor numbers by promoting their insertion into the plasma membrane. Although this has not been directly shown in the present work, we suggest that BDNF specifically regulates the expression of HVA but not of LVA Ca^{2+} channels. The data presented in Fig. 7 favour this hypothesis. It is worth noting that transgenic animals over-expressing BDNF have an aberrant cortical architecture (Ringstedt *et al*., 1998). Whether the BDNF-dependent modulation of I_{Ca} reported in the present study is the mechanism by which this neurotrophin influences the cortical maturation and architecture remains to be determined.

Recent data indicate the existence of a functional coupling between the glutamatergic and the BDNF/trkB systems (Marini *et al*., 1998; Xiong *et al*., 2002). For instance, glutamate promotes the release of BDNF from cerebellar granule cells (Jiang *et al*., 2003). However, K252a, which prevents the BDNF-dependent regulation of I_{Ca} (Fig. 8), fails to suppress the glutamate-dependent I_{Ca} inhibition. We found no evidence for the presence of such crosstalk between the glutamatergic and the BDNF systems in embryonic cortical neurons kept in culture.

The external factors glutamate and BDNF act by recruiting different receptors, NMDA or trkB, respectively. Although they both modulate I_{Ca} via a BAPTA-sensitive step they do not exert the same actions on I_{Ca}. We propose that an influx of Ca^{2+} through NMDA channels non-specifically affects LVA and HVA I_{Ca} whereas an influx via SKF-96365-sensitive channels preferentially regulates HVA I_{Ca}. Although SKF-96365 exerts multiple cellular actions, it is generally described as an inhibitor of voltage-independent Ca^{2+} channels (Clementi & Meldolesi, 1996) currently used to block TRPC channels and agonist-dependent Ca^{2+} entry. For instance, a chronic SKF-96365 treatment mimics antisense TRPC treatment on neural stem cell proliferation (Pla *et al*., 2005). SKF-96365 also completely blocks the BDNF- as well as the netrin-1-dependent effect on guidance of growth cones, a mechanism mediated by TRPC channels (Li *et al*., 2005; Wang & Poo, 2005). We therefore propose that BDNF modulates I_{Ca} via SKF-96365-sensitive TRPC channels. The fact that glutamate and BDNF do not have additive effects on HVA I_{Ca} indicates that they share a common Ca^{2+}-dependent step.

External factors such as neurotransmitters or growth factors can influence not only the migration (Behar *et al*., 1997, 1999; Medina *et al*., 2004) but also the electrophysiological properties of embryonic cortical neurons. Whether the glutamate- or BDNF-dependent I_{Ca} modulation is the mechanism by which these modulators influence neuronal migration remains to be verified. Nevertheless, the experiments described herein indicate that the electrical properties of embryonic neurons may critically depend on their local microenvironment. In addition, the glutamate- or BDNF-dependent down-regulation of I_{Ca} of embryonic neurons could play a neuroprotective role.

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Abbreviations

BDNF, brain-derived neurotrophic factor; DIV, days *in vitro*; E, embryonic day; HVA, high-voltage activated; I_{Ca}, voltage-gated calcium channels; LVA, low-voltage activated; (S)-AMPA, (S)-α-amino-3-hydroxy-5methyl-4-osoxale-propionic acid; *trans*- ±-ACPD, trans-(±)-1-amino-(1S,3R)-cyclopentanedi-carboxylic acid; TRPC, transient receptor potential protein of C subtype; TTX, tetrodotoxin; ω-CTx, ω-conotoxin GVIA.

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