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Nicotine-induced phosphorylation of ERK in mouse primary cortical neurons: evidence for involvement of glutamatergic signaling and CaMKII

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Abstract

Extracellular signal-regulated kinase (ERK) is activated *in vivo* in a number of brain areas by nicotine and other drugs of abuse. Here we show that nicotine stimulation of cultured mouse cortical neurons leads to a robust induction of ERK phosphorylation that is dependent on nicotine concentration and duration of exposure. Calcium/calmodulin-dependent protein kinase II activity is necessary for nicotine-induced ERK phosphorylation and neither cAMP-dependent protein kinase or protein kinase C appear to be involved. Activity of glutamate receptors, L-type voltage-gated calcium channels, and voltage-gated sodium channels are also required for nicotine-induced ERK phosphorylation. Nicotine-induced ERK phosphorylation was inhibited by high concentrations of mecamylamine, however it was not blocked by other broad nicotinic acetylcholine receptor (nAChR) inhibitors (including hexamethonium and chlorisondamine) or nAChR subtype selective inhibitors (such as methyllycaconitine, alpha-bungarotoxin, dihydro-beta-erythroidine, and α-conotoxin Au1B). In accord with these pharmacological results, nicotine-induced ERK phosphorylation was normal in primary cultures made from β2 or α7 nAChR subunit knockout mice. The α3/β4 nAChR agonist cytisine did not induce ERK phosphorylation suggesting that α3/β4 nAChRs were not involved in this process. Taken together, these data define a necessary role for glutamatergic signaling and calcium/calmodulin-dependent protein kinase II in nicotine-induced ERK phosphorylation in cortical neurons and do not provide evidence for the involvement of classical nAChRs.

Keywords: cortical neurons, primary neuronal cultures.
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Nicotine, like many other drugs of abuse, is known to activate extracellular signal-regulated kinase or mitogen-activated protein kinase (ERK or MAPK) *in vivo* (Brunzell *et al.* 2003; Valjent *et al.* 2004). Although nicotine-induced ERK phosphorylation has been demonstrated *in vivo* (Brunzell *et al.* 2003; Valjent *et al.* 2004), in cell culture lines (Nakayama *et al.* 2001; Dajas-Bailador *et al.* 2002; Huang *et al.* 2005), and in cultured hippocampal neurons (Dajas-Bailador *et al.* 2002) there is not a clear understanding of the molecular signaling pathway that results in nicotine-induced ERK phosphorylation in cortical neurons.

Acute injection of many drugs of abuse (including nicotine) produces a characteristic neuronal pattern of ERK phosphorylation, however co-injection of the dopamine D_1 receptor antagonist SCH23390 significantly attenuates drug-induced ERK phosphorylation in many brain areas. Interestingly, with co-injection of nicotine and SCH23390, significant increases in ERK phosphorylation remained in multiple cortical regions, indicating that nicotine-induced ERK phosphorylation in the cortex is dopamine D_1 receptor-independent (Valjent *et al.* 2004). Therefore, nicotine may act directly on cortical neurons to yield increased ERK phosphorylation, but the mechanism of this effect is currently unknown.

Investigations into the mechanism of nicotine-induced ERK phosphorylation have been performed in cell culture systems where the molecular components of signaling pathways are more accessible. ERK phosphorylation has been demonstrated after cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and calcium/calmodulin-

dependent protein kinase (CaMK) activation (Sweatt 2004), however which of these pathways is activated in cortical neurons has not been defined. Nicotine-induced ERK phosphorylation involves α7 nicotinic acetylcholine receptor (nAChRs) and PKA in SH-SY5Y cells and hippocampal neurons (Dajas-Bailador *et al.* 2002), and α3/β4 nAChRs and CaMK in PC12 cells (Nakayama *et al.* 2001, 2006).

In the current study, we sought to define the molecular pathway involved in D_1 dopamine receptor-independent, nicotine-induced ERK phosphorylation in cortical neurons. We addressed this question in experiments using primary cortical neuronal cultures. Interneuronal communication in these cultures may play a vital role in nicotine-induced ERK phosphorylation. Nicotine can increase release of many different classes of neurotransmitters, including glutamate (Toth *et al.* 1992; Gray *et al.* 1996), GABA (Lena and Changeux 1997), dopamine (Marshall *et al.* 1996), glycine (Lopez *et al.* 2001), and norepinephrine (Leslie *et al.* 2002). In primary hippocampal cultures, nicotine has been shown to enhance release of glutamate and GABA (Radcliffe *et al.* 1999). Therefore, nicotine-induced ERK phosphorylation in primary cortical neuronal cultures may be subsequent to nicotine-induced neurotransmitter release.

In the experiments described here, we demonstrate that nicotine does increase phosphorylation of ERK in primary cortical neuronal cultures, characterize nicotine-induced ERK phosphorylation over a range of concentrations and durations of exposure, explore the intracellular signaling pathway which results in ERK phosphorylation after primary cortical neuronal cultures are exposed to nicotine, examine the contribution of glutamate receptors to nicotine-induced ERK phosphorylation, and examine the contribution of specific nAChR classes to nicotine-induced ERK phosphorylation.

Experimental procedures

Animals

C57Bl/6J (Jackson Labs, Bar Harbor, ME, USA), α7 nAChR subunit knockout mice, and β2 nAChR subunit knockout mice were used to generate primary cultures and were genotyped by tail biopsy and PCR as has been described previously (Picciotto *et al.* 1995; Herber *et al.* 2004). Knockout mice were backcrossed more than 10 generations onto the C57Bl/6J background. Mice from Jackson Labs were given at least 7 days to habituate to the colony room before any experimental manipulations were initiated. Mice were between 2- and 7-months-old at the time of use. All mice were group housed with a maximum of five per cage in a colony room maintained at 22°C on a 12 h light/dark cycle (lights on at 7:00 AM), with food and water available *ad libitum*, unless otherwise noted. All animal procedures were in strict accordance with *NIH Care and Use of Laboratory Animals Guidelines* and were approved by the Yale University Animal Care and Use Committee.

Reagents

Nicotine hydrogen tartrate (Sigma, St Louis, MO, USA), S(-) nicotine and R(+) nicotine (Chemische Laboratorien, Dr Christoph Mark, Worms, Germany) or cytisine (Sigma) were dissolved in phosphate-buffered saline (PBS) pH 7.4 and placed at −20°C until use. Inhibitors were incubated with the cultures for the following durations before nicotine stimulation: mecamylamine (20 min; Sigma), methyllycaconitine (MLA, 20 min; Sigma and Calbiochem, San Diego, CA), α-bungarotoxin (α-BTX, 20 min; Sigma and Calbiochem), dihydro-beta-erythroidine (DHβE, 20 min; Sigma), U0126 (20 min; Calbiochem), H89 (30 min; Calbiochem), KT5720 (30 min; Calbiochem), Rp-cAMPs (a cAMP analog which decreases PKA activity, 15 min; Sigma), Gö6983 (30 min; Sigma), BAPTA (15 min; Calbiochem), nifedipine (15 min; Sigma), diltiazem (15 min; Sigma), dantrolene (30 min; Sigma), KN93 (30 min; Sigma), Stö609 (60 min; Sigma), autocamtide-2-related inhibitory peptide (AIP, 60 min; Sigma), tetrodotoxin (TTX, 20 min; Calbiochem), D-2-amino-5-phosphonovaleric acid (AP5, 20 min; Calbiochem), and 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, 15 min; Sigma). Most inhibitors were used at a stock concentration of 1000× of the desired stimulating concentration.

Cell culture

Mixed cortical cultures were made from wild type, α7 knockout, or β2 knockout mice at embryonic day 17 as described previously (Stevens *et al.* 2003). Wild type and β2 knockout cultures were made from an entire litter, whereas α7 knockout cultures were made from single fetal mice generated from heterozygous matings and were genotyped after plating because pregnancies could not be obtained from α7 knockout × knockout matings. Neurons were dissociated by incubating minced cortices in papain, dispase and DNAse (0.01% papain; Worthington, Freehold, NJ, USA), 0.1% dispase (Roche Products, Hertfordshire, UK), and 0.01% DNaseI (Sigma) in Hanks buffered salt solution (Invitrogen, San Diego, CA, USA) with penicillin-streptomycin (Invitrogen) for 15 min at 37°C, triturating cells with a glass pipette, incubating for 15 min at 37°C, and triturating cells with a fire-polished glass pipette. The cell-suspension was centrifuged at 500 *g* for 5 min at 25°C and the cell pellet was resuspended in Neurobasal medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), B27 supplement (Invitrogen), sodium pyruvate, L-glutamine, penicillin–streptomycin, and HEPES. Dissociated cortical neurons were cultured on poly-L-lysine coated six-well plates at 0.65×10^6 cells/2 mL/well. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. Within 20 h of plating, the media was removed and replaced with supplemented Neurobasal medium without FBS. Cells were then fed on day 7 *in vitro* (1 mL of medium was removed per well and replaced with 1.5 mL of supplemented Neurobasal medium without FBS) and stimulated on day 14 *in vitro*. Care was taken in dissection to remove all meninges and choroid plexus and use of embryonic day 17 fetal mice, thus glial cells represented <5% of the overall population.

Culture stimulations and sample collection

On day 14 *in vitro* media was removed and 1.5 mL media was quickly replaced to ensure a consistent stimulating volume. Inhibitors were added prior to nicotine stimulations (as detailed above) and were present during nicotine stimulations. Immediately

following stimulation, the media was removed, wells were washed with ice-cold PBS, PBS was removed by suction and cell lysis buffer [50 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, 1% sodium dodecyl sulfate (SDS), and 1 mmol/L PMSF] was added. The plates were then incubated at 4°C on a tilting shaker for at least 20 min before the lysate was collected and frozen at −80°C until western blotting.

Western blotting

Approximately 10 μg of each sample was separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Blots were blocked with 5% milk in Tris-buffered saline with 0.05% (v/v) Tween-20 (TBS-T) for 60 min at 25°C, washed three times in TBS-T for 8 min, and then incubated at 4°C overnight in primary antibody diluted in TBS-T [anti-ERK (rabbit) and/or anti-pERK (mouse): 1 : 2000; Cell Signaling Technology, Danvers, MA, USA]. Blots were washed three times in TBS-T and incubated for 60 min at 25°C in secondary antibody (Alexa-Fluor 680 goat anti-mouse IgG: 1 : 5000; Molecular Probes, Invitrogen, Carlsbad, CA, USA and IRDye800 goat anti-rabbit IgG: 1 : 5000; Rockland Immunochemicals, Gilbertsville, PA, USA) in TBS with 0.1% Tween and 0.001% SDS. Blots were washed three times in TBS-T and two times in water before scanning with an Odyssey Infrared Scanner (Li-Cor Biosciences, Lincoln, NE, USA). The scans were performed with the following scan parameters: intensity = 5.0, for each wavelength (700 and 800 nm), resolution = 169, quality = medium, and focus offset = 0 mm. The scanned intensity of the two secondary antibodies' fluorophores was then used by the Odyssey Software v1.2 (Li-Cor Biosciences) to produce individual images for each wavelength, and a pseudo-color overlay of the 700 (red) and 800 nm (green) scans. The area of each band was selected for quantitation and the integrated intensity of the 700 and 800 nm wavelengths was measured by the Odyssey Software without investigator manipulation. Since Li-Cor uses the absolute intensities for quantitation, the pseudocolor images are reflections of the absolute value measured by the program. The integrated intensity values were then exported to Excel (Microsoft, Redmond, WA, USA) for further analysis.

Statistical analyses

At least two replicates (wells) for each condition were obtained from each culture and averaged before statistical analysis. Each '*n*' represents an independent culture (the average of multiple wells within that culture). All statistical analyses were carried out using SPSS v12.0 (SPSS Inc., Chicago, IL, USA). ANOVA tests were performed for nicotine, cytisine, and each inhibitor with its corresponding control groups. All significant main effects and interactions were followed up with the least significant difference *post hoc* test. A value of $p < 0.05$ was considered significant for all comparisons.

Results

Nicotine induces ERK phosphorylation in a concentration- and time-dependent manner

The ability of nicotine to increase ERK phosphorylation in primary cortical cultures has not been characterized, there-fore we examined nicotine-induced ERK phosphorylation across a range of concentrations of nicotine and durations of exposure (Fig. 1a and b). Acute nicotine application induced a rapid increase in ERK phosphorylation in cultured cortical neurons that was concentration- and time-dependent. The highest level of ERK phosphorylation was observed at the first time point (5 min) and then decayed as the duration of nicotine exposure increased to 120 min. ERK1 (p44 ERK) and ERK2 (p42 ERK) exhibited a similar pattern of phosphorylation, however ERK2 exhibited greater phosphorylation and was chosen for analysis in all subsequent experiments. Total levels of ERK1 (p44 ERK) and ERK2 (p42 ERK) did not change under any concentration or exposure time for nicotine (data not shown). Phosphorylated ERK2 was normalized to total ERK2 and expressed as a ratio; an ANOVA showed main effects of nicotine concentration [$F(3,167) = 20.157$, $p < 0.001$] and time of exposure to nicotine [$F(4,167) = 8.976$, $p < 0.001$] on pERK/ERK.

Levels of phosphorylated ERK did not change significantly following application of 1 μmol/L nicotine, however concentrations of 10 μmol/L nicotine or above resulted in significant increases in phosphorylated ERK. For the 10 μmol/L nicotine concentration, phosphorylated ERK returned to basal levels by 60 min. However, application of 50 and 100 μmol/L nicotine extended the duration of ERK phosphorylation beyond 60 min, returning to basal levels by 120 min.

The maximal increase in ERK phosphorylation was observed with 100 μmol/L nicotine applied for 5 min. This condition was therefore chosen for experiments to investigate the signaling pathway leading from nAChR activation to the phosphorylation of ERK. As expected, pre-treatment with the ERK kinase [MAPK (or ERK) kinase] inhibitor U0126 effectively blocked the ability of nicotine to cause an increase in ERK phosphorylation [$F(3,8) = 5.282$, $p = 0.027$] (Fig. 1c).

CaMKII is an upstream kinase involved in nicotine-induced ERK phosphorylation in primary cortical cultures

To identify the upstream kinase(s) necessary for nicotine-induced ERK phosphorylation, we examined the effect of multiple kinase inhibitors on the ability of nicotine to increase ERK phosphorylation. The pan-CaMK inhibitor KN93 significantly attenuated nicotine-induced ERK phosphorylation in a concentration-dependent manner [$F(5,31) = 14.531$, $p < 0.001$] (Fig. 2a). Investigation into which CaMK may be responsible for the effect of KN93 revealed that the specific CaMKII inhibitor AIP blocked nicotine's effect, similar to KN93 [$F(5,26) = 39.467$, $p < 0.001$] (Fig. 2a). Conversely, the CaMK kinase inhibitor Stö609 (which inhibits activation of CaMKI/IV) did not decrease nicotine-induced ERK phosphorylation [$F(3,12) = 5.791$, $p < 0.02$] (Fig. 2a).
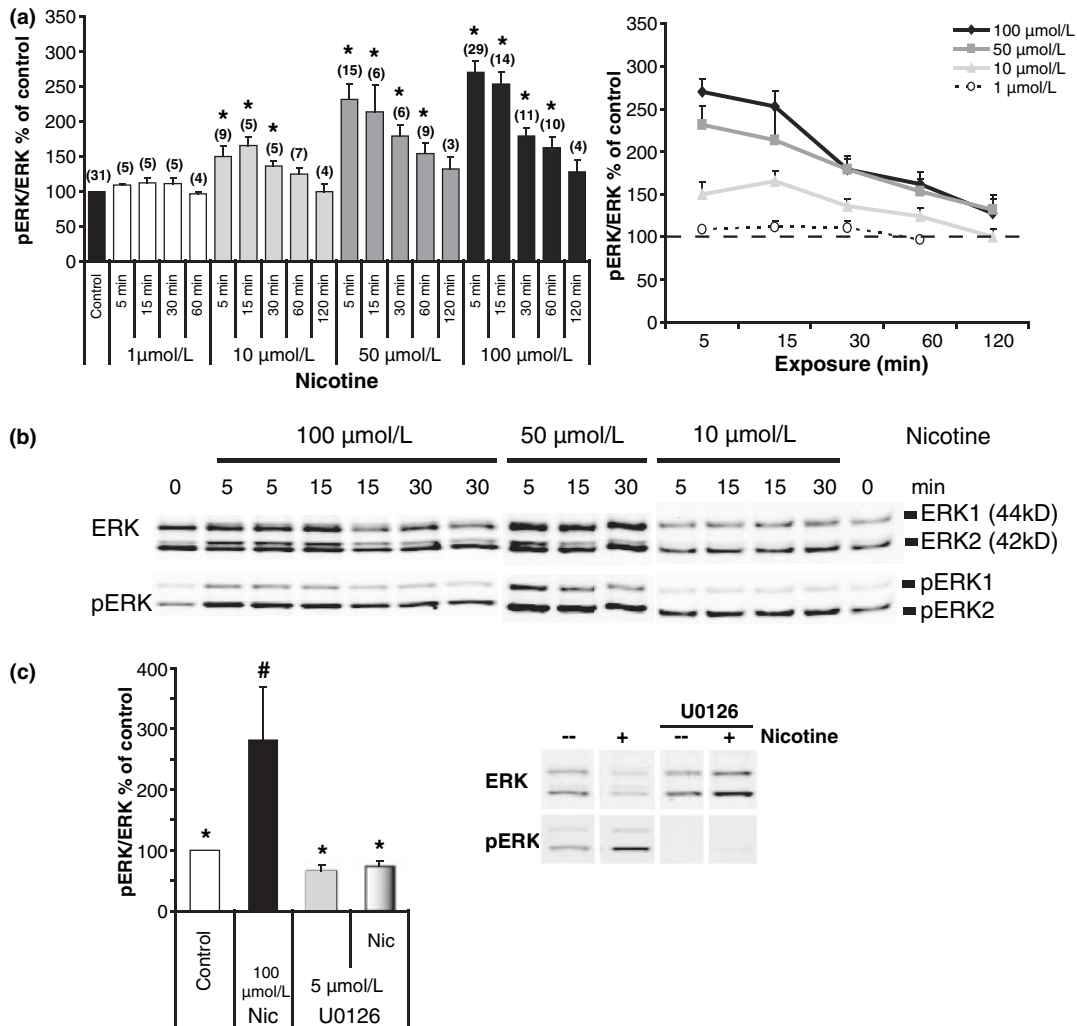
Fig. 1 Nicotine elicits an increase in ERK phosphorylation in a time- and concentration-dependent manner. (a) Quantitation of the ratio of pERK2 to ERK2 (42 kDa). Mean +SEM, the number of independent cultures per condition are indicated, *indicates significant difference from control ($p < 0.05$). (b) Representative western blots of total and phosphorylated ERK1 and ERK2 from primary cortical cultures stimulated with nicotine. The total ERK antibody recognizes phosphorylated and unphosphorylated forms of ERK, therefore a double band is seen when ERK is heavily phosphorylated (for example, ERK2 in the 100 μmol/L nicotine, 5 min condition). (c) The increase in ERK phosphorylation observed with 100 μmol/L nicotine for 5 min is blocked by pre-treatment with the mitogen-activated protein kinase (or ERK) kinase inhibitor U0126. Representative western blots are shown. Mean + SEM, the number of cultures per condition is shown, *indicates a significant difference from the 100 μmol/L, 5 min nicotine condition, #indicates a significant difference from the control condition ($p < 0.05$).

It has been demonstrated that nAChR stimulation can activate PKA and PKC (Dajas-Bailador *et al.* 2002; Dajas-Bailador and Wonnacott 2004), which are both potentially upstream of ERK activation. To determine whether PKA or PKC activity is necessary for the ability of nicotine to induce ERK phosphorylation, we examined the effect of PKA and PKC inhibitors in wild type cortical neuronal cultures. The PKA antagonist H89 did not significantly attenuate nicotine-induced ERK phosphorylation at concentrations of the inhibitor specific for PKA [$F(5,28) = 14.313$, $p < 0.001$] (Fig. 2b). Confirming H89's inability to block nicotine-induced ERK phosphorylation via PKA antagonism, KT5720 {a PKA inhibitor, [$F(5,19) = 15.369$, $p < 0.001$], Fig. 2b} and Rp-cAMPs {[$F(5,18) = 10.647$, $p < 0.001$], Fig. 2b} had no effect on ERK phosphorylation after nicotine stimulation. Additionally, the PKC inhibitor Gö6983 had no effect on nicotine-induced ERK phosphorylation [$F(5,14) = 5.496$, $p < 0.005$]. The apparent increase in phosphorylated ERK observed when Gö6983 was applied alone did not reach statistical significance ($p > 0.25$) (Fig. 2c). Taken together, these data suggest that CaMKII is the primary upstream kinase necessary for
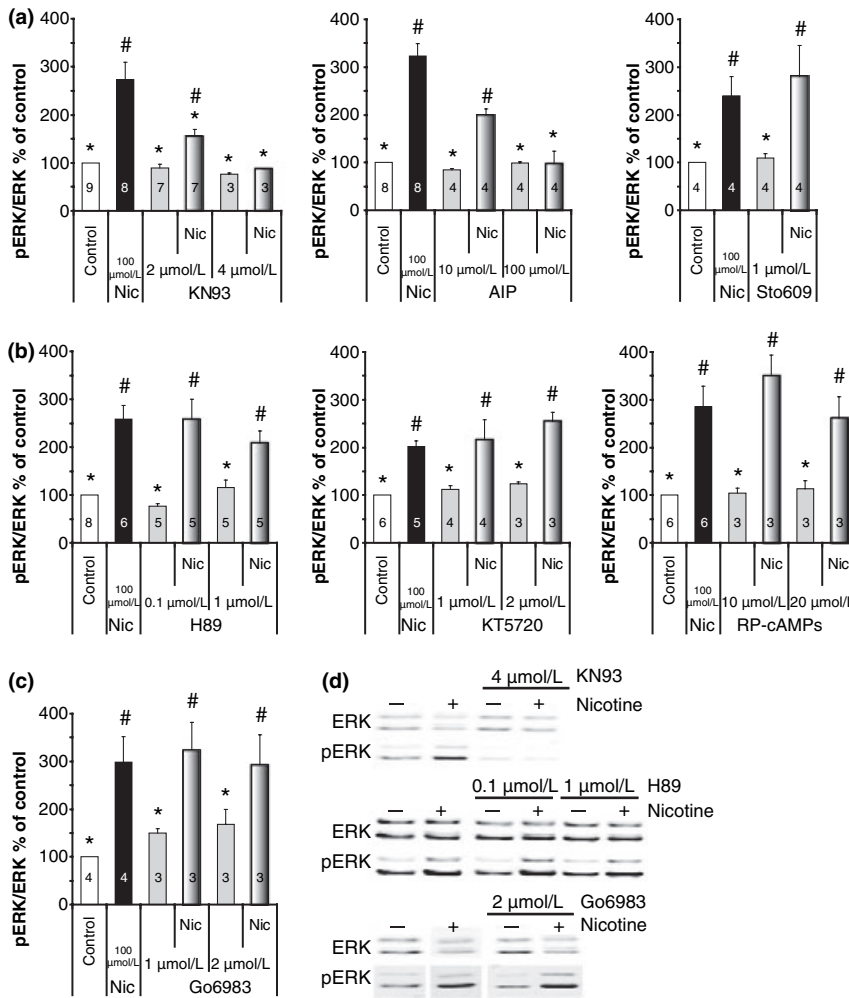
Fig. 2 Nicotine-induced increases in ERK phosphorylation are dependent on CaMKII activity and not on PKA or PKC activity. (a) Both KN93 and AIP significantly attenuate nicotine-induced ERK phosphorylation, whereas Stö609 does not affect nicotine-induced ERK phosphorylation. (b) Pre-treatment with either H89, KT5720, or RP-cAMPs does not reduce nicotine-induced ERK phosphorylation. (c) Pre-treatment with the PKC inhibitor Gö6983 does not significantly attenuate nicotine-induced ERK phosphorylation in primary cortical cultures. Mean + SEM, the number of cultures per condition is shown, *indicates a significant difference from the 100 μmol/L ($p < 0.05$), 5 min nicotine condition, #indicates a significant difference from the control condition ($p < 0.05$). (d) Representative western blots are shown.

nicotine-induced ERK phosphorylation in primary cortical cultures.

Calcium is necessary for nicotine-induced ERK phosphorylation

The activation of CaMKII is critically dependent on an increase in intracellular calcium and subsequent activation of calmodulin. We confirmed that calcium is required for nicotine-induced ERK phosphorylation by demonstrating that chelating free calcium with BAPTA results in a complete inhibition of nicotine-induced ERK phosphorylation [$F_{(3,10)} = 13.091$, $p = 0.001$] (Fig. 3a). Additionally, the L-type voltage-gated calcium channel inhibitors nifedipine [$F_{(5,29)} = 24.479$, $p < 0.001$] and diltiazem [$F_{(3,8)} = 14.493$, $p = 0.001$] significantly attenuated the increase in phosphorylated ERK seen after 5 min exposure to 100 μmol/L nicotine (Fig. 3b), indicating that L-type voltage-gated calcium channels may be important for nicotine-induced ERK phosphorylation. Intracellular calcium release from the endoplasmic reticulum via ryanodine receptor activation

is not involved in nicotine-induced ERK phosphorylation as dantrolene (a ryanodine receptor antagonist) did not attenuate the increase in phosphorylated ERK after 100 μmol/L nicotine application [$F_{(3,12)} = 18.558$, $p < 0.001$] (Fig. 3c).

Endogenous glutamatergic transmission and neuronal activity is required for nicotine-induced ERK phosphorylation

Primary cortical neuronal cultures are synaptically active after 14 days *in vitro* and express glutamate receptors (Jones and Baughman 1991). To silence all spontaneous action potentials in our cultures, we pretreated the neurons with the voltage-gated Na^{+} channel blocker TTX and found that it completely inhibited nicotine-induced increases in phosphorylated ERK [$F_{(3,12)} = 11.244$, $p = 0.001$] (Fig. 4a). Nicotine is known to increase glutamate release from axon terminals (Toth *et al.* 1992; Gray *et al.* 1996; Lopez *et al.* 2001; Wang *et al.* 2006) and glutamate receptor activity can modulate ERK phosphorylation (Sweatt 2004). Therefore,
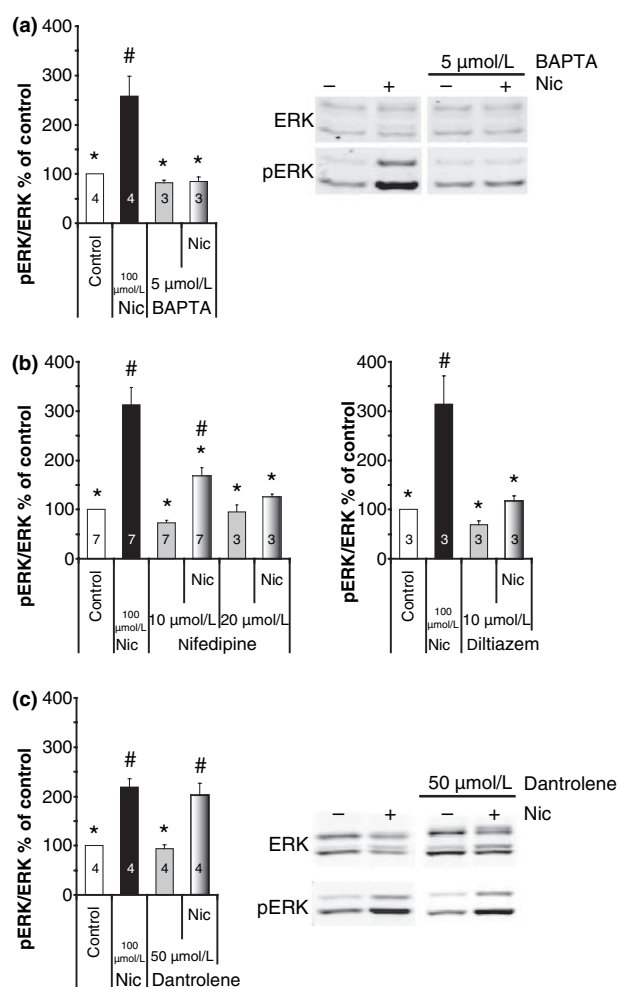
Fig. 3 Calcium entry via L-type voltage-gated calcium channels is necessary for nicotine-induced ERK phosphorylation. (a) BAPTA, a calcium chelator, significantly blocks nicotine-induced ERK phosphorylation. Representative western blots are shown. (b) Pre-treatment with the L-type voltage-gated calcium channel blockers nifedipine and diltiazem significantly inhibits nicotine-induced ERK phosphorylation. (c) The ryanodine receptor antagonist dantrolene does not attenuate the ability of nicotine to induce ERK phosphorylation. Representative western blots are shown. Mean + SEM, the number of cultures per condition is shown, *indicates a significant difference from the 100 μmol/L ($p < 0.05$), 5 min nicotine condition, #indicates a significant difference from the control condition ($p < 0.05$).

glutamate receptor activity may be involved in the ability of nicotine to increase ERK phosphorylation, either by providing basal excitation of the neurons with which the nAChR activity reaches a level that is sufficient to induce ERK phosphorylation, or by nicotine-induced glutamate release resulting in direct effects at glutamate receptors to initiate signaling pathways that subsequently lead to increased ERK phosphorylation (Sweatt 2004). The gluta-

mate receptor antagonists CNQX (an alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/Kainate receptor antagonist) and AP5 [an N-methyl-D-aspartic acid (NMDA) receptor antagonist] were used to examine whether glutamate receptor activity is necessary for the increase in phosphorylated ERK observed with nicotine application. Pre-treatment with either CNQX [$F(3,16) = 8.407$, $p = 0.001$] or AP5 [$F(3,12) = 10.855$, $p = 0.001$] completely blocked the ability of nicotine to increase ERK phosphorylation (Fig. 4b and c). Interestingly, these data indicate that glutamate receptor activity, coincident with nicotine application, is required for increased ERK phosphorylation in cortical neurons.

Are nAChRs involved in nicotine-induced ERK phosphorylation?

It has been thought that nicotine acts predominantly via activation of nAChRs to initiate the signaling pathway that leads to increased ERK phosphorylation. The two most prominent nAChR classes in the brain are the homomeric α7 and heteromeric, high-affinity α4/β2 nAChRs. Additionally, α7 nAChRs have been implicated in nicotine-induced ERK phosphorylation in hippocampal neurons (Dajas-Bailador et al. 2002). We therefore stimulated wild type, α7 nAChR subunit knockout, and β2 nAChR subunit knockout cultures with nicotine in the absence or presence of nAChR antagonists. The non-competitive and non-selective nAChR antagonist mecamylamine inhibited nicotine-induced ERK phosphorylation in a concentration-dependent manner in wild type cultures [$F(7,49) = 22.668$, $p < 0.001$] (Fig. 5a). In order to determine whether nicotine was acting specifically at one class of nAChR to cause an increase in ERK phosphorylation, more specific antagonists were tested. None of the competitive nAChR-specific antagonists [α7 nAChR-specific: MLA and α-BTX; $F(5,40) = 22.685$, $p < 0.001$], heteromeric nAChR-specific: DHβE [$F(3,15) = 14.345$, $p < 0.001$], or α3/β4 nAChR-specific: α-conotoxin Au1B [$F(3,15) = 65.329$, $p < 0.001$]] significantly attenuated the increase in phosphorylated ERK observed when 100 μmol/L nicotine was applied for 5 min (Fig. 5b). Additionally, these nAChR antagonists did not decrease nicotine-induced ERK phosphorylation when cultures were stimulated with 10 μmol/L nicotine for 5 min (data not shown). Stimulation of wild type cultures with cytisine (an α3/β4 nAChR selective agonist) did not cause an increase in ERK phosphorylation (Fig. 5c and d). These data indicate that if nAChRs are necessary for nicotine-induced ERK phosphorylation, one particular class of nAChRs is not likely to be necessary for nicotine-induced ERK phosphorylation. Instead, multiple nAChRs may be able to initiate the signaling cascade that results in increased ERK phosphorylation.

To confirm the results obtained utilizing pharmacological inhibitors, cultures were made from knockout mice lacking
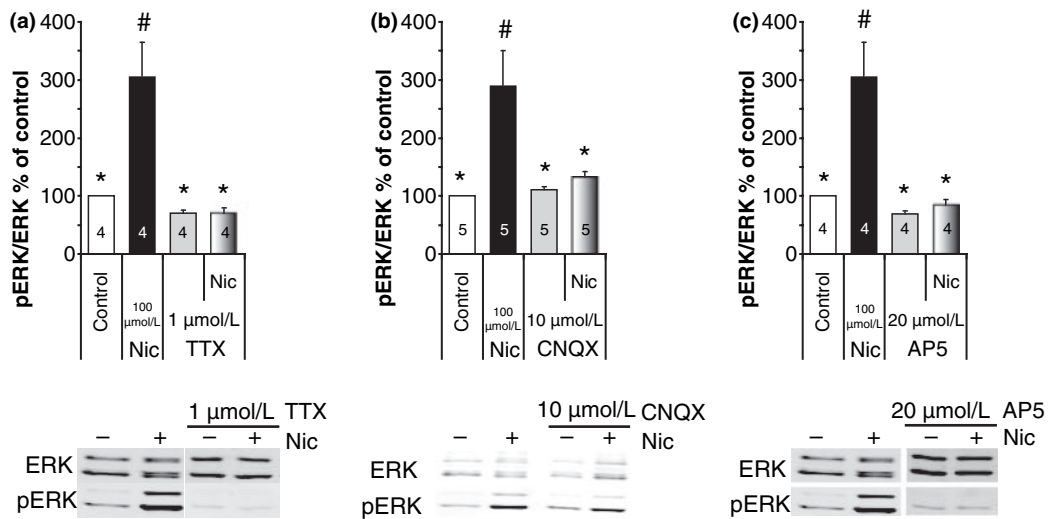
Fig. 4 Voltage-gated sodium channels and glutamate receptor activation is necessary for nicotine-induced ERK phosphorylation. (a) TTX abolishes nicotine-induced ERK phosphorylation. (b) The AMPA receptor antagonist CNQX significantly blocks nicotine-induced ERK phosphorylation. (c) The NMDA receptor antagonist AP5 significantly inhibits nicotine-induced ERK phosphorylation. Representative western blots are shown. Mean + SEM, the number of cultures per condition is shown, *indicates a significant difference from the 100 μmol/L, 5 min nicotine condition ($p < 0.05$), #indicates a significant difference from the control condition ($p < 0.05$).

either the α7 or β2 nAChR subunit and nicotine-induced ERK phosphorylation was assessed across concentration and duration of exposure (Fig. 6a and b). A time × dose × genotype ANOVA revealed a main effect of time [$F(4,280) = 16.169$, $p < 0.001$] and a main effect of concentration [$F(3,280) = 31.827$, $p < 0.001$], however no main effect of genotype [$F(2,280) = 2.038$, $p > 0.05$], and no interactions ($p > 0.05$). Therefore, the genetic deletion of either the α7 or β2 nAChR subunit alone does not alter the ability of nicotine to induce an increase in ERK phosphorylation in primary neuronal cortical cultures. Similar to wild type cultures, pre-treatment of α7 knockout cultures with DHβE [$F(3,8) = 106.629$, $p < 0.001$] or β2 knockout cultures with α-BTX [$F(3,8) = 32.842$, $p < 0.001$] did not inhibit nicotine-induced ERK phosphorylation (Fig. 6c). Additionally, the ability of mecamylamine to block nicotine-induced ERK phosphorylation (in a concentration-dependent manner) was preserved in both the α7 and β2 knockout cultures (data not shown).

The data indicate that inhibition or genetic deletion of a specific class of nAChRs does not alter nicotine-induced ERK phosphorylation. Multiple nAChR classes may be able to initiate the nicotinic signaling cascade leading to increased ERK phosphorylation, therefore we examined the effect of inhibiting multiple classes of nAChRs simultaneously. A mixture of α-BTX, α-conotoxin Au1B and DHβE was unable to decrease nicotine-induced ERK phosphorylation [$F(3,10) = 70.778$, $p < 0.001$] (Fig. 7a). Pre-treatment with the general nAChR antagonists hexamethonium or chlorisondamine before nicotine stimulation was equally ineffective

in blocking the increase in ERK phosphorylation observed in primary cortical cultures after nicotine stimulation [$F(3,14) = 56.009$, $p < 0.001$ and $F(3,10) = 18.672$, $p < 0.001$, respectively] (Fig. 7b and c). Stimulation of mouse cortical cultures with optically pure S(-) or R(+) nicotine resulted in increased levels of ERK phosphorylation at the 5 min [$F(4,10) = 5.338$, $p < 0.05$] and 30 min [$F(4,10) = 4.633$, $p < 0.05$] time points. The S(-) and R(+) nicotine enantiomers each increased ERK phosphorylation to a level equivalent to stimulation with the nicotine (Sigma) used for all other experiments, with no significant difference observed between them ($p > 0.15$) (Fig. 7d).

The concentrations of mecamylamine that produce a decrease in nicotine-induced ERK phosphorylation are sufficient to block nAChRs, however NMDA receptors are also likely to be antagonized at these concentrations (O'Dell and Christensen 1988; Papke *et al.* 2001). Therefore, we cannot determine whether the inhibitory effect of mecamylamine on nicotine-induced ERK phosphorylation is due to its action at nAChRs as the NMDA receptor antagonist AP5 also blocks this effect, however nicotine is not known to activate the NMDA receptor directly. Taken together, these data do not identify a classic nAChR-mediated mechanism that initiates the signaling cascade leading to increased ERK phosphorylation after acute application of nicotine to primary cortical cultures. Instead, the data presented here indicate that several classes of nAChRs (including α7, β2 subunit-containing, and α3/β4 nAChRs) are not necessary for nicotine-induced ERK phosphorylation in primary cortical neurons.
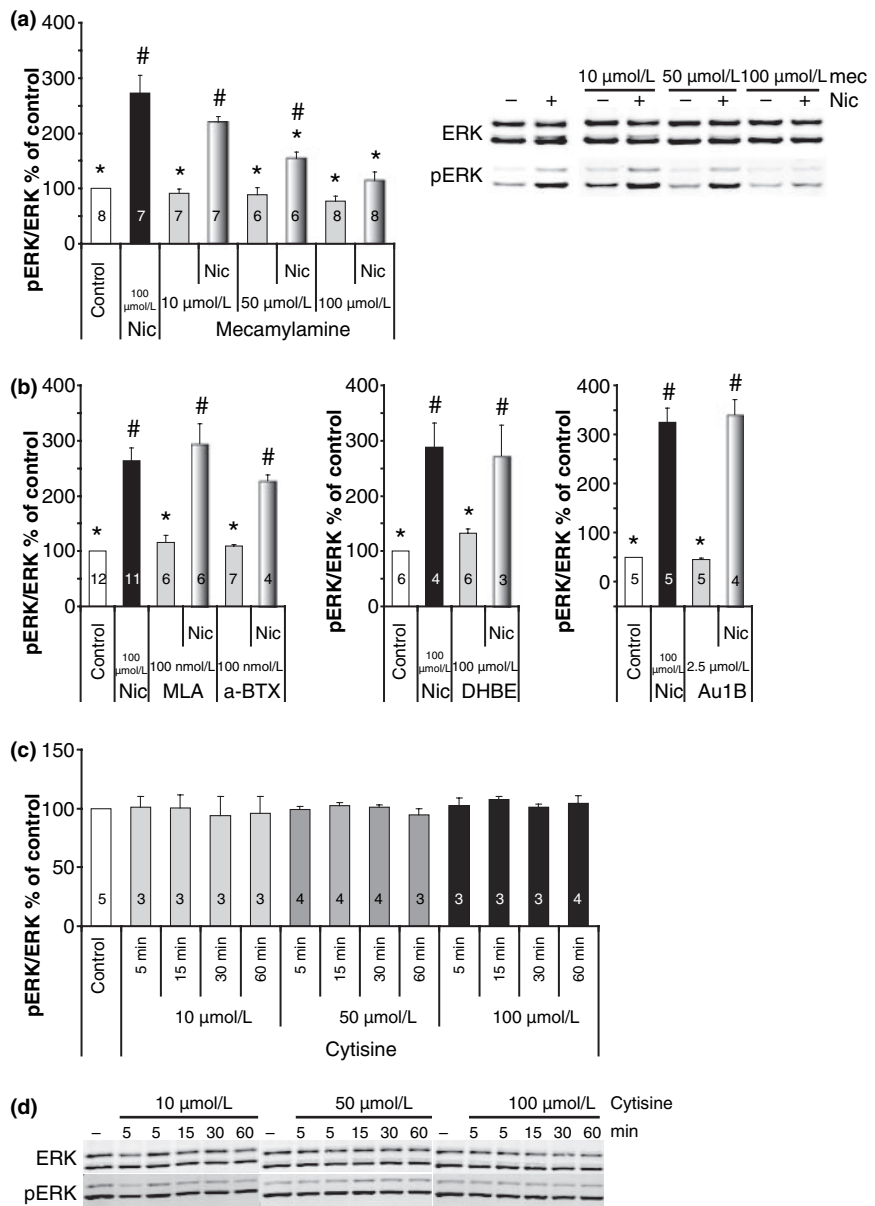
Fig. 5 Nicotine-induced ERK phosphorylation is blocked by the non-selective nAChR antagonist mecamylamine, but not by antagonists that are specific for subtypes of nAChRs. (a) The non-competitive nAChR antagonist mecamylamine dose-dependently decreases the increase in pERK observed with nicotine application. Representative western blots are shown. (b) Pretreatment with either MLA or α-BTX (α7 nAChR-specific antagonists) does not affect nicotine-induced ERK phosphorylation. Similarly, pre-treatment with either DHβE (an antagonist that is selective for heteromeric nAChRs) or α-conotoxin Au1B (a selective antagonist for α3/β4 nAChRs) does not inhibit nicotine-induced ERK phosphorylation. Mean + SEM, the number of cultures per condition is shown, *indicates a significant difference from the 100 μmol/L, 5 min nicotine condition ($p < 0.05$), #indicates a significant difference from the control condition ($p < 0.05$). (c) Stimulation with the α3/β4 nAChR-selective agonist cytisine does not increase ERK phosphorylation in primary cortical cultures. (d) Representative western blots from cultures stimulated with cytisine are shown.

Discussion

Nicotine induces a rapid and robust increase in ERK phosphorylation in primary cortical cultures that is both concentration- and time-dependent. We also show that the signaling pathway resulting in nicotine-induced ERK phosphorylation in primary cortical neurons involves voltage-gated sodium channels, glutamate receptor signaling, calcium, L-type voltage-gated calcium channels, and CaM-KII. In addition, activation of known nAChR subtypes does not appear to be responsible for nicotine-induced ERK phosphorylation, indicating that nicotine may be acting through a novel, undefined mechanism in cultured cortical neurons. Neither the α7 nor the β2 nAChR subunit is

required to observe increased levels of phosphorylated ERK after nicotine stimulation, and pharmacological evidence indicates that activation of α3/β4 nAChRs alone is not necessary or sufficient to increase ERK phosphorylation.

Calcium/calmodulin-dependent protein kinase II activity is necessary for nicotine-induced ERK phosphorylation in cortical neurons because the non-specific CaMK inhibitor KN93 and the CaMKII inhibitor AIP block, whereas Stö609 (a CaMK kinase inhibitor, subsequently blocking activation of CaMKI/IV) does not alter nicotine's ability to increase ERK phosphorylation. These data are consistent with findings in PC12 cells with respect to the magnitude of nicotine-induced ERK phosphorylation observed and the involvement of calmodulin and CaMK (Nakayama *et al.*
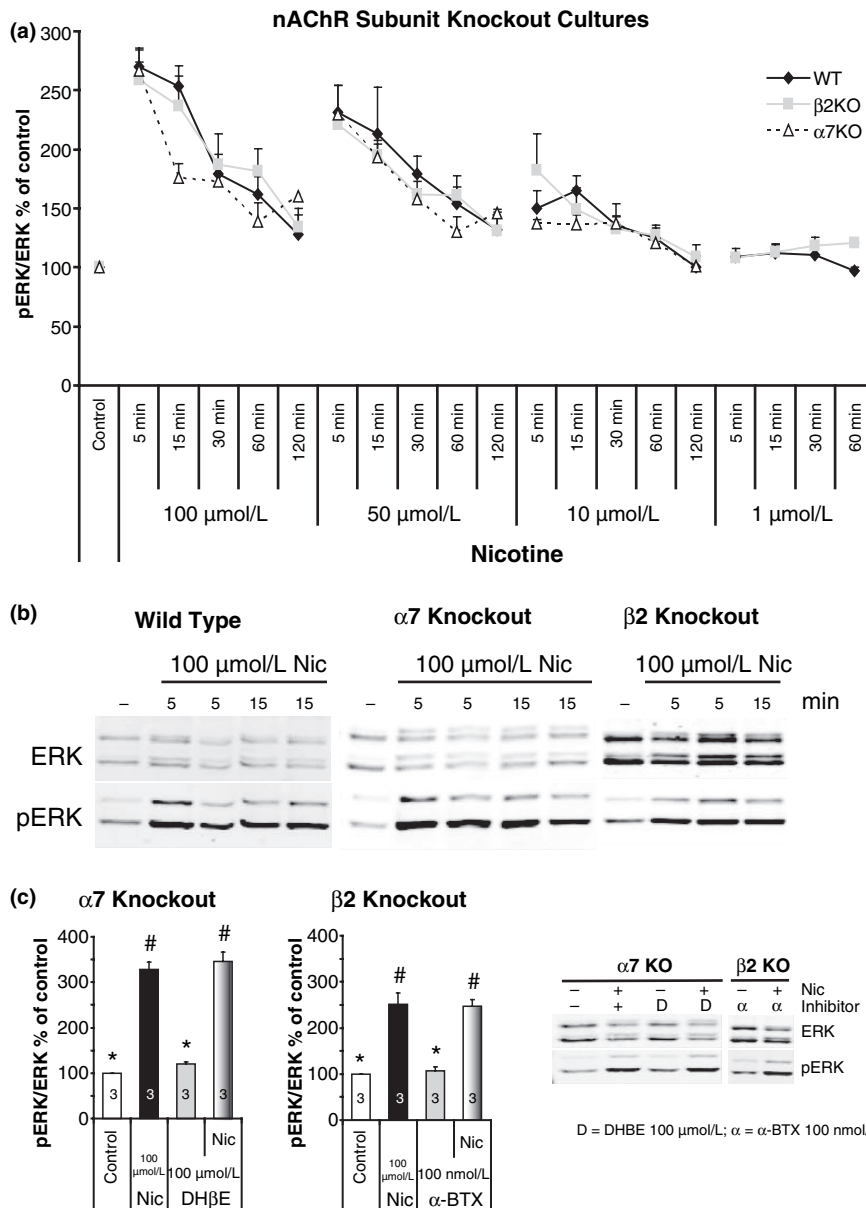
Fig. 6 (a) Quantitation of pERK/ERK in primary cortical cultures made from α7 or β2 nAChR subunit knockout mice. There is no significant difference due to genotype. Mean + SEM, $n \geq 3$ independent cultures per condition. (b) Representative western blots showing ERK and pERK immunoreactivity in samples obtained from wild type and knockout cultures stimulated with nicotine. (c) α7 KO cultures show normal increases in ERK phosphorylation when pretreated with DHβE.

Additionally, β2 KO cultures that are pre-treated with α-BTX demonstrate normal nicotine-induced ERK phosphorylation. Representative western blots are shown. Mean + SEM, the number of cultures per condition is shown, *indicates a significant difference from the 100 µmol/L, 5 min nicotine condition ($p < 0.05$), #indicates a significant difference from the control condition ($p < 0.05$).

2001); however nicotine-induced ERK phosphorylation in SH-SY5Y cells and rat hippocampal neurons is fundamentally different in the magnitude of response and the requirement of PKA activity (Dajas-Bailador *et al.* 2002). PKA or PKC activity is not involved in nicotine-induced ERK phosphorylation in primary cortical neurons. There is a profound difference in the nAChR subtypes expressed in the

hippocampus and cortex. α7* nAChRs are expressed at highest levels in the hippocampus, whereas mRNA encoding the α4 subunit is extremely low in this brain area and α4/β2* nAChRs are more highly expressed in the cortex than in the hippocampus (Seguela *et al.* 1993; Picciotto *et al.* 1995; Orr-Urtreger *et al.* 1997; Marubio *et al.* 1999). Thus, the report that hippocampal neurons require PKA activation (Dajas-
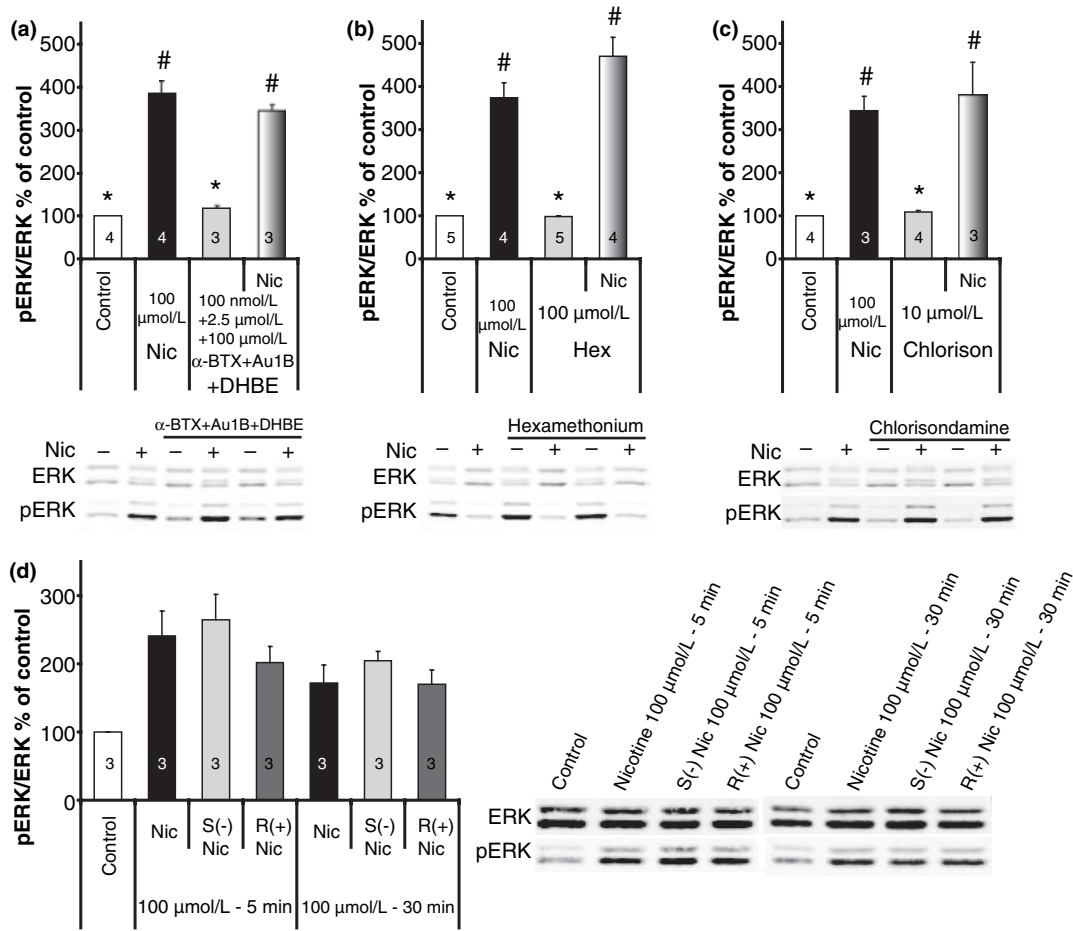
Fig. 7 (a) A combination of the nAChR antagonists α-BTX, α-cono-toxin Au1B, and DHβE does not inhibit nicotine-induced ERK phos-phorylation. (b) Pre-treatment with the general nAChR antagonist hexamethonium does not decrease nicotine-induced ERK phosphor-ylation. (c) The general nAChR antagonist chlorisondamine does not inhibit nicotine-induced ERK phosphorylation. (d) Stimulation of mouse cortical cultures with optically pure S(-) or R(+) nicotine pro-duces an increase in ERK phosphorylation at 5 and 30 min, and is equivalent to stimulation with the racemic mixture of nicotine. Repre-sentative western blots are shown. Mean + SEM, the number of cul-tures per condition is shown, *indicates a significant difference from the 100 μmol/L, 5 min nicotine condition ($p < 0.05$), #indicates a sig-nificant difference from the control condition ($p < 0.05$).

Bailador et al. 2002), whereas nicotine-induced ERK phos-phorylation in our cortical cultures requires CaMKII activa-tion (and is independent of PKA) may be the result of inherent differences between these neuronal classes based on species (rat vs. mouse) or brain region of origin (hippocam-pal vs. cortical), or by the stage of neuronal maturation at which the experiments were conducted (8 vs. 14 days in vitro), which is likely to result in large differences in synapse maturity (Weiss et al. 1986).

The increase in ERK phosphorylation observed after nicotine stimulation was dependent on calcium, as in rat hippocampal neurons (Dajas-Bailador et al. 2002), and influx of calcium via L-type voltage gated calcium chan-nels, similar to nicotine-induced ERK phosphorylation in PC12 cells (Nakayama et al. 2001). The results with nifedipine and diltiazem must be interpreted cautiously

however, because these inhibitors have also been shown to inhibit nAChR activity (Donnelly-Roberts et al. 1995; Herrero et al. 1999; Wheeler et al. 2006). It is unlikely that the inhibition observed with nifedipine and diltiazem is due to nAChR antagonism as the only nAChR antagonist that decreased nicotine-induced ERK phosphorylation was mecamylamine (also an NMDA receptor antagonist) and all other nAChR antagonists did not alter nicotine-induced ERK phosphorylation. Interestingly, the ryanodine receptor antagonist dantrolene did not affect nicotine-induced ERK phosphorylation, indicating that release of calcium from intracellular stores is not necessary for nicotine to increase ERK phosphorylation. It is likely, however, that calcium influx through L-type calcium channels is critical for the activation of calmodulin and subsequently CaMKII, up-stream of ERK phosphorylation.

An interesting result of this report is that nicotine-induced ERK phosphorylation in cortical neurons requires the activity of both alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and NMDA receptors and voltage-gated sodium channels. There are two likely explanations for this interaction. Basal glutamatergic tone may be necessary for nicotine to excite the neurons beyond some threshold that is necessary for ERK phosphorylation or, alternatively, nicotine may stimulate release of glutamate from axon terminals, which in turn activates glutamate receptors and the intracellular signaling pathway that leads to an increase in ERK phosphorylation. These hypotheses are not mutually exclusive, and both may occur. Glutamate receptor activation is known to induce ERK phosphorylation in primary cortical neuronal cultures (Chandler *et al.* 2001; Mao *et al.* 2004). The finding that the voltage-gated sodium channel blocker TTX completely inhibits nicotine-induced ERK phosphorylation suggests that the overall activity of cortical neurons is critical for their ability to respond to nicotine.

Nicotine may act at nAChRs to activate the signaling cascade that results in increased ERK phosphorylation, however we have found a surprising lack of evidence implicating an involvement of the classes of nAChRs thought to be most prevalent in cortical neurons. The general nAChR antagonist mecamylamine inhibited nicotine-induced ERK phosphorylation in a concentration-dependent manner, however, at the concentrations of mecamylamine where the nicotine effect is inhibited this antagonist is no longer specific for nAChRs and could also be blocking NMDA receptor function (Papke *et al.* 2001). Because the NMDA receptor antagonist AP5 can completely abolish nicotine-induced ERK phosphorylation, it is possible that the effect of mecamylamine is not due to its action at nAChRs. This interpretation is supported by the observation that two other general nAChR antagonists (hexamethonium and chlorisondamine) do not attenuate nicotine-induced ERK phosphorylation.

The two most abundant nAChR classes in the brain are the α7 homomeric and β2 subunit-containing heteromeric nAChRs. It has previously been reported that α7 nAChRs are necessary for nicotine-induced ERK phosphorylation in rat hippocampal neurons (Dajas-Bailador *et al.* 2002). Nicotine-induced ERK phosphorylation was completely unaffected in cortical cultures made from either α7 nAChR subunit or β2 nAChR subunit knockout mice. This surprising result was supported with data from pharmacological nAChR inhibitors that are specific for either α7 nAChRs (MLA and a-BTX) or β2-containing heteromeric nAChRs (DHβE) that demonstrated no inhibition of nicotine-induced ERK phosphorylation in wild type cultures or in the α7 and β2 knockout cultures, indicating that redundancy between the α7 and β2 subunit-containing nAChRs is unlikely.

Nakayama *et al.* (2006) have demonstrated that the α3/β4 class of nAChRs is responsible for nicotine-induced ERK phosphorylation in PC12 cells, both by mimicking the effects of nicotine with an agonist that is selective for α3/β4 nAChRs (cytisine; Mulle and Changeux 1990) and by blocking them with an antagonist that is selective for these nAChRs (18-methoxycoronaridine) (Nakayama *et al.* 2006). Contrary to the findings in PC12 cells, cytisine did not induce ERK phosphorylation in primary cortical neuronal cultures and α-conotoxin Au1B (an α3/β4 nAChR-specific antagonist) did not inhibit nicotine-induced ERK phosphorylation, indicating that α3/β4 nAChR activation is not sufficient to increase ERK phosphorylation in this system. Similar to what was observed with other general nAChR antagonists (hexamethonium and chlorisondamine), the application of the selective nAChR antagonists α-BTX, α-conotoxin Au1B, and DHβE in combination did not block the ability of nicotine to increase ERK phosphorylation in wild type cultures.

Finally, stimulation of mouse cortical cultures with either the *S*(-) or *R*(+) enantiomer of nicotine alone increased ERK phosphorylation levels, although there was a trend for the *S*(-) enantiomer to be somewhat more effective ($p = 0.19$). The previously reported nicotine enantiomer-specific effects at different subtypes of nAChRs are mixed. The function of the *S*(-) and *R*(+) enatiomers was found to be equivalent at the neuromuscular junction (Ikushima *et al.* 1982), but the *S*(-) form is more potent than *R*(+) nicotine in increasing blood pressure (Ikushima *et al.* 1982), antinociception, spontaneous activity, and rotarod performance (Martin *et al.* 1983). There are two conflicting reports on the stereoselectivity of the effects of nicotine on dopamine release from striatal synaptosomes with one study reporting the *S*(-) enantiomer as more potent (Rapier *et al.* 1988) and another, using higher doses, reporting equivalence between the two enantiomers (Connelly and Littleton 1983). The lack of strong stereoselectivity observed here is consistent with the idea that nicotine-induced ERK phosphorylation in cortical neurons may be mediated through a molecule that is not a known central nAChR.

Taken together these data outline a signaling pathway in primary cortical neurons, initiated by nicotine stimulation and involving voltage-gated sodium channels, glutamate receptors, calcium, L-type voltage-gated calcium channels, CaMKII, and MAPK (or ERK) kinase to result in increased ERK phosphorylation. We do not find evidence for a significant role for the α7, α4/β2, or α3/β4 nAChR subtypes in nicotine-induced ERK phosphorylation in primary cortical cultures, implicating a non-classical nAChR-mediated mechanism that is yet to be explained. Nicotine could act through a receptor not previously identified as responsive to nicotine, or it could have a more direct effect [similar to nicotine's ability to reduce free radical generation by binding directly to complex I of the respiratory chain in

mitochondria, independent of nAChRs (Cormier *et al.* 2001; Xie *et al.* 2005)].

Acknowledgements

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