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Depolarization recruits DCC to the plasma membrane of embryonic cortical neurons and enhances axon extension in response to netrin-1

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Abstract

The netrin-1 receptor Deleted in Colorectal Cancer (DCC) is required for the formation of major axonal projections by embryonic cortical neurons, including the corpus callosum, hippocampal commissure, and cortico-thalamic tracts. The presentation of DCC by axonal growth cones is tightly regulated, but the mechanisms regulating DCC trafficking within neurons are not well understood. Here, we investigated the mechanisms regulating DCC recruitment to the plasma membrane of embryonic cortical neurons. In embryonic spinal commissural neurons, protein kinase A (PKA) activation recruits DCC to the plasma membrane and enhances axon chemoattraction to netrin-1. We demonstrate that PKA activation similarly recruits DCC and increases embryonic cortical neuron axon extension, which, like spinal commissural neurons, respond to netrin-1 as a chemoattractant. We then determined if depolarization might recruit DCC to the plasma membrane. Neither netrin-1 induced axon extension, nor levels of plasma membrane DCC, were altered by depolarizing embryonic spinal commissural neurons with elevated levels of KCl. In contrast, depolarizing embryonic cortical neurons increased the amount of plasma membrane DCC, including at the growth cone, and increased axon outgrowth evoked by netrin-1. Inhibition of PKA, phosphatidylinositol-3-kinase, protein kinase C, or exocytosis blocked the depolarization-induced recruitment of DCC and suppressed axon outgrowth. Inhibiting protein synthesis did not affect DCC recruitment, nor were the distributions of trkB or neural cell adhesion molecule (NCAM) influenced by depolarization, consistent with selective mobilization of DCC. These findings identify a role for membrane depolarization modulating the response of axons to netrin-1 by regulating DCC recruitment to the plasma membrane.

Keywords: cortical neuron, deleted in colorectal cancer, netrin-1, PI3-kinase, PKA, PKC.

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Netrins are a small family of secreted axon guidance cues that attract some axons and repel others during neural development. Deleted in Colorectal Cancer (DCC), a type 1 single pass transmembrane protein, is a receptor for netrin-1 that is required for the chemoattractant response (Keino-Masu *et al.* 1996; Fazeli *et al.* 1997). UNC5A-D, the four mammalian UNC-5 homologues, make up a second family of receptors for netrin-1 and are required for repellent responses (Ackerman *et al.* 1997; Leonardo *et al.* 1997; Hong *et al.* 1999; Burgess *et al.* 2006; Dillon *et al.* 2007).

A gradient of netrin-1 protein directs commissural axons to the floor plate at the ventral midline of the embryonic spinal cord (Kennedy *et al.* 1994, 2006). In embryonic spinal commissural neurons, DCC is localized both at the plasma membrane and sequestered within an intracellular vesicular pool (Bouchard *et al.* 2004). Activating protein kinase A (PKA) recruits DCC to the plasma membrane of spinal commissural growth cones and enhances both axon extension and axon turning in response to netrin-1 (Bouchard *et al.* 2004; Moore and Kennedy 2006). Additionally,

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Abbreviations used: BSA, bovine serum albumin; CREB, cyclic AMP response element binding protein; DCC, deleted in colorectal cancer; FSK, Forskolin; HBSS, Hanks balanced salt solution; NCAM, neural cell adhesion molecule; PBS, phosphate-buffered saline; PDL, poly-D-lysine; PKA, protein kinase A; PKC, protein kinase C.

activating PKCα triggers UNC5A endocytosis, reducing netrin-1-mediated growth cone collapse and switching chemorepulsion to chemoattraction (Williams *et al.* 2003; Bartoe *et al.* 2006). The response of axonal growth cones to netrin-1 is also influenced by phosphatidylinositol-3-kinase (PI3-kinase), phospholipase C-gamma, mitogen-activated protein kinases, and protein synthesis (Ming *et al.* 1999, 2001, 2002; Campbell and Holt 2001; Forcet *et al.* 2002).

Netrin-1 also guides mammalian cortical neuron axon extension: it directs cortico-thalamic projections and is required for the formation of corpus callosum and hippocampal commissure (Serafini *et al.* 1996; Metin *et al.* 1997). Here, using primary cultures of embryonic mouse cortical neurons, we demonstrate that activating PKA recruits DCC to the plasma membrane and enhances axon extension, consistent with previous findings using embryonic spinal commissural neurons (Bouchard *et al.* 2004). We then tested the hypothesis that depolarization might recruit vesicles containing DCC to the plasma membrane. Depolarizing embryonic rat spinal commissural neurons with elevated concentrations of KCl did not significantly alter netrin-1-induced axon extension or levels of plasma membrane DCC. In contrast, depolarizing embryonic cortical neurons significantly increased plasma membrane levels of DCC and axon outgrowth evoked by netrin-1. Pharmacological inhibition of either PKA, phosphatidylinositol-3-kinase (PI3-kinase), protein kinase C (PKC), or exocytosis blocked the KCl-induced increase in cell surface DCC and suppressed axon outgrowth. These findings identify a role for membrane depolarization modulating the response of axons to netrin-1 by regulating the presentation of DCC at growth cones of embryonic rat cortical neurons.

Material and methods

Reagents

The following antibodies were used: monoclonal DCC antibodies against extracellular (G92-13) or intracellular (G97-449) epitopes of DCC, obtained from PharMingen (Mississauga, ON, Canada), function blocking monoclonal DCC antibody, DCC_{FB} (AF5, 2.7 mg/mL in phosphate-buffered saline, PBS) from Calbiochem (LaJolla, CA, USA); monoclonal anti-netrin-1 (MAB1109, R&D Systems, Mineapolis, MN, USA); polyclonal anti-VAMP2 (AB5856, Chemicon/Millipore Biosciences, Temecula, CA, USA); polyclonal anti-NCAM (AB5032, Chemicon/Millipore Biosciences); anti-phospho cyclic AMP response element binding protein (CREB) (Ser133) (1B6), anti-CREB, anti-phospho Akt (Ser473), anti-Akt, and anti-phospho PKC_{pan} (Ser660) from Cell Signalling Technology Inc. (Beverly, MA, USA). Polyclonal anti-$trkB_{ECD}$ was provided by Dr. Louis Reichardt (University of California, San Francisco, CA, USA).

Chelerythrine, cycloheximide, forskolin, LY294002, poly-D-lysine, Tetanus Toxin, and wortmannin were obtained from Sigma-Aldrich (Missisauga, ON, Canada); KT5720 and SQ22536 from Calbiochem; Neurobasal, Hanks balanced salt solution (HBSS) media, B27, and N2 supplements from Invitrogen Canada (Burlington, ON, Canada); and L-glutamine and Penstrep from Bio Media (Drummondville, QC, Canada). Recombinant netrin-1 protein was purified from a HEK293T cell line secreting netrin-1 as described (Shirasaki *et al.* 1996).

Embryonic cortical neuron culture

Staged pregnant CD1 mice were obtained from Charles River (St-Constant, QC, Canada). The cortices of embryonic day 15 mice were isolated by microdissection and dissociated to produce a suspension of single cells. In brief, cortices were incubated at 37°C for 15 min in a trypsin solution (0.25%, Invitrogen, Canada) in non-supplemented Neurobasal. Dnase (0.02%) was then added at 25°C. Tissue was triturated four times sequentially with three different sized Pasteur pipettes (large, medium, and small tip) to yield a suspension of single cells.

Dissociated cells were plated and then cultured for either 2 days (~25 000 cells/well, growth cone analysis) or 6 days (~50 000 cells/well, neurite analysis) in 24-well plates (Sarstedt, St-Leonard, QC, Canada). Cells were grown in the wells on 12 mm round glass coverslips (No. 0 Deckgläser, Carolina Biological, NC, USA) that had been coated with poly-D-lysine (PDL) (70–150 kDa, 20 μg/mL). The cells were cultured in Neurobasal media containing: 1X B27, 1X N2, 0.4 mM Glutamine, 1 unit/mL penicillin, and 1 μg/mL streptomycin. Inhibitors (1 mM SQ22536, 200 nM KT5720, 1 μM wortmannin, 33 μM LY294002, 10 μM chelerythrine, 1.6 nM tetanus toxin, or 100 μM cycloheximide) or their respective vehicles were added to medium 15 min before the addition of netrin-1 or its respective vehicle. Fifteen minutes after, the medium was supplemented either with 50 mM KCl, 10 μM Forskolin (FSK) or their respective vehicles following a time course (0, 5, 15, 30, and 60 min).

Immunocytochemistry

For immunostaining, cortical neuron cultures were washed with ice-cold PBS, pH 7.4, and fixed with ice-cold 4% paraformaldehyde in PBS, pH 7.4. They were blocked with 2% goat serum and 2% bovine serum albumin (BSA) in PBS, pH 7.4 for 2 h at 25°C.

Antibodies were used in blocking solution, 2% goat serum, 2% BSA in PBS overnight at 4°C at the following dilutions: monoclonal anti-DCC_{IN} 1 : 500; monoclonal anti-DCC_{EX} 1 : 500; monoclonal anti-netrin-1 1 : 500; polyclonal anti-VAMP2 1 : 500; and polyclonal anti-$trkB_{ECD}$ 1 : 500. Cultures were subsequently washed with PBS and incubated with secondary antibodies conjugated to Alexa Fluor 546 or 488 (1 : 1000) in blocking solution (Molecular Probes, Eugene, OR, USA). Cell nuclei were stained with Hoechst 33258 (Sigma–Aldrich). Coverslips were mounted in Geltol (Thermo Electron Corporation, Pittsburgh, PA, USA) on glass slides and grayscale micrographs were taken using a Carl Zeiss Axiovert microscope (Oberkochen, Germany), a 20X or 100X objective lens, and a MagnaFire CCD camera (Optronics, Goleta, CA, USA).

Quantification of surface receptor density using immunofluorescence

All micrographs used for quantification were taken using the same microscope, objective lens, and exposure time to allow comparison of measurements. Fluorescence was quantified using Northern Eclipse image analysis software (Empix Imaging Inc, Mississauga,

ON, Canada). For image analysis of neurites and growth cones, both fluorescent and differential contrast (DIC) images were taken. Fluorescent intensity per micrometer squared was quantified and expressed as mean ± SEM for every condition. The statistical significance was evaluated by a two-way analysis of variance with a Sheffe *post hoc* test (Systat, Chicago, IL, USA). In the presence of an interaction between the different groups, one-way analyses of variance were used for each group. Probability values of $p < 0.05$ were considered significant.

Surface biotinylation and western blot analysis

For biotinylation of cell surface proteins, E15 mouse cortical neurons were plated and cultured for 6 days at a density of ~2 500 000 cells per 10 cm PDL-coated tissue culture dish. Cells were cultured in Neurobasal medium containing 1X B27, 1X N2, 0.4 mM Glutamine, 1unit/mL penicillin, and 1 μg/mL streptomycin. The cells were treated with various inhibitors (1 mM SQ22536, 200 nM KT5720, 1 μM wortmannin, 33 μM LY294002, 10 μM chelerythrine, 1.6 nM tetanus toxin, or 100 μM cycloheximide) or the respective vehicles of the inhibitors for 15 min.

To test the effect of depolarization or elevating cAMP, neurons were exposed for 15 min to 10 μM FSK, 50 mM KCl or vehicles. Cell surface proteins were biotinylated and examined by western blot analysis either with anti-DCC_{EX}, anti-DCC_{IN}, anti-$trkB_{ECD}$, or anti-NCAM as follows. Briefly, after treatment, cells were washed with ice-cold PBS containing 0.1 μM calcium chloride and 1 μM magnesium chloride (pH 7.4) to halt protein trafficking. Surface biotinylation was performed by adding EZ-Link Sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA), 5 mL per plate at 0.5 mg/mL in PBS at 4°C for 30 min (Lisanti *et al.* 1989). This solution was removed, and the reaction quenched by the addition of 5 mL of 10 mM ice-cold glycine in PBS at 4°C for two periods of 10 min. Subsequently, cells were washed two times with 5 mL ice-cold PBS and lysed by the addition of RIPA buffer [150 mM NaCl, 20 mM Tris, pH 8.0, 1%, NP-40 (USB Corp., Cleveland, OH, USA), 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA]. Biotinylated proteins were precipitated with streptavidin–agarose (Pierce) and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 8% gels. After electrophoresis, proteins were transferred to nitrocellulose membrane (Hybond ECL, Amersham Pharmacia Biotech, Baie d'Urfé, QC, Canada) and blocked for 2 h by incubation in 5% non-fat dry milk in PBST (PBS containing 0.1% Tween 20). Western blot analysis was performed using anti-DCC_{IN} (1 : 2000), anti-DCC_{EX} (1 : 2000), anti-$trkB_{ECD}$ (1 : 2000), or anti-NCAM (1 : 5000) overnight at 4°C. After 2 h of incubation with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), bands were visualized using the Western Lighting Chemiluminescence Reagent Plus kit (Perkin-Elmer, Boston, MA, USA). Immunoreactivity was imaged with a ScanJet 5300C (Hewlett Packard Canada, Mississauga, ON, Canada). Densitometry and quantification of the relative levels of DCC protein were performed on scanned images of immunoblots using NIH Image software (National Institutes of Health, Bethesda, MD, USA).

Western blot analysis for phosphorylated CREB, Akt, PKC

E15 mouse cerebral cortices were dissociated and neurons plated and cultured for 6 days at a density of ~250 000 cells per 35 mm PDL-coated tissue culture dish. Cells were cultured in neurobasal medium containing 1X B27, 1X N2, 0.4 mM glutamine, 1 unit/mL penicillin, and 1 μg/mL streptomycin. The cells were treated with various inhibitors (1 mM SQ22536, 200 nM KT5720, 1 μM wortmannin, 33 μM LY294002, 10 μM chelerythrine, 1.6 nM tetanus toxin, or 100 μM cycloheximide) or their respective vehicles for 15 min.

To test the effects of elevating cAMP and depolarization, neurons were exposed for 15 min to 10 μM FSK, 50 mM KCl or their respective vehicle. After treatment, cells were washed once with ice-cold PBS (pH 7.4) and lysed with Laemmli sample buffer. Total cell extracts were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 12% gels. After electrophoresis, proteins were transferred to nitrocellulose membrane and blocked for 2 h by incubation in 5% milk/TBST. Western blot analysis was performed using antibodies against CREB (1 : 1000), phosphoCREB (1 : 1000), Akt (1 : 1000), phosphoAkt (1 : 1000), and phospho-PKC_{pan} (1 : 1000) in 5% BSA, 1X TBS, 0.1% tween 20 overnight at 4°C. Immunoreactivity was visualized and quantified as described above.

Ratiometric calcium imaging

E15 mouse cortical neurons were isolated and cultured as described above but were plated on glass bottom culture dishes (MatTEK, Ashland, MA, USA). E13 rat spinal commissural neurons were isolated and cultured as described (Bouchard *et al.* 2004), and also plated on MatTEK dishes. At 2 DIV, neurons were loaded for approximately 45 min with 5 μM *fura*-2AM from Sigma–Aldrich (Oakville, ON, Canada), diluted in 1.5 mL of neurobasal medium, with 0.1% Pluronic F-127 (Invitrogen) to facilitate loading. The cells were then washed twice with heated air media (500 mL of air media is composed of: 250 mL L15 medium, 225 mL HBSS, 10 mL B27, 9 mL of 1 M HEPES, 1.25 mL of 200 mM glutamine, 0.9 mL of 14.3 mM beta-2-mercaptoethanol in ddH_2O, 0.5 mL penicillin/streptomycin, and 2.5 mL of 1 M glucose). For ratiometric calcium measurements, fluorescent images were acquired sequentially at 340 nm and 380 nm wavelengths from a defined region of interest encompassing the cell soma. The fluorescent intensities of the 340 nm images were then divided by the intensity value of the 380 nm images to obtain a ratiometric value. Imaging was carried out with a 40X oil immersion objective, air media heated to 30°C by a Warner Inline Heating Instrument Model TC-324B (Warner Instruments, CT, USA), using a Nikon Eclipse TE2000U epifluorescence microscope with a Nikon XPS-100 lamp (Tokyo, Japan), a Coolsnap HQ cooled CCD camera (Photometrics, Tusson, AZ, USA) with filter switching controlled by a Lambda 10-2 optical filter changer (Sutter Instruments, Novata, CA, USA) and MetaMorph software (Molecular Devices, Downingtown, PA, USA). A baseline of calcium signal activity was recorded for 100 s. Following the baseline measurement, 100 μL of air media was injected as a negative control, and images collected for the following 100 s. Subsequently, the same volume of KCl solution was added, generating a final concentration of 50 mM KCl in the dish. The overall baseline measurements were normalized to a value of 1.

Embryonic cortical explant culture

Cortical explants were microdissected from E13 rat telencephalic vesicles and cultured in three-dimensional collagen gels as described

(Metin *et al.* 1997). Inhibitors (1 mM SQ22536, 200 nM KT5720, 1 μM wortmannin, 33 μM LY294002, 10 μM chelerythrine, or 1.6 nM tetanus toxin), DCC function blocking antibody (30 ng/mL–10 μg/mL), or vehicle were added to the medium 15 min before the addition of netrin-1. Following 15 min of treatment, the medium was supplemented either with 50 mM KCl, 10 μM FSK or their respective vehicles. All drugs were present throughout the experiment. Explants were cultured at 37°C for 16 h in Neurobasal containing 10% heat inactivated fetal bovine serum, 2 mM glutamine, 1 unit/mL penicillin, and 1 μg/mL streptomycin.

Measurements of neurite extension from cortical explants

Photomicrographs were taken using a Carl Zeiss Axiovert microscope, phase-contrast optics, a 20X objective lens, and a MagnaFire CCD camera, and analysed using Northern Eclipse image analysis software. The total number and length of all axon fascicles emerging from each explant was measured as described (Serafini *et al.* 1994) and expressed as a mean ± SEM. Statistical significance of differences between means was evaluated by a one-way analysis of variance with Sheffe *post hoc* test (Systat).

Results

Netrin-1 directs cortical axon guidance and promotes axon outgrowth (Metin *et al.* 1997). PKA activation regulates the response of neuronal growth cones to netrin-1, causing some cell types to switch between chemoattraction and chemorepulsion, and increasing the sensitivity of responsiveness in others (Ming *et al.* 1997; Moore and Kennedy 2006). We previously reported that PKA activation recruits DCC to the plasma membrane of embryonic spinal commissural axons, increasing axon outgrowth and turning in response to netrin-1 (Bouchard *et al.* 2004; Moore and Kennedy 2006). To determine if this mechanism functions in other neuronal cell types, we utilized primary cultures of dissociated embryonic day 15 (E15) mouse cortical neurons (2 DIV). Widespread expression of DCC by neurons was detected in these cultures (Fig. 1a), consistent with previous studies demonstrating DCC expression by cortical neurons that pioneer the corpus callosum during late embryogenesis in the mouse (Shu *et al.* 2000). PKA-induced recruitment of DCC to the plasma membrane of embryonic spinal commissural neurons can be blocked by application of tetanus toxin, an inhibitor of exocytosis that acts by cleaving v-SNAREs (Schiavo *et al.* 1992). DCC and VAMP2, a tetanus toxin sensitive v-SNARE, were both expressed by embryonic cortical neurons (Fig. 1). The distribution of DCC immunoreactivity visualized using confocal microscopy was consistent with DCC being distributed both at the neuronal plasma membrane and within an intracellular pool of vesicles. Overlapping distributions of punctate VAMP2 and DCC immunoreactivities were detected in cell bodies (Fig. 1b and c) and growth cones (Fig. 1c and d), suggesting that DCC may be a cargo protein within a subset of VAMP2 vesicles in embryonic mouse cortical neurons. Immunostaining cortical neurons following 6 DIV revealed readily detectable expression of DCC and netrin-1 in the cultures (Fig. 1e).

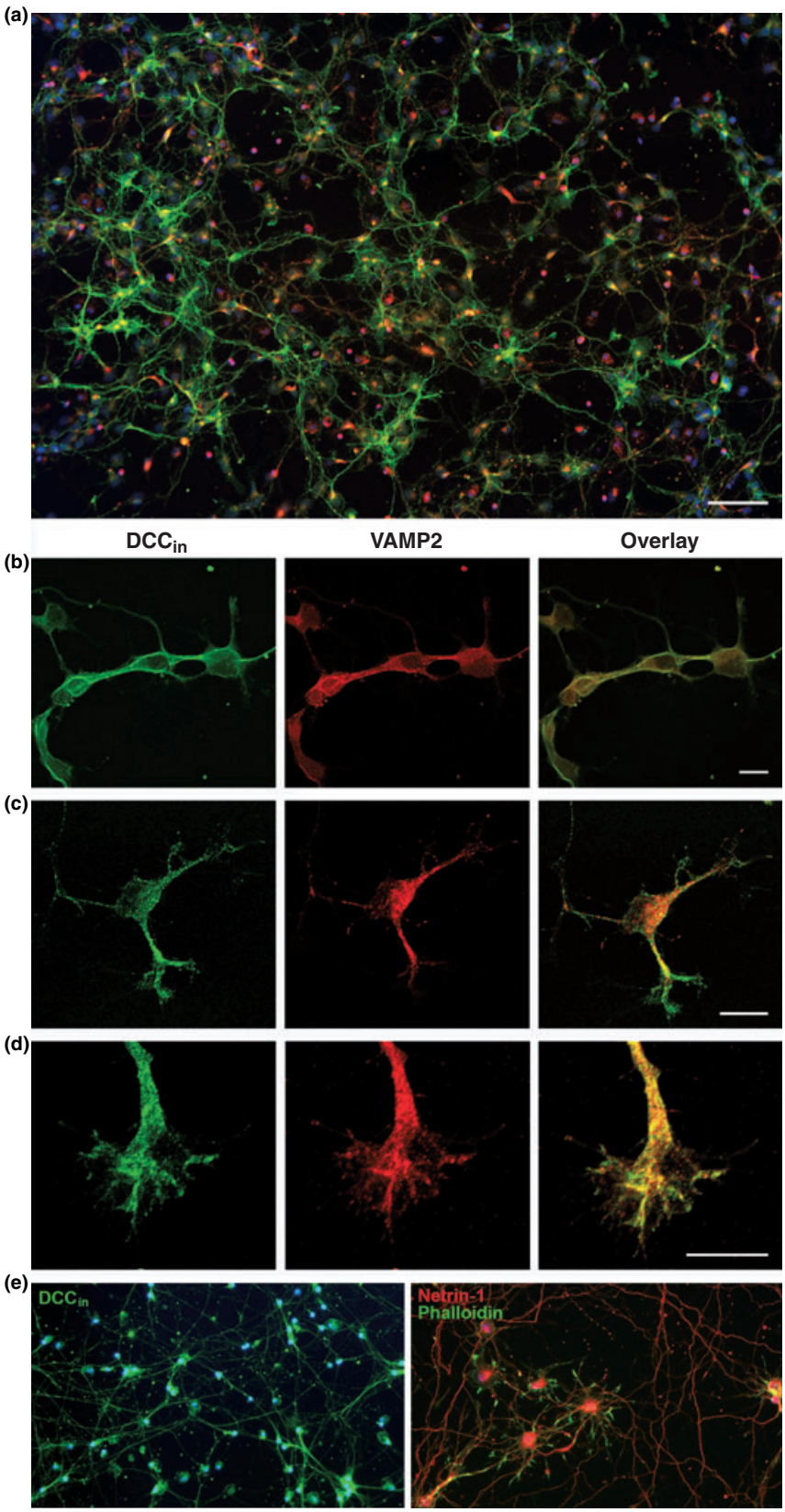
PKA activation regulates DCC recruitment in embryonic cortical neurons

To determine if DCC trafficking to the plasma membrane of cortical neurons might be regulated similarly to embryonic spinal commissural neurons, cultures of cortical neurons were fixed and immunostained using conditions that did not permeabilize the plasma membrane, allowing the selective visualization of cell surface DCC protein, as described (Bouchard *et al.* 2004). Briefly, neurons dissociated from E15 cortices were cultured for either 2 or 6 days, then fixed with 4% paraformaldehyde, and immunocytochemical analyses performed in the absence of detergent to avoid permeabilizing the cell membrane.

Forskolin activates adenylate cyclase, increasing intracellular cAMP, which in turn enhances the activity of PKA (Nairn *et al.* 1985). Following treatment with 10 μM FSK, non-permeabilized cortical neurons were immunostained with αDCC_{EX}, a monoclonal antibody against an extracellular epitope of DCC, to visualize DCC on the neuronal surface. Increased DCC immunoreactivity was detected along neurites (Fig. 2a) and on growth cones (Fig. 2b and c) following 5, 15, 30, and 60 min of FSK treatment. Identical FSK exposure did not alter immunoreactivity using an antibody that binds the extracellular domain of trkB (Fig. 2c).

To further investigate the mechanism underlying the effect of FSK, cultured cortical neurons were exposed to different enzyme inhibitors for 15 min before the addition of FSK to the media. To confirm that FSK was acting via adenylate cyclase, we blocked cyclase activity using 1 mM SQ22536, a selective inhibitor of adenylate cyclase (Fabbri *et al.* 1991; Goldsmith and Abrams 1991; Tamaoki *et al.* 1993). SQ22536 completely blocked the increase in DCC surface immunoreactivity (Fig. 2a–c), consistent with the effect of FSK being due to adenylate cyclase activation. Next, to confirm that the cAMP produced by the adenylate cyclase was activating PKA, cortical neurons were pre-treated with 200 nM KT5720, a selective inhibitor of PKA (Kase *et al.* 1987). KT5720 abolished the increase in DCC surface immunoreactivity produced by FSK (Fig. 2a–c). Together, these findings indicate that PKA activation is essential for FSK-induced recruitment of DCC.

Protein kinase A activation could enhance the amount of DCC on the neuronal surface by increasing either *dcc* transcription or the translation of *dcc* mRNA, or by raising the rate of insertion of DCC protein recruited from an intracellular store to the plasma membrane. Testing the hypothesis that DCC might be recruited from an intracellular pool, we found that 1.6 nM tetanus toxin blocked the FSK-



induced increase in plasma membrane DCC immunoreactivity (Fig. 2a–c).

A time course analysis detected a significant increase in cell surface DCC within 5 min following the addition of FSK (Fig. 2a and b), indicating that the increase in DCC protein on the neuronal surface is likely too rapid to be accounted for by increased transcription or translation of *dcc* mRNA. Consistent with this conclusion, we found that 100 μM cycloheximide, a concentration sufficient to inhibit protein synthesis (Twiss and Shooter 1995), had no effect on the FSK-induced increase in DCC at the cell surface (Fig. 2a–c).

PKA activation induces DCC translocation via a mechanism that requires exocytosis

The increase in cell surface DCC immunofluorescence described above could be produced either by increased plasma membrane DCC protein, or potentially by clustering DCC protein that was previously distributed more diffusely in the plasma membrane before treatment. To differentiate between these two possibilities, plasma membrane DCC was assessed directly by biotinylating cell surface proteins and quantifying the relative amount of DCC present on the neuronal surface in different conditions. Neurons isolated from the cortex of E15 mice were cultured for 6 days as described above, and the cells then treated for 15 min with SQ22536, KT5720, tetanus toxin, cycloheximide or the respective vehicles for each of these agents. Cultures were then exposed for 15 min to 10 μM FSK or vehicle control, in order to elevate intracellular cAMP. Cell surface proteins were then biotinylated, isolated, and examined by western blot analysis. As shown (Fig. 2d), a single band with the molecular weight of full-length DCC (~180 kDa) was detected by αDCC_{IN}, a monoclonal antibody that binds an intracellular epitope of DCC. A band of the same molecular weight was also detected using αDCC_{EX} (not shown). Analysis of biotinylated proteins indicated that in the presence of FSK, the amount of cell surface DCC was increased ~2.5-fold, in comparison to control (Fig. 2d). When cells were pre-treated with SQ22536, KT5720, or tetanus toxin prior to FSK, cell surface DCC remained not significantly different from control (Fig. 2d). In contrast, inhibition of protein synthesis with cycloheximide did not affect the FSK-induced increase in plasma membrane DCC (Fig. 2d). Levels of surface trkB or NCAM were not affected by cAMP elevation in these cells under these conditions (Fig. 2d).

To monitor PKA activation, we assayed the relative level of CREB phosphorylation induced by FSK in cortical neurons. As shown in Fig. 2(e), increased CREB phosphorylation was detected in response to FSK compared to control, which was blocked by SQ22536 and KT5720 but not by tetanus toxin or cycloheximide. In all conditions, the total amount of CREB protein was comparable, as indicated by western blotting with an antibody insensitive to CREB phosphorylation (Fig. 2e).

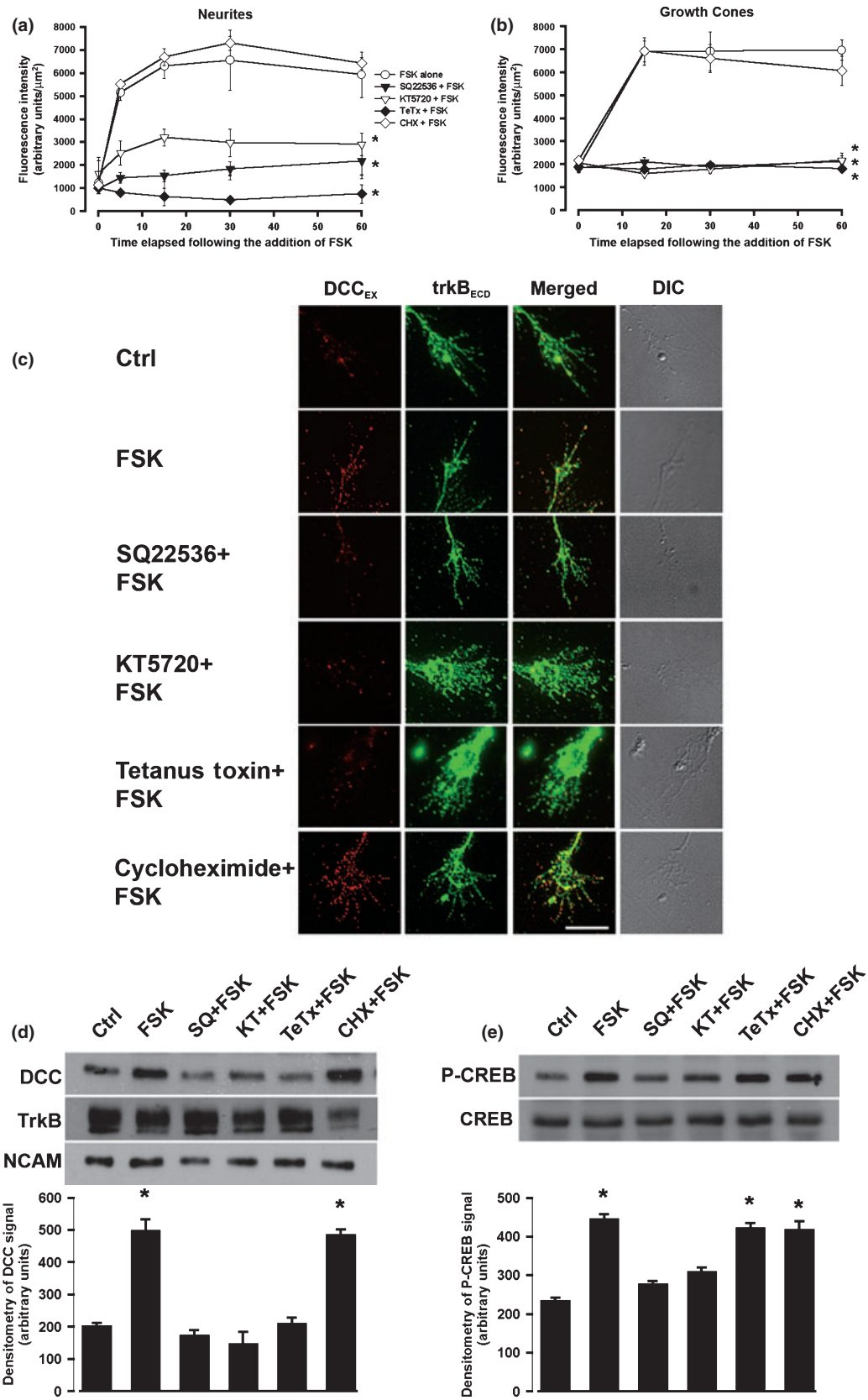
Together, these results support the conclusion that increasing the intracellular concentration of cAMP recruits DCC from a pre-existing vesicular pool to the plasma membrane.

PKA-induced DCC recruitment promotes cortical axon outgrowth

Consistent with the findings reported by Metin *et al.* (1997), application of recombinant netrin-1 protein evoked axon outgrowth from explants of E13 rat neocortex. Using this as a functional assay, we tested the hypothesis that PKA activation would cause DCC protein to be recruited to the plasma membrane of cortical axons and promote axon outgrowth. Explants of E13 rat cortex were cultured in the presence of 10 μM FSK with and without a sub-maximal concentration of netrin-1 (50 ng/mL). Following 16 h in culture, application of FSK alone did not significantly enhance axon outgrowth. In contrast, FSK plus netrin-1 produced a dramatic increase in axon outgrowth compared to application of netrin-1 alone (Fig. 3a–c).

To investigate the mechanism underlying the action of FSK, cortical explants were exposed to different enzyme inhibitors 15 min before the addition of netrin-1, thus 30 min prior to the addition of FSK to the media, and then cultured for 16 h. To verify that the effect of FSK was acting through adenylate cyclase, 1 mM SQ22536 was applied. SQ22536 completely blocked the increase in axon outgrowth caused by FSK in the presence of netrin-1 (Fig. 3a–c), demonstrating that FSK exerts its effect on axon length via the activation of adenylate cyclase and production of cAMP.

Fig. 1 DCC and VAMP2 expression by 2 DIV E15 mouse cortical neurons. (a) Epifluorescence image of E15 mouse cortical neurons grown for 2 DIV (DCC green, anti-IgG mouse antibody conjugated to Alexa 488; VAMP2 red, anti-IgG rabbit antibody conjugated to Alexa 546; nuclei stained blue with Hoechst dye; 20X objective; Carl Zeiss Axiovert 100 microscope; MagnaFire CCD camera; scale bar corresponds to 50 μm). (b–d) Confocal imaging of E15 cortical neurons illustrating the distributions of DCC and VAMP2, shown separately and in overlay (Carl Zeiss 510 confocal microscope, 100X 1.4 n.a. objective; scale bars correspond to 10 μm). (b) DCC and VAMP2 distributions in a cluster of neurons illustrating immunoreactivity in the cell body cytoplasm and along neurites. (c) A single neuron illustrating DCC and VAMP2 immunoreactivity in the cytoplasm of the cell body, along neurites, associated with the plasma membrane, and within growth cones. (d) Higher magnification reveals a partially overlapping punctate distribution of DCC and VAMP2 within a growth cone. (e) Epifluorescence imaging of E15 mouse cortical neurons grown for 6 DIV. The left panel illustrates DCC immunoreactivity, green, and Hoechst dye, blue. The right panel illustrates netrin-1 immunoreactivity, red; phalloidin staining for F-actin, green; and Hoechst dye, blue. 20X objective lens; Carl Zeiss Axiovert 100 microscope; MagnaFire CCD camera; scale is the same as in panel (a).



The PKA inhibitor KT5720 blocked the effect of FSK (Fig. 3a–c) indicating that PKA is required to produce the netrin-1-dependent increase in axon outgrowth evoked by FSK. Notably, these inhibitors reduced the level of axon outgrowth to that evoked by netrin-1 without FSK, but not to the level of the limited spontaneous axon outgrowth observed in the absence of netrin-1. This suggests that FSK does not enhance DCC function, but that one pool of DCC protein is present on the surface of the cell in its baseline state, and that the effect of FSK is to mobilize additional DCC to the membrane.

We then tested the hypothesis that recruitment of DCC from an intracellular vesicular pool enhances netrin-1-dependent axon outgrowth using the exocytosis inhibitor tetanus toxin. Sixteen hours of treatment with 1.6 nM tetanus toxin reduced axon outgrowth induced by netrin-1 with FSK to the level evoked by netrin-1 alone. These findings are consistent with PKA enhancement of netrin-1-induced axon outgrowth requiring v-SNARE-dependent vesicle fusion with the plasma membrane (Fig. 3a–c).

The results described above suggest that FSK activates adenylate cyclase, leading to the activation of PKA, and enhancing the netrin-1-dependent outgrowth of cortical axons via a mechanism that requires the recruitment of vesicles to the plasma membrane. To determine if the increase in axon outgrowth evoked by FSK required DCC, cortical explants were exposed to increasing concentrations of DCC function blocking monoclonal antibody (αDCC_{FB}, from 30 ng/mL to 10 μg/mL) 15 min before the addition of netrin-1, thus 30 min before the addition of FSK to the media and then cultured for 16 h. αDCC_{FB} blocks netrin-1-dependent commissural axon outgrowth *in vitro* (Keino-Masu *et al.* 1996). In the presence of FSK and netrin-1, αDCC_{FB} blocked axon outgrowth in a concentration-dependent manner (Fig. 3d and e), indicating that the increased netrin-1-dependent axon outgrowth induced by FSK requires DCC.

KCl-induced depolarization does not recruit DCC to the plasma membrane or alter commissural axon outgrowth

Activity and depolarization influence the chemotropic response of the growth cones of embryonic *Xenopus laevis* spinal cord neurons as they turn toward a source of netrin-1 (Ming *et al.* 2001). To determine if depolarization might similarly influence embryonic rat spinal commissural axon outgrowth, we tested the effect of 50 mM KCl in the presence or absence of netrin-1 (50 ng/mL or 100 ng/mL). Following 16 h of culture, no difference was found between axon outgrowth in groups treated with KCl alone versus control, or netrin-1 plus KCl versus netrin-1 alone (Fig. 4b), indicating that in these conditions, depolarization does not affect netrin-1-dependent outgrowth of embryonic rat spinal commissural neurons.

Using the monoclonal antibody DCC_{EX}, we then examined the distribution of cell surface DCC in commissural neurons that had been treated with either 50 mM KCl alone or with 100 ng/mL netrin-1 and 50 mM KCl (Fig. 4a). Following treatment with KCl alone, no increase in surface DCC immunoreactivity was observed (Fig. 4a). Consistent with the lack of an effect of depolarization on commissural axon outgrowth, the small increase in DCC cell surface immunoreactivity observed in the group pre-treated with 100 ng/mL netrin-1 and 50 mM KCl was the same as that evoked by netrin-1 alone (Fig. 4a). None of these treatments altered the cell surface distribution of TAG-1 or trkB immunoreactivity measured in these cells (data not shown).

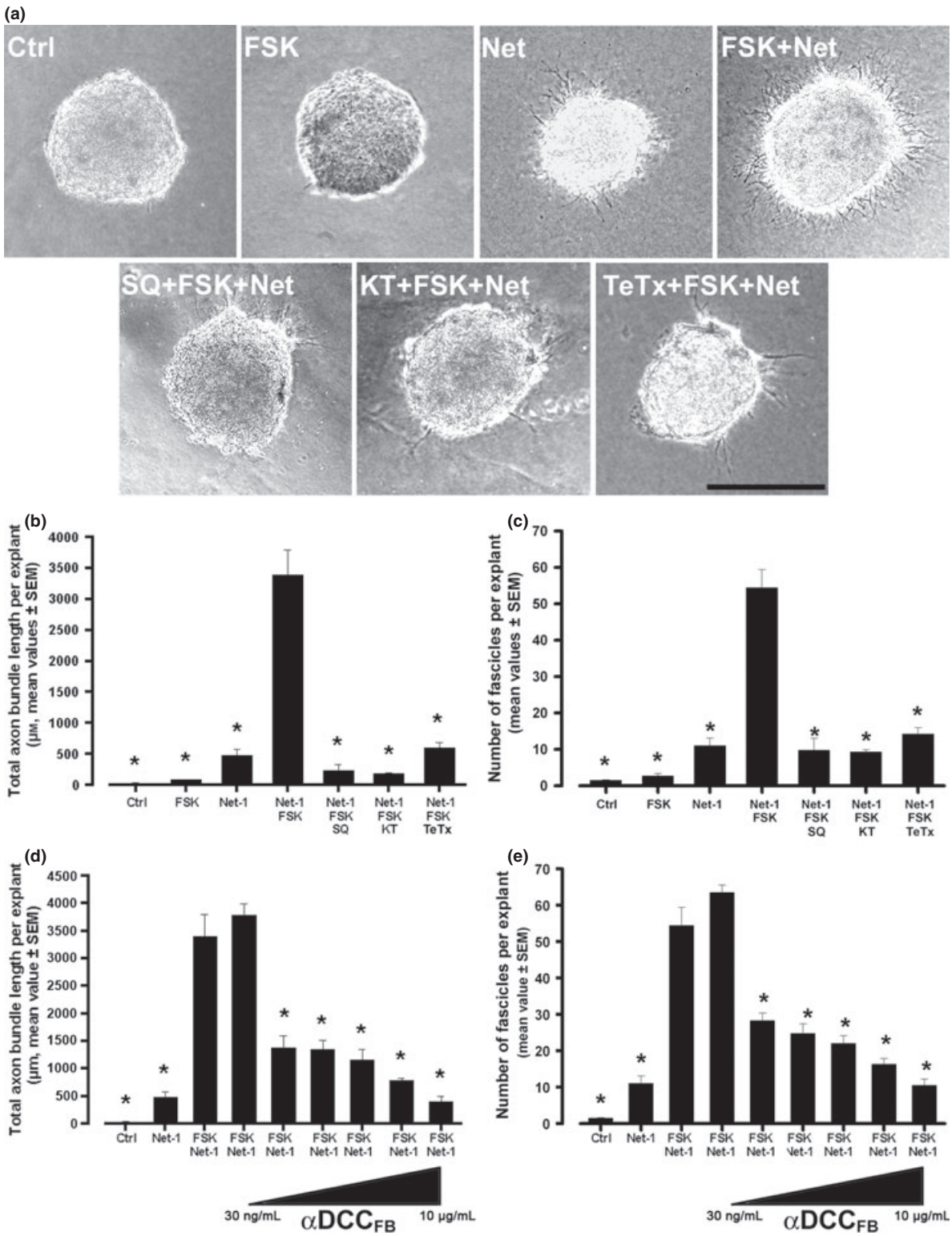
KCl-induced depolarization recruits DCC to the plasma membrane of embryonic cortical neurons

Although KCl-induced depolarization did not measurably influence DCC recruitment in embryonic rat spinal commissural neurons, we tested the possibility that it might influence the response of embryonic cortical neurons. Using monoclonal antibody DCC_{EX} on unpermeabilized cells, we examined the distribution of cell surface DCC in dissociated cortical neurons that had been depolarized using 50 mM KCl. Following KCl treatment, significantly increased cell surface DCC immunoreactivity was observed along neurites (Fig. 5a) and on growth cones (Fig. 5b and c). In contrast, this treatment did not significantly alter cell surface trkB immunoreactivity (Fig. 5c).

Electrical stimulation modifies the response of growth cones, in part, by increasing the concentration of intracellular

Fig. 2 DCC recruitment to the plasma membrane is regulated by PKA activation. Cortical neurons were cultured for 2 DIV (growth cones) or 6 DIV (neurites) prior to a 15 min treatment with 1 mM SQ22536, 200 nM KT5720, 1.6 nM tetanus toxin, or vehicle controls. FSK (10 μM) was then added for 5, 15, 30, or 60 min, cells were fixed, not permeabilized, and immunostained with anti-DCC_{EX} or anti-$trkB_{ECD}$. Primary antibodies were detected using Alexa 546 or Alexa 488-conjugated secondary antibodies. Panels a and b illustrate quantification of DCC fluorescence along neurites and the growth cone surface, respectively. Values are mean ± SEM for an n of 8–12 per condition. The asterisk indicates $p < 0.05$ versus 10 μM FSK alone. Panel c presents representative images of growth cones stained with DCC_{EX} and $trkB_{ECD}$. Scale bar corresponds to 10 μm. In panel d, cell surface proteins were biotinylated and then isolated using streptavidin-agarose beads. Western blot analysis utilized antibodies directed against DCC_{IN} (~180 kDa), $trkB_{ECD}$ (~145 kDa), and NCAM (200 kDa). Histograms present quantification of the optical density of DCC immunoreactivity. Values are mean ± SEM for an n of 4 per condition. The asterisk indicates $p < 0.05$ versus control. Panel e shows western blots of total cell extracts analysed with antibodies raised against the phosphorylated form (P-CREB) and total CREB (~43 kDa). The histogram shows quantification of the optical density of P-CREB immunoreactivity. Values are mean ± SEM for an n of 5 per condition. The asterisk indicates $p < 0.05$ versus control.

cAMP (Ming *et al.* 2001). To investigate the mechanism underlying the effect of depolarization on the amount of DCC presented by the cell, we exposed cultures of cortical neurons to different enzyme inhibitors 15 min before the addition of KCl to the media. To determine if KCl was acting via adenylate cyclase, cyclase activity was inhibited using 1 mM SQ22536, which completely blocked the increase in DCC cell surface immunoreactivity (Fig. 5a–c). To confirm



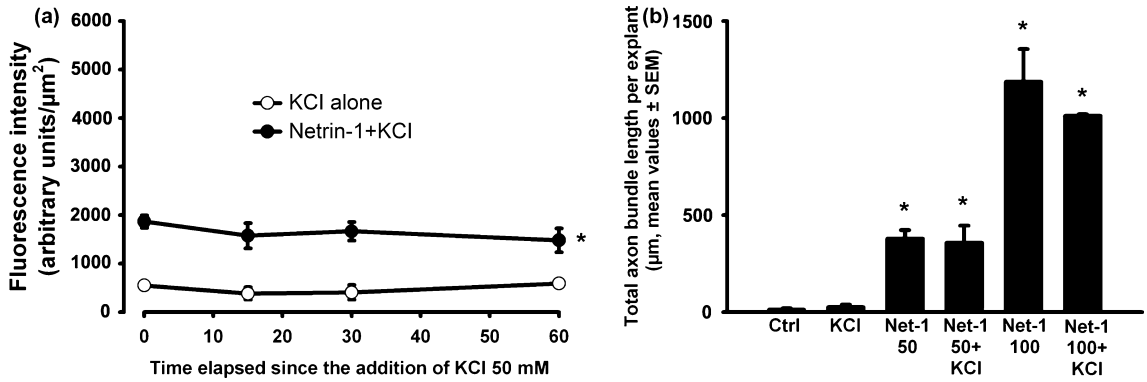


Fig. 4 Depolarization does not alter axon outgrowth or recruit DCC to the plasma membrane of embryonic spinal commissural neurons. Microdissected explants of E13 rat spinal cord were dissociated and spinal commissural neurons cultured for 2 DIV prior to treatment for 15 min with 50 ng/mL netrin-1. Cells were depolarized by the addition of 50 mM KCl for 5, 15, 30 or 60 min. Panel (a) illustrates quantification of growth cone surface DCC fluorescence intensity (mean ± SEM, $n = 10–15$ per condition. *, indicates $p < 0.01$ vs. application of 50 mM KCl). Explants of E13 rat dorsal spinal cord were cultured for 16 h under the following conditions: control, 50 mM KCl, 50 ng/mL netrin-1, 50 ng/mL netrin-1 plus 50 mM KCl, 100 ng/mL netrin-1, and 100 ng/mL netrin-1 plus 50 mM KCl. Panel (b) illustrates quantification of total axon bundle outgrowth per explant. *, indicates $p < 0.01$ versus control or KCl alone conditions.

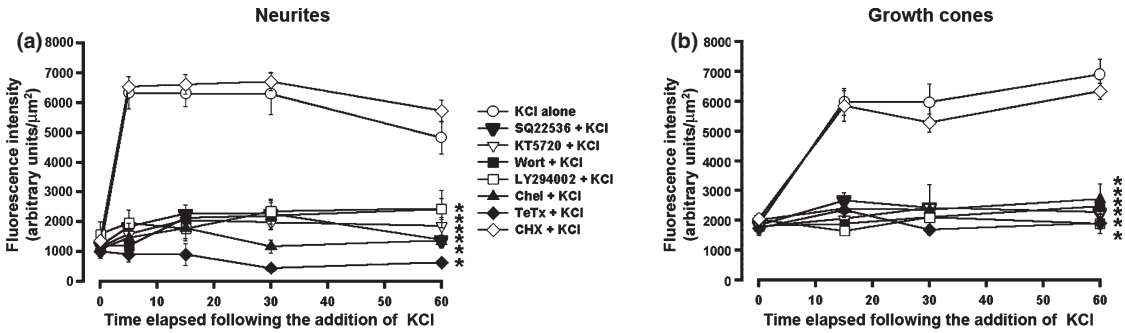
that the cAMP produced by the adenylate cyclase was activating PKA, cortical neurons were pre-treated with 200 nM KT5720, which abolished the increase in DCC cell surface immunoreactivity produced by KCl (Fig. 5a–c). These findings indicate that PKA activation is essential for the depolarization-induced translocation of DCC.

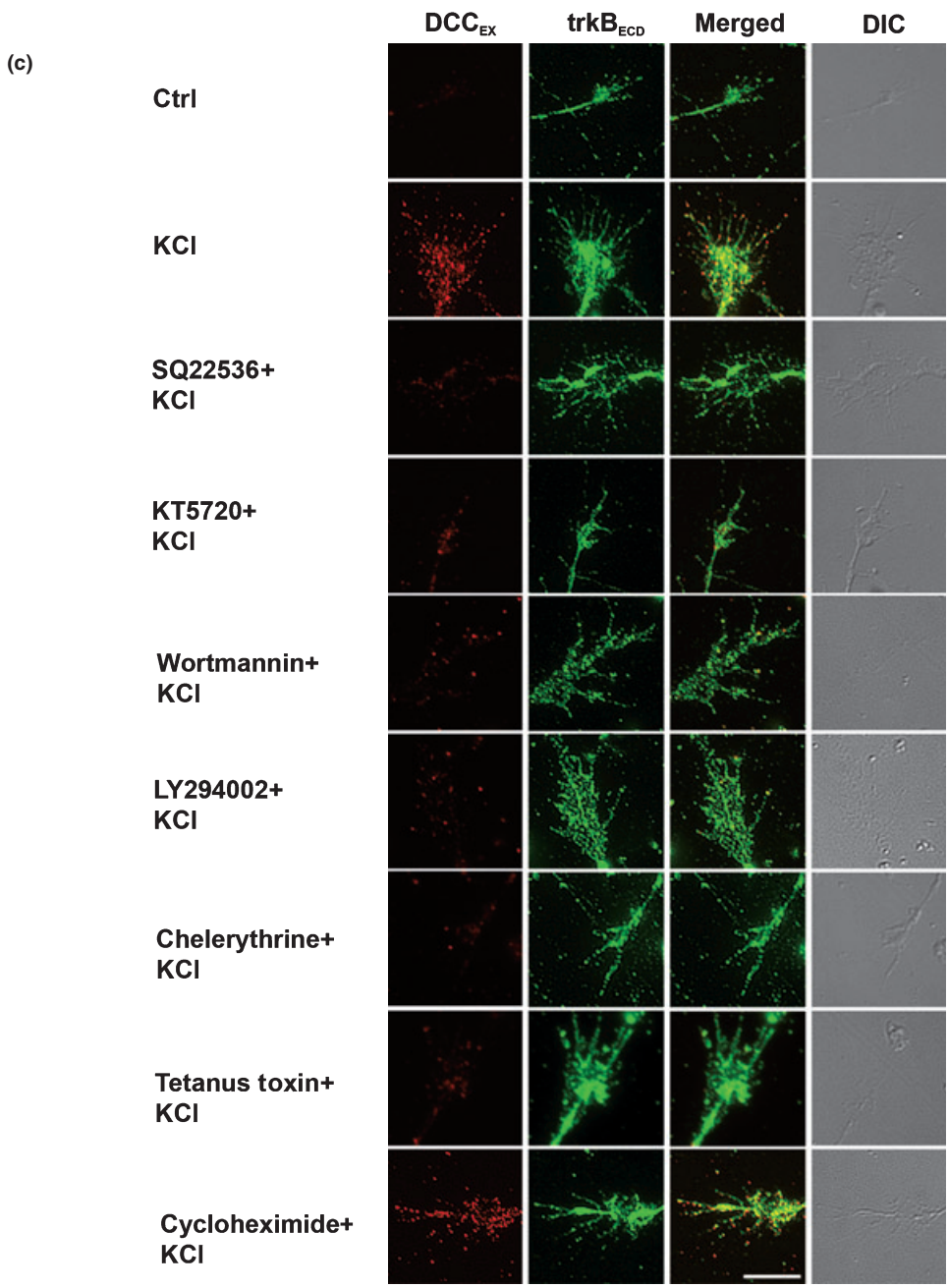
As described above, PKA activation might increase the amount of DCC on the neuronal surface through a number of different mechanisms: by increasing the transcription of *dcc* or the translation of *dcc* mRNA, or by recruiting DCC to the plasma membrane from an intracellular store. Addition of tetanus toxin (1.6 nM) blocked the KCl induced increase in surface DCC immunoreactivity (Fig. 5a–c), consistent with the increase being the result of vesicle mediated insertion of DCC into the plasma membrane. Additionally, we found that application of 100 μM cycloheximide, an inhibitor of protein synthesis, had no effect on the KCl-induced increase in DCC on the cell surface (Fig. 5a–c), indicating that the increase detected is protein synthesis independent, consistent with recruitment from a pre-existing intracellular pool.

Previous studies indicated that PI3-kinase cytoplasmic signaling contributes to netrin-1 mediated axon chemoattraction (Ming *et al.* 1999). To investigate the possible contribution of this pathway to DCC recruitment, cultures of cortical neurons were exposed to PI3-kinase inhibitors (Quevedo *et al.* 2000; Yagyu *et al.* 2001; Yamaguchi *et al.* 2001; Liu *et al.* 2005) 15 min before the addition of KCl to the media. Application of PI3-kinase inhibitors wortmannin (1 μM) or LY294002 (33 μM) completely blocked the KCl-induced increase in DCC surface immunoreactivity (Fig. 5a–c), consistent with the effect of KCl requiring active PI3-kinase.

PI3-kinase is a well established modulator of PKB/Akt and protein kinase C (PKC) signaling (Herbert *et al.* 1990; Singh *et al.* 1993), and depolarization has been shown to activate PKB/Akt signaling as a result of PI3-kinsae activation in sympathetic neurons (Vaillant *et al.* 1999). To determine if PKC might contribute to KCl-induced DCC translocation, cortical neurons were pre-treated with 10 μM chelerythrine, a competitive inhibitor of the PKC catalytic domain (Herbert *et al.* 1990). Chelerythrine abolished the increase in DCC surface immunoreactivity produced by KCl (Fig. 5a–c), consistent with PKC activity being required for DCC translocation induced by depolarization. These findings

Fig. 3 Protein kinase A-dependent recruitment of DCC to the plasma membrane promotes netrin-1-induced cortical neuron axon outgrowth. Panel (a) illustrates E13.5 rat cortical explants embedded in a collagen matrix and cultured for 16 h in the following conditions: control, 10 μM FSK, 50 ng/mL netrin-1, netrin-1 plus FSK. Explants treated with netrin-1 and FSK were cultured with 1 mM SQ 22536, 200 nM KT 5720, or 1.6 nM tetanus toxin. Scale bar = 100 μm. Panels (b) and (c) illustrate quantification of cortical neuron axon outgrowth evoked by netrin-1 following addition of 10 μm FSK in the presence of various pharmacological inhibitors. Histograms in (b) correspond to the mean of the total axon bundle length per explant and in (c) the mean total number of fascicles per explant (± SEM, 10–30 explants per condition, *, indicates $p < 0.05$ vs. netrin-1 plus FSK group). Panels (d) and (e) illustrate axon outgrowth evoked by netrin-1 (50 ng/ml) plus FSK (10 μM) when explants were cultured with DCC function blocking antibody at concentrations from 30 ng/mL to 10 μg/mL. Histograms in (d) correspond to the mean of total axon bundle length per explant and in (e) the mean total number of fascicles per explant (± SEM, $n = 8–12$ explants per condition. *, indicates $p < 0.05$ vs. netrin-1 plus FSK group).





provide evidence that depolarization increases the amount of DCC on the neuronal surface by recruiting DCC protein to the plasma membrane through a mechanism dependent on active PKA, PI3-kinase, and PKC.

We then assessed the difference found between embryonic cortical neurons and embryonic spinal commissural neurons, investigating the lack of DCC recruitment in response to 50 mM KCl-induced depolarization in the former. Ratiometric calcium imaging was used to assess calcium concentrations in these two populations of neurons following treatment with KCl. Calcium levels were imaged in cortical neurons cultured for 2 DIV and loaded with *fura*-2AM. Depolarization following bath application of KCl rapidly triggered a significant increase in the concentration of intracellular calcium in cortical neurons (Fig. 6a, c and e). In 2 DIV embryonic spinal commissural neurons bath application of KCl induced a substantially smaller increase in the average level of intracellular calcium (Fig. 6b, d, and f). These findings provide evidence that spinal commissural neurons, at this early stage of development, exhibit a reduced capacity to respond to plasma membrane depolarization relative to embryonic cortical neurons, and that this may underlie the lack of DCC plasma membrane recruitment in these cells.

Depolarization recruits DCC in a PKA, PI3-Kinase, and PKC-dependent manner

The immunocytochemical findings described above are consistent with a KCl-induced selective translocation of DCC to the plasma membrane of neurites (Fig. 5a) and growth cones (Fig. 5b and c). We assessed this hypothesis biochemically by biotinylating cell surface proteins and quantifying the relative amount of DCC protein present on the neuronal surface in different conditions. Neurons dissociated from E15 mouse cortices were cultured for 6 days as described above, and then the cells treated for 15 min with SQ22536, KT5720, wortmannin, LY294002, chelerythrine, tetanus toxin, cycloheximide or their respective vehicles. Cultures were then exposed for 15 min to 50 mM KCl to depolarize the cells. Following these treatments, cell surface proteins were biotinylated, isolated, and examined by western blot analysis using αDCC_{IN}, $\alpha trkB_{ECD}$, and αNCAM. As shown (Fig. 7a), αDCC_{IN} detected a single ~180 kDa band, the molecular weight of full-length DCC. The same band was detected using αDCC_{EX} (not shown). Analysis of biotinylated proteins indicated that in the presence of KCl, the amount of cell surface DCC was increased by ~4-fold in comparison with control (Fig. 7a). When pre-treated with SQ22536, KT5720, wortmannin, LY294002, chelerythrine or tetanus toxin prior to KCl, the level of plasma membrane DCC did not differ significantly from controls (Fig. 7a). Inhibition of protein synthesis with cycloheximide did not affect DCC recruitment (Fig. 7a). Unlike the regulated presentation of DCC, in these cells under these conditions, the amounts of surface trkB and NCAM were not affected by depolarization (Fig. 7a).

To evaluate the possibility that treatment with KCl may lead to activation of PKA, PI3-kinase, and PKC in cortical neurons, we measured the phosphorylation of CREB, Akt, and PKC, respectively. Increased CREB phosphorylation was observed in response to KCl compared with controls and this was blocked by SQ22536 and KT5720 (Fig. 7b). KCl produced an increase in Akt phosphorylation, which was abolished by wortmannin and LY294002 (Fig. 7c). Phosphorylation of PKC was also increased by addition of KCl (Fig. 7d), and this was blocked by chelerythrine (Fig. 7d).

Western blot analysis indicated that the total amount of CREB or Akt proteins was comparable across all conditions (Fig. 7b and c). Together, these findings suggest that depolarization increases the amount of DCC on the neuronal surface via a mechanism that is dependent on the activation of PKA, PI3-kinase, and PKC.

PKA, PI3-K, and PKC-dependent DCC plasma membrane recruitment induced by depolarization promotes cortical axon outgrowth

We then determined if cell membrane depolarization would promote cortical neuron axon outgrowth by recruiting DCC protein to the plasma membrane. Explants of E13 rat cortex were cultured in the presence of 50 mM KCl with or without 50 ng/mL netrin-1, a subsaturating concentration. Following 16 h of culture, KCl alone did not enhance axon outgrowth. In contrast, KCl plus 50 ng/mL netrin-1 produced a significant increase in axon outgrowth compared with explants exposed to netrin-1 alone (Fig. 8a–c).

To characterize the mechanisms underlying the action of KCl-induced depolarization, cortical explants were exposed to different enzyme inhibitors 15 min before the addition of netrin-1, thus 30 min before the addition of KCl to the

Fig. 5 DCC recruitment to the plasma membrane induced by depolarization is regulated by PKA, PI3-kinase, and PKC. Cortical neurons were plated onto PDL-coated glass coverslips and cultured for 2 DIV (growth cones) or 6 DIV (neurites) prior to treatment for 15 min with 1 mM SQ 22536, 200 nM KT 5720, 1 μM wortmannin, 33 μM LY294002, 10 μM chelerythrine, 1.6 nM tetanus toxin, or 100 μM cycloheximide. Following these treatments, cells were depolarized using 50 mM KCl for 5, 15, 30 or 60 min. Cultures were then fixed without permeabilization. Cells were immunostained with antibodies against DCC or trkB directed against extracellular epitopes. Primary antibodies were detected using Alexa 488 or Alexa 546-conjugated secondary antibodies. Scale bar = 10 μm. Panels (a) and (b) illustrate quantification of DCC fluorescence along neurites (a) and on growth cones (b) following different treatments. Values represent the mean ± SEM, $n = 10–19$ samples per condition. *p-value < 0.05 versus KCl alone. Panel (c) presents representative images of immunostaining using DCC_{EX} and $trkB_{ECD}$ antibodies on growth cones.

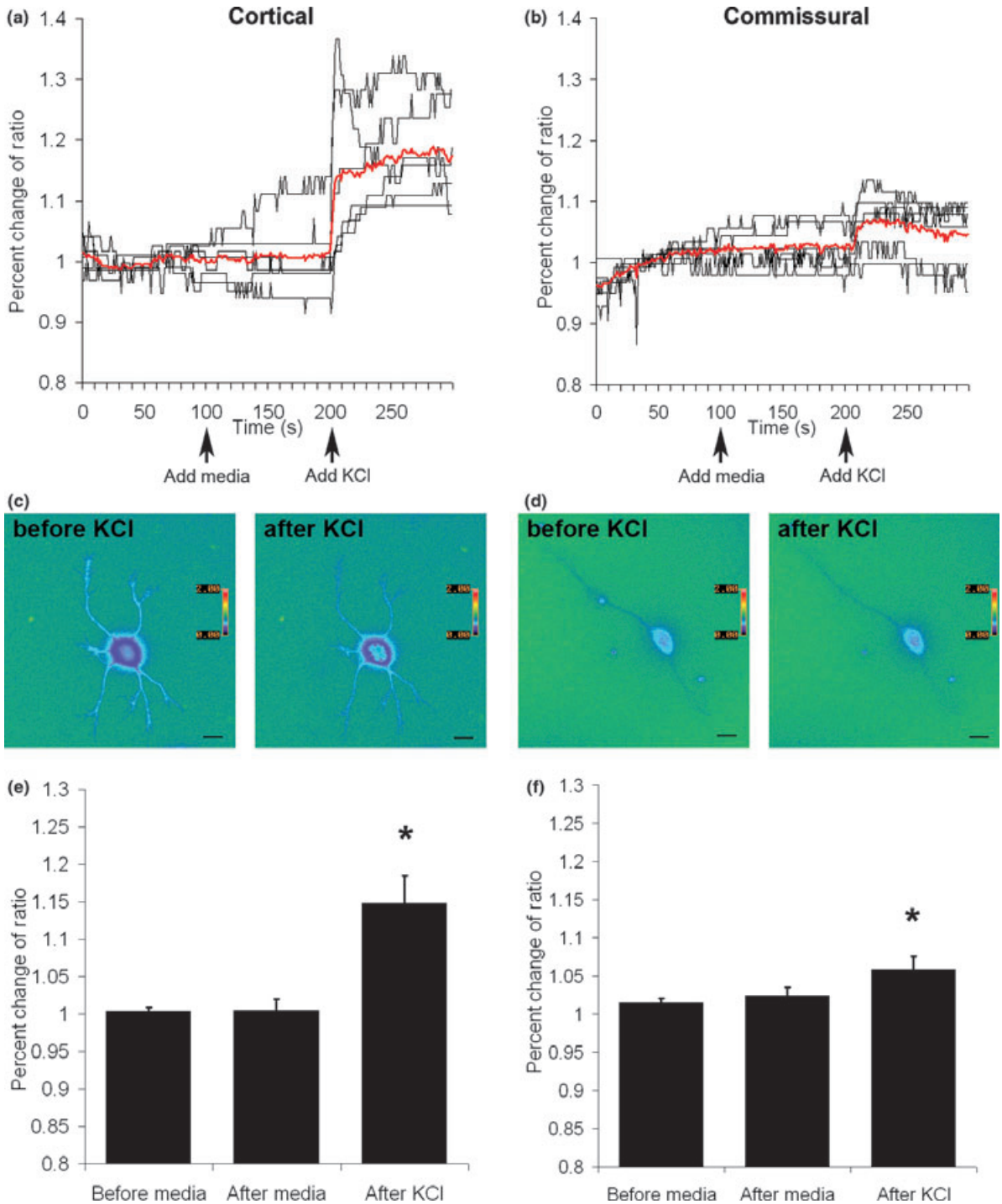


Fig. 6 Ratiometric imaging of intracellular calcium following KCl-induced depolarization. Intracellular calcium monitored with fura-2AM ratiometric imaging in embryonic cortical neurons (a, c and d) and embryonic spinal commissural neurons (b, d and f), all 2 DIV. Panels (a) and (b) illustrate baseline measurements collected for 200 s, with the injection of media alone control at the 100 s time point. At 200 s, KCl was injected to a final concentration of 50 mM, and measurements continued for an additional 100 s. The black lines represents measures for individual cells ($n = 6$). The red line illustrates the mean value derived from these 6 measures. Panels (c) and (d) show a cortical neuron and a commissural neuron, respectively, 10 s before (left panel) and 10 s after (right panel) the addition of KCl. Color bar indicates ratio measurement scale. Scale bar indicates 10 μm. Histograms for cortical (e) and commissural (f) neurons illustrate the mean corrected ratio measurements of the cells for the 50 s period immediately before addition of control media, the 50 s period immediately after the addition of control media, and 50 s after the addition of KCl. Error corresponds to SEM ($^*p < 0.05$, $n = 6$).

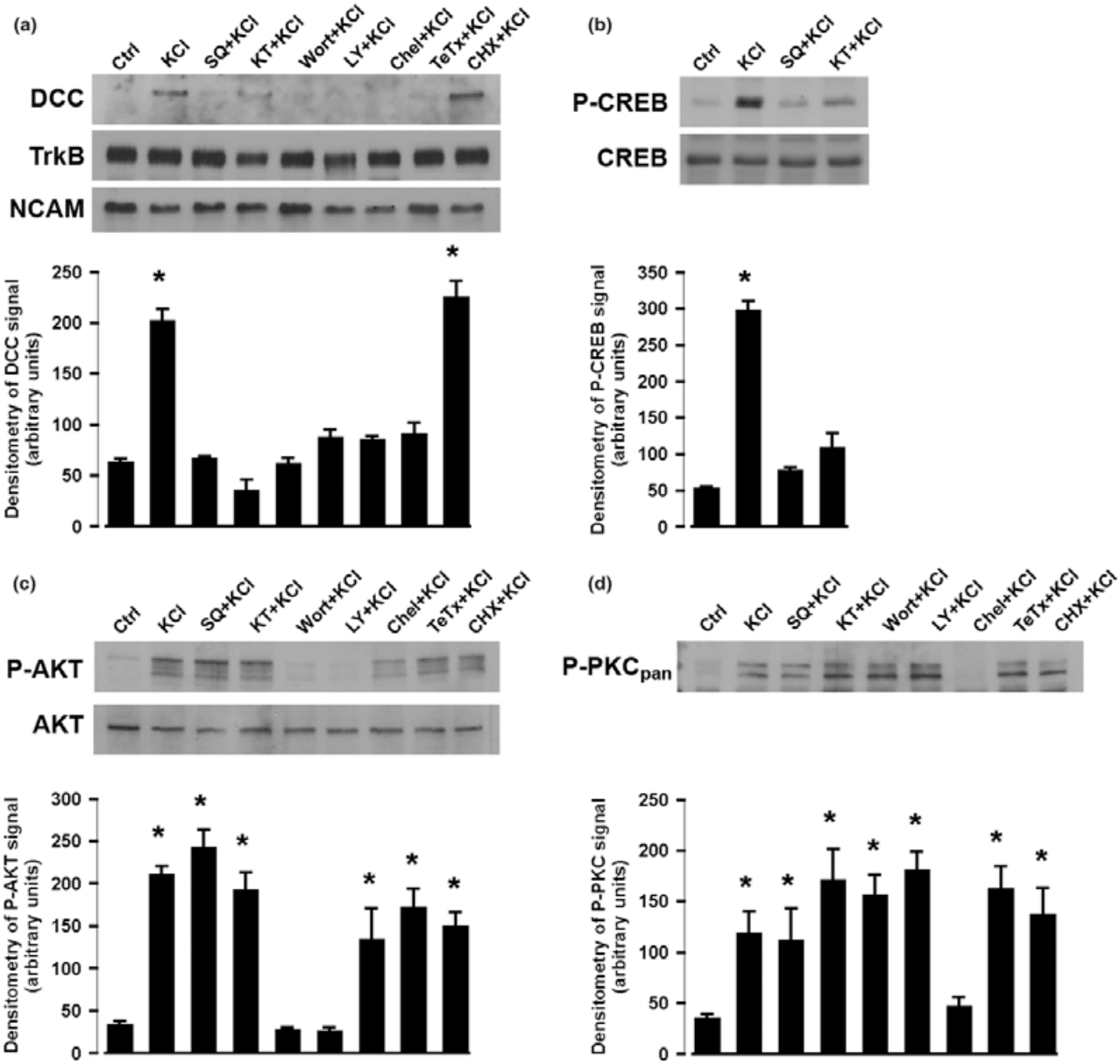
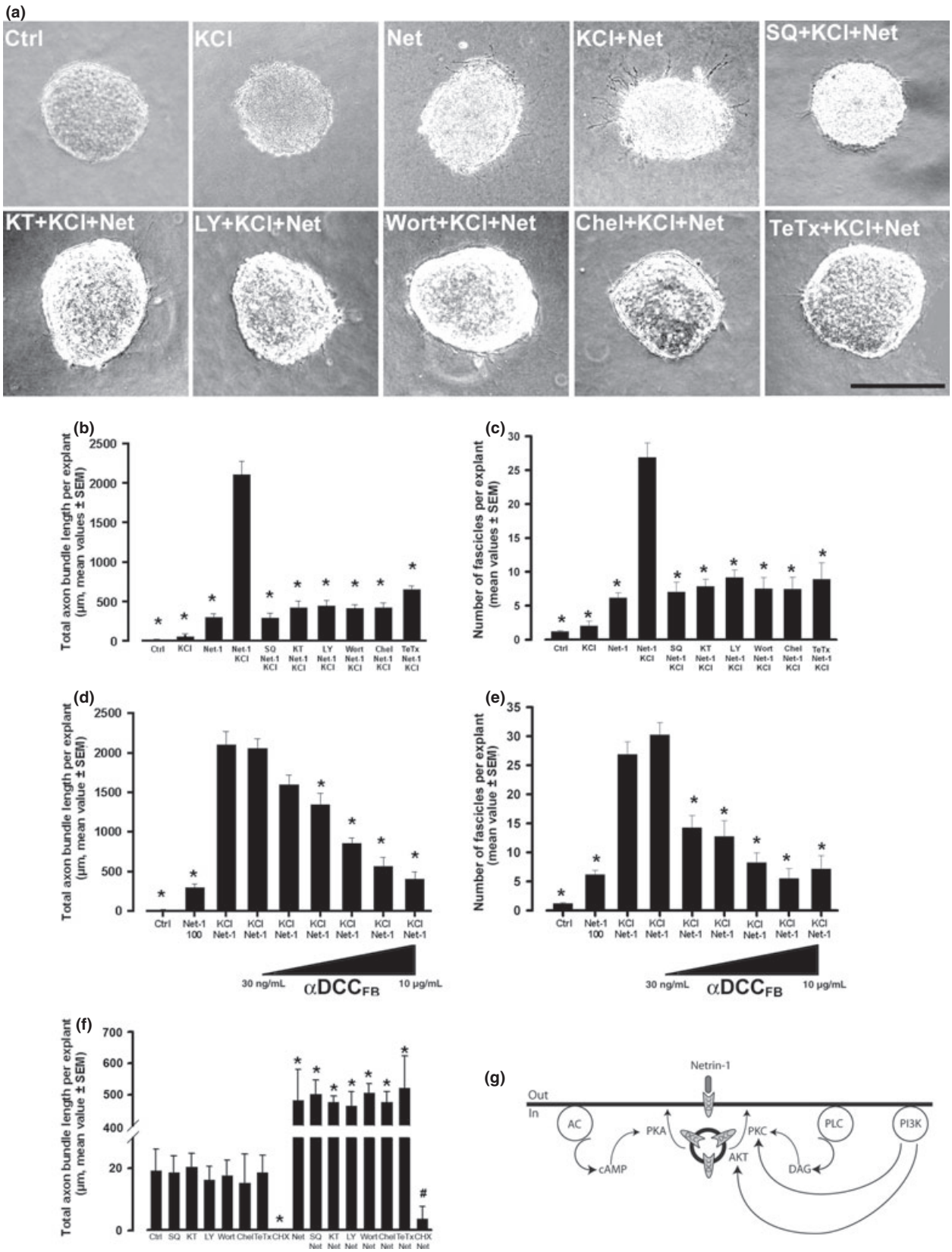


Fig. 7 Depolarization-induced recruitment of DCC to the plasma membrane requires PKA, PI3-kinase, and PKC. Cortical neurons were plated on PDL-coated 100 mm (biotinylation assay) or 35 mm (phosphorylation assay) culture dishes and cultured for 6 days prior to treatment for 15 min with 1 mM SQ 22536, 200 nM KT 5720, 1 μM wortmannin, 33 μM LY294002, 10 μM chelerythrine, 1.6 nM tetanus toxin, 100 μM cycloheximide, or their respective vehicles. Following these treatments, cells were depolarized with 50 mM KCl for 30 min. In panel (a), cell surface proteins were biotinylated and then isolated using streptavidin-agarose beads. Biotinylated proteins were visualized by western blot analyses with antibodies against DCC_{IN} (~180 kDa), $trkB_{ECD}$ (~145 kDa), or NCAM (~200 kDa). The histograms present quantification of the optical density of DCC immunoreactivity (mean ± SEM, $n = 5$ per condition, *, indicates $p < 0.05$ vs. control). Panels (b), (c), and (d) illustrate respectively western blots of total cell extracts analysed with antibodies against phospho-CREB, phospho-Akt, phospho-PKC, and against total CREB and Akt. CREB (~ 43 kDa), Akt (~ 60 kDa), or PKC_{pan} (~ 78, 80, 82, 85 kDa). Histograms illustrate quantification of the optical density of P-CREB (panel b), P-Akt (panel c), and P-PKC_{pan} (panel d) immunoreactivities (mean ± SEM, $n = 5$ per condition, *, indicates $p < 0.05$ vs. control).

media, and then cultured for 16 h. To verify that KCl activated adenylate cyclase, 1 μM SQ22536 was applied. This completely blocked the increase in axon outgrowth caused by KCl in the presence of netrin-1, demonstrating that KCl enhances netrin-1-induced axon outgrowth via activation of adenylate cyclase. To confirm the involvement of PKA, cortical explants were pre-treated with 200 nM KT5720. This blocked the effect of KCl plus netrin-1



(Fig. 8a–c), indicating that PKA activity is required to produce the netrin-1-dependent increase in axon outgrowth evoked by KCl.

To determine if the KCl induced increase in axon outgrowth required PI3-kinase activity, either 1 μM wortmannin or 33 μM LY294002 were applied. Both inhibitors completely blocked the increase in axon outgrowth caused by KCl in the presence of netrin-1 (Fig. 8a–c), demonstrating that the effect of KCl requires PI3-kinase activity. Similarly, the involvement of PKC was assessed by adding 10 μM chelerythrine to the media. Chelerythrine blocked the increase in axon outgrowth caused by KCl in the presence of netrin-1 (Fig. 8a–c), indicating that the effect of KCl requires PKC activity. Consistent with the assays of plasma membrane DCC described above, these inhibitors reduced the level of axon outgrowth to that evoked by netrin-1 without KCl, and not to the background level of outgrowth found in the absence of netrin-1. This provides evidence that these inhibitors do not indiscriminately block axon outgrowth, nor do they block the signalling required for netrin-1 mediated axon outgrowth, but rather that they exert a more specific effect on the enhancement of axon outgrowth caused by KCl.

Following co-application of 1.6 nM tetanus toxin for 16 h with netrin-1 and KCl, axon extension was reduced to a level not significantly different from netrin-1 alone, consistent with the KCl-induced increase requiring recruitment of DCC to the plasma membrane from an intracellular store (Fig. 8a–c).

To confirm that the enhancement of axon outgrowth evoked by KCl required DCC, cortical explants were exposed to increasing concentrations of DCC function blocking monoclonal antibody (anti-DCC_{FB}, from 30 ng/mL to 10 μg/mL) 15 min before the addition of netrin-1, thus 30 min before the addition of KCl to the media, and cultured for 16 h. In the presence of KCl and netrin-1, the DCC function blocking monoclonal antibody, DCC_{FB}, blocked axon outgrowth in a concentration-dependent manner (Fig. 8d and e), indicating that the increased netrin-1-dependent axon outgrowth induced by KCl requires DCC. Figure 8(f) illustrates a series of controls, indicating that, with the exception of inhibiting protein synthesis with cycloheximide, treatment of E13 cortical telencephalic explants with the various inhibitors for 16 h in the absence of KCl-induced depolarization had no effect on either the basal level of background axon outgrowth, or outgrowth evoked by 50 ng/mL netrin-1. These findings indicate that the inhibitors are not exerting a non-specific influence that inhibits either axon extension or cell survival. The results described above provide evidence that depolarization activates PKA, PI3-kinase, and PKC, and that this contributes to potentiating netrin-1-dependent outgrowth of cortical axons via a mechanism that requires recruitment of DCC from an intracellular store to the plasma membrane.

Discussion

Multiple lines of evidence indicate that neuronal activity can influence axon guidance (Fields 1994; McLaughlin *et al.* 2003; Hanson and Landmesser 2004; Nicol *et al.* 2007). For example, neuronal activity regulates the selection of appropriate cortical targets by thalamo-cortical axons (Catalano and Shatz 1998) and influences the formation of layer-specific intracortical connections by cortical pyramidal neurons (Dantzker and Callaway 1998). Specifically with regard to netrin-1, studies carried out *in vitro* indicate that a brief train of action potentials delivered to frog spinal neurons enhanced netrin-1-induced chemoattraction (Ming *et al.* 2001). These authors demonstrated that the enhancement of chemoattraction by activity was dependent on a Ca^{2+}-dependent increase in the concentration of cAMP.

Increasing cytosolic cAMP and activating PKA has been demonstrated to recruit proteins from intracellular vesicles to the plasma membrane: in neurons, these include acetylcholine

Fig. 8 Protein kinase A, PI3-kinase, and protein kinase C-dependent recruitment of DCC induced by depolarization promotes cortical axon outgrowth. Panel (a) illustrates cortical explants microdissected from E13.5 rat telencephalic vesicles, embedded in a collagen matrix, and cultured for 16 h under control conditions, with 50 mM KCl, with 50 ng/mL recombinant netrin-1, or with netrin-1 plus KCl. Netrin-1 plus KCl treated explants were cultured with 1 mM SQ 22536, 200 nM KT 5720, 1 μM wortmannin, 33 μM LY294002, 10 μM chelerythrine or 1.6 nM tetanus toxin (scale bar = 100 μm). Panel (b) and (c) illustrate histograms of the mean of the total axon bundle length per explant ± SEM (b) and the mean total number of axon fascicles per explant ± SEM (c) in the various conditions. In panels (d) and (e), netrin-1 plus KCl pretreated explants were cultured with DCC function blocking antibody (αDCC_{FB}) at an increasing concentration from 30 ng/mL to 10 μg/mL. Histograms illustrate the mean total axon bundle length per explant ± SEM (d) and the mean total number of axon fascicles per explant ± SEM (e), with 4–8 explants per condition (*$p < 0.05$ vs. netrin-1 plus KCl). Panel (f) illustrates background levels of axon outgrowth (16 hr culture), and outgrowth evoked by 50 ng/mL netrin-1, with and without pharmacological inhibitors, present for the entire culture period. Only the protein synthesis inhibitor cycloheximide (CHX) reduced outgrowth below controls. All other inhibitors exhibited outgrowth either not significantly different from background or from 50 ng/mL netrin-1 (*$p < 0.05$ vs. control group and # $p < 0.05$ vs netrin-1). Panel (g) presents a model illustrating the influence of depolarization on the recruitment of DCC to the plasma membrane. Activation of adenylate cyclase (AC) as a result of depolarization increases the concentration of cytosolic cAMP, triggering a PKA-dependent recruitment of DCC to the plasma membrane. In cortical neurons, depolarization also activates protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3-kinase), leading to DCC recruitment. (PLC: phospholipase C, DAG: diacylglycerol).

receptors (Laufer and Changeux 1987) and the neurotrophin receptor trkB (Meyer-Franke *et al.* 1998). We previously reported that DCC is distributed both on the cell surface and in a pre-existing intracellular vesicular pool in embryonic rat spinal commissural neurons (Bouchard *et al.* 2004). Increasing the intracellular concentration of cAMP or activating PKA in these cells produced a netrin-1-dependent increase in the amount of plasma membrane DCC, including at the growth cone, and increased commissural axon outgrowth and chemoattractive turning in response to netrin-1 (Bouchard *et al.* 2004; Moore and Kennedy 2006). These findings identified DCC recruitment to the plasma membrane as a mechanism underlying the role of PKA as a modulator that regulates the response of neuronal growth cones to netrin-1.

Here we report that membrane depolarization increases netrin-1 evoked axon outgrowth and recruits DCC to the neuronal plasma membrane of embryonic cortical neurons. The depolarization-induced increase in netrin-1-dependent axon outgrowth and the increase in plasma membrane DCC were blocked by inhibitors of adenylate cyclase, PKA, PI3-kinase, or PKC. These findings provide evidence that membrane depolarization recruits DCC to the plasma membrane via a mechanism that requires PKA, PI3-Kinase, and PKC pathways and that this enhances axon growth (Fig. 8g). The level of plasma membrane DCC was well correlated with the extent of axon outgrowth evoked by netrin-1. Additionally, tetanus toxin, which selectively blocks VAMP1/2-dependent recruitment of vesicles to the plasma membrane, reduced netrin-1-induced outgrowth to a level not significantly different from control. We conclude that the rapid mobilization of DCC to the plasma membrane, induced by depolarization, is a major contributing mechanism underlying the depolarization-induced enhancement of cortical axon outgrowth to netrin-1.

Differences between cortical neurons and spinal commissural neurons

Although DCC recruitment to the plasma membrane occurs in both embryonic spinal commissural neurons and cortical neurons, we detected two major differences between these two cell types. First, embryonic cortical neurons express netrin-1, while embryonic spinal commissural neurons do not (Fig. 1; Manitt *et al.* 2001; Kennedy *et al.* 2006). In commissural neurons, in order for PKA activation to recruit DCC, addition of netrin-1 was required. In the absence of netrin-1, increased cell surface DCC was not detected. We have previously suggested that this may be due to netrin-1 stabilizing DCC at the plasma membrane following translocation (Bouchard *et al.* 2004), although we do not rule out roles for other mechanisms. Here, a consequence of the embryonic cortical neurons expressing netrin-1 is that recruitment of plasma membrane DCC did not require exogenous netrin-1, presumably because the autocrine source of netrin-1 is sufficient.

The second major difference detected was that embryonic cortical neurons respond to increasing extracellular KCl by translocating DCC to the plasma membrane, while commissural neurons did not. This suggests that in spite of DCC recruitment in commissural neurons depending on similar intracellular signalling proteins as cortical neurons, some component of the depolarization sensitive mechanism is missing or blocked in the commissural cells. The embryonic spinal commissural neurons examined here are sensory interneurons that ultimately form spinal-thalamic projections (Colamarino and Tessier-Lavigne 1995), and must extend an axon a substantial distance along the developing spinal cord before reaching their target and forming a synapse. In contrast, the axon of a cortical neuron may, in some cases, synapse on cells relatively nearby. Although a significant increase in intracellular calcium was detected in commissural neurons, the relatively modest increase compared with the response of cortical neurons suggests that the capacity of commissural neurons to respond to depolarization, at this early stage of development, is less well developed than in cortical neurons.

Depolarization rapidly increases plasma membrane DCC

Depolarization increased plasma membrane DCC levels by approximately four-fold, as measured by DCC accessible to surface biotinylation. To investigate the mechanism underlying the depolarization-induced increases in plasma membrane DCC, we first determined if PKA was implicated. Increased intracellular Ca^{2+} concentration, induced by depolarization, can influence the intracellular concentration of cAMP by increasing Ca^{2+}-dependent adenylate cyclase activity (Eliot *et al.* 1989; Zhang and Poo 2001). Additionally, a voltage sensitive, but Ca^{2+}-independent adenylate cyclase (Reddy *et al.* 1995) has also been described, raising the possibility that changes in membrane potential could also increase the concentration of cAMP, through signaling mechanisms independent of elevated intracellular Ca^{2+}.

Kinase activation and protein phosphorylation make multiple contributions to axon growth and guidance. Key roles for PI3-kinase in neurite extension and guidance have been demonstrated in a number of neuronal cell types. With regard to the findings presented here, PI3-kinase has been previously shown to modulate axon chemoattraction to netrin-1 (Ming *et al.* 1999), and multiple PKC isoforms influence neurite extension in several neuronal cell types (Hsu *et al.* 1989; Heacock and Agranoff 1997; Kolkova *et al.* 2000; Rosdahl *et al.* 2002).

In *Xenopus* embryonic neuronal cultures and intact embryos, the nerve growth-promoting action of cAMP/PKA is mediated in part by the phosphorylation of synapsins (Kao *et al.* 2002). Synapsins are avid substrates for multi-site phosphorylation by several protein kinases, including PKA (Czernik *et al.* 1987; Kao *et al.* 2002), PKC (Browning and

Dudek 1992; Rebas *et al.* 1995; Iwata *et al.* 1997), Ca^{2+}/calmodulin-dependent kinase II (Czernik *et al.* 1987), mitogen-activated protein kinase (Jovanovic *et al.* 1996, 2000; Matsubara *et al.* 1996), and Cdk5 (Matsubara *et al.* 1996). Moreover, depolarization, induced by high potassium concentration, increases synapsin phosphorylation (Rodnight and Perrett 1986). Regulation of protein trafficking by PKA and PKC-dependent increases in synapsin phosphorylation suggests a possible mechanism that may underlie DCC mobilization; however, while this is an intriguing hypothesis, it remains to be demonstrated that synapsins influence DCC recruitment to the plasma membrane.

Depolarization-dependent recruitment of DCC in development

The present observations raise the question of whether the growth or targeting of the cortical neuron growth cones *in vivo* to netrin-1 is normally regulated by cell depolarization. Developing neurons may receive synaptic inputs at their dendrites and become electrically active, even while their axons are still in the process extending toward their target (Milner and Landmesser 1999; O'Donovan 1999). In the developing chick spinal cord, motorneurons exhibit regular bursts of activity from early embryonic stages before their growing axons reach target muscles (Milner and Landmesser 1999). Furthermore, tetrodotoxin blockade of activity resulted in aberrant patterns of axon targeting in the developing cortex (Catalano and Shatz 1998; Dantzker and Callaway 1998). As such, neuronal spiking and depolarization, both spontaneous and evoked, may contribute to axonal path finding and targeting during development.

Our current findings provide evidence that depolarization regulates chemotropic axon guidance responses made by cortical neurons. Intracellular and plasma membrane DCC was readily detected in growth cones, cell bodies, and the axons of cortical neurons. Robust depolarization-mediated increases in plasma membrane DCC were detected throughout these cells: at the cell body, along processes, and at the growth cones. These findings suggest that activity-dependent recruitment of DCC to the neuronal plasma membrane may influence a diverse array of responses to netrin-1 during development, including both process extension and axon branch formation.

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References

Ackerman S. L., Kozak L. P., Przyborski S. A., Rund L. A., Boyer B. B. and Knowles B. B. (1997) The mouse rostral cerebellar malformation gene encodes an UNC-5-like protein. *Nature* **386**, 838–842.

Bartoe J. L., McKenna W. L., Quan T. K., Stafford B. K., Moore J. A., Xia J., Takamiya K., Huganir R. L. and Hinck L. (2006) Protein interacting with C-kinase 1/protein kinase Calpha-mediated endocytosis converts netrin-1-mediated repulsion to attraction. *J. Neurosci.* **26**, 3192–3205.

Bouchard J. F., Moore S. W., Tritsch N. X., Roux P. P., Shekarabi M., Barker P. A. and Kennedy T. E. (2004) Protein kinase A activation promotes plasma membrane insertion of DCC from an intracellular pool: A novel mechanism regulating commissural axon extension. *J. Neurosci.* **24**, 3040–3050.

Browning M. D. and Dudek E. M. (1992) Activators of protein kinase C increase the phosphorylation of the synapsins at sites phosphorylated by cAMP-dependent and Ca2+/calmodulin-dependent protein kinase in the rat hippocampal slice. *Synapse* **10**, 62–70.

Burgess R. W., Jucius T. J. and Ackerman S. L. (2006) Motor axon guidance of the mammalian trochlear and phrenic nerves: dependence on the netrin receptor Unc5c and modifier loci. *J. Neurosci.* **26**, 5756–5766.

Campbell D. S. and Holt C. E. (2001) Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. *Neuron* **32**, 1013–1026.

Catalano S. M. and Shatz C. J. (1998) Activity-dependent cortical target selection by thalamic axons. *Science* **281**, 559–562.

Colamarino S. A. and Tessier-Lavigne M. (1995) The role of the floor plate in axon guidance. *Annu. Rev. Neurosci.* **18**, 497–529.

Czernik A. J., Pang D. T. and Greengard P. (1987) Amino acid sequences surrounding the cAMP-dependent and calcium/calmodulin-dependent phosphorylation sites in rat and bovine synapsin I. *Proc. Natl Acad. Sci. USA* **84**, 7518–7522.

Dantzker J. L. and Callaway E. M. (1998) The development of local, layer-specific visual cortical axons in the absence of extrinsic influences and intrinsic activity. *J. Neurosci.* **18**, 4145–4154.

Dillon A. K., Jevince A. R., Hinck L., Ackerman S. L., Lu X., Tessier-Lavigne M. and Kaprielian Z. (2007) UNC5C is required for spinal accessory motor neuron development. *Mol. Cell. Neurosci.* **35**, 482–489.

Eliot L. S., Dudai Y., Kandel E. R. and Abrams T. W. (1989) Ca^{2+}/calmodulin sensitivity may be common to all forms of neural adenylate cyclase. *Proc. Natl Acad. Sci. USA* **86**, 9564–9568.

Fabbri E., Brighenti L. and Ottolenghi C. (1991) Inhibition of adenylate cyclase of catfish and rat hepatocyte membranes by 9-(tetrahydro-2-furyl)adenine (SQ 22536). *J. Enzyme Inhib.* **5**, 87–98.

Fazeli A., Dickinson S. L., Hermiston M. L. *et al.* (1997) Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. *Nature* **386**, 796–804.

Fields R. D. (1994) Regulation of neurite outgrowth and immediate early gene expression by patterned electrical stimulation. *Prog. Brain Res.* **103**, 127–136.

Forcet C., Stein E., Pays L., Corset V., Llambi F., Tessier-Lavigne M. and Mehlen P. (2002) Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. *Nature* **417**, 443–447.

Goldsmith B. A. and Abrams T. W. (1991) Reversal of synaptic depression by serotonin at Aplysia sensory neuron synapses involves activation of adenylyl cyclase. *Proc. Natl Acad. Sci. USA* **88**, 9021–9025.

Hanson M. G. and Landmesser L. T. (2004) Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules. *Neuron* **43**, 687–701.

Heacock A. M. and Agranoff B. W. (1997) Protein kinase inhibitors block neurite outgrowth from explants of goldfish retina. *Neurochem. Res.* **22**, 1179–1185.

Herbert J. M., Augereau J. M., Gleye J. and Maffrand J. P. (1990) Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.* **172**, 993–999.

Hong K., Hinck L., Nishiyama M., Poo M. M., Tessier-Lavigne M. and Stein E. (1999) A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. *Cell* **97**, 927–941.

Hsu L., Jeng A. Y. and Chen K. Y. (1989) Induction of neurite outgrowth from chick embryonic ganglia explants by activators of protein kinase C. *Neurosci. Lett.* **99**, 257–262.

Iwata S., Hewlett G. H. and Gnegy M. E. (1997) Amphetamine increases the phosphorylation of neuromodulin and synapsin I in rat striatal synaptosomes. *Synapse* **26**, 281–291.

Jovanovic J. N., Benfenati F., Siow Y. L., Sihra T. S., Sanghera J. S., Pelech S. L., Greengard P. and Czernik A. J. (1996) Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. *Proc. Natl Acad. Sci. USA* **93**, 3679–3683.

Jovanovic J. N., Czernik A. J., Fienberg A. A., Greengard P. and Sihra T. S. (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. *Nat. Neurosci.* **3**, 323–329.

Kao H. T., Song H. J., Porton B., Ming G. L., Hoh J., Abraham M., Czernik A. J., Pieribone V. A., Poo M. M. and Greengard P. (2002) A protein kinase A-dependent molecular switch in synapsins regulates neurite outgrowth. *Nat. Neurosci.* **5**, 431–437.

Kase H., Iwahashi K., Nakanishi S., Matsuda Y., Yamada K., Takahashi M., Murakata C., Sato A. and Kaneko M. (1987) K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. *Biochem. Biophys. Res. Commun.* **142**, 436–440.

Keino-Masu K., Masu M., Hinck L., Leonardo E. D., Chan S. S., Culotti J. G. and Tessier-Lavigne M. (1996) Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. *Cell* **87**, 175–185.

Kennedy T. E., Serafini T., de l. T. Jr and Tessier-Lavigne M. (1994) Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**, 425–435.

Kennedy T. E., Wang H., Marshall W. and Tessier-Lavigne M. (2006) Axon guidance by diffusible chemoattractants: a gradient of netrin protein in the developing spinal cord. *J. Neurosci.* **26**, 8866–8874.

Kolkova K., Novitskaya V., Pedersen N., Berezin V. and Bock E. (2000) Neural cell adhesion molecule-stimulated neurite outgrowth depends on activation of protein kinase C and the Ras-mitogen-activated protein kinase pathway. *J. Neurosci.* **20**, 2238–2246.

Laufer R. and Changeux J. P. (1987) Calcitonin gene-related peptide elevates cyclic AMP levels in chick skeletal muscle: possible neurotrophic role for a coexisting neuronal messenger. *EMBO J.* **6**, 901–906.

Leonardo E. D., Hinck L., Masu M., Keino-Masu K., Ackerman S. L. and Tessier-Lavigne M. (1997) Vertebrate homologues of C. elegans UNC-5 are candidate netrin receptors. *Nature* **386**, 833–838.

Lisanti M. P., Le Bivic A., Sargiacomo M. and Rodriguez-Boulan E. (1989) Steady-state distribution and biogenesis of endogenous Madin-Darby canine kidney glycoproteins: evidence for intracellular sorting and polarized cell surface delivery. *J. Cell Biol.* **109**, 2117–2127.

Liu F., Gong X., Zhang G., Marquis K., Reinhart P. and Andree T. H. (2005) The inhibition of glycogen synthase kinase 3beta by a metabotropic glutamate receptor 5 mediated pathway confers neuroprotection to Abeta peptides. *J. Neurochem.* **95**, 1363–1372.

Manitt C., Colicos M. A., Thompson K. M., Rousselle E., Peterson A. C. and Kennedy T. E. (2001) Widespread expression of netrin-1 by neurons and oligodendrocytes in the adult mammalian spinal cord. *J. Neurosci.* **21**, 3911–3922.

Matsubara M., Kusubata M., Ishiguro K., Uchida T., Titani K. and Taniguchi H. (1996) Site-specific phosphorylation of synapsin I by mitogen-activated protein kinase and Cdk5 and its effects on physiological functions. *J. Biol. Chem.* **271**, 21108–21113.

McLaughlin T., Torborg C. L., Feller M. B. and O'Leary D. D. (2003) Retinotopic map refinement requires spontaneous retinal waves during a brief critical period of development. *Neuron* **40**, 1147–1160.

Metin C., Deleglise D., Serafini T., Kennedy T. E. and Tessier-Lavigne M. (1997) A role for netrin-1 in the guidance of cortical efferents. *Development* **124**, 5063–5074.

Meyer-Franke A., Wilkinson G. A., Kruttgen A., Hu M., Munro E., Hanson M. G. Jr, Reichardt L. F. and Barres B. A. (1998) Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. *Neuron* **21**, 681–693.

Milner L. D. and Landmesser L. T. (1999) Cholinergic and GABAergic inputs drive patterned spontaneous motoneuron activity before target contact. *J. Neurosci.* **19**, 3007–3022.

Ming G. L., Song H. J., Berninger B., Holt C. E., Tessier-Lavigne M. and Poo M. M. (1997) cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19**, 1225–1235.

Ming G., Song H., Berninger B., Inagaki N., Tessier-Lavigne M. and Poo M. (1999) Phospholipase C-gamma and phosphoinositide 3-kinase mediate cytoplasmic signaling in nerve growth cone guidance. *Neuron* **23**, 139–148.

Ming G., Henley J., Tessier-Lavigne M., Song H. and Poo M. (2001) Electrical activity modulates growth cone guidance by diffusible factors. *Neuron* **29**, 441–452.

Ming G. L., Wong S. T., Henley J., Yuan X. B., Song H. J., Spitzer N. C. and Poo M. M. (2002) Adaptation in the chemotactic guidance of nerve growth cones. *Nature* **417**, 411–418.

Moore S. W. and Kennedy T. E. (2006) Protein kinase A regulates the sensitivity of spinal commissural axon turning to netrin-1 but does not switch between chemoattraction and chemorepulsion. *J. Neurosci.* **26**, 2419–2423.

Nairn A. C., Hemmings H. C. Jr and Greengard P. (1985) Protein kinases in the brain. *Annu. Rev. Biochem.* **54**, 931–976.

Nicol X., Voyatzis S., Muzerelle A., Narboux-Neme N., Sudhof T. C., Miles R. and Gaspar P. (2007) cAMP oscillations and retinal activity are permissive for ephrin signaling during the establishment of the retinotopic map. *Nat. Neurosci.* **10**, 340–347.

O'Donovan M. J. (1999) The origin of spontaneous activity in developing networks of the vertebrate nervous system. *Curr. Opin. Neurobiol.* **9**, 94–104.

Quevedo C., Alcazar A. and Salinas M. (2000) Two different signal transduction pathways are implicated in the regulation of initiation factor 2B activity in insulin-like growth factor-1-stimulated neuronal cells. *J. Biol. Chem.* **275**, 19192–19197.

Rebas E., Lachowicz A. and Lachowicz L. (1995) Estradiol and pregnenolone sulfate could modulate PMA-stimulated and Ca^{2+}/calmodulin-dependent synaptosomal membrane protein phosphorylation from rat brain *in vivo*. *Biochem. Biophys. Res. Commun.* **207**, 606–612.

Reddy R., Smith D., Wayman G., Wu Z., Villacres E. C. and Storm D. R. (1995) Voltage-sensitive adenylyl cyclase activity in cultured neurons. A calcium-independent phenomenon. *J. Biol. Chem.* **270**, 14340–14346.

Rodnight R. and Perrett C. (1986) Protein phosphorylation and synaptic transmission: receptor mediated modulation of protein kinase C in a rat brain fraction enriched in synaptosomes. *J. Physiol. (Paris)* **81**, 340–348.

Rosdahl J. A., Mourton T. L. and Brady-Kalnay S. M. (2002) Protein kinase C delta (PKCdelta) is required for protein tyrosine phosphatase mu (PTPmu)-dependent neurite outgrowth. *Mol. Cell. Neurosci.* **19**, 292–306.

Schiavo G., Benfenati F., Poulain B., Rossetto O., Polverino d. L., DasGupta B. R. and Montecucco C. (1992) Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature* **359**, 832–835.

Serafini T., Kennedy T. E., Galko M. J., Mirzayan C., Jessell T. M. and Tessier-Lavigne M. (1994) The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. *Cell* **78**, 409–424.

Serafini T., Colamarino S. A., Leonardo E. D., Wang H., Beddington R., Skarnes W. C. and Tessier-Lavigne M. (1996) Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* **87**, 1001–1014.

Shirasaki R., Mirzayan C., Tessier-Lavigne M. and Murakami F. (1996) Guidance of circumferentially growing axons by netrin-dependent and -independent floor plate chemotropism in the vertebrate brain. *Neuron* **17**, 1079–1088.

Shu T., Valentino K. M., Seaman C., Cooper H. M. and Richards L. J. (2000) Expression of the netrin-1 receptor, deleted in colorectal cancer (DCC), is largely confined to projecting neurons in the developing forebrain. *J. Comp. Neurol.* **416**, 201–212.

Singh S. S., Chauhan A., Brockerhoff H. and Chauhan V. P. (1993) Activation of protein kinase C by phosphatidylinositol 3,4,5-trisphosphate. *Biochem. Biophys. Res. Commun.* **195**, 104–112.

Tamaoki J., Chiyotani A., Takeyama K., Yamauchi F., Tagaya E. and Konno K. (1993) Relaxation and inhibition of contractile response to electrical field stimulation by Beraprost sodium in canine airway smooth muscle. *Prostaglandins* **45**, 363–373.

Twiss J. L. and Shooter E. M. (1995) Nerve growth factor promotes neurite regeneration in PC12 cells by translational control. *J. Neurochem.* **64**, 550–557.

Vaillant A. R., Mazzoni I., Tudan C., Boudreau M., Kaplan D. R. and Miller F. D. (1999) Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. *J. Cell Biol.* **146**, 955–966.

Williams M. E., Wu S. C., McKenna W. L. and Hinck L. (2003) Surface expression of the netrin receptor UNC5H1 is regulated through a protein kinase C-interacting protein/protein kinase-dependent mechanism. *J. Neurosci.* **23**, 11279–11288.

Yagyu K., Kitagawa K., Irie T., Wu B., Zeng X. T., Hattori N. and Inagaki C. (2001) Amyloid beta proteins inhibit Cl(-)-ATPase activity in cultured rat hippocampal neurons. *J. Neurochem.* **78**, 569–576.

Yamaguchi A., Tamatani M., Matsuzaki H., Namikawa K., Kiyama H., Vitek M. P., Mitsuda N. and Tohyama M. (2001) Akt activation protects hippocampal neurons from apoptosis by inhibiting transcriptional activity of p53. *J. Biol. Chem.* **276**, 5256–5264.

Zhang L. I. and Poo M. M. (2001) Electrical activity and development of neural circuits. *Nat. Neurosci.* **4** (Suppl), 1207–1214.

