RAPID REPORT

Calcium-sensing mechanism in TRPC5 channels contributing to retardation of neurite outgrowth

Hui Hui[1], Damian McHugh[2], Meredith Hannan[3], Fanning Zeng[2], Shang-Zhong Xu[2], Saeed-ul-Hassan Khan[1], Robert Levenson[3], David J. Beech[2] and Jamie L. Weiss[1]

[1] *Biomedical Science, University of Sheffield, Sheffield S10 2TN, UK*
[2] *Institute of Membrane and Systems Biology, University of Leeds, Leeds LS2 9JT, UK*
[3] *Department of Pharmacology, Penn State College of Medicine, Hershey, PA 17033, USA*

The calcium- and sodium-permeable transient receptor potential channel TRPC5 has an inhibitory role in neuronal outgrowth but the mechanisms governing its activity are poorly understood. Here we propose a mechanism involving the neuronal calcium sensor-1 (NCS-1) protein. Inhibitory mutants of TRPC5 and NCS-1 enhance neurite outgrowth similarly. Mutant NCS-1 does not inhibit surface-expression of TRPC5 but generally suppresses channel activity, irrespective of whether it is evoked by carbachol, store depletion, lanthanides or elevated intracellular calcium. NCS-1 and TRPC5 are in the same protein complex in rat brain and NCS-1 directly binds to the TRPC5 C-terminus. The data suggest protein–protein interaction between NCS-1 and TRPC5, and involvement of this protein complex in retardation of neurite outgrowth.

(Received 2 December 2005; accepted after revision 2 February 2006; first published online 9 February 2006)
Corresponding author D. J. Beech: Institute of Membrane and Systems Biology, Garstang Building, University of Leeds, Leeds LS2 9JT, UK. Email: d.j.beech@leeds.ac.uk

Development of the nervous system and plasticity of neurons for learning and memory require tightly controlled and directed neuronal outgrowth mechanisms (Henley & Poo, 2004; Waites *et al.* 2005). The underlying molecular mechanisms are emerging and recently the importance of the calcium- and sodium-permeable TRPC channels, homologues of the *Drosophila melanogaster* transient receptor potential protein (Voets *et al.* 2005; Ramsey *et al.* 2006), became apparent (Greka *et al.* 2003; Shim *et al.* 2005; Wang & Poo, 2005; Li *et al.* 2005; Gomez, 2005). The finding that TRPC channels have such roles is consistent with reports indicating calcium dependence of growth cone extension and turning (Henley & Poo, 2004; Bolsover, 2005). One of the TRPC channels involved is TRPC5. TRPC5 has an inhibitory impact on neurite extension and a rapid vesicular trafficking mechanism regulated by growth factors (Greka *et al.* 2003; Bezzerides *et al.* 2004). Furthermore, the *TRPC5* gene was identified in a region of the human X-chromosome associated with non-syndromic mental retardation (Sossey-Alaoui *et al.* 1999) and thus there is a putative genetic as well as functional link with neuronal development. Activation mechanisms for TRPC5 are poorly understood but a consistent observation has been the dependence of TRPC5 on intracellular Ca^{2+} and modest activation by small elevations of Ca^{2+} above resting levels (Okada *et al.* 1998; Strubing *et al.* 2001; Zeng *et al.* 2004). We were motivated to elucidate the mechanism underlying this positive effect of Ca^{2+} and so investigated the potential role of neuronal Ca^{2+} sensor-1 (NCS-1) (Burgoyne & Weiss, 2001; Weiss & Burgoyne, 2002). NCS-1 is associated with X-linked mental retardation (Bahi *et al.* 2003), senses Ca^{2+} in the same concentration range as TRPC5 (Burgoyne & Weiss, 2001; Weiss & Burgoyne, 2002), is a known modulator of other types of ion channel (Weiss & Burgoyne, 2002), and is expressed endogenously in neurites (Olafsson *et al.* 1997; Angaut-Petit *et al.* 1998).

Methods

Cell culture and transfection

Tetracycline-inducible expression of human TRPC5 (Zeng *et al.* 2004) and human FLAG-epitope-tagged TRPM2 (McHugh *et al.* 2003) in HEK293 cells has been described. Cells were grown in Dulbecco's modified Eagle's medium–F_{12} (Invitrogen) supplemented with 10% fetal bovine serum and penicillin (50 units ml^{-1}) and streptomycin (0.5 mg ml^{-1}) at 37°C in a 5% CO_2 incubator. One microgram of wild-type (WT) or dominant negative (DN) mutant (E120Q) NCS-1 in the

Hui Hui and Damian McHugh contributed equally to this work.

DOI: 10.1113/jphysiol.2005.102889

pcDNA3(−) plasmid was transfected into cells using the Fugene 6 transfection reagent (Roche, Lewes, UK). The fluorescent protein plasmid pDsRed2-N1 (0.1 μg) (Clontech, Palo Alto, CA, USA) was cotransfected to act as an indicator of transfection. PC12 cells were obtained from the European collection of cell cultures (ECACC no. 88022401) and cultured according to the instructions supplied. Cells were plated on collagen type IV-coated coverslips placed in 12-well plates. Cells were transfected with the cDNAs using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. DNTRPC5 is a triple alanine mutation of the conserved LFW sequence in the ion pore; dominant negative function was demonstrated previously (Strubing *et al.* 2003) and confirmed by us in the HEK-TRPC5 cells (data not shown). We obtained PC12 cell transfection efficiency up to 70%. NGF (Invitrogen) was added at 50 ng ml^{-1} after 2 days. Western blot analysis confirmed successful over-expression of all transfected plasmids. The cells were allowed to differentiate in the presence of NGF for 3 days and a more neuronal phenotype could be seen (i.e. neurite formation) before fixing for immunocytochemistry. Anti-NCS-1 (rabbit) (Biomol/Affiniti, Exeter, UK) or affinity purified anti-Frequenin (chicken) (Rockland Immunochemicals) was used at 1 : 1000 for overexpression and at 1 : 500 to detect endogenously expressed NCS-1. TUJ1 neuronal class III β-tubulin monoclonal (Covance) was used at 1 : 500.

Reverse transcriptase-PCR (RT-PCR)

Total RNA was extracted from PC12 cells using the RNeasy kit (Qiagen) and subjected to oligo(dT)-primed reverse transciption. Rat TRPC5 PCR primers were (5′–3′): forward, ACCTCTCATCAGAACCATGCCA; reverse TGCATGAGCAAGTCACAGGCCT. Rat TRPC4 primers were (5′–3′): forward, TCTGCAGATATCTCT-GGGAAGAATGC; reverse, AAGCTTTGTTCGAGCAAA-TTTCCACTC. PCR was 94°C for 2 min followed by 35 cycles of 94°C (30 s), 60°C (30 s), 72°C (30 s), and finally 72°C for 7 min.

Image analysis and neurite outgrowth quantification

Representative PC12 cell images were picked blind at random for each experimental group from at least three separate transfections performed on different days. The numbers of neurites were counted per cell and neurite length was measured directly from the cell body to the end of the neurite. We analysed 40 cells for each transfected plasmid and the averaged values as mean ± S.E.M. were graphed and compared to control cells transfected with vector (pcDNA3.1 (−)) alone.

Immunoprecipitation and Western blotting

Thirty-six hours after induction of TRex-TRPC5 or TRex-TRPM2 cells with tetracycline (1 μg ml^{-1}), the cells were washed 3 times in chilled phosphate-buffered saline (PBS), harvested by scraping, and centrifuged for 5 min at 500 *g*. If the cells were not used immediately, the cell pellet was snap frozen in liquid N_2 and stored at −80°C. Cells were homogenized at 4°C in ice-cold lysis buffer containing (mM): Tris–acetate 20, pH 7; EDTA 1, sodium β-glycerophosphate 10, sodium orthovanadate 1, Triton X-100 1%, sucrose 270, protease inhibitor tablet (Roche), benzamidine 1 and β-mercaptoethanol 0.1%. Rats (> postnatal day 12) were killed by cervical dislocation according to Schedule 1 procedures outlined in the Code of Practice, UK Animals (Scientific Procedures) Act 1986. Rat brain membranes were isolated with an additional ultracentrifugation step. The membrane lysates were centrifuged at 4°C in a microcentrifuge and the protein concentration of the supernatants assayed by the Bradford method using BSA as a standard. For immunoprecipitation experiments, 600 μg of lysate was precleared with protein G–Sepharose agarose (Amersham Biosciences, UK) by incubating for 1 h at 4°C on a rotating mixer. To 500 μg of precleared cell/brain lysate was added 4 μg anti-NCS-1 for 2 h. Immune complexes were recovered by the addition of 30 μl of protein G–Sepharose agarose with further mixing overnight at 4°C. The tubes were briefly centrifuged and the pelleted protein-G immune complex was boiled for 5 min after adding 50 μl of sample buffer (Sigma, UK). The tubes were spun once more and 25 μl of the supernatant was resolved by 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked in PBS 1 × with 0.1% TWEEN and 3% milk powder prior to incubation for 1 h with primary antibodies. Anti-TRPC5 antibodies were a custom-made, affinity-purified antibody generated in chicken (to peptide VFETWGEACDLLMHKWGDGQ) or a commercial antibody generated in rabbit (Sigma, UK). Monoclonal anti-FLAG M2 antibody was from Sigma. Blots were incubated with the appropriate secondary antibody coupled to horseradish peroxidase before being washed 3 times in PBS followed by ECL detection.

Biotinylation experiments

A Cell Surface Protein Biotinylation and Purification Kit (Pierce) was used according to the manufacturer's instructions. Briefly PC12 cells overexpressing TRPC5 in combination with DN or WT NCS-1 were labelled with Sulfo-NHS-SS-Biotin. Cells were lysed and Bradford assays were used to determine the same amount of EZ-link sulfo-NHS-SS-biotin labelled protein for binding to the immobilized NeutrAvidin gel, bounds proteins were released by incubating with sample buffer, equal amounts

were run on 10% SDS-PAGE and subjected to Western blot analysis with anti-TRPC5 to look for changes in surface expression.

Directed yeast two-hybrid screen

Constructs were generated by PCR amplification and verified by DNA sequencing. A cDNA encoding the complete NCS-1 (accession no. NM_014286) open reading frame (ORF) was constructed in the DNA-binding domain vector pAS2-1 (BD Biosciences Clontech) and used as bait. cDNAs encoding carboxyl-terminal truncations of human TRPC5 (accession no. AF054568) were constructed in the DNA-activation domain vector pACT2 (BD Biosciences Clontech) and separately used as prey. Bait and prey constructs were sequentially transformed into yeast strain MaV103 using a lithium acetate protocol (Lin, 2001). Transformants were identified via growth on −Leu/−Trp selection plates. Protein–protein interactions were detected using α-galactosidase assays according to the manufacturer's instructions (BD Biosciences). Positive interactions were identified by growth of yeast on selection plates (−Leu/−Trp) and expression of α-galactosidase activity by cleavage of 5-bromo-4-chloro-3-indolyl-α-D-galactopyranoside (X-α-gal; Calbiochem).

GST-pulldown

A cDNA encoding the complete NCS-1 ORF was constructed in pGEX-4T-1 (Amersham Pharmacia) to generate GST-tagged NCS-1 (NCS-1-GST). The third cytoplasmic loop of the dopamine D2L receptor (amino acids 211–373) was constructed in pGEX-4T-1 to generate a GST-tagged D2L receptor (D2LIC3-GST) and was used as a negative control. Carboxyl-terminal truncations of hTRPC5 were subcloned in the vector pET30C (Novagen) to generate a set of S-tagged constructs. All fusion proteins were induced in *E. coli* strain BL-21 (DE3) and purified using glutathione-Sepharose beads (Amersham) according to the manufacturer's instructions. Eluted proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was probed for the presence of S-tagged carboxyl-terminal hTRPC5 fusion proteins using an anti-S-tag polycolonal antibody (1 : 5000 dilution) conjugated to horseradish peroxidase (Novagen). Immunoreactivity was detected by ECL.

Ca^{2+} imaging and patch-clamp recording

Experiments were performed as previously described (Zeng *et al.* 2004). The standard bath solution contained (mM): NaCl 130, KCl 5, D-glucose 8, Hepes 10, $MgCl_2$ 1.2, $CaCl_2$ 1.5; pH was titrated to 7.4 with NaOH. The patch pipette solution contained (mM): CsCl 100, Hepes 10, Na_2ATP 5, EGTA 10, $MgCl_2$ 2; $CaCl_2$ was included at 0 (0 nM), 4.3 mM (100 nM), 6.9 mM (300 nM), 8.173 mM (600 nM) and 8.84 mM (1000 nM) to obtain the unbound Ca^{2+} concentrations in parentheses after titration to pH 7.2 and adjustment of osmolarity to 290 mosmol l^{-1} with mannitol. Unbound Ca^{2+} concentrations were calculated using EQCAL software (Biosoft, Cambridge, UK). Standard bath solution was used for all recordings (see Ca^{2+} imaging). 2-Aminoethoxydiphenyl borate (2-APB, Sigma) was prepared as a 75-mM stock in 100% dimethylsulphoxide.

Data analysis

Averaged data are given as means ± S.E.M. Statistical analysis was by paired or unpaired Student's *t* test, as appropriate ($^*P < 0.05$; $^{**}P \leq 0.001$; n.d. denotes 'not different').

Results

Labelling of the PC12 neuroendocrine cell line with anti-NCS-1 (Fig. 1*A*) shows endogenous expression of NCS-1 in cell bodies, where characteristic perinuclear localization of NCS-1 is observed (O'Callaghan *et al.* 2002). Endogenous expression of NCS-1 is also detected in neurites (Fig. 1*A* and *B*). To explore the role of NCS-1 we expressed a dominant negative (DN) NCS-1 containing the E120Q mutation, which inactivates the third EF-hand (Weiss *et al.* 2000). The DN NCS-1 enhances neurite extension (Fig. 1*C–F*, cf. *A*, *B* and *K*) whereas exogenous wild-type (WT) NCS-1 has little effect (Fig. 1*G–H*, cf. *A*, *B* and *K*), suggesting endogenous NCS-1 is normally saturating and serving to inhibit neurite outgrowth. This effect is reminiscent of that reported for DN mutant TRPC5 where hippocampal neurons over-expressing DN TRPC5 had significantly longer neurites (Greka *et al.* 2003). The effect of DN TRPC5 (Greka *et al.* 2003; Strubing *et al.* 2003) on neurite extension was reproduced in PC12 cells (Fig. 1*J* cf. *I* and *K*), which express *TRPC5* but not *TRPC4* endogenously (see Supplemental material, Fig. S1a and b). There was no significant effect on neurite number (data not shown). Notably, the effects of DNNCS-1 and DNTRPC5 are non-additive (Fig. 1*K*). One possible explanation for lack of additivity is that NCS-1 and TRPC5 are involved in a common mechanism.

Rapid vesicular trafficking of TRPC5 to the surface membrane of neuronal growth cones has been suggested (Bezzerides *et al.* 2004). However, DNNCS-1 does not reduce but rather slightly increases the amount of TRPC5 at the plasma membrane (Fig. 2*A*), suggesting endogenous NCS-1 has no effect on, or inhibits, forward trafficking – a tendency that would not explain its inhibitory effect on neurite extension (Fig. 1*K*).

Alternatively, NCS-1 might directly affect the function of TRPC5. To explore this possibility we used a tetracycline-inducible HEK-293 cell expression system in

which functional TRPC5 signals are clearly distinguished from background signals (Zeng *et al.* 2004) and where there is endogenous NCS-1 expression as demonstrated by Western blotting (see Supplemental material, Fig S1c). Using this cell system we previously showed TRPC5 activity is evoked by a multiplicity of signals, including G-protein-coupled receptor agonists, store depletion and external lanthanides including gadolinium (Zeng *et al.* 2004). Ca^{2+}-imaging experiments show that DNNCS-1 inhibits TRPC5 activated by any of these signals (Fig. 2*B* and *C*), suggesting NCS-1 has a general role in TRPC5 function.

TRPC5 shows modest activation in response to 200 nM intracellular Ca^{2+} (Ca_i^{2+}) (Strubing *et al.* 2001; Zeng *et al.* 2004), which is in the range of NCS-1's Ca^{2+}-binding affinity (Burgoyne & Weiss, 2001). Activation of TRPC5 by other means also depends on intracellular Ca^{2+}: for example, activation by gadolinium is graded according to Ca_i^{2+} (Fig. 2*D*) and fails when Ca_i^{2+} is buffered to very low levels by EGTA (Supplemental material, Fig S2). These observations suggest that TRPC5 function has a general dependence on physiological Ca_i^{2+} – as if Ca^{2+} has a permissive role. We therefore explored the hypothesis that the function of TRPC5 depends on the Ca^{2+}-sensing capability of NCS-1. In order to buffer Ca_i^{2+} at specific concentrations we performed whole-cell patch-clamp recordings, controlling Ca^{2+} via the patch pipette. DNNCS-1 inhibits TRPC5 current when Ca^{2+} in the patch pipette is 300 or 600 nM but has weak effects outside this Ca^{2+} range (Fig. 2*D*). These data are consistent

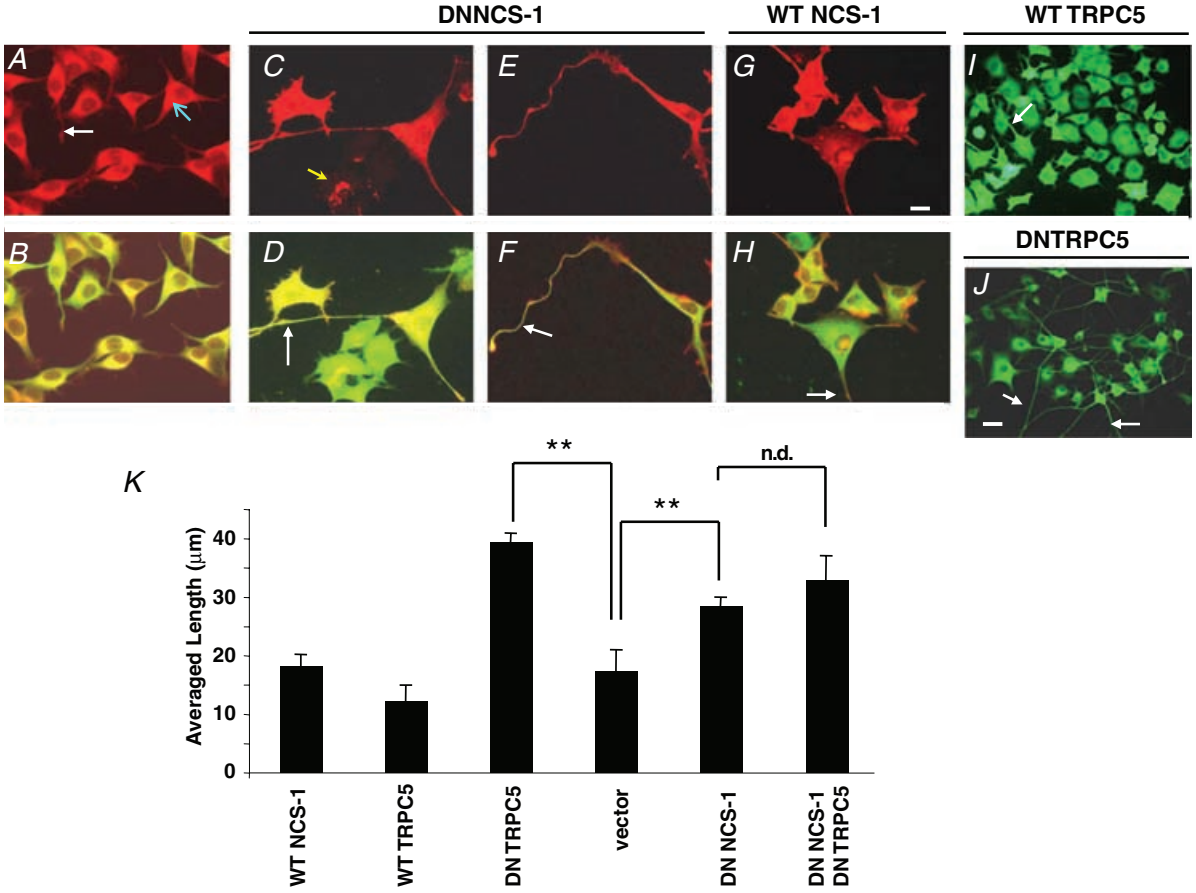

Figure 1. Overlapping effects of TRPC5 and NCS-1 on neurite outgrowth
A–J, images of PC12 cells showing fluorescence after antibody labelling for NCS-1 (*A–H*), with colabelling for β-tubulin (*B*, *D*, *F*, *H*), or labelling for β-tubulin alone (*I–J*). *A* and *B*, native PC12 cells without transfection showing endogenous NCS-1 and overlay with antitubulin staining. In the absence of primary antibody or with preimmune staining, fluorescence was absent (data not shown). *C–H*, cells were transfected with plasmids encoding dominant negative (DN) NCS-1 (*C–F*), wild-type (WT) NCS-1 (*G* and *H*), WT TRPC5 (*I*) or DNTRPC5 (*J*). *C* and *D* and *E* and *F* are two examples of the same experiment. The scale bar in *G* is 5 μm and applies to *A–H*; the bar in *J* is 10 μm and applies to *I* and *J*. White arrows point to neurite extensions. The blue arrow (*A*) points to perinuclear NCS-1, which is characteristic. The yellow arrow in *C* points to an untransfected cell. *K*, mean ± S.E.M. ($n = 40$, per condition) neurite length (μm) for PC12 cells transfected with cDNA constructs as indicated, and where 'control' is the vector without insert.

with NCS-1 acting as a sensor underlying Ca^{2+} dependence of TRPC5.

To determine if NCS-1 acts as a direct Ca^{2+} sensor for TRPC5 we investigated whether there is physical association between the two proteins. In TRPC5 over-expressing cells, TRPC5 occurs in the precipitate pulled down by anti-NCS-1 antibody (Fig. 3*A*). Similarly, anti-NCS-1 antibody pulls-down endogenous TRPC5 in rat brain (Fig. 3*B*). These data demonstrate that NCS-1 and TRPC5 exist in the same protein complex. NCS-1 specificity was suggested by the failure of anti-NCS-1 antibody to pull down another Ca^{2+}-dependent TRP channel, TRPM2 (Supplemental material, Fig S3). To test for a direct interaction between NCS-1 and TRPC5 we performed yeast two-hybrid assays, focusing on the C-terminus of TRPC5. A positive interaction was detected in eight independent experiments (Fig. 3*C*). In control experiments we failed to detect an interaction between NCS-1 and protein 4.1N (data not shown). In order to better define the NCS-1 interaction site, truncated fragments of TRPC5 C-terminus were tested; the data suggest predominant interaction with proximal C-terminus but do not exclude other regions (Fig. 3*C*). As an independent test of these findings, *in vitro* GST pull-down assays were performed (Fig. 3*D*). The entire C-terminal fragment (619–973) exhibited binding to the D2L dopamine receptor, which had been included as a negative control. However, proximal and distal TRPC5 C-terminal fragments showed specificity – failing to bind D2L but showing robust interaction with NCS-1 (Fig. 3*D*).

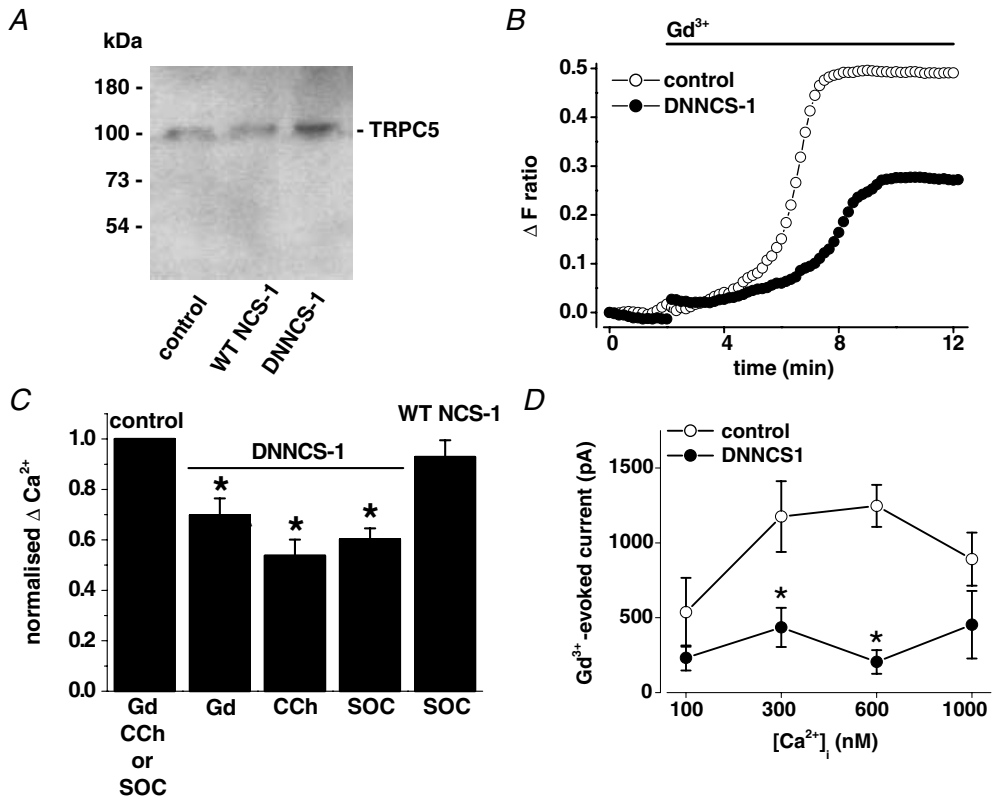

Figure 2. NCS-1 affects TRPC5 function at the membrane
A, typical of 3 independent experiments, Western blot labelled with anti-TRPC5 antibody showing output from the surface biotinylation assay. PC12 cells were transfected with TRPC5 in combination with cDNA encoding WT NCS-1, DNNCS-1, or without the NCS-1 insert (control). *B–D*, data for HEK 293 cells induced to express TRPC5. *B*, illustrative examples of simultaneous measurements of intracellular Ca^{2+} in two cells, one transfected with DNNCS-1, the other not (control). Ca^{2+} concentration is shown as the change (Δ) in fura-PE3 fluorescence (*F*) ratio above the base-line. TRPC5 activity was evoked by 0.1 mM gadolinium (Gd^{3+}). *C*, as for *B* but normalized mean data ($n \geq 123$ cells each from 6 to 11 independent experiments). Each experiment was paired, comparing the response to a TRPC5 activator without (control) or with NCS-1 (as DN or WT). Activators were: 0.1 mM Gd^{3+} (Gd); 0.1 mM carbachol (CCh); or Ca^{2+}-readdition after pretreatment with 1 μM thapsigargin (SOC; store-operated channel). *D*, mean whole-cell patch-clamp data showing amplitudes of currents evoked at +80 mV by 10 μM Gd^{3+} 10-min after starting the whole-cell recording. The Ca^{2+} concentration in the patch pipette is indicated on the *x*-axis. For each concentration, currents were compared on the same day in cells transfected with DsRed2 (control) or DsRed2 plus DNNCS-1 ($n = 3$–9 cells per point).

Collectively the data suggest NCS-1 is a direct protein partner of TRPC5.

Discussion

Other Ca^{2+} sensor proteins have also been associated with TRPC5 (Tang *et al.* 2001; Kinoshita-Kawada *et al.* 2005; Ordaz *et al.* 2005). The most widely studied of these, calmodulin, binds the C-terminus of all TRPC family members (Tang *et al.* 2001). Over-expression of calmodulin enhances activation of TRPC5 by thrombin or carbachol (Ordaz *et al.* 2005; Kim *et al.* 2006). Furthermore, there is an inhibitory impact of the Ca^{2+} sensor CaBP1 (Kinoshita-Kawada *et al.* 2005). Therefore, TRPC5 is a channel receiving both positive (calmodulin and NCS-1) and negative (CaBP1) impact from Ca^{2+}-sensing proteins and the relative balance of these will be important for the final functional outcome.

There are seven TRPC family members, with six being expressed in human. Based on analogies with the structurally related voltage-gated potassium channels it is thought four TRP proteins come together to form a single channel, often involving mixtures of different TRP subtypes. TRPC5, for example, interacts with TRPC1 (Strubing *et al.* 2001). However, TRPC1 was not detected in neuronal growth cones (Greka *et al.* 2003) implying TRPC5 exists as a homotetramer in this context. The other possible partner is the functionally similar TRPC4 (Plant & Schaefer, 2003), although we have not been able to detect RNA-encoding TRPC4 in PC12 cells (see Supplemental material, Fig S1b).

We suggest TRPC5 and NCS-1 form a direct and functional protein partnership, with NCS-1 having a positive impact on TRPC5 and thus Ca^{2+} influx. It is envisaged that NCS-1 mediates the permissive role of intracellular Ca^{2+} on TRPC5 function, regulating the

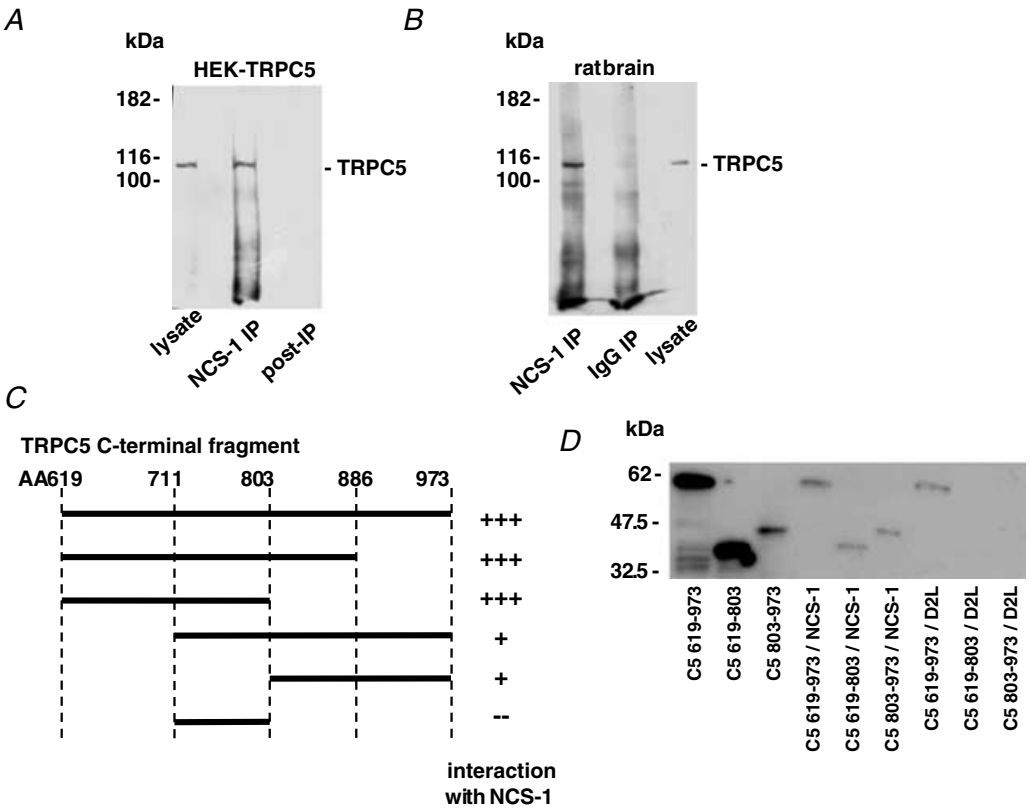

Figure 3. TRPC5 interacts physically with NCS-1
A, Western blot showing protein labelled by anti-TRPC5 antibody. Immunoprecipitation (IP) with anti-NCS-1 antibody revealed TRPC5. *B*, similar to *A* but for endogenous proteins from rat brain membrane lysate, showing IP with anti-NCS-1 antibody, but not control IgG antibody, pulls-down TRPC5. *C*, schematic summary of yeast 2-hybrid data. On the left, representations of the C-terminal TRPC5 fragments used in each assay (AA, amino acid): On the right, representations of the strength of the interaction with NCS-1 (+++, strong interaction; –, no interaction). *D*, Western blot showing a typical result from the GST-pull-down assays. Lanes are: TRPC5-S-Tag (AA619-973); TRPC5-S-Tag (AA 619–803); TRPC5-S-Tag (AA 803–973); TRPC5-S-Tag (AA 619–973)/NCS-1-GST; TRPC5-S-Tag (AA 619–803)/NCS-1-GST; TRPC5-S-Tag (AA 803–973)/NCS-1-GST; TRPC5-S-Tag (AA 619–973)/D2LIC3-GST; TRPC5-S-Tag (AA 619–803)/D2LIC3-GST; TRPC5-S-Tag (AA 803–973)/D2LIC3-GST.

capacity of TRPC5 to respond to activators with high efficacy. Although positive with regard to Ca^{2+} influx, this partnership is inhibitory for neurite outgrowth, consistent with previous but independent reports for TRPC5 and NCS-1 (Angaut-Petit *et al.* 1998; Greka *et al.* 2003; Bezzerides *et al.* 2004). Optimal ranges of Ca^{2+} are required for neuronal outgrowth with differential actions of Ca^{2+} on neurite extension (Henley & Poo, 2004; Bolsover, 2005). Synaptic plasticity is a Ca^{2+}-mediated process and NCS-1 has been implicated in short-term synaptic plasticity as well as learning and memory (Gomez *et al.* 2001). Our study suggests NCS-1 is a Ca^{2+} sensor that enables TRPC5, via various activating signals at the plasma membrane, to link Ca^{2+} transients to their effects on growth cone extension. It is therefore proposed that the TRPC5–NCS-1 partnership has a role in neuronal development and plasticity – perhaps either as a negative feedback mechanism or a mechanism to retard neuronal outgrowth prior to synapse formation.

References

Angaut-Petit D, Toth P, Rogero O, Faille L, Tejedor FJ & Ferrus A (1998). Enhanced neurotransmitter release is associated with reduction of neuronal branching in a *Drosophila* mutant overexpressing frequenin. *Eur J Neurosci* **10**, 423–434.

Bahi N, Friocourt G, Carrie A, Graham ME, Weiss JL, Chafey P, Fauchereau F, Burgoyne RD & Chelly J (2003). IL1 receptor accessory protein like, a protein involved in X-linked mental retardation, interacts with Neuronal Calcium Sensor-1 and regulates exocytosis. *Hum Mol Genet* **12**, 1415–1425.

Bezzerides VJ, Ramsey IS, Kotecha S, Greka A & Clapham DE (2004). Rapid vesicular translocation and insertion of TRP channels. *Nat Cell Biol* **6**, 709–720.

Bolsover SR (2005). Calcium signalling in growth cone migration. *Cell Calcium* **37**, 395–402.

Burgoyne RD & Weiss JL (2001). The neuronal calcium sensor family of Ca^{2+}-binding proteins. *Biochem J* **353**, 1–12.

Gomez T (2005). Neurobiology: channels for pathfinding. *Nature* **434**, 835–838.

Gomez M, De Castro E, Guarin E, Sasakura H, Kuhara A, Mori I, Bartfai T, Bargmann CI & Nef P (2001). Ca^{2+} signaling via the neuronal calcium sensor-1 regulates associative learning and memory in *C. elegans*. *Neuron* **30**, 241–248.

Greka A, Navarro B, Oancea E, Duggan A & Clapham DE (2003). TRPC5 is a regulator of hippocampal neurite length and growth cone morphology. *Nat Neurosci* **6**, 837–845.

Henley J & Poo MM (2004). Guiding neuronal growth cones using Ca^{2+} signals. *Trends Cell Biol* **14**, 320–330.

Kim MT, Kim BJ, Lee JH, Kwon SC, Yeon DS, Yang DK, So I & Kim KW (2006). Involvement of calmodulin and myosin light chain kinase in the activation of mTRPC5 expressed in HEK cells. *Am J Physiol Cell Physiol* (in press).

Kinoshita-Kawada M, Tang J, Xiao R, Kaneko S, Foskett JK & Zhu MX (2005). Inhibition of TRPC5 channels by Ca^{2+}-binding protein 1 in *Xenopus* oocytes. *Pflugers Arch* **450**, 345–354.

Li Y, Jia YC, Cui K, Li N, Zheng ZY, Wang YZ & Yuan XB (2005). Essential role of TRPC channels in the guidance of nerve growth cones by brain-derived neurotrophic factor. *Nature* **434**, 894–898.

Lin R, Karpa KD, Kabbani N, Goldman-Rakic P & Levenson R (2001). Dopamine D2 and D3 receptors are linked to the actin cytoskelton via interaction with filamin A.. *Proc Natl Acad Sci USA* **98**, 5258–5263.

McHugh D, Flemming R, Xu SZ, Perraud AL & Beech DJ (2003). Critical intracellular Ca^{2+} dependence of transient receptor potential melastatin 2 (TRPM2) cation channel activation. *J Biol Chem* **278**, 11002–11006.

O'Callaghan DW, Ivings L, Weiss JL, Ashby MC, Tepikin AV & Burgoyne RD (2002). Differential use of myristoyl groups on neuronal calcium sensor proteins as a determinant of spatio-temporal aspects of Ca^{2+} signal transduction. *J Biol Chem* **277**, 14227–14237.

Okada T, Shimizu S, Wakamori M, Maeda A, Kurosaki T, Takada N, Imoto K & Mori Y (1998). Molecular cloning and functional characterization of a novel receptor-activated TRP Ca^{2+} channel from mouse brain. *J Biol Chem* **273**, 10279–10287.

Olafsson P, Soares HD, Herzog KH, Wang T, Morgan JI & Lu B (1997). The Ca^{2+} binding protein, frequenin is a nervous system-specific protein in mouse preferentially localized in neurites. *Brain Res Mol Brain Res* **44**, 73–82.

Ordaz B, Tang J, Xiao R, Salgado A, Sampieri A, Zhu MX & Vaca L (2005). Calmodulin and calcium interplay in the modulation of TRPC5 channel activity. Identification of a novel C-terminal domain for calcium/calmodulin-mediated facilitation. *J Biol Chem* **280**, 30788–30796.

Plant TD & Schaefer M (2003). TRPC4 and TRPC5: receptor-operated Ca^{2+}-permeable nonselective cation channels. *Cell Calcium* **33**, 441–450.

Ramsey IS, Delling M & Clapham DE (2006). An introduction to TRP channels. *Annu Rev Physiol* (in press).

Shim S, Goh EL, Ge S, Sailor K, Yuan JP, Roderick HL, Bootman MD, Worley PF, Song H & Ming GL (2005). XTRPC1-dependent chemotropic guidance of neuronal growth cones. *Nat Neurosci* **8**, 730–735.

Sossey-Alaoui K, Lyon JA, Jones L, Abidi FE, Hartung AJ, Hane B, Schwartz CE, Stevenson RE & Srivastava AK (1999). Molecular cloning and characterization of TRPC5 (HTRP5), the human homologue of a mouse brain receptor-activated capacitative Ca^{2+} entry channel. *Genomics* **60**, 330–340.

Strubing C, Krapivinsky G, Krapivinsky L & Clapham DE (2001). TRPC1 and TRPC5 form a novel cation channel in mammalian brain. *Neuron* **29**, 645–655.

Strubing C, Krapivinsky G, Krapivinsky L & Clapham DE (2003). Formation of novel TRPC channels by complex subunit interactions in embryonic brain. *J Biol Chem* **278**, 39014–39019.

Tang J, Lin Y, Zhang Z, Tikunova S, Birnbaumer L & Zhu MX (2001). Identification of common binding sites for calmodulin and inositol 1,4,5-trisphosphate receptors on the carboxyl termini of Trp channels. *J Biol Chem* **276**, 21303–21310.

Voets T, Talavera K, Owsianik G & Nilius B (2005). Sensing with TRP channels. *Nat Chem Biol* **1**, 85–92.

Waites CL, Craig AM & Garner CC (2005). Mechanisms of vertebrate synaptogenesis. *Annu Rev Neurosci* **28**, 251–274.

Wang GX & Poo MM (2005). Requirement of TRPC channels in netrin-1-induced chemotropic turning of nerve growth cones. *Nature* **434**, 898–904.

Weiss JL, Archer DA & Burgoyne RD (2000). Neuronal Ca^{2+} sensor-1/frequenin functions in an autocrine pathway regulating Ca^{2+} channels in bovine adrenal chromaffin cells. *J Biol Chem* **275**, 40082–40087.

Weiss JL & Burgoyne RD (2002). Sense and sensibility in the regulation of voltage-gated Ca^{2+} channels. *Trends Neurosci* **25**, 489–491.

Zeng F, Xu SZ, Jackson PK, McHugh D, Kumar B, Fountain SJ & Beech DJ (2004). Human TRPC5 channel activated by a multiplicity of signals in a single cell. *J Physiol* **559**, 739–750.

Acknowledgements

We thank the Wellcome Trust, BBSRC and Royal Society for support.

Supplemental material

The online version of this paper can be accessed at:
DOI: 10.1113/jphysiol.2005.102889
http://jp.physoc.org/cgi/content/full/jphysiol.2005.102889/DC1
and contains supplemental material consisting of three figures:
Figure S1. Endogenous expression of *TRPC* and NCS-1
Figure S2. Effect of Ca^{2+}-buffering on TRPC5 current
Figure S3. Lack of association of NCS-1 with TRPM2

This material can also be found as part of the full-text HTML version available from http://www.blackwell-synergy.com