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## Nerve growth factor enhances voltage-gated Na<sup>+</sup> channel activity and transwell migration in Mat-LyLu rat prostate cancer cell line

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### Abstract

The highly dynamic nature of voltage-gated Na<sup>+</sup> channel (VGSC) expression and its controlling mechanism(s) are not well understood. In this study, we investigated the possible involvement of nerve growth factor (NGF) in regulating VGSC activity in the strongly metastatic Mat-LyLu cell model of rat prostate cancer (PCa). NGF increased peak VGSC current density in a time- and dose-dependent manner. NGF also shifted voltage to peak and the half-activation voltage to more positive potentials, and produced currents with faster kinetics of activation; sensitivity to the VGSC blocker tetrodotoxin (TTX) was not affected. The NGF-induced increase in peak VGSC current density was suppressed by both the pan-trk antagonist K252a, and the protein kinase A (PKA) inhibitor KT5720. NGF did not affect the Nav1.7 mRNA level, but the total VGSC  $\alpha$ -subunit protein level was upregulated. NGF potentiated the cells' migration in Transwell assays, and this was not affected by TTX. We concluded that NGF upregulated functional VGSC expression in Mat-LyLu cells, with PKA as a signalling intermediate, but enhancement of migration by NGF was independent of VGSC activity.

### Keywords

Mat-LyLu; NGF; PKA; Prostate cancer; Voltage-gated Na<sup>+</sup> channel

## INTRODUCTION

Voltage-gated Na<sup>+</sup> channels (VGSCs) are expressed not just in 'excitable' tissues (nerve and muscle), but also in a variety of 'non-excitable' cells, including lymphocytes (DeCoursey et al., 1985), endothelial cells (Gordienko and Tsukahara, 1994), fibroblasts (Bakhrarov et al., 1995), and glial cells (Chiu and Ritchie, 1984). Importantly, VGSC expression is highly dynamic, although the underlying mechanisms are not well understood (Diss et al., 2004). Nerve growth factor (NGF) is a member of the neurotrophin (NT) family of secreted proteins, which are well known for their involvement in neuronal growth promotion, survival, differentiation, plasticity and functional maintenance (Kovalchuk et al., 2004; Lu et

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al., 2005). Among the functional targets of NGF signalling are VGSCs (Dib-Hajj et al., 1998; Hilborn et al., 1997; Toledo-Aral et al., 1995). NGF may be associated with a variety of downstream signalling intermediates, including protein kinase A (PKA) (D'Arcangelo et al., 1993; Kalman et al., 1990).

VGSC upregulation has been found in human prostate cancer (PCa) in vitro and in vivo, and correlated with metastatic progression (Diss et al., 2005). In vitro, VGSC activity has been shown to potentiate a variety of cell behaviours associated with the metastatic cascade, including morphological development and cellular process extension (Fraser et al., 1999), galvanotaxis (Djamgoz et al., 2001), lateral motility (Fraser et al., 2003), endocytic membrane activity (Krasowska et al., 2004; Mycielska et al., 2003), gene expression including activity-dependent regulation (Brackenbury and Djamgoz, 2006; Mycielska et al., 2005), and invasion (Bennett et al., 2004; Grimes et al., 1995; Laniado et al., 1997; Smith et al., 1998). These findings imply that VGSCs are tonically active in metastatic PCa cells, and enhance metastatic cell behaviour. In fact, functional VGSC expression was considered to be “necessary and sufficient” for potentiation of PCa cell invasiveness (Bennett et al., 2004).

In the strongly metastatic Dunning rat PCa Mat-LyLu cell line, VGSC/Nav1.7  $\alpha$ -subunit mRNA was upregulated over 1000-fold, compared to the isogenic weakly metastatic AT-2 cells (Diss et al., 2001). Although the mechanism responsible for the VGSC upregulation is not yet known, serum concentration was found to modify VGSC current amplitude and kinetics, raising the possibility of modulation of VGSC expression/activity by growth factor(s) (Ding and Djamgoz, 2004). Interestingly, prostate contains one of the highest levels of NGF outside the nervous system (MacGrogan et al., 1992; Murphy et al., 1984). NGF also plays a significant role in proliferation, differentiation and apoptosis in a variety of cancers (Nakagawara, 2001). As regards PCa, it has been shown that anchorage-independent growth of weakly metastatic human LNCaP cells can be stimulated by NGF (Delsite and Djakiew, 1999). Furthermore, NGF secretion has been detected in the strongly metastatic DU145 and PC-3 cell lines, and this enhanced the cells' invasive capacity in vitro (Djakiew et al., 1993; Geldof et al., 1997).

The available data, taken together, would raise the possibility, therefore, that NGF could be involved in VGSC regulation in PCa cells. In the present study, we investigated this possibility, using the rat Mat-LyLu cell model of metastatic PCa.

## MATERIALS AND METHODS

### Cell culture and pharmacological treatments

Mat-LyLu cells were cultured as described before (Grimes and Djamgoz, 1998). Prior to addition of any pharmacological agent, including NGF, cells were seeded in normal medium for 24 h, then washed 5 times in serum-free RPMI 1640. For some migration assays, cells were maintained for an additional 24 h in serum-free RPMI 1640 prior to addition of compounds. The following agents were used, added to serum-free RPMI 1640: NGF (1-100 ng/ml; Alomone), K252a (100 nM; Calbiochem), KT5720 (500 nM; Calbiochem), tetrodotoxin (TTX; 1  $\mu$ M; Alomone). K252a is a general inhibitor of trk (including NGF) receptors (Mallei et al., 2004; Shimazu et al., 2005; Turner et al., 2004). KT5720 has

previously been shown to inhibit PKA in a range of cells, including cancer (Ungefroren et al., 1997; Yang et al., 2003; Yoshida et al., 2005). We have previously shown that KT5720 would completely inhibit PKA activity in Mat-LyLu cells (Brackenbury and Djamgoz, 2006). All pharmacological agents used were non-toxic at their working concentrations, as described previously (Fraser et al., 2003).

## Electrophysiology

Whole-cell patch clamp recordings were performed on single cells as described previously (Grimes and Djamgoz, 1998). Three voltage-clamp protocols were used (holding potential =  $-100$  mV):

1. Basic current-voltage (I-V) protocol: Cells were depolarised to test potentials within the range  $-70$  to  $+70$  mV in 5 mV steps. The test pulse duration was 60 ms; the interpulse duration was 2 s.
2. Steady-state inactivation protocol: Prepulses in the range  $-130$  to  $-10$  mV were applied in 10 mV steps for durations of 1 s. A test pulse of  $-10$  mV was immediately applied for 80 ms. The interpulse duration was 2 s.
3. TTX protocol: One test pulse of  $-10$  mV (duration 60 ms) was applied while cell was perfused with normal external bath solution. TTX (1 nM - 6  $\mu$ M) was applied to the cell for 30 s and then the test pulse was repeated. Reversibility of the effect of TTX was tested after returning to normal external bath solution and presenting a final test pulse.

Recordings were obtained from up to 20 cells per condition, from at least 3 repeat treatments. Data from individual dishes were combined to provide an overall mean and standard error (SEM).

## Real-time PCR

Extraction of total RNA, cDNA synthesis and real-time PCR were performed as before (Mycielska et al., 2005). Cytochrome b5 reductase (Cytb5R) gene, shown previously to be unchanged in rat PCa, was the 'internal' control gene (Diss et al., 2001). The following primer pairs were used:

1. Nav1.7: 5'-TTCATGACCTTGAGCAACCC-3' and 5'-TCTCTTCGAGTTCCTTCCTG-3'; annealing temperature, 60 °C;
2. Cytb5R: 5'-ACACGCATCCCAAGTTTCCA-3' and 5'-CATCTCCTCATTACGAAGC-3'; annealing temperature, 60 °C.

The threshold amplification cycles were determined using the Opticon Monitor 2 software (MJ Research) and then analysed by the  $2^{-CT}$  method (Livak and Schmittgen, 2001). The level of Nav1.7 mRNA was compared relative to untreated control cells, for three separate treatments.

## Western blotting

Western blots were performed as described previously (Laniado et al., 1997). The primary antibodies used and their dilutions were as follows:

1. Pan-VGSC antibody (1 µg/ml; Upstate), and
2. Anti-actinin antibody (1 µl/ml; Sigma).

The secondary antibodies were peroxidase-conjugated swine anti-rabbit, and goat anti-mouse, respectively (Dako). Densitometric analysis was performed using Image- Pro Plus software (Media Cybernetics). Signal intensity was normalised to anti- actinin antibody as a loading control/reference, for at least 3 separate treatments.

### Immunocytochemistry and confocal microscopy

Immunocytochemistry was performed as described previously (Chioni et al., 2005). Cells were labelled first with fluorescein isothiocyanate (FITC)-conjugated concanavalin A (Sigma) for 20 min as a plasma membrane marker, and then permeabilised in saponin (0.1 %) for 5 min. Cells were incubated with the pan-VGSC primary antibody for 1 h. The secondary antibody was Alexa567-conjugated goat anti-rabbit IgG (Dako) and mounting was in Vectashield (Vector Laboratories). Samples were viewed using a Leica DM IRBE microscope (with a ×100 objective) with a confocal laser scanner (Leica TCS-NT with Ar/Kr laser).

### Digital image analysis

Densitometric analysis was performed using the LCS Lite software (Leica), as follows:

1. Protein distribution was determined using the “straight line profile” function drawn across the cytoplasm avoiding the nucleus, as described previously (Brackenbury and Djamgoz, 2006). Signal intensity in plasma membrane region, set to cover 1.5 µm inward from the edge of concanavalin A staining, was compared with cytoplasmic signal intensity within the central 30 % of the line profile. Measurements were taken from 6 cells (randomly chosen) per condition, for three repeat treatments.
2. Cell surface VGSC expression was determined using the “freeform line profile” function drawn around the cell surface, determined by concanavalin A staining. Measurements were taken from 30 cells per condition, for three repeat treatments.
3. Internal protein level was assessed in 4.8 µm<sup>2</sup> rectangular sections using the “area histogram” function. Measurements were taken from 30 cells per condition, for three repeat treatments.

### Migration assay

In order to achieve steady-states, cells were maintained for 24 h in serum-free medium, prior to treatment with NGF (20 ng/ml) and/or TTX (1 µM) for a further 24 h. Cells ( $1.5 \times 10^5$  cells/ml) were then plated onto 12 µm-pore Transwell filters in a 12-well plate, according to the manufacturer’s instructions (Corning), in a 0.1-1 % FBS chemotactic gradient. The number of cells migrating over 7 h was determined using the MTT assay (Grimes et al., 1995). Results were compiled as the mean of five repeats containing at least two platings.

## Curve fitting and data analysis

Conductance-voltage relationships and curve fitting were performed as described previously (Ding and Djamgoz, 2004). TTX dose-response data were fitted using Excel (Microsoft) and Origin (OriginLab, Northampton, MA) software to a Langmuir adsorption isotherm:

$$y=1/(1+[TTX]/IC_{50})$$

where  $IC_{50}$  is the concentration of TTX for 50 % VGSC blockage. All quantitative data are presented as means  $\pm$  standard errors, unless stated otherwise. Statistical significance was determined with Student's t test, or ANOVA followed by Newman-Keuls post hoc analysis, as appropriate. Results were considered significant at  $P < 0.05$  (\*).

## RESULTS

### NGF increased VGSC functional expression in a dose-dependent manner

Pre-incubation of Mat-LyLu cells in NGF (20 ng/ml) for 24 h significantly increased peak VGSC current density by 62 %, from  $-30.8 \pm 5.6$  pA/pF to  $-49.9$  pA/pF ( $P < 0.05$ ;  $n = 20$  for each; Figure 1A, B). Lower (1 ng/ml) and higher (100 ng/ml) concentrations of NGF had no effect (Figure 1B). Accordingly, 20 ng/ml NGF was adopted as the working concentration in the remainder of the experiments. Short-term (30 min) incubation with 20 ng/ml NGF had no effect on peak VGSC current density.

The effect of 24 h incubation with NGF (20 ng/ml) on VGSC characteristics was studied further (Table I). NGF significantly depolarised voltage for current peak ( $V_p$ ) from  $-5.0 \pm 2.1$  mV to  $2.3 \pm 2.1$  mV ( $P < 0.05$ ;  $n = 20$  for each) and also depolarised the half-activation voltage ( $V_{1/2}$ ) from  $-19.7 \pm 1.6$  mV to  $-15.3 \pm 1.4$  mV ( $P < 0.05$ ;  $n = 20$  for each; Figures 1C, 2A). There was no effect on activation voltage, activation slope factor, or steady-state inactivation (Table I). There was a window current between  $-55$  mV and  $-10$  mV in control cells, and this changed slightly to between  $-50$  mV and  $-20$  mV in NGF-treated cells (Figure 2A inset). The NGF treatment partially reduced the voltage dependency of time to peak ( $T_p$ ; Figure 2B, Table I). There was no effect on fast ( $\tau_f$ ) and slow ( $\tau_s$ ) time constants of inactivation at 10 mV, derived from double-exponential fits (Table I). Similarly, NGF had no effect on the cells' TTX sensitivity profile (Figure 2C), and the  $IC_{50}$  was unchanged at  $\sim 21$  nM (Table I).

Incubation with K252a (100 nM), a pan-trk receptor inhibitor (Mallei et al., 2004; Shimazu et al., 2005; Turner et al., 2004) for 24 h had no effect on peak VGSC current density ( $P = 0.19$ ;  $n = 20$  for each). However, co-application of K252a with NGF (20 ng/ml) blocked the increasing effect of NGF on peak current density ( $P = 0.58$  compared with K252a alone;  $n > 19$  for each).

In summary, the 24 h NGF treatment upregulated VGSC functional expression. TTX sensitivity was unchanged, consistent the VGSC isoform expression profile remaining the same.

### **Inhibition of PKA abrogated the effect of NGF on VGSC current enhancement**

Treatment with KT5720 (500 nM), a PKA inhibitor (Brackenbury and Djamgoz, 2006; Cabell and Audesirk, 1993) for 24 h also had no effect on peak VGSC current density ( $P = 0.51$ ;  $n > 19$  for each; Figure 3). However, when co-applied with NGF (20 ng/ml), KT5720 blocked the effect of NGF on peak VGSC current density ( $P = 0.54$ , cf. KT5720 alone;  $P < 0.01$  cf. NGF alone;  $n > 19$ ; Figure 3).

These data are consistent with NGF increasing VGSC functional expression via PKA activity. We next analysed at what level(s) (mRNA, protein, post-translational modification) the VGSC upregulation occurred.

### **NGF had no effect on Nav1.7 mRNA expression but increased VGSC protein level**

Mat-LyLu cells were treated with NGF and/or K252a for 24 h, after which the mRNA level of Nav1.7, the predominant VGSC isoform expressed in PCa cells (Diss et al., 2001), was assessed by real-time PCR. NGF (20 ng/ml) did not significantly affect the Nav1.7 mRNA level ( $P = 0.53$ ;  $n = 3$ ; Figure 4A). Similarly, K252a (100 nM) had no effect, either when applied alone, or when co-applied with NGF ( $P = 0.56$  and  $0.30$ , respectively;  $n = 3$ ; Figure 4A).

Treatment with NGF for 24 h increased the total VGSC  $\alpha$ -subunit protein level, detected by Western blot with a pan-VGSC antibody, by 49 % ( $P < 0.05$ ;  $n = 3$ ; Figure 4B). Consistent with this, confocal immunocytochemistry with the pan-VGSC antibody also revealed that NGF increased the VGSC  $\alpha$ -subunit protein level (Figure 5A). After treatment with NGF for 24 h, the level of internal and plasma membrane VGSC  $\alpha$ -subunit protein levels increased by  $35 \pm 0.5$  % and  $59 \pm 0.5$  %, respectively ( $P < 0.001$  for both;  $n = 90$  cells for each; Figure 5A-C).

The distribution of VGSC immunoreactivity along cellular cross-sections was quantified and two regions were compared: (1) 'plasma membrane' and (2) 'internal' (Brackenbury and Djamgoz, 2006). The relative level of VGSC protein in both regions was unaffected by the NGF treatment, consistent with there being no change in the trafficking ( $P = 0.48$  and  $P = 0.93$ , respectively;  $n = 20$  cells for each; Figure 5D). We concluded that NGF increased VGSC  $\alpha$ -subunit protein expression, without affecting the Nav1.7 mRNA level, or the cycling balance of VGSC proteins.

### **NGF increased migration in vitro**

Pre-treatment of Mat-LyLu cells with NGF (20 ng/ml) for 24 h increased migration by 49 % ( $P < 0.05$ ;  $n = 12$ ; Figure 6). In contrast, under these conditions, TTX (1  $\mu$ M) had no effect compared to control ( $P = 0.134$ ;  $n = 12$ ). In the presence of TTX, NGF could still increase migration ( $P < 0.001$  cf. TTX alone;  $n = 12$ ). In fact, there was no difference between the effects of NGF alone, and NGF + TTX ( $P = 0.83$ ;  $n = 12$ ). It was concluded that in serum-free growth medium, NGF increased migration, without any VGSC involvement.

## DISCUSSION

The main conclusions of this study are as follows: (1) NGF increased total and plasma membrane VGSC protein levels in Mat-LyLu cells, without affecting the level of Nav1.7 mRNA, or the balance of protein cycling. (2) NGF increased VGSC current density, via trk receptor(s). (3) The NGF-induced increase in VGSC current density was dependent on PKA activity. (4) NGF increased migration in vitro, independent of VGSC activity.

### Involvement of trk receptors and PKA

Treatment with NGF for 24 h increased VGSC peak current density in a bell-shaped dose-dependent manner, as reported before for growth factors (e.g. Meng et al., 2006; Neal et al., 2003; Wei et al., 2004). Co-application of the pan-trk receptor inhibitor K252a (Mallei et al., 2004; Shimazu et al., 2005) with NGF prevented the NGF-induced increase in VGSC current density, consistent with the NGF effect being mediated by trk receptor activation. TrkA receptors were previously shown to be expressed in various Dunning cell lines and the K252a analogue CEP-751 inhibited their in vivo growth (Dionne et al., 1998).

Importantly, in Mat-LyLu cells, the NGF-induced increase in VGSC current density was blocked completely by the PKA inhibitor KT5720 (Figure 3). It has also been reported elsewhere that NGF could increase VGSC functional activity by activating PKA. For example, in PC12 cells, treatment with NGF or activation of PKA (with forskolin or 8-Br-cyclic AMP) for 1-10 days, increased VGSC current density (Bouron et al., 1999; Furukawa et al., 1993), and this could be via activation of PKA (D'Arcangelo et al., 1993; Kalman et al., 1990).

### Effects of NGF on VGSC mRNA and protein levels

Treatment for 24 h with NGF did not significantly affect the mRNA level of Nav1.7, the predominant isoform expressed in Mat-LyLu cells (Diss et al., 2001). However, similar treatment increased the total VGSC protein level and membrane current density. These data suggested that NGF induced de novo VGSC protein synthesis, also consistent with 'short-term' (30 min) treatment having no effect. The extent of the VGSC protein increase was similar for both the cytoplasm and the plasma membrane. This could be due to the following: (1) The effect of NGF was transcriptional, but included de novo synthesis of mRNA of VGSC isoform(s) other than Nav1.7. For example, NGF has previously been shown to induce Nav1.2 mRNA expression in PC12 cells (D'Arcangelo et al., 1993). (2) The effect of NGF on transcription of Nav1.7 and/or other VGSC isoforms was transient, and occurred prior to the PCR assay at 24 h. (3) The NGF effect was mainly post-transcriptional, upregulating the level of VGSC proteins. Whilst these could include Nav1.7, TTX-resistant VGSCs (Nav1.5, Nav1.8 and Nav1.9) can be ruled out since the  $IC_{50}$  for TTX did not change. Regulation of mRNA and protein levels may be separate and independent (Gu et al., 2006; Martin and Zukin, 2006; Orphanides and Reinberg, 2002; Pfeiffer and Huber, 2006; Ropponen et al., 2001; Schedel et al., 2004; Sola et al., 1999). Furthermore, mRNA localisation/degradation, and translational control processes may be involved (Ben Fredj et al., 2004; St Johnston, 2005; Tiedge et al., 1999). Interestingly, PKA itself has been found to control localised protein synthesis from docked mRNA (Smith et al.,

2005). (4) The NGF/PKA-induced upregulation of VGSC protein level was caused by an increase in protein stability, e.g. by reducing ubiquitination (Hino et al., 2005). Further work would be required to evaluate these possibilities, and to confirm these effects in other PCa cell lines, including those of human origin.

### NGF, VGSC activity and control of migration

Overall, the data taken together with the published evidence are consistent with NGF increasing VGSC functional expression in Mat-LyLu cells, as follows:

NGF → Trk receptor → PKA activation (Bouron et al., 1999; D'Arcangelo et al., 1993; Kalman et al., 1990) → VGSC  $\alpha$ -subunit protein upregulation → increased functional VGSC availability (Wada et al., 2004; Yuhi et al., 1996; Zhou et al., 2000). This scheme does not exclude the possibility that the pro-migratory effect of NGF could occur through PKA.

Pre-treatment of Mat-LyLu cells in serum-free growth medium with NGF for 24 h enhanced migration by ~50 %, in general agreement with the potentiating role of NGF in PCa metastasis (Geldof et al., 1997; Montano and Djamgoz, 2004; Sortino et al., 2000). Interestingly, similar pre-treatment with TTX did not significantly reduce migration in the presence or absence of NGF, suggesting that in serum-free conditions, VGSC activity was not involved in potentiating migration, and that the enhancement by NGF was independent of VGSC activity. However, other ion channels may play a role, e.g. voltage-gated  $K^+$  channels (Kim et al., 2004; O'Grady and Lee, 2005). In contrast, we have previously shown that when Mat-LyLu cells were grown in 1 % serum, TTX inhibited migration (Brackenbury and Djamgoz, 2006). Thus, different serum factor(s) may be required for the VGSC-dependent potentiation of migration and/or the NGF-induced upregulation of VGSC activity could enhance other component(s) of the metastatic cascade.

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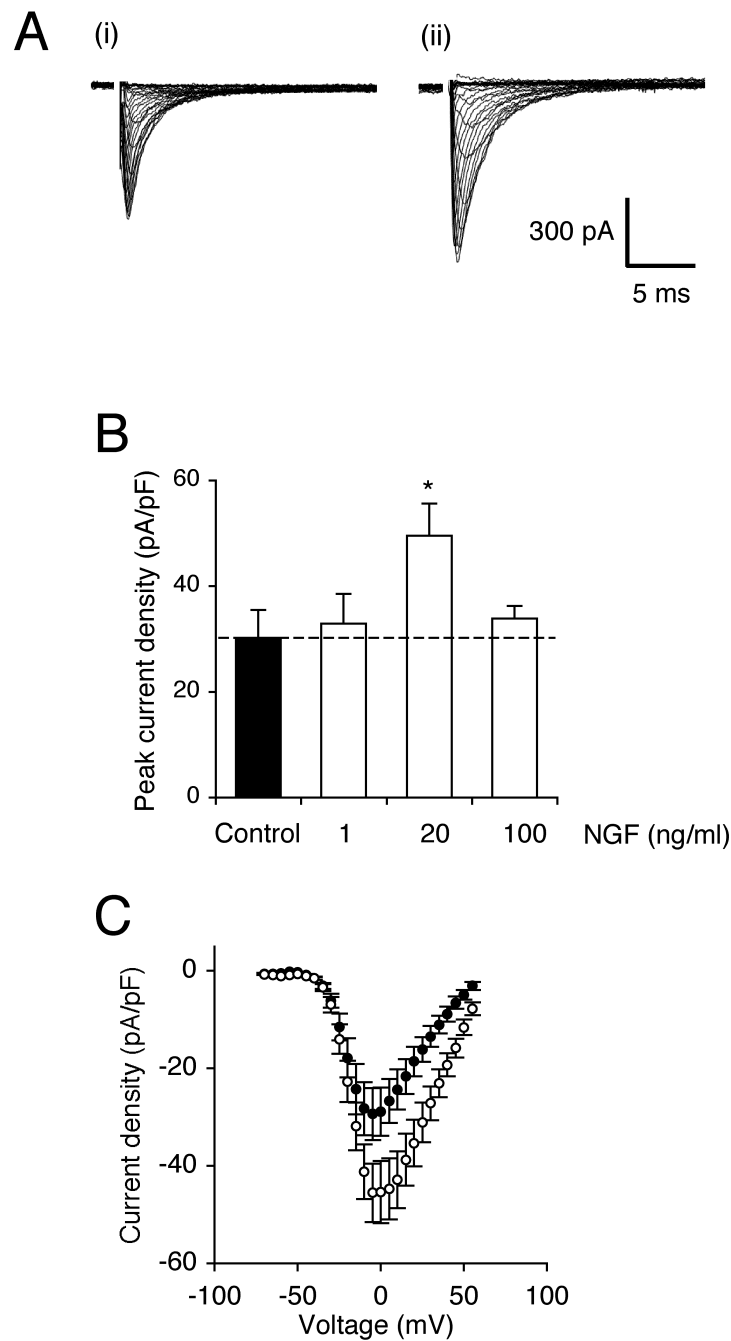


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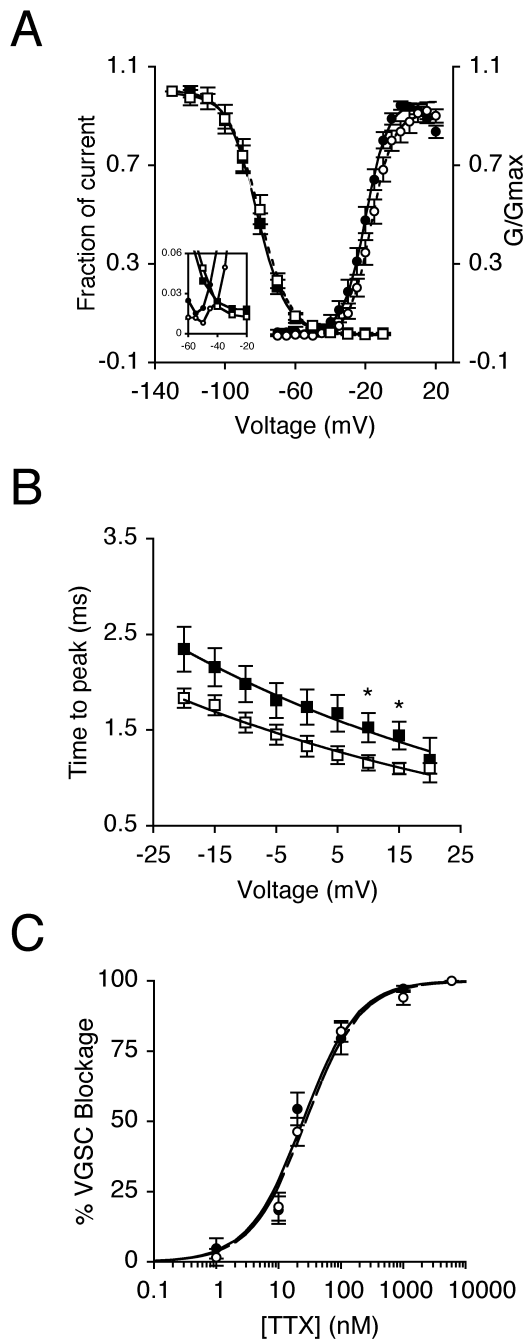
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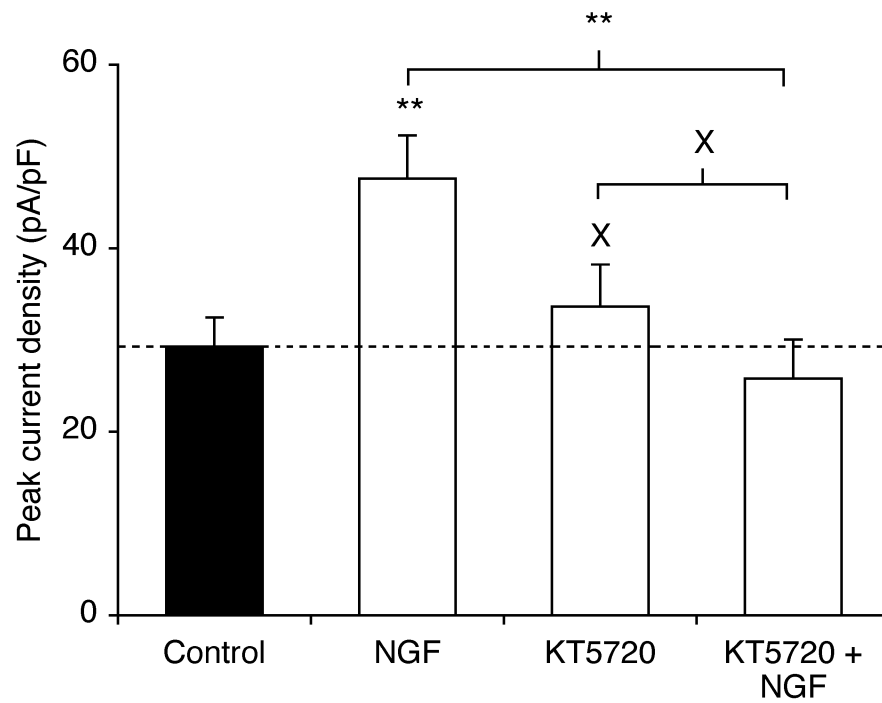
**Figure 1. NGF increased peak VGSC current density.**

(A) Typical whole-cell VGSC currents elicited by 60 ms depolarising voltage pulses between  $-70$  mV and  $+70$  mV applied from a holding potential of  $-100$  mV: (i) a control cell; (ii) a cell pre-treated with 20 ng/ml NGF for 24 h. (B) Quantitative comparison of peak current densities recorded in control cells and cells pre-treated with 1-100 ng/ml NGF for 24 h. (C) Mean current-voltage relationships for control cells (dark circles) and cells pre-treated with 20 ng/ml NGF for 24 h (light circles). Data are presented as mean  $\pm$  SEM ( $n = 20$ ). Significance: (\*)  $P < 0.05$ ; ANOVA with Newman-Keuls.



**Figure 2. NGF decreased time to peak but did not affect TTX sensitivity.** (A) Mean availability- voltage (squares) and relative conductance ( $G/G_{max}$ )-voltage relationships (circles) for control cells (dark symbols) and cells pre-treated with 20 ng/ml NGF for 24 h (light symbols). Control (solid lines) and NGF data (dotted lines) are fitted with Boltzmann functions. Inset magnifies a window in which current is activated and not fully inactivated. (B) Dependence of time to peak on membrane voltage for control cells (dark squares), and cells treated with 20 ng/ml NGF for 24 h (light squares). Control (solid line) and NGF data (dashed line) are fitted with single exponential functions. (C) Reduction

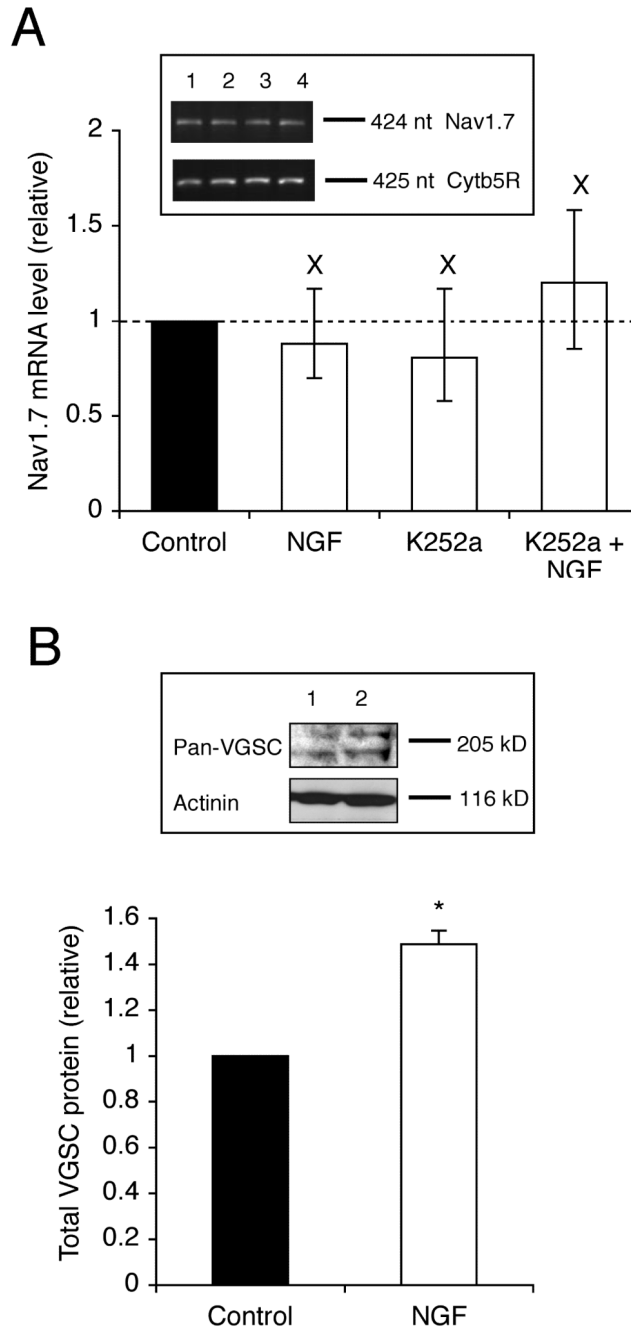
of VGSC current by TTX for control cells (dark circles), and cells treated with 20 ng/ml NGF for 24 h (light circles). Control (solid line) and NGF data (dashed line) are fitted to Langmuir adsorption isotherms. Data are presented as mean  $\pm$  SEM ( $n > 17$ ). Significance: (\*)  $P < 0.05$ ; Student's t test.



**Figure 3. The PKA inhibitor KT5720 reversed the potentiating effect of NGF on peak current density.**

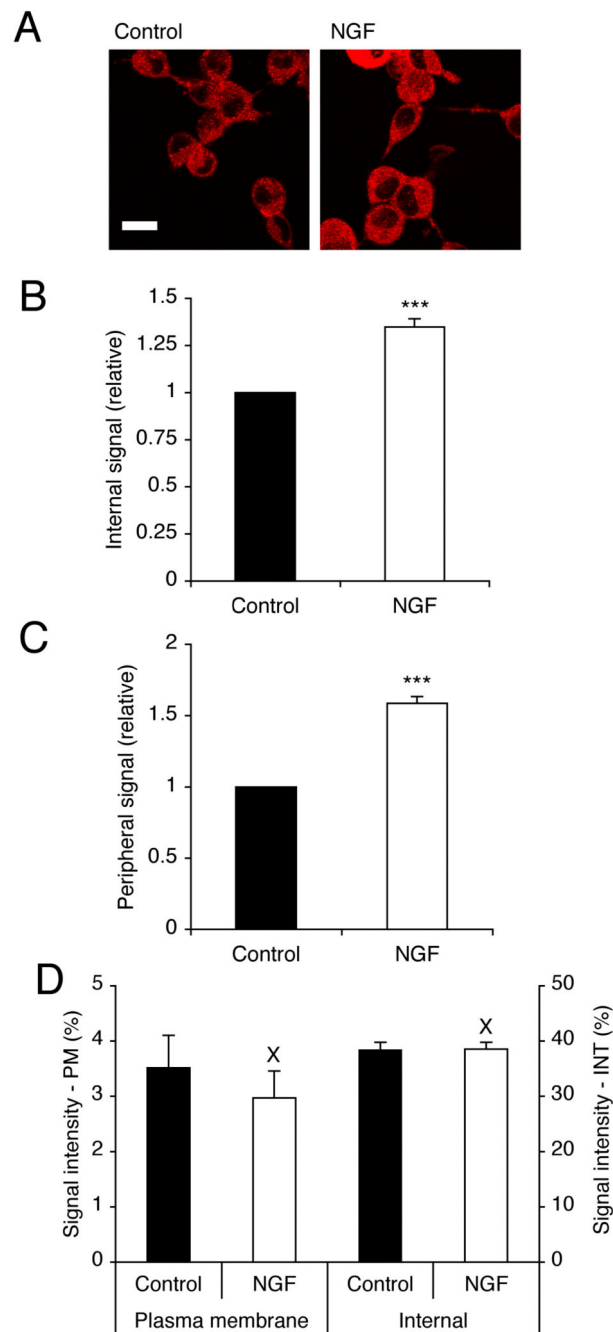
Peak current densities were recorded after pre-treatment for 24 h in control conditions, or with NGF (20 ng/ml) and/or KT5720 (500 nM). Data are presented as mean and SEM (n > 19). Significance: (X)  $P > 0.05$  cf. control; (\*)  $P < 0.05$ ; ANOVA with Newman-Keuls.





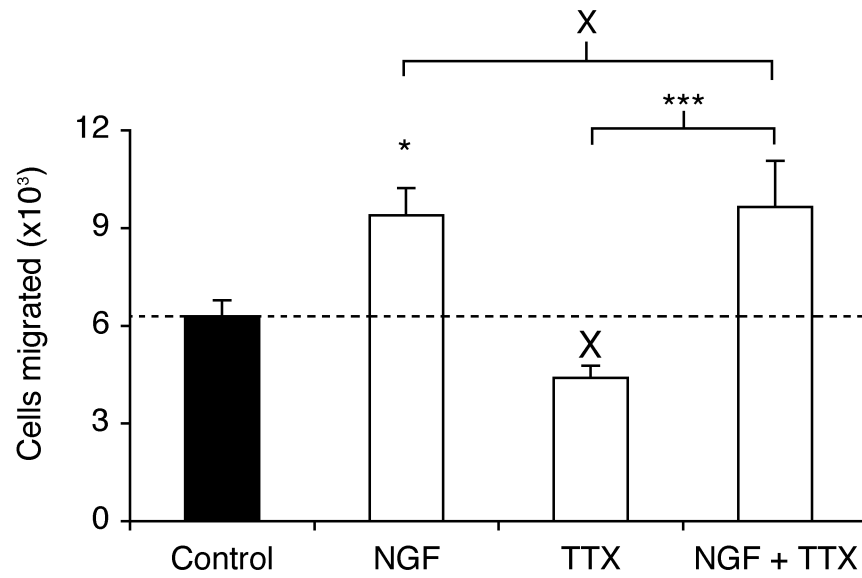
**Figure 4. NGF did not affect the Nav1.7 mRNA level, but increased the total VGSC protein level.** (A) Relative Nav1.7 mRNA levels in control cells and cells treated for 24 h with NGF (20 ng/ml), K252a (100 nM), and NGF (20 ng/ml) + K252a (100 nM). The Nav1.7 level was normalised to cytochrome-b5 reductase (Cytb5R) by the  $2^{-Ct}$  method. Errors are propagated through the  $2^{-Ct}$  analysis. Inset, typical gel images of PCR products for Nav1.7 and Cytb5R. Lanes: 1, control; 2, pre-treated for 24 h with NGF (20 ng/ml); 3, K252a (100 nM); 4, NGF (20 ng/ml) + K252a (100 nM). (B) Relative total VGSC protein level in control cells and cells treated with NGF (20 ng/ml) for 24 h. The VGSC  $\alpha$ -subunit

protein level was normalised to the actinin control. Inset, Western blot with 60  $\mu$ g of total protein per lane from cells treated with or without NGF (20 ng/ml) for 24 h, using a pan-VGSC antibody, and an actinin antibody as a control for loading. Data are presented as mean and SEM (n = 3). Significance: (X)  $P > 0.05$ ; (\*)  $P < 0.05$ ; (A) ANOVA with Newman-Keuls; (B) Student's t test.



**Figure 5. NGF increased VGSC immunoreactivity internally and at the plasma membrane.** (A) Typical confocal images of control cells, and cells treated with NGF (20 ng/ml) for 24 h, immunolabelled with pan-VGSC  $\alpha$ -subunit antibody. Scale bar, 15  $\mu$ m. (B) Relative internal VGSC protein level in control cells, and cells treated with NGF (20 ng/ml) for 24 h. (C) Relative peripheral VGSC protein level in control cells, or cells treated with NGF (20 ng/ml) for 24 h. (D) VGSC protein distribution along subcellular cross-sections (%). Left-hand bars, 1.5  $\mu$ m sections measured inward from edge of concanavalin A staining; Right-hand bars, middle 30 % of cross-section. PM, plasma membrane; INT, internal. Data are

presented as mean and SEM (B, C n = 90; D, n = 20). Significance: (X)  $P > 0.05$ ; (\*\*\*)  $P < 0.001$ ; Student's t test.



**Figure 6. NGF increased the relative number of cells migrating through a Transwell chamber over 7 h.**

Cells were grown for 24 h in serum-free medium, or with NGF (20 ng/ml), TTX (1  $\mu$ M), or NGF (20 ng/ml) + TTX (1  $\mu$ M), prior to the Transwell assay. Data are presented as mean and SEM (n = 12). Significance: (X)  $P > 0.05$ , (\*)  $P < 0.05$ ; ANOVA with Newman-Keuls.

**Table 1**  
**Effect of 24 h treatment with NGF (20 ng/ml) on VGSC characteristics in Mat-LyLu cells**

Parameter <sup>a</sup>	Control	NGF	P
I <sub>p</sub> (pA/pF)	-30.8±5.6	-49.9±6.2	0.03*
V <sub>a</sub> (mV)	-41.3±1.5	-40.8±1.2	0.77
V <sub>p</sub> (mV)	-5.0±2.1	2.3±2.1	0.02*
Activation V <sub>1/2</sub> (mV)	-19.7±1.6	-15.3±1.4	0.04*
Activation k (mV)	5.4±0.4	5.4±0.3	0.99
Inactivation V <sub>1/2</sub> (mV)	-84.2±2.9	-80.6±1.7	0.30
Inactivation k (mV)	-7.0±0.5	-8.0±0.3	0.11
T <sub>p</sub> at 10 mV(msec)	1.7±0.2	1.2±0.1	0.04*
T <sub>f</sub> at 10 mV(msec)	0.9±0.1	0.8±0.04	0.46
T <sub>s</sub> at 10 mV(msec)	4.7±0.4	4.8±0.2	0.79
IC <sub>50</sub> (nM)	21.4±4.8	20.7±5.9	0.93

<sup>a</sup>I<sub>p</sub>, peak current density; V<sub>a</sub>, activation voltage; V<sub>p</sub>, voltage at current peak; V<sub>1/2</sub>, half-(in)activation voltage; k, slope factor; T<sub>p</sub>, time to peak; T<sub>f/s</sub>, fast/slow time constant of inactivation. IC<sub>50</sub>, concentration of TTX at which 50% of VGSC current is inhibited. Data expressed as mean ±SEM (n = 6).

\* P < 0.05; Student's *t*-test.