ENaC PROTEINS ARE REQUIRED FOR NGF-INDUCED NEURITE GROWTH

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Neurite growth is required for nervous system development and repair. Multiple signals, including neurotrophic factors and intact mechanosensing mechanisms, interact to regulate neurite growth. Degenerin/Epithelial Na⁺ Channel (DEG/ENaC) proteins have been identified as putative mechanosensors in sensory neurons. Recently, others have shown the neurotrophic factor Nerve Growth Factor (NGF), stimulates expression of Acid-Sensing Ion Channel (ASIC) molecules, members of the DEG/ENaC family. However, it is unknown if NGF regulates ENaC expression or if ENaC expression is required for neurite formation. Therefore, the aims of this study are to determine if ENaC expression is 1) regulated by NGF and 2) required for NGF-induced neurite growth in PC12 cells. We found NGF induced expression of β and γ but not αENaC. Tyrosine kinase A (TrkA) receptor blockade abolished NGF-induced β and γENaC expression and neurite formation. Disruption of ENaC expression using 1) pharmacological blockade with benzamil, a specific ENaC inhibitor, 2) siRNA and 3) dominant-negative ENaC molecules, inhibited NGF-induced neurite formation. These data indicate NGF-TrkA regulation of ENaC expression may be required for neurite growth and may suggest a novel role for DEG/ENaC proteins in neuronal remodeling and differentiation.

**Key Words:** Mechanosensation, degenerins, neurotrophins, TrkA, PC12 cells.
INTRODUCTION

Neurite growth is required for development of the nervous system, target organ innervation, neural control of organ systems and nerve regeneration following neuronal injury. Neurotrophic factors are well studied regulators of neurite growth and include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and insulin growth factor (5, 11, 26). NGF stimulated neurite growth is primarily mediated by activation of high-affinity tyrosine kinase A (TrkA) receptors, and to a smaller extent, activation of low-affinity p75 receptors (3, 8). However, the down-stream mechanisms by which neurotrophic factors stimulate neurite formation are not fully understood.

One potential mechanism is neurotrophins may induce expression of putative mechanosensory elements, which permit interactions between neurons and their extracellular environment. Three lines of evidence support this possibility. First, mechanosensitive proteins are found at the site of growth in nerve endings and growth cones (4, 25, 27, 28, 43). Second, normal neurite formation and elongation require activation of mechanosensitive proteins (17, 32, 39). Third, neurotrophic factors upregulate putative mechanosensory proteins in sensory neurons (7, 29, 30). These data suggest mechanosensitive elements contribute to neurite growth and may be regulated by neurotrophic factors.

Members of the Degenerin/Epithelial Na⁺ Channel (DEG/ENaC) protein family have been identified as potential components of mechanosensitive ion channel complexes involved in baroreception, touch sensation and proprioception in sensory neurons (1, 9,
Despite the fact that Acid-Sensing Ion Channel (ASIC) transcripts, DEG/ENaC family members, are NGF targets (10, 29, 30), the role of mechanosensory DEG/ENaC proteins in neurite formation has never been addressed. Therefore, we wanted to address the hypothesis that NGF induces neurite formation by stimulating expression of mechanosensory ENaC proteins. To address this hypothesis, we determined if 1) NGF increases expression of ENaC message and protein, 2) NGF-induced ENaC expression is mediated by TrkA receptor activation, and 3) disruption of ENaC and ASIC1 expression prevents NGF-induced neurite growth in neuronal PC12 cells. Our results suggest ENaC expression is regulated by NGF-TrkA/p75 receptor interactions and required for NGF-induced neurite growth.
MATERIALS AND METHODS

*PC12 Cell Culture.* Rat PC12 cells (ATCC, Manassas, VA), a neuronal cell model commonly used to study neurite formation, were maintained in 75cm² culture flasks in culture medium [Kaighn’s modification of Ham’s F12 medium (F12K, Invitrogen, Carlsbad, CA) supplemented with 1.5g/L sodium bicarbonate, 15% horse serum, 2.5% fetal bovine serum and 1% penicillin/streptomycin] at 37°C, 5% CO₂. For all experiments, PC12 cells were plated at 2.5 x 10⁵ cells/ml on 100 mm² culture dishes or collagen-coated 8-well glass slides. To determine NGF regulation, culture medium was supplemented with NGF (10 - 100 ng/ml) for 24 h, followed by reverse-transcriptase polymerase chain reaction (RT-PCR) or immunostaining to detect ENaC message or protein as described below. To determine if TrkA or p75 receptors mediate the response, anti-TrkA and anti-p75 receptor antibodies (2 ng/ml; Abcam, Cambridge, MA) were added to the NGF supplemented media. Both antibodies have been used previously to block TrkA and p75 receptor activation (30). Cultures were then immunostained to detect changes in α and β ENaC protein or assayed for neurite growth as described below.

*Reverse Transcription Polymerase Chain Reaction (RT-PCR).* To determine if NGF induces α, β and γ ENaC transcript expression, we used RT-PCR. Total RNA was isolated from harvested cells using RNA STAT-60 (Tel-Test Inc, Friendswood, TX), DNase treated (Ambion, Austin, TX) and quantified by spectrophotometry. RNA (1 µg) was reverse transcribed using random hexamers and Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega, Madison, WI). cDNA was amplified using the following
primer sequences: \[\text{\texttt{\textsuperscript{5}rENaC, 5'-AGTACCTCAGCTACCCAGTGAGC-3'}}\] and \[\text{\texttt{5'-TTGACCGGGACATGCTACCAT-3'}}\]; \[\text{\texttt{\textsuperscript{5}rENaC, 5'-CAAGAAGAAGGCCATGTGTG-3'}}\] and \[\text{\texttt{5'-GTACTGGAAGGGGCTGGAAT-3'}}\]; and \[\text{\texttt{\textsuperscript{5}rENaC, 5'-AATCCTTACAGATACTG-3'}}\] and \[\text{\texttt{5'-TTCTTTTCTCATACTGATG-3'}}\]. Samples were preheated on a Robocycler (Stratagene, La Jolla, CA) to 94°C for 3 min, then cycled 40 times at 94°C for 20s, 60°C (\[\text{\texttt{\textsuperscript{5}rENaC}}\]) or 55°C (\[\text{\texttt{\textsuperscript{5}rENaC}}\] and \[\text{\texttt{\textsuperscript{5}rENaC}}\]) for 20s, and 72°C for 1 min. As negative controls, the reverse transcriptase was omitted from the reverse transcription reaction. PCR products were separated by electrophoresis, visualized with ethidium bromide and sequenced to confirm identity. Expected PCR product sizes are as follows: \[\text{\texttt{\textsuperscript{5}rENaC}}\], 900 bp; \[\text{\texttt{\textsuperscript{5}rENaC}}\], 177 bp; and \[\text{\texttt{\textsuperscript{5}rENaC}}\], 376 bp.

**Semi-quantitative Immunostaining.** To study the effects of NGF and neurotrophin receptor blockade and siRNA on protein expression, we used semi-quantitative immunostaining. In order to use immunofluorescence in a semi-quantitative manner, we used extreme care to maintain uniform experimental conditions. To minimize sampling variability, the following steps were taken. All samples (control and experimental) were 1) run in parallel and treated identically, 2) examined under identical conditions and 3) repeated at least three times. Antigen-affinity purified rabbit anti-\[\text{\texttt{\textsuperscript{5}rENaC}}\] and sheep anti-\[\text{\texttt{\textsuperscript{5}rENaC}}\] antibodies (1:100) have been used previously for immunolabeling (13, 14). Rabbit anti-ASIC1 was obtained from Chemicon (Temecula, CA). Cells were plated on collagen coated 8-well glass slides as described and incubated with NGF (10 ng/ml – 100 ng/ml) for 24 hours to induce neurite formation. Immediately following, all samples were washed in PBS, fixed in 4% paraformaldehyde for 10 minutes, rinsed with PBS.
Samples were air dried on 37°C block to adhere cells to slide. After drying, samples were re-equilibrated in PBS and blocked with 5% normal donkey serum (NDS) for 1 hour. Cells were then co-incubated with anti-ENaC antibodies in 5% NDS (rabbit anti-[ENaC and sheep anti-[ENaC, 1:100) overnight at 4°C, washed with PBS and incubated with fluorescence-labeled secondary antibodies [Cy3-conjugated donkey anti-rabbit F(ab)_2', 1:100 (Jackson ImmunoResearch Laboratories, West Grove, PA); and Alexa 488-conjugated donkey anti-sheep IgG, 1:1000, Molecular Probes, Eugene, OR] for 1 hour. Samples were rinsed with PBS and cover slipped with Gel Mount (Biomeda, Foster City, CA). Immunolabeling was examined using a laser scanning confocal microscope (Leica Microsystems, Exton, PA). Fluorescence intensity was normalized for cell size and analyzed as Relative Fluorescence Units (RFU)/cell area (μm^2).

**Neurite Growth Assay.** Neurite growth was assessed using a standard neurite growth assay (23, 24). PC12 cells were plated on collagen-coated 8 well cover-glass slides as described. Cells were examined using transmitted light-confocal microscopy and cells with neurites twice the length of the soma were considered to have neurites. Neurite growth was quantified as percent of cells with neurites. Clumped cells were eliminated from the analysis. Approximately 100 neurons per group were included in the analysis.

To confirm TrkA and p75 receptor involvement in NGF-stimulated neurite formation, cells plated on 8 well slides were co-incubated with NGF and anti-TrkA and/or p75 receptor antibodies as described earlier. To determine if pharmacological ENaC inhibition blocks neurite formation, cells were treated with NGF (50 ng/ml) in the presence of benzamil (100 nM – 10 μM; Biomol, Plymouth Meeting, PA), a specific
ENaC inhibitor, for 24 h. To determine if expression disruption of specific ENaC molecules blocks NGF-induced neurite formation, cultures were transfected with \[ \Delta \] and/or \[ \Delta \] ENaC siRNA or dominant-negative molecules and maintained for 48-72 h. Neurite formation was induced with NGF (50 ng/ml, 24 h) and assessed using a standard neurite growth assay described.

**siRNA. and Dominant-Negative Fusion Molecules.** To determine if specific DEG/ENaC proteins are required for NGF-induced neurite formation, we knocked down message and protein using siRNA and dominant-negative technology.

Validated siRNA molecules, directed to \[ \Delta r \] ENaC (Scnn_1b, cat. # 16804), \[ \Delta f \] ENaC (Scnn_1g, cat. # 16704) and rASIC1 (ACCN2, cat. #52768, were purchased from Ambion. As a negative control, we used a non-targeting siRNA control molecule that activates the RNA Inducing Silencing Complex (RISC, cat.# D 001210-02, Dharmaco, Chicago, IL). Quantization of knockdown was performed using semi-quantitative immunofluorescence as detailed previously.

Dominant-negative constructs for ENaC proteins (\[ \Delta 41X \] and \[ \Delta 160X \]) were generated by standard PCR cloning techniques. The extreme N-terminal coding regions of \[ \Delta \] and \[ \Delta f \] ENaC cDNA were ligated into Enhanced Green Fluorescent Protein expression vector (pEGFP-C1, BD Biosciences Clontech, Palo Alto, CA) at EcoRI and BamHi restriction sites. Gene fusion regions were sequenced to confirm the N-terminal coding regions were in frame with EGFP. A diagram of the expression constructs is shown in Figure 5A. Dominant negative constructs encoding similar regions of ENaC and other DEG family members are known to effectively silence their expression (1, 21).
Dominant-negative and siRNA molecules were transfected into PC12 cells using lipid-mediated transfection.

*Lipid-Mediated Cell Transfection.* To study the effect of decreased ENaC expression on NGF-induced neurite formation, PC12 cells were transfected with siRNA or dominant-negative EGFP fusion molecules using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were grown on collagen-coated, 8-well glass slides at 2.5 x 10⁵ cells/ml. Cells were transfected upon reaching 70% confluence. Lipofectamine 2000 was complexed with 1 μg/ml cDNA or 100 ng/ml siRNA, at an oligonucleotide: Lipofectamine ratio of 1:2 in Opti-MEM I medium for 4 h. After this, cultures were supplemented with culture medium and maintained for 48-72 h before addition of NGF.

*Statistical Analyses.* Statistical analyses were performed with Sigma Stat 3.0 (SPSS Inc., Chicago, IL). Groups were compared using analysis of variance (ANOVA) and Student Newman-Keuls post hoc tests were performed to determine statistical differences among groups (p ≤ 0.05).
RESULTS

NGF Induces Expression of $\beta$ and $\gamma$ENaC in PC12 Cells. To determine if NGF stimulated ENaC message in PC12 cells, we used reverse transcriptase-polymerase chain reaction (RT-PCR). As shown in Figure 1A, in NGF unstimulated cells, we could not detect expression of $\alpha$, $\beta$ or $\gamma$ENaC transcripts, even following a second round of PCR with nested primers (data not shown). However, after stimulation with NGF (50 ng/ml, 24 h), we were able to detect expression of $\beta$ and $\gamma$ENaC, but not $\alpha$ENaC message. These data indicate NGF induces $\beta$ENaC and $\gamma$ENaC expression at the transcriptional level. We observed similar results with immunostaining as shown in Figure 1B and 1C. In unstimulated cells, $\alpha$ and $\gamma$ENaC relative immunofluorescence intensity was not different from the no-primary antibody negative control, however exposure to NGF increased $\beta$ and $\gamma$ENaC immunofluorescence intensity remarkably (23, 24) (Figure 1B and 1C, respectively). These data show NGF induces $\beta$ and $\gamma$ENaC message and protein in PC12 cells.

TrkA Receptors Primarily Mediate NGF-Induced $\beta$ and $\gamma$ENaC Expression and Neurite Growth. To determine if TrkA or p75 receptors mediate NGF-induced ENaC expression, $\beta$ and $\gamma$ENaC immunolabeling was examined in PC12 cells treated with NGF (10 – 100 ng/ml) in the presence or absence of specific TrkA or p75 antibodies. As shown in Figures 2A and 2B, NGF produces a concentration-dependent increase in $\beta$ and $\gamma$ENaC immunofluorescence intensities (white bars) compared to unstimulated cells. TrkA receptor blockade (grey bars) prevents NGF-induced increases in $\beta$ and $\gamma$ENaC expression with NGF. To a lesser extent, p75 receptor blockade (black bars) also
inhibited ENaC immunolabeling at all NGF concentrations. Unexpectedly, p75 receptor blockade at the highest NGF concentration (100 ng/ml) significantly increased NGF-induced ENaC immunofluorescence intensity.

As shown by others, NGF (50 ng/ml, 24 h) induces neurite formation in approximately 40% of cells, which was prevented by TrkA, and to a lesser extent p75, receptor blockade (Figure 2C).

**Disruption of ENaC Activity Prevents NGF-Induced Neurite Growth.** To determine if ENaC expression is required for neurite formation, we studied NGF-induced neurite growth following ENaC inhibition. We used three different approaches to disrupt ENaC expression; 1) pharmacological blockade of ENaC activity with benzamil (6, 20), 2) gene-silencing using siRNA and 3) protein silencing using expression of dominant-negative molecules.

**ENaC Inhibition with Benzamil Blocks Neurite Formation.** As shown in Figure 3, benzamil (100 nM – 10 μM) prevented NGF-induced neurite formation in a concentration-dependent manner. These data suggest ENaC activity is required for NGF-induced neurite formation.

**\[\alpha\text{ENaC} and \gamma\text{ENaC}, but not ASIC1, siRNA Molecules Block Neurite Formation.** To determine if \[\alpha\text{ENaC}, \gamma\text{ENaC} and ASIC1 molecules are required for neurite formation, we silenced \[\alpha\] and/or \[\gamma\] ENaC gene expression using siRNA. As shown in Figure 4, siRNA molecules to \[\alpha\text{ENaC}, \gamma\text{ENaC} and ASIC1 (Figure 4A and B) suppress expression of their respective proteins. Transfection of \[\alpha\] and/or \[\gamma\] ENaC siRNA molecules inhibited neurite formation compared to negative controls [Lipofectamine alone and non-targeting, RISC-activating
siRNA molecules (Figure 4C)]. These data indicate expression of β and γ ENaC, but not ASIC1, transcripts are required for NGF-mediated neurite formation.

**Expression of Dominant-Negative Constructs Inhibits NGF-Induced Neurite Formation.**

Our third approach to silencing ENaC expression used dominant-negative β and γ ENaC molecules. Transfected cells were identified and visualized using EGFP and fluorescence microscopy. Transfection efficiency, based on EGFP fluorescence, was estimated at approximately 80% (Figure 5B). Compared to EGFP transfected PC12 cells, expression of the β and/or γ ENaC dominant-negative molecules (EGFPβENaC141X and EGFPγENaC160X, respectively) nearly abolished NGF-induced neurite formation (Figure 5C). Combined with our findings on benzamil and β/γ ENaC siRNA molecules, these data suggest β and γ ENaC expression is required for NGF-mediated neurite growth.
DISCUSSION

Mechanosensory Processes Contribute to Neurite Formation. Neurotrophic factor initiation of neurogenesis is well established (5, 11, 26). Yet the down-stream signaling events mediating neurite formation and remodeling are poorly understood. Studies from other labs indicate mechanosensitive processes are required for normal neurite formation. For example, gadolinium (stretch-activated channel blocker) or integrin disruption, block neurite formation (36, 42). Although evidence suggests 1) expression of ASIC transcripts, DEG/ENaC family members, are targets of NGF regulation (10, 29, 30), and 2) DEG/ENaC channels may function as mechanosensors in sensory neurons (1, 9, 13, 15, 33, 35), the role of DEG/ENaC proteins as mechanosensors in neurite formation has never been addressed. In the current investigation, we present two major findings addressing the role of ENaC proteins in neurite formation. First, $\alpha$ and $\beta$ENaC expression is regulated by NGF-TrkA/p75 receptor interactions. Second, ENaC expression is required for neurite formation in neuronal PC12 cells. These data provide evidence DEG/ENaC proteins may contribute to neurite formation.

DEG/ENaC Cation Channel Family. DEG/ENaC proteins are expressed in a diverse range of tissues and species where many are known to form cation-selective ion channels. In epithelial tissue, $\alpha$, $\beta$ and $\gamma$ENaC proteins form a non-voltage gated, sodium selective channel that contributes to sodium and water transport in the kidney, lung and colon (18, 22, 38). A growing body of evidence suggests DEG/ENaC proteins may also play a role in mechanosensation (1, 9, 13, 15, 33, 35). Some DEG/ENaC members are expressed at the site of mechanotransduction in mechanosensitive tissues, such as sensory neurons and
vascular smooth muscle, and disruption of DEG/ENaC activity, using pharmacological inhibition or knock-out animal models, alters mechanosensitive responses in some tissues (13, 15, 34, 35).

**NGF-TrkA/p75 Receptor Interactions Stimulate ENaC Expression in Neurons.** In unstimulated PC12 cells, we were unable to detect $\alpha$, $\beta$ or $\gamma$ ENaC transcript or protein expression. However, upon stimulation with NGF, we were able to detect transcript and protein expression for $\beta$ and $\gamma$ but not $\alpha$ ENaC. This expression pattern has also been observed in sensory neurons (13, 15). Since Mamet et al. (29, 30) have also shown NGF-TrkA receptor interactions stimulate transcription of ASIC molecules, it is not surprising that expression of ENaC molecules is also regulated by NGF.

NGF-induced neurite growth is primarily mediated via high-affinity TrkA receptors (2, 8, 31); however activation of low-affinity p75 receptors can augment the TrkA-mediated response (16, 19). Here, we present similar findings: most of the effects of NGF are mediated by TrkA receptor activation. Unexpectedly, p75 receptor blockade augmented $\beta$ but not $\gamma$ ENaC immunolabeling, but only at the highest NGF concentration (100 ng/ml). This suggests p75 receptor activation may differentially regulate $\gamma$ and $\gamma$ENaC protein, at least at higher concentrations. Although the signal transduction pathway mediating NGF induced ENaC expression is not known, it may involve activation of phosphoinositide-3-kinase (PI3K) and serum and glucocorticoid-inducible kinase (SGK) pathways, since the same pathways are activated by NGF-TrkA activation and known to up-regulate ENaC expression (12, 37, 40, 41, 44).

**Disruption of ENaC Expression Blocks Neurite Formation.** To determine if ENaC molecules contribute to NGF induced neurite formation, we evaluated neurite formation
in NGF-stimulated cells following blockade of ENaC expression using three approaches. In the first approach, we blocked channel activity with benzamil (100 nM – 10 μM) specific ENaC inhibitor at low doses. In the second and third approaches, we blocked transcript and protein expression using β and γ ENaC siRNA and dominant-negative molecules, respectively. All approaches yielded similar results; disruption of ENaC expression blunts neurite formation. Disruption of ASIC1 expression did not significantly inhibit neurite formation and suggests ASIC1 does not play a pivotal role in neurite formation in-vitro. These data provide evidence that ENaC expression is required for NGF-mediated neurite growth.

Despite our findings that ENaC inhibition with siRNA and dominant-negative molecules has a profound effect on neurite formation in-vitro; it is unlikely DEG/ENaC molecules play a prominent role in development of the nervous system since ENaC null mice display no gross neuro-developmental defects. The presence of less obvious neurological defects in β and γ ENaC null mice has not been evaluated. Alternatively, ENaC proteins may not play a role in neurogenesis, but rather participate in neuronal regeneration following injury or degenerative diseases such as multiple sclerosis.

In summary, our data demonstrate NGF-TrkA/p75 receptor interactions induce β and γ ENaC expression, which are required for neurite formation. We speculate β and γ ENaC proteins may be components of mechanosensitive ion channel complexes and transduce extracellular matrix-membrane interactions, i.e. permit the neurite to “feel” its way through its environment. The results of these studies provide insight into the mechanisms underlying neurite formation and may contribute to our understanding of neuronal repair following injury and degenerative diseases.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1: NGF induces ENaC and ENaC expression in PC12 cells. RT-PCR analysis (A) shows NGF (50 ng/ml, 24h) stimulates ENaC and ENaC, but not ENaC message, in PC12 cells. Immunostaining shows NGF (50 ng/ml, 24h) also profoundly increases ENaC (B) and ENaC (C) protein expression in PC12 cells. Data, expressed as Relative Immunofluorescence Intensity (RFU) / cell area (mm²), are mean ± SE; *, p ≤ 0.05, significantly different from no-NGF control. Representative immunostaining images are shown. Experiments were repeated at least three times.

Figure 2. NGF-Induced ENaC and ENaC Expression is Primarily Mediated Via the TrkA Receptor. NGF increased ENaC (A) and ENaC (B) immunofluorescence in a concentration-dependent manner (white bars). TrkA receptor blockade (grey bars) with TrkA antibody (2 ng/ml) prevented NGF-induced ENaC and ENaC immunofluorescence. Blocking p75 receptor (black bars) with anti-p75 antibody (2 ng/ml) produced a smaller decrease in ENaC, but not in ENaC immunofluorescence. C. TrkA receptor blockade also profoundly inhibited NGF-induced neurite growth, while p75 receptor blockade decreased NGF-mediated neurite growth to a lesser but significant extent. Data are mean ± SE. *, p ≤ 0.05, significantly different from NGF control.

Figure 3. Benzamil Prevents NGF-Induced Neurite Growth. The specific ENaC blocker, benzamil, decreased NGF-induced neurite growth in PC12 cells in a
concentration-dependent manner. Representative images are shown for some groups. Data are mean ± SE. *, p ≤ 0.05, significantly different from NGF control.

Figure 4. Silencing βENaC and γENaC, but not ASIC1, Message Inhibits NGF-Induced Neurite Growth. A. Quantitation of siRNA on βENaC, γENaC and ASIC1 protein expression. siRNA molecules silenced the respective protein expression to or near background signal levels, as indicated by the light grey dashed line. B. Representative images of βENaC, γENaC and ASIC1 immunlabeling 48 h following siRNA transfection. C. Transfection with 100 ng/ml β and/or γENaC siRNA prevented NGF-induced neurite growth compared to the Lipofectamine and non-targeting siRNA controls. ASIC1 siRNA did not disrupt neurite formation. Data are mean ± SE. *, p ≤ 0.05, significantly different from NGF control.

Figure 5. Disrupting βENaC and γENaC Protein Inhibits NGF-Induced Neurite Growth. A. Diagram of dominant-negative constructs. A CMV promoter drives expression of Enhanced Green Fluorescent Protein (EGFP) fused to the N-terminal portion of βENaC or γENaC. B. Representative images show lipid-mediated transfection with EGFP-labeled βENaC (EGFPβ41X) and γENaC (EGFPγ160X) dominant-negative molecules had a transfection efficiency of approximately 80%, based on EGFP-transfected cells. C. Transfection with 1 μg/ml EGFPβ41X and EGFPγ160X prevented NGF-induced neurite growth. Data are mean ± SE. *, p ≤ 0.05, significantly different from NGF control.
Figure 1

A

ENaC: □ □ □
NGF:       - +       - +       - +

500bp -

B

[Graph showing ENaC RFU/area]

Negative control  - NGF  + NGF

n=97  n=99

C

[Graph showing ENaC RFU/area]

Negative control  - NGF  + NGF

n=103  n=102  n=99
Figure 2

A

- Control
- Anti-p75 (2 ng/ml)
- Anti-TrkA (2 ng/ml)

NGF Concentration (ng/ml)

B

- Control
- Anti-p75 (2 ng/ml)
- Anti-TrkA (2 ng/ml)

NGF Concentration (ng/ml)

C

- % Cells with Neurites

NGF (ng/ml) 0 50 50 50 50
Anti-p75 (ng/ml) 0 0 2 0 2
Anti-TrkA (ng/ml) 0 0 0 2 2
Figure 3

% Cells with Neurites

Control  -  100 nM  1 µM  10 µM

Benzamil

50 ng/ml NGF

NGF 50  NGF 50 + Benz 100nM  NGF 50 + Benz 10µM

40 µm
Figure 4

A

![Graph showing ENaC RFU](image)

B

![Images of RISC siRNA and ENaC siRNA](image)

C

![Graph showing % Cells with Neurites](image)
Figure 5

A

Dominant-Negative Constructs

<table>
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<tr>
<th></th>
<th>CMV promoter</th>
<th>EGFP</th>
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<tr>
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<td>EGFP(_{41X})</td>
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B

Figure 5B shows images of cells expressing different constructs. The images display the expression of EGFP\(_{41X}\) and EGFP\(_{L160X}\) under various conditions.

C

The graph in Figure 5C represents the percentage of cells with neurites under different conditions, with cDNA and NGF concentrations. The x-axis shows cDNA concentration (1μg/ml) and NGF concentration (ng/ml), while the y-axis represents the percentage of cells with neurites.