Application Note

# Establishment of Localized RNA Interference in the Axons and Growth Cones of Dorsal Root Ganglion Neurons

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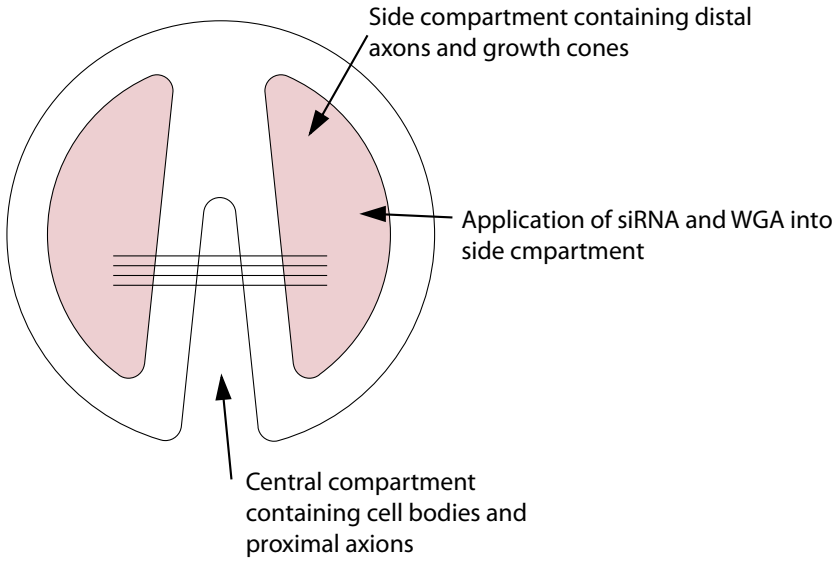
## Introduction

Translation of mRNA has been shown to occur locally in the axons and growth cones of developing neurons.[1] However, the cellular mechanisms regulating localized mRNA translation have not been clearly elucidated. Towards this end,

a study in the Journal of Neuroscience investigated whether several RNA interference (RNAi)-associated proteins, which together could form an important regulatory apparatus, are found in the developing axons and growth cones of dorsal root ganglion (DRG) neurons.[2] The researchers used immunofluorescence to determine if the following RNAi-related proteins were present in isolated DRG neuron axons: Dicer, argonaute-3 and argonaute-4, and fragile X mental retardation protein (FMRP). Also, they transfected siRNA into the distal axons of the DRG neurons to establish that the RNAi pathway is functional in axons and growth cones.

**Figure 1. Schematic Diagram of Campenot Chamber Culture Set-Up for Isolation of Distal DRG Axons**




## Methods and Materials

### Establishment of primary DRG neuron culture in Campenot chambers

Primary Rat E15 DRG explants were isolated and grown in Campenot chambers to isolate axons and growth cones for subsequent procedures.[3] The explants were grown in the central compartment of the three-compartment Campenot chambers on Permenox® slides precoated with poly-D-lysine, collagen and laminin (See Figure 1). A gradient of nerve growth factor was established to induce growth of axons into the side compartments, which were sealed by application of a thin silicon grease layer to the plastic slides. After 5 days in vitro, axons had grown into the side compartments.

### RNAi related protein detection by Immunofluorescence

Immunofluorescence was performed using anti-Dicer (Genetex, San Antonio, TX), anti-argonaute-3 and anti-argonaute 4 antibodies (Abcam, Cambridge, UK), anti-FMRP (Chemicon, Temecula, CA), and AlexaFluor 488- and 568-labeled secondary antibodies (Invitrogen, Eugene, OR). All images were captured using a Nikon (Tokyo, Japan) TE2000 microscope.

### siRNA Transfections

To establish the presence of localized functional RNAi, siRNA targeting RhoA mRNA was transfected into the Campenot chamber side compartments containing the isolated DRG axons. RhoA mRNA is itself axonally localized and its local translation is implicated

**Figure 2. RNAi-related proteins are localized within DRG neuron axons**

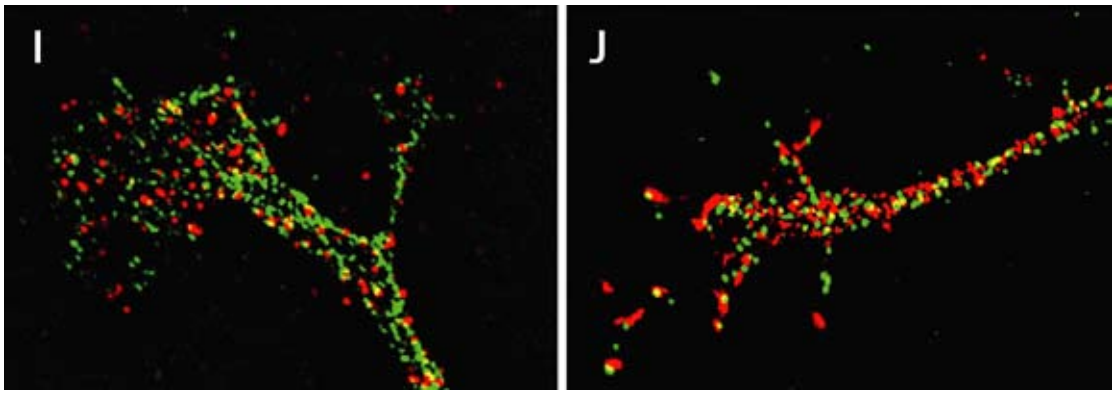



Figure 2. I. Immunofluorescent microscopy confirms the presence of: Dicer (Red) and Argonaute 3 (green); J. Argonaute 4 (green) and FMRP (red) in distal axons of DRG neurons. Punctate appearance confirms co-localization of these proteins and suggests local functioning RNA interference.

in induction of growth cone collapse via signalling mediated by the axonal guidance molecule Semaphorin 3A. The axons were transfected with RhoA-specific or non-targeting siRNA using the GeneSilencer® siRNA Transfection Reagent (Genlantis, San Diego, CA) following the manufacturer's guidelines.

### Post-Transfection analysis

After 3 days in culture, the cells were fixed and permeabilized. Eight hours prior to fixation, Alexa 488–wheat germ agglutinin (WGA) (Invitrogen) was added to the side chambers to label the axons. Forty-eight hours post-transfection, the following tests were performed: fluorescent microscopy to determine siRNA delivery efficiency, fluorescent in situ hybridization (FISH) to measure RhoA transcripts, and fluorescent labeling using GAP-43 to quantify RhoA protein. Additionally, analysis of growth cone collapse was performed utilizing phase-contrast microscopy with photos taken before and after addition of 450 ng/ml of Semaphorin 3A or vehicle.

## Results

RNAi related proteins are localized within DRG neuron axons

Immunoflourescent microscopy confirmed the presence of the following RNAi associated proteins in DRG axons and growth cones: Dicer, argonaute-3 and argonaute-4, and FMRP (Figure 2). Furthermore, these proteins were co-localized in in punctate granular particles suggesting an active role for the RNAi pathway in regulating localized mRNA translation.

### Knockdown of RhoA mRNA by siRNA Transfection

Transfection of siRNA targeting RhoA into distal axons inhibited localized Semaphorin 3A-mediated growth cone collapse (Figure 3b). Successful delivery of siRNA into axons was confirmed by fluorescent microscopy using non-specific FITC-labeled siRNA. Transfection of RhoA-specific siRNA in Campenot chamber side compartments resulted in >75% reduction in RhoA mRNA levels in distal axons (Figure 3a and data not shown). The absence of RhoA mRNA knockdown by transfection of the control siRNA indicates that siRNA transfection by itself did not induce a nonspecific mRNA degradation.

## Discussion

This study demonstrates that RNAi-related proteins function locally within the isolated axons and growth cones of DRG neurons. Immunofluorescent staining confirmed the presence of the RNAi-related proteins, Dicer, argonaute-3, argonaute-4, and FMRP. Efficient transfection of RhoA-targeted siRNA using the GeneSilencer® siRNA Transfection Reagent results

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**Figure 3. Knockdown of RhoA by GeneSilencer-mediated transfection of RhoA-targeted siRNA into axons of DRG neurons.**

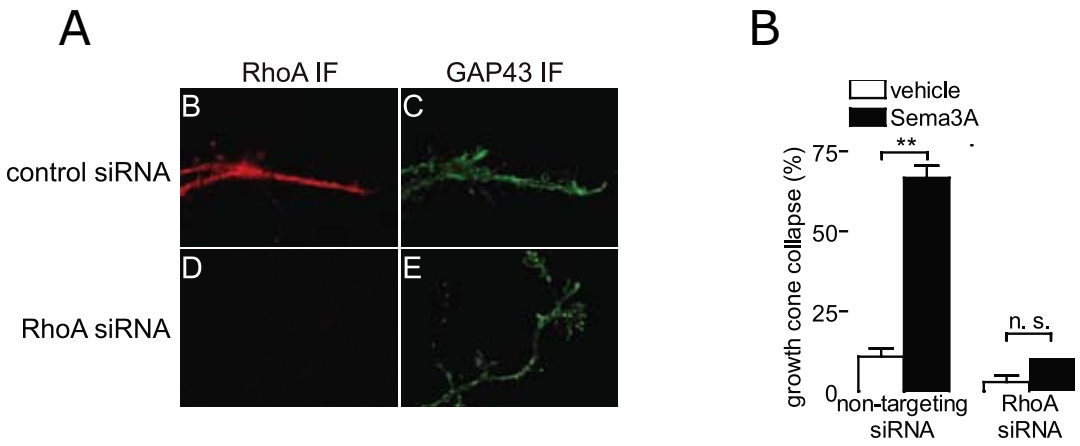


Figure 3: Transfection of RhoA specific siRNA into DRG neuron distal axons was performed using the GeneSilencer siRNA Transfection Reagent (Genlantis). A. Efficient RNAi was confirmed by the absence of red immunostained RhoA in the RhoA siRNA chamber D compared to the presence of the red stained RhoA in the control siRNA chamber B. In chambers C & E, the axons were visualized using green GAP-43 immunofluorescence. B. FISH signal quantification confirms siRNA-mediated knock-down of RhoA mRNA (right) blocks Semaphorin 3A-mediated growth cone collapse vs. non-targeting siRNA (left).

in reduction of Sempaphorin 3A-mediated growth cone collapse and demonstrates that RNAi proteins function locally in distal axons. These results show functional and potent RNAi in axons and identify an effective approach to spatially regulate mRNA transcripts at a subcellular level in neuronal processes.

## References:


1. Eng H, Lund K, Campenot RB (1999) Synthesis of ß-tubulin, actin, and other proteins in axons of sympathetic neurons in compartmented cultures. *J.Neurosci* **19**:1–9.
2. Hengst, U. Cox, L.J., Macosko, E.Z. and Jaffrey, S.R. (2006) Functional and Selective RNA Interference in Developing Axons and Growth Cones. *J.Neurosci.* **26**(21): 5727–5732.
3. Campenot, RB (1977) Local control of neurite development by nerve growth factor. *Proc Natl Acad Sci USA* **74**:4516–4519.


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