**Background:**

In our previous investigations of the role of the extracellular matrix (ECM) in promoting neurite growth we have observed that a permissive laminin (LN) substrate stimulates differential growth responses in subpopulations of mature dorsal root ganglion (DRG) neurons. DRG neurons expressing Trk and p75 receptors grow neurites on a LN substrate in the absence of neurotrophins, while isolectin B4-binding neurons (IB4+) do not display significant growth under the same conditions. We set out to determine whether there was an expression signature of the LN-induced neurite growth phenotype. Using a lectin binding protocol IB4+ neurons were isolated from dissociated DRG neurons, creating two groups - IB4+ and IB4-. A small-scale microarray approach was employed to screen the expression of a panel of ECM-associated genes following dissociation (t=0) and after 24 hr culture on LN (t=24LN). This was followed by qRT-PCR and immunocytochemistry of selected genes.

**Methods:**

# **DRG neuron separation and culture**

# Animal procedures were approved by the Animal Care Committee at Memorial University of Newfoundland in accordance with The Canadian Council on Animal Care (CCAC). For each biological replicate, DRGs were collected from six young adult Sprague Dawley rats (4-7 weeks old), cleaned of attached nerve roots and connective tissue, pooled and enzymatically dissociated using our standard protocol [[27](#_ENREF_27)]. Approximately 95% of non-neuronal cells were removed using a 4 x 4 minute centrifugations at 50xg in a 15ml centrifuge tube prior to the separation procedure. IB4+ binding DRG neurons were separated from the total DRG neuronal population using our established magnetic separation protocol that utilizes Streptavidin-Dynabeads coated with biotinylated isolectin B4 from *Banderaea simplicifolia* (Sigma, cat#L2140) to produce two populations of DRG neurons [[27](#_ENREF_27)]. Briefly, neurons are incubated with the lectin-coated beads, and neurons that bind the lectin are subsequently collected using a magnet (IB4+ neurons). The IB4+ selected cells are detached from the beads after a DNase treatment which degrades the DNA linker that attaches the streptavidin to the beads. The supernatant contains the remaining neuronal populations, the IB4- group.

For mRNA analysis, t=0 samples were collected from IB4+ and IB4- populations of neurons immediately after the separation procedure for total RNA extraction. Alternatively, t=24LN samples were collected from the two populations after 24 hr culture on laminin (LN, 20 g/ml) coated 35mm culture dishes at 37°C. Neurons were maintained in serum-free Neural Basal Media (NBM) with B27 supplement and mitotic inhibitors.

# **RNA collection and preparation**

Total RNA was extracted from each sample using an RNeasy plus micro kit (Qiagen). For t=0 samples, the IB4+ and non-selected neurons were pelleted after the separation procedure for 5 minutes at 300xg. The supernatant was then removed and the pellet was resuspended in 350μl of Buffer RLT. For t=24LN samples, the culture medium was removed and 350μl of Buffer RLT was added to each well of a 6-well culture plate to directly lyse the neurons. The well was then scraped with a rubber policeman and the sample was transferred from the well to a 1.5 ml centrifuge tube. The lysed samples were processed through the remainder of the RNeasy Plus Micro protocol as per the manufacturers manual. Total RNA was quantified using a NanoDrop 1000 spectrophotometer (ThermoScientific) and stored at -80˚C.

Biotinylated cRNA was prepared using the True-Labeling-AMP 2.0 kit (SABiosciences) using 600 ng of total RNA. Labeled cRNA was purified employing the ArrayGrade cRNA Cleanup kit (SABiosciences) and quantified with a NanoDrop 1000 spectrophotometer (ThermoScientific).

# **GEArray hybridization**

Gene expression was analyzed using Gearray oligo microarrays (SABiosciences) that were designed to analyze the mRNA expression levels of genes that encode for extracellular matrix attachment and adhesion molecules and remodeling enzymes (extracellular and lysosomal proteases) that were specific for rat tissues (Supplementary Table 1). These arrays are nylon membranes containing a printed matrix of 60-mer oligonucleotide probes specific to each gene. During the course of the experiment, the manufacturer created an updated version of the array. Thus two arrays were used for the experiments (ORN 0.13 and ORN 0.13.2). Although the arrays individually analysed the expression of 133 genes, due to small differences between the gene lists of each array, we ultimately analysed 144 genes. Three experiments were performed with ORN 0.13 and four experiments with ORN 0.13.2. This caused the number of biological replicates to vary for each gene. For *Itgb1*, *Icam1*, *Adamts1*, *Lamb1*, *Fn1* and *Spp1* the n values are 5-6, while for *Ctsh*, *Plaur* and *Plat* n=3. GEarray membranes were hybridized with the biotin-labeled cRNA target and 0.75 ml of GEarray hybridization solution overnight at 60˚C. The hybridization solution was then removed and the array membrane was washed with 2XSSC, 1%SDS for 15 min at 60˚C. Blocking and detection were performed at room temperature using the Chemiluminescent Detection kit (SA Biosciences). The membrane was exposed to film for 20 sec to 5 min, and films were developed and scanned to create digital images. Gene expression was indicated by the density of a hybridized “spot” for each gene. The digital images for each array were uploaded to the SABiosciences online GEarray Expression Analysis Suite.

This software calculated the intensity of the hybridization spots for each gene on the array. Spot intensities (densitometry) for each gene were subtracted from background and normalized to housekeeping gene expression levels (1.0) to calculate the absolute value of expression for each gene. Gene expression levels ranged from 0.02 (faint spot, very low expression) to 1.34 (bleeding spot, very high expression). The housekeeping genes [peptidylpropyl isomerase A, ribosomal protein L32, lactate dehydrogenase, aldolase A, glyceraldehydes-3-phosphate dehydrogenase and a biotinylated artificial sequence 2 complementary sequence] were located on the top left corner and along the lower edge of the array. The absence of the expression of particular genes may indicate that the expression level is below the detection limit of the array technique. Relative quantities were determined by expressing normalized values relative to IB4- t=0 or IB4+ t=0. Genes that had relative changes in expression greater than 1.5 fold or less than 0.60 fold were labeled as differentially expressed. The spot intensities were also confirmed visually. Statistical differences between normalized spot intensities of the two neuron populations at both timepoints, and within populations from t=0 to t=24LN, were determined using ANOVA with Tukey post hoc testing and/or Students t-test.