Glioma invasion mediated by the p75 neurotrophin receptor ($p75^{NTR}$/CD271) requires regulated interaction with PDLIM1

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Supplementary Figure Legends

Supplementary Figure S1. Depletion of plasma membrane cholesterol using cholesterol oxidase or Methyl β cyclodextran abrogates glioma invasion. Histogram shows the number of cells that migrated through a collagen coated transwells in the absence (no treatment) or presence of cholesterol oxidase (Chol Ox) or Methyl β cyclodextran (M-β-CD). Asterisks (*,**) indicate $p > 0.01$ versus non-treated U87R cells (one-way ANOVA with the Neuman-Keuls post-test).

Supplementary Figure S2. Flow cytometric analysis of p75S303G expressing glioma cells using a monoclonal antibody specific to the extracellular domain (ECD) of $p75^{NTR}$ confirmed plasma membrane expression and correct topography. Asterisks (*, **) indicate $p < 0.01$ as compared to U87pcDNA (one-way ANOVA with the Neuman-Keuls post-test). The MESF units represented the antibody binding capacity (ABC). (MESF: the molecules of equivalent soluble fluorochrome).

Supplementary Figure S3. Glioma-derived-BDNF was found localized to the cell membrane of cells expressing wild type or mutant (S303G) $p75^{NTR}$. BDNF present in the conditioned medium or cell lysates were analyzed by ELISA. BDNF was found in the cell-associated fraction of U87 cells expressing both wild type and mutant $p75^{NTR}$ (S303G), but not in the U87pcDNA cells. Asterisks (*, **) indicate $p < 0.01$ as compared to U87pcDNA (one-way ANOVA with the Neuman-Keuls post-test).

Supplementary Figure S4. Flow cytometric analysis of U87 glioma cells expressing PDZ-binding domain mutants (ΔSPV; SPM) of $p75^{NTR}$ using a monoclonal antibody specific to the extracellular domain (ECD) of $p75^{NTR}$ confirmed plasma membrane expression and correct topography. Asterisk (*) indicate $p > 0.05$ and asterisks (**, ***)

indicate $p > 0.01$ versus U87 pcDNA cells (one-way ANOVA with the Neuman-Keuls post-test).

Supplementary Figure S5. Glioma-derived-BDNF was found localized to the cell membrane of cells expressing wild type or PDZ-binding domain mutants (ΔSPV; SPM) of $p75^{NTR}$. BDNF present in conditioned media, or cell lysates, was analyzed by ELISA. Increased levels of BDNF were found in the cell-associated fraction of U87 cells expressing both wild-type and mutant $p75^{NTR}$ ((ΔSPV; SPM), as compared to the U87pcDNA cells. Asterisk (*) indicate $p > 0.01$ and double asterisks (**, ***) indicate $p > 0.05$ versus non-treated U87pcDNA (one-way ANOVA with the Neuman-Keuls post-test).

Supplementary Figure S6. Identification of PDLIM1 by LC-MS/MS analysis. Using Mascot software v2.3, PDLIM1 was identified from the MS/MS spectrum. Shown is the peptide fragment identifying PDLIM1 as a $p75^{NTR}$ interacting protein.

Supplementary Figure S7. $p75^{NTR}$ and PDLIM1 interaction was confirmed by immunoprecipitation. U87 glioma cells expressing empty vector (pcDNA), full-length $p75^{NTR}$ (U87p75) or the mutant $p75^{NTR}$ receptors ΔSPV or S303G (SG) were immunoprecipitated using a $p75^{NTR}$ antibody followed by Western blot analysis for PDLIM1 and $p75^{NTR}$ (left panel). Input cell lysates were assessed by Western blot analysis for expression of $p75^{NTR}$ and PDLIM1. Actin was used as a protein loading control. Right panel shows immunoprecipitation of U87 glioma cells expressing an empty vector (pcDNA), $p75^{NTR}$, ΔSPV, or the intracellular domain of $p75^{NTR}$ (ICD) with a PDLIM1 antibody followed by Western blot analysis for $p75^{NTR}$. Expression of the $p75^{NTR}$ intracellular domain (ICD) alone is sufficient for PDLIM1 binding. Input cell lysates were assessed by Western blot analysis for expression of $p75^{NTR}$ and PDLIM1. Actin was used as a protein loading control.

Supplementary Figure S8. PDLIM1 associates with $p75^{NTR}$ via the C-terminal PDZ-binding motif in patient-derived primary glioma and human metastatic melanoma. Immunoprecipitation with a PDLIM1 antibody co-precipitated $p75^{NTR}$ in $p75^{NTR}$-expressing glioma cell lines (U87, U118, U251) (left panel). U87 glioma cells expressing

empty vector (pcDNA), p75NTR or ΔSPV are also shown. Input cell lysates were assessed by Western blot analysis for expression of $p75^{NTR}$ and PDLIM1. Actin was used as a protein loading control. Interaction of PDLIM1 with $p75^{NTR}$ was observed in human metastatic melanoma cell lines 70W and 3S5 (right panel).

Supplementary Materials and Methods

Depletion of lipid raft

To assess the role of lipid rafts in glioma invasion, 1.8 U/ml of Cholesterol oxidase or 5 mM of Methyl β cyclodextran were added to the upper chambers containing the cells while the invasion assays was performed. The treated cells were incubated for 4 hrs under normal culture conditions, and migrated cells were counted.

Quantification of $p75^{NTR}$ cell surface expression

Expression of $p75^{NTR}$ in U87 cells expressing the full length or mutant $p75^{NTR}$ proteins was measured using fluorescently labeled anti-$p75^{NTR}$ monoclonal antibody and quantified by comparison with a standard curve generated with Simply Cellular anti-mouse IgG microspheres (Bangs Laboratories) analyzed in parallel according to manufacturer instructions. This analysis assesses antibody binding capacity (ABC) corresponding to the total number of monoclonal antibody binding sites/cell.

ELISA

1 x 10^5 cells of U87 glioma cells were allowed to condition culture media for 4 days. The conditioned media was then collected, concentrated by centrifugation using Amicon-Ultra-3 (Millipore). The remaining cells were washed with ice-cold PBS, and total cellular lysates were extracted as described for the Western blot analysis. Protein quantification was performed using the Brad-Ford assay (Bio-Rad), and BDNF ELISA (R&D Systems) was performed as per the company protocol. Briefly, MaxiSorp ELISA plates (Nalge Nunc International) were coated with monoclonal anti-human BDNF (R&D

Systems), nonspecific binding was blocked, and serial dilutions of recombinant human BDNF (R&D Systems), conditioned media or cell lysate was added. Bound antigen was detected using the corresponding biotinylated antibody, streptavidin-HRP, and a tetramethylbenzidine substrate (R&D Systems). Absorbance was measured at 450 nm.

Cell lines

Human Metastatic melanoma cells 3S5 and 70W (98-99) were obtained from Dr. Robert Kerbel Mount Sinai Research Institute, Toronto Canada. Glioma cell lines (U118, U251) and melanoma cell lines were grown in Dulbecco's modified eagle's medium (DMEM, Gibco-BRL, Bethesada, MD, USA) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL) at 37^{o}C in a humidified 5% CO_2 incubator. Stable transfectants of glioma cells were maintained in identical media with 400 μg/ml of G418 (Sigma, St. Louis, MO, USA). Glioma cell lines ectopically expressing $p75^{NTR}$ were established as described previously (Johnston et al, 2007).