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Modulation of Angiopoietin 2 release from endothelial cells and angiogenesis by the synaptic protein Neuroligin 2

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ABSTRACT

The synaptic protein Neuroligin 2, similarly to its isoform Neuroligin 1, is produced by endothelial cells, but its activity in the vascular context remains unknown. This study aimed at verifying the hypothesis that Neuroligin 2, in parallel with its extraneuronal involvement in pancreatic beta cells exocytosis, modulated cytokine release from endothelial cells and consequently angiogenesis.

We used in vitro approaches to modulate Neuroligin 2 expression and Neuroligin 2 null mice to test our hypotheses. In vitro, upon VEGF stimulation, Neuroligin 2 silencing strongly reduces Angiopoietin 2 release in the medium and increases the endothelial cell retention of Weibel Palade Bodies, the specialized organelles that store Angiopoietin 2 and various other cytokines. On the contrary, Neuroligin 2 overexpression almost depletes cells of Weibel Palade Bodies, independent of VEGF. In vivo, both the retina and tumor xenografts grown in NLGN2- null mice display an immature vasculature, with lower pericyte coverage and lower Tie2 phosphorylation. At the molecular level NLGN2 colocalizes with its neuronal partner collibystin, a CDC42 guanine nucleotide exchange factor, which is also expressed by endothelial cells and in turn modulates Angiopoietin 2 release.

Neuroligin 2, an inhibitory synaptic protein, modulates a peculiar aspect of vascular function and could represent a novel target of therapy in various fields, from tumor angiogenesis to vascular diseases.

1. Introduction

Neuroligin 1 (NLGN1) is a synaptic protein expressed on endothelial cells (ECs) that modulates angiogenesis and EC adhesion in cooperation with the laminin-binding integrin alpha6 beta1 [1]. Nonetheless, another Neuroligin isoform, Neuroligin 2 (NLGN2), is also highly produced by ECs, but its vascular activities do not overlap with those of NLGN1 [1]. Interestingly, outside of the CNS, NLGN2 is involved in insulin release from pancreatic β cells [2].

Angiopoietin 2 (Ang2) is a vascular mediator with diverse activities, ranging from vessel sprouting to *vascular permeability and* inflammation [3] that is stored in secretory organelles of ECs called Weibel–Palade bodies (WPB) [4] along with von Willebrand factor (vWF) and a number of other vascular regulators. Vascular endothelial growth factor (VEGF) is an agonist of Ang2 release from ECs with a prominent role in tumor angiogenesis and vascular remodeling [3]. Here, we report the finding that endothelial NLGN2 modulates the VEGF-induced Ang2 and vWF release from ECs in vitro and in vivo, in cooperation with a neuronal Guanine Exchange Factor (GEF) for the small GTPase CDC42. Moreover, NLGN2 null mice display an altered vascular pattern and reduced pericyte coverage of vessels both in the neonatal retina and in subcutaneous tumors. These phenotypes are compatible with the lack of an agonistic activity of Ang2 towards its receptor Tie2.

2. Materials and methods

Animals: Mice lacking NLGN2 (Nlgn2^{tm1}Bros/J - Jackson Laboratory), previously described [5], were maintained on a mixed C57BL/6 x 129 background (designated B6; 129) in the animal

Abbreviations: EC, endothelial cells; Ang2, angiopoietin 2; NLGN1, Neuroligin 1; NLGN2, Neuroligin 2; PKC, Protein Kinase C; PKA, Protein Kinase A; CB, collybistin; WPB, Weibel–Palade bodies.

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facility of the Institute (IRCC) under standard housing conditions. Mice were sacrificed at P5 and retinas were isolated for analysis. PCR amplification was used for the genotype characterization using the forward common primer (5′GTGAGCTGAATCTTATGGTTAGATGGG3′), the wild-type reverse primer: (5′CCCATCAGTGTACCATTCCCTAAA3′), and the mutant reverse primer (5′GAGCGCGCGCGGCGGAGTTGTTGAC3′). All animal experiments were performed in compliance with guidelines governing the care of laboratory mice of the University of Torino Committee on Animal Research.

Antibodies: Goat polyclonal Human Angiopoietin-2 (AF623), Monoclonal Mouse Human Collybistin/ARHGEF9 (Clone #809309, MAB 7848) and Rabbit Polyclonal Human/Mouse Phospho-Tie-2 (Y992) (AF2720) antibodies were obtained from R&D Systems.

The Rabbit Polyclonal Anti-Human Von Willebrand Factor (A0082) was from Dako.

The mouse anti-HA (sc-7392) was purchased from Santa-Cruz. The Rabbit anti-HA (H6908) and the Isolectin B4 FITC-conjugated from Bandeiraea Simplicifolia were purchased from Sigma.

The Rabbit anti-NG2 Chondroitin Sulfate Proteoglycan Polyclonal antibody was from Millipore.

The Anti-Active Cdc 42 Mouse Monoclonal Antibody (catalog number 26905) was obtained from ewEast Biosciences.

siRNA and cDNA transient transfection: ECs (2×10^6) were plated in 10 cm^2 dishes and transfected in opti-MEM® I + GlutaMAX™ (Gibco by Life Technologies) by using the Lipofectamine™ (2 mg/ml) and Plus Reagent (3 mg/ml) (Invitrogen) with 3 μg of DNA. After 3 h at 37 °C the opti-MEM® I was replaced with EGM-2. For transient down-regulation, ECs were seeded in EGM-2, in 6 well plate ($1{,}2 \times 10^5$ cells/well). ECs were transfected for the next two days with 200 pmol of each ON-TARGET plus siRNAs (Dharmacon): siRNA NLGN2_B09 (J-008843-09) recognizing the 5′UTR and the ORF sequences of human NLGN2, siRNA NLGN1_A5 recognizing the CDS sequences of human NLGN1 or with Non-targeting siRNA#1 (Scrambled), by using the Oligofectamine (Invitrogen) according to manufacturer's protocol.

Endothelial cells infection: The lentiviruses NLGN2 shRNA, Collybistin shRNA (XM_377014-TRCN0000047617) and CTRL shRNA (RNAi Consortium library SIGMA) were produced as described [6]. One day before the infection, ECs were seeded at concentration of $3{,}5 \times 10^4$ cells/ml in a 100 mm tissue culture dish. Then the cells were transduced for 36 h with the lentiviral particles in the presence of 8 μg/ml Polybrene (Sigma-Aldrich). Before starting every experiment, the efficiency of cell infection was determined by analyzing gene downregulation by qRT-PCR.

In vitro cord formation assay: For in vitro cord formation BME was added to each well at a concentration of 8 mg/ml and incubated at 37 °C for 30 min to allow gel formation. Scramble control and NLGN2 silenced ECs ($2{,}3 \times 10^4$/well) were plated onto Matrigel in the presence of Recombinant Human $VEGF_{165}$ (50 ng/ml) (R&D systems-catalog number 293-VE) or VEGF plus angiopoietin 2 (0,2 μg/ml) (R&D systems-catalog number 623-AN) and incubated for 4 h at 37 °C in 5% CO_2 humidified atmosphere. Cells organization was examined (Leica Microsystem, Heerbrugg, Switzerland) and photographed. The lengths of the capillary-like structures were quantified with the imaging software winRHIZO Pro (Regent Instruments Inc.).

Retinal whole mount staining and confocal imaging analysis: Mouse retinas dissection and whole mount immunostaining were performed as previously described [7] with modifications. Eyes were harvested at P5 and fixed in 4% paraformaldehyde (PFA) for 1 h in ice. Retinas were dissected and washed briefly with DPBS (Sigma) three times and incubated overnight at 4 °C in DPBS containing 0.5% Triton X-100 and 0.2% BSA. The retinas were rinsed briefly with DPBS containing 1% Triton X-100 and then incubated overnight at 4 °C in DPBS 1% Triton X-100 with specific antibodies against: Isolectin B4 (40 μg/ml); von Willebrand Factor (vWF 1:200); Phospho-Tie-2 (pTie2 1:50); Neural/glial antigen 2 (NG2 1:50). The retinas were rinsed in DPBS for 2 h and then incubated for 1 h at RT in DPBS 1% Triton X-100 containing the appropriate secondary antibody Alexa Fluor conjugate (1:200 Alexa Invitrogen Molecular Probes®) and stained with DAPI (1:5000) for 45 min. Retinas were then washed in DPBS for 1 h, fixed again with 4% PFA for 30 min at RT and flat-mounted onto a glass coverslip. Images were acquired using a confocal laser-scanning microscope (TCS SP2 with DM IRE2; Leica) and processed using Adobe Photoshop®.

Retina images were quantified with Image Processing & Analysis in Java software (Image J) as follows: the immunoreactivity of each antigen was calculated as the surface area stained by the corresponding antibody normalized on the total surface vascular area visualized by Isolectin B4. For each quantification at least 5 mice/genotype were analyzed.

Endothelial cells immunostaining and signal quantification: ECs plated on gelatin-coated coverslips were fixed either with 4% PFA or with −20 °C Methanol for 10 min at room temperature (RT), permeabilized with 0.2% Triton−X in PBS for 8 min at RT and saturated with 10% Donkey Serum/1% BSA in PBS for 40 min at RT. Slides were incubated with the primary antibodies: mouse anti-HA (1:100), rabbit anti-HA (10 μg/ml); rabbit anti-vWF (1:200); goat anti-Angiopoietin 2 (1:50) and mouse anti-Collybistin (1:50) in PBS 10% Donkey Serum/1% BSA for 1 h at RT and with the appropriate Alexa Fluor secondary antibodies in PBS 5% Donkey Serum for 45 min at RT. Finally, cells were stained with DAPI (1:10000) for 10 min at room temperature and mounted with immunofluorescence mounting medium (Dako).

Confocal images were acquired using a confocal laser-scanning microscope (TCS SP2 with DM IRE2; Leica). Fluorescent antibody signal was calculated with the Image Processing & Analysis in Java software (ImageJ) measuring the mean pixel intensity of maximum-projected confocal image stacks.

Enzyme-Linked Immunosorbent Assay (ELISA): Cell culture supernatants of NLGN2 overexpressing ECs in basal condition, or NLGN2 silenced ECs treated with vehicle, PMA (50 ng/ml) or VEGF (50 ng/ml) were collected for the analysis of Angiopoietin 2 release. The enzyme-linked immunosorbent assay (ELISA) (Quantikine Human Angiopoietin-2 ELISA kit -R&D Systems Europe, Ltd, Abingdon, UK) was used following the instruction manual. Each sample was measured in triplicate, and the OD values were measured at 450 nm using Synergy HT Microplate Reader. Wavelength correction were made at 540 nm.

Xenografts: WT and NLGN2KO C57BL/6J mice, 5 weeks of age, were subcutaneously inoculated with $2{,}5 \times 10^5$ B16 melanoma cells. Xenograft Tumors were excised when they reached around 1000 mm^3. Immunofluorescence analysis of OCT embedded tumors were performed with Isolectin and Neural/Glial antigen 2 antibodies.

Images were acquired using a confocal laser-scanning microscope (TCS SP2 with DM IRE2; Leica) and quantified with Image J as follow: the immunoreactivity of each antigen was calculated as the surface area stained by the corresponding antibody normalized on the total surface vascular area visualized by Isolectin B4. For each quantification at least 6 tumors/genotype were analyzed.

Statistical analysis: Upon verification of normal distribution, the statistical significance of raw data between the groups in each experiment was evaluated using the unpaired Student's *t*-test or ANOVA followed by the Bonferroni post-test. Results are expressed as means ± standard error of the mean (SEM) when derived from averaged experiments, or means ± standard deviation (SD) when derived from several data points of one experiment. *n* represents the number of individual experiments. The asterisks and circles */°,