Lyon Neuroscience Research Center
ONCOFLAM Team

De Rossi P[1,2]., Bardin M[1,2]., Chounlamountri N[1,2]., Benetollo C[2,3]., Honnorat J[1,2]., Seugnet L[1,5]., Salin PA[2,4]., Meissirel C[1,2].

1) Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 1028, Centre National de la Recherche Scientifique Unité Mixte de Recherche 5292, Neurooncology and Neuroinflammation, Lyon Neuroscience Research Center, F-69000 Lyon, France; 2) University Lyon 1, F-69000 Lyon, France ; 3) INSERM Unité 1028, Centre National de la Recherche Scientifique Unité Mixte de Recherche 5292, Functional neurogenomics and optogenetics; 4) INSERM Unité 1028, Centre National de la Recherche Scientifique Unité Mixte de Recherche 5292, Physiopathology of the Sleep Neuronal Networks, Lyon Neuroscience Research Center, F-69000 Lyon, France ; 5) INSERM Unité 1028, Centre National de la Recherche Scientifique Unité Mixte de Recherche 5292, Integrative Physiology of Brain Arousal Systems, Lyon Neuroscience Research Center, F-69000 Lyon, France.

VEGF modulates NMDA receptor function and synaptic localization in the hippocampus

Lyon Neuroscience Research Center

Introduction

The vascular endothelial growth factor (VEGF) is well known to play a critical role during vascular development but recent evidence indicates that it also regulates key neuronal processes in the nervous system, such as neurogenesis, hippocampal synaptic plasticity, learning and memory. However, little is known about the underlying molecular mechanisms through which VEGF regulates hippocampal synaptic transmission and plasticity. Recently, we showed a novel interaction between the glutamate receptor NMDA (NMDAR) and the VEGF receptor VEGFR2 during neuronal migration in the developing cerebellum. As NMDAR have been widely implicated in synaptic transmission and plasticity, we hypothesized that VEGF signaling might regulate NMDAR function at hippocampal synapses. Using a multisciplinary approach based on electrophysiological, biochemical and immunocytochemical techniques, we explored the impact of VEGF on NMDAR traffic and responses at hippocampal synapses.

Method

Electrophysiological recordings: Whole cell patch clamp recordings were performed on acute coronal hippocampal slices from P15 C57/Bl6 mice and CA1 or CA3 pyramidal cells were recorded in the voltage-clamp mode. The perfusion solution contained picrotoxin and NBQX to isolate NMDARs mediated responses, which were further characterized with specific GluN2B and GluN2A expressing NMDAR antagonists. A stimulating electrode was placed near the proximal apical dendrite of the recorded cell and VEGF was applied locally. All data were expressed as mean ± SEM and a paired student t test was used for statistical analysis.

Cell surface immunostaining: After 15 DIV, low density hippocampal cell cultures were treated with NMDA (50 µM), VEGF (50ng/ml) or NMDA + VEGF for 15min. Hippocampal cells were fixed with 4% PFA/4% for 4 minutes at RT, and specific antibodies were applied in a non permeabilizing buffer to allow cell surface immunolabeling of Flk1, GluN2B or GluR1. Immunostaining of synaptic sites were performed after a permeabilizing step using Synapsin or PSD95 specific antibodies.

Image acquisition and quantitative analysis of cell surface immunostaining: Immunostained hippocampal cells were selected based on their pyramidal morphology. Images were acquired using a Zeiss microscope equipped with apotome technology and analyzed using Image J softaware. The regions of interest (ROI) were delineated on the apical dendrite of pyramidal cells and the density of receptor clusters was quantified in each ROI. Co-localized clusters were identified using the co-localization plug-in of Image J and their location further validated for each channel. Synaptic clusters were defined as being colocalized with a synapsin or PSD95 cluster. All data were expressed as mean ± SEM and a one way ANOVA was used with post hoc t-test for statistical analysis.

PSD enrichment: After treatments, 15 DIV neurons were incubated in cold buffer containing 0.32 M sucrose and 10 mM HEPES, pH 7.4. Homogenates were cleared two times at 1000 g for 10 min to remove nuclei and large debris (P1). The resulting supernatants were concentrated at 12 000 g for 20 min to obtain a crude membrane fraction (P2), which was rinsed twice (4 mM HEPES, 1 mM EDTA, pH=7.4; 20 min at 12 000g). Then, it was incubated (20 mM HEPES, 100 mM NaCl, 0.5% triton X, pH= 7.2) for 15 min and centrifuged at 12 000 g for 20 min to pellet the synaptosomal membrane fraction (LP1). The supernatant was considered the non-postsynaptic density membrane fraction (non-PSD), sometimes referred to as the triton soluble fraction. The pellet was then solubilized (20 mM HEPES, 0.15 mM NaCl,1% triton X100, 1% deoxycholic acid, 1% SDS, pH= 7.5) for 1 h and centrifuged at 10 000 for 15min. The supernatant contained the postsynaptic density fraction (PSD) or triton insoluble fraction. The integrity of non-PSD and PSD fractions was verified by immunoblotting for PSD-95 which was enriched in PSD fraction. All buffers were supplemented with orthovanadate and protease inhibitors cocktail (Complete mini tablets, Roche).

Results

VEGF and VEGFR2 expression in the hippocampus

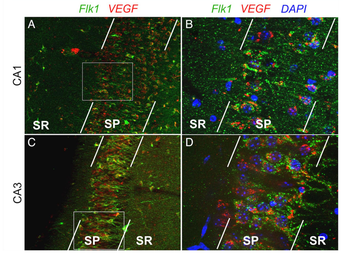


Figure 1: **VEGF and VEGFR2 expression in the CA1 and CA3 regions of the hippocampus:** VEGF (in red) and VEGFR2 (in green) are expressed in the Stratum Radiatum (SR) and Stratum Pyramidale (SP) in CA1 (A and B) and CA3 (C and D).

VEGF effect on NMDAR mediated synaptic activity in hippocampal neurons?

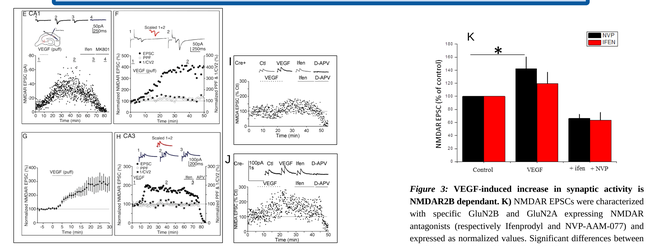


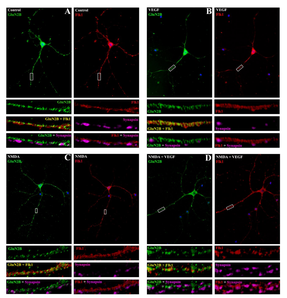
Figure 2: VEGF-induced increase in NMDAR-mediated synaptic transmission in hippocampal CA1 pyramidal cells. **E)** Representative time course of the VEGF effect on NMDAR-mediated Excitatory Postsynaptic Currents (EPSCs) at Schaffer collateral-CA1 synapses, in baseline condition (1), after VEGF application (2), ifenprodyl (3) and MK801 (4) treatments. Note the enhancement of EPSCs in presence of VEGF and their blockade when the NMDAR antagonist ifenprodyl and the open blocker MK801 were applied **F)** Paired Pulse facilitation in CA1; **G)** Summary graph of the impact of VEGF on NMDAR mediated responses in CA1 (N = 8), showing a 3 fold increase in the EPSC amplitude after VEGF local application **H)** Paired Pulse Facilitation in CA3. **I and J)** Representative time course of the VEGF effect on NMDAR-mediated EPSCs recorded in Cre+ VEGFR2 lox/- and Cre- VEGFR2 lox/- *mice.*

Figure 3: **VEGF-induced increase in synaptic activity is NMDAR2B dependant. K)** NMDAR EPSCs were characterized with specific Glu2B and Glu2A expressing NMDAR antagonists (respectively Ifenprodyl and NVP-AAM-077) and expressed as normalized values. Significant differences between conditions are indicated by (*) for p<0.05.

→ VEGF increases NMDAR2B mediated responses in pyramidal cells through Flk1 signaling

Results

VEGFR2 and NMDAR interaction: impact on synapse formation and NMDAR synaptic targeting



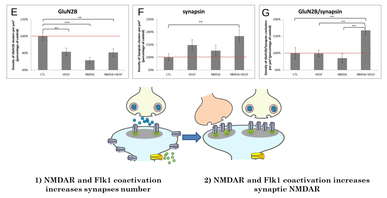


Figure 7: **Quantitative analysis of NR2B, VEGFR2 and synapsin cluster distribution: E-G)** Graphs showing GluN2B and synapsin cluster densities, as well as GluN2B/synapsin co-clusters densities. All values were normalized to control and represented as mean ± SEM, n=17, 12, 10, 8 pyramidal cells, respectively in control, VEGF, NMDA and NMDA+VEGF conditions. Data shown are derived from 2 independent experiments. Significant difference between conditions are indicating by (*) for p<0.05; (**) for p<0.01; (***) for p<0.001.

Figure 5: **Cell surface expression of NMDAR2B and VEGFR2 in hippocampal pyramidal cells: A)** Pseudo-confocal image of a representative hippocampal pyramidal cell immunostained for GluN2B (green) and VEGFR2 (red), in control condition. White boxes indicate ROI on the apical dendrite and represented below for each immunostaining. **B, C, D,** Representative immunostainings after VEGF, NMDA or combined NMDA and VEGF treatments.

→ Co-activation of NMDAR and VEGFR2 triggers synapse formation and NMDAR2B synaptic targeting

VEGFR2 and NMDAR interaction : impact on AMPA receptor expression and synaptic targeting

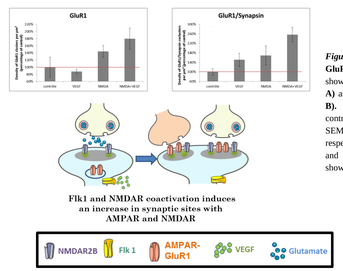
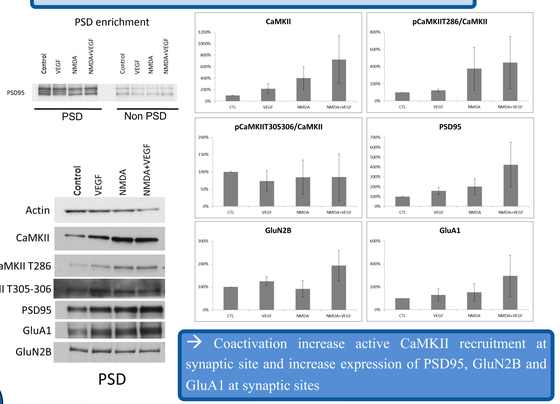


Figure 8 : **Quantitative analysis of GluR1 cluster distribution.** Graphs showing GluR1 cell surface expression **A)** and GluR1/Synapsin colocalisation **B).** All values were normalized to control and represented as mean ± SEM, n=3, 5, 4, 5 pyramidal cells, respectively in control, VEGF, NMDA and NMDA+VEGF conditions. Data shown are derived from 2 experiment.

→ Co-activation of NMDAR and VEGFR2 increases cell surface expression and synaptic targeting of AMPAR-GluR1

Impact of NMDAR and VEGFR2 co-activation on the postsynaptic organisation of hippocampal synapses



→ Coactivation increase active CaMKII recruitment at synaptic site and increase expression of PSD95, GluN2B and GluA1 at synaptic sites

Role of NMDAR and VEGFR2 interaction in Flies Phototaxic behavior

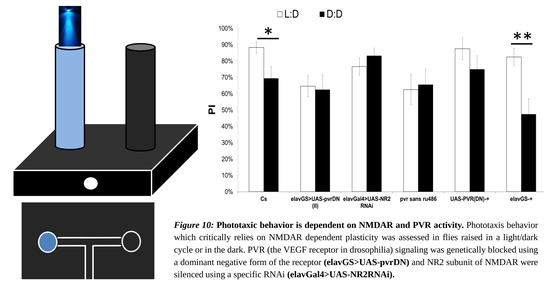
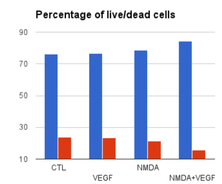


Figure 10: **Phototaxic behavior is dependent on NMDAR and PVR activity.** Phototaxis behavior which critically relies on NMDAR dependent plasticity was assessed in flies raised in a light/dark cycle or in the dark. PVR (the VEGF receptor in drosophilia) signaling was genetically blocked using a dominant negative form of the receptor **(elavGS>UAS-pvrDN)** and NR2 subunit of NMDAR were silenced using a specific RNAi **(elavGal4>UAS-NR2RNAi).**

→ The phototaxic behavior relies on a molecular mechanim which critically depends on NMDAR and PVR functions highlighting the VEGFR2-NMDAR crosstalk in neuronal plasticity



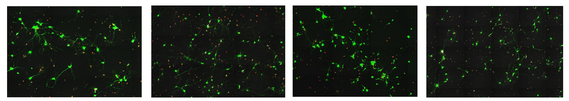


Figure 6: **Live/dead cell assay:** To determine the impact of the previously described treatments on cell viability, a fluorescent-based assay was used for live (green) /dead cell (red) discrimination. Quantitative analyses were performed and normalized to the total cell number by condition.

→ No cell viability difference between treatments

Conclusion

Our results revealed that VEGFR2 is expressed by pyramidal cells in the CA1 and CA3 regions of the hippocampus, indicating that endogenous VEGF can involved in pyramidal cell function in the hippocampus. Whole-cell patch clamp data demonstrated that VEGF potentiates NMDAR mediated synaptic transmission through a postsynaptic modulation of NMDAR 2B function. We further showed that NMDAR and VEGFR2 co-activation is a critical event in this postsynaptic modulation because it induces synapse formation and synaptic targeting of GluN2B expressing NMDAR. In addition, this coincident activation of NMDAR and VEGFR2 triggers the expression of well known synaptic plasticity markers. Altogether, our results demontrated a new VEGF-dependent mechanism which controls NMDAR localization and function at hippocampal synapses. Such a molecular mechanism can be relevant for hippocampal form of long-term potentiation and memory and could be more largely involved in different types of NMDAR dependent plasticity behavior.

Contact:
Dr Claire MEISSIREL:
claire.meissirel@inserm.fr
+33 4 57 78 78 04