Second congress on the Eph/ephrin system

Parma, Italy

May 3-4, 2018

State of the art, challenges and opportunities
Scientific committee:

Massimiliano Tognolini, University of Parma, Italy

Alessio Lodola, University of Parma, Italy

Elena Pasquale, SBP Medical Discovery Institute, La Jolla, USA

Yoshiro Maru, Tokyo Women's Medical University, Tokyo, Japan

Organizing committee

Miriam Corrado, University of Parma, Italy

Carmine Giorgio, University of Parma, Italy

Alessio Lodola, University of Parma, Italy

Massimiliano Tognolini, University of Parma, Italy

Ilaria Zanotti, University of Parma, Italy

Cover picture courtesy of Comune di Parma, Foto Glamour.
### Thursday 3 May 2018

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<td><strong>8.30-13.00</strong></td>
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<td><strong>8.50-9.00</strong></td>
<td>M. Tognolini - Welcome and introductory remarks</td>
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<td><strong>9.00-9.25</strong></td>
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<td>Sanford-Burnham Prebys Medical Discovery Institute, La Jolla, USA</td>
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### Structural biology and trafficking
*Chair: M. Tognolini (Italy) - M. Henkemeyer (USA)*

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<td>Case Western Reserve University, Cleveland, USA</td>
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<td>Spatiotemporal Regulation of EphA2 Receptor Oligomerization: Insights from PIE-FCCS Single Molecule Live Cell Analyses</td>
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<td><strong>9.50-10.10</strong></td>
<td>S. Lahaie</td>
<td>McGill University, Montréal, QC, Canada</td>
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<td>HD-PTP, an ESCRT protein, is required for EphB2 forward signalling in cell cytoskeletal dynamics and axon guidance</td>
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<td><strong>10.10-10.35</strong></td>
<td>J. P. Himanen</td>
<td>Memorial Sloan-Kettering Cancer Center, New York, USA</td>
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<td>Functional Relevance of the Head-to-Head vs Head-to-Tail Eph-Eph Interactions for Receptor Activation</td>
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<td><strong>10.35-10.55</strong></td>
<td>S. Ojosnegros</td>
<td>California Institute of Technology, Pasadena, CA, USA</td>
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<td>A new dynamic model for the activation of the Eph receptor based on live-cell brightness analysis</td>
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<td><strong>10.55-11.30</strong></td>
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### Biology and Physiology
*Chair: Y. Maru (Japan) – B. Wang (USA)*

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<td>EphB-EphrinB Bidirectional Signaling in the Nervous System and Beyond</td>
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<td><strong>11.55-12.20</strong></td>
<td>R. Lamprecht</td>
<td>University of Haifa, Israel</td>
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<td>The role of EphB2 in memory formation</td>
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<td>12.20-12.45</td>
<td>J. Wu</td>
<td>University of Montreal, Montreal, Canada</td>
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<td>12.45-13.10</td>
<td>A. Davy</td>
<td>CNRS, Université de Toulouse, Toulouse, France</td>
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**Eph and cancer**

*Chairs: E. Pasquale (USA) – B. Day (Australia)*

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<td>Y. Maru</td>
<td>Tokyo Women’s Medical University, Tokyo, Japan</td>
<td>Analysis of soluble forms of ephrin-A1</td>
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<td>14.55-15.20</td>
<td>J. Chen</td>
<td>Vanderbilt University, Nashville, USA</td>
<td>EphA2 RTK in cancer and metabolism</td>
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<td>15.20-15.40</td>
<td>P. W. Janes</td>
<td>Monash University, Clayton, Australia</td>
<td>Inducible knock-down of endogenous EphA3 in mice reveals novel roles for EphA3 in the inflammatory tumour microenvironment</td>
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<td>15.40-16.00</td>
<td>S. Karam</td>
<td>University of Colorado, Aurora, CO, USA</td>
<td>Inhibition of EphB4-ephrin-B2 interaction remodels the tumor immune microenvironment in head and neck cancers.</td>
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**Eph and other pathologies**

*Chairs: J. Chen (USA) – E. Barocelli (Italy)*

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<td>16.30-16.50</td>
<td>C. Cheng</td>
<td>The Scripps Research Institute, La Jolla, CA, USA</td>
<td>EphA2 and ephrin-A5 maintain distinct eye lens epithelial cell populations</td>
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<td>16.50-17.10</td>
<td>L. Poppe</td>
<td>KU Leuven-University of Leuven, Leuven, Belgium</td>
<td>Impact of EphA4 ablation on cognitive function and disease pathology in a mouse model of Alzheimer’s disease</td>
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<td>17.10-17.30</td>
<td>D. Poitz</td>
<td>TU Dresden, Germany</td>
<td>Stop-and-go: ephrinA1 in endothelial migration and proliferation</td>
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**Evening**

**SOCIAL EVENT**

**Friday 4 May 2018**

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<td>9.00-11.00</td>
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**Targeting the Eph/ephrin system**

*Chairs: M. Mor (Italy) – J.P. Himanen (USA)*

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<td>11.00-11.25</td>
<td>A. Lodola</td>
<td>University of Parma, Parma, Italy</td>
<td>Overview on the pharmacological tools to target Eph/ephrins</td>
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<td>11.25-11.50</td>
<td>B. Day</td>
<td>Queensland Institute of Medical Research, Brisbane, Australia</td>
<td>EphA3 a functional targetable receptor for adult and pediatric brain cancer</td>
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<td>11.50-12.10</td>
<td>M. Leone</td>
<td>Institute of Biostructures and Bioimaging (CNR), Napoli, Italy</td>
<td>Peptides targeting the Sam domain of EphA2 and its interactome</td>
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<td>12.10-12.35</td>
<td>M. Pellecchia</td>
<td>University of California Riverside, Riverside, CA, USA</td>
<td>Chemical biology strategies for targeting the EphA2 and EphA4 ligand binding domains: applications in neurodegeneration and oncology</td>
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<td>12.35-12.55</td>
<td>A. Bedini</td>
<td>University of Bologna, Bologna, Italy</td>
<td>Mu opioid receptor (MOR) activation by morphine in neuronal cell models is dampened by ephrinB1-induced signaling and may be rescued by novel EphB1 receptor peptide antagonists</td>
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<td>Farewell</td>
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Eph receptors and ephrins in 2018
E.B. Pasquale
Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California, USA.

More than 30 years after the discovery of Eph (now EphA1) as the founding member of the Eph family of receptor tyrosine kinases, research on the 14 Eph receptors and the 8 cell surface-anchored ephrin ligands is still revealing new intriguing findings. Eph receptors and ephrins typically interact at sites of cell-cell contact, where they trigger bidirectional signals (forward signals through the receptor tyrosine kinase activity and reverse signals through the ephrin cytoplasmic region). Soluble monomeric ephrins released from the cell surface by proteases can also activate some Eph receptors. More recently discovered Eph receptor/ephrin signaling modalities include regulation of EphA2 signaling through serine phosphorylation, incorporation of Eph receptors into exosomes that can stimulate ephrin reverse signaling in distant cells, and regulation of Eph receptor function by ephrin-induced extracellular tyrosine phosphorylation. Furthermore, structural and biophysical approaches are offering new insights into different types of Eph receptor oligomeric assemblies in the plasma membrane, which could have different signaling ability.

Eph receptors and ephrins have been implicated in a multitude of diverse physiological and pathological processes. Besides the “traditional” roles of the Eph/ephrin system in tissue development/organization and in adult nervous system function, new roles have emerged in immune function, the cardiovascular system, cancer including cancer metabolism, neurodegeneration and some infections. However, much work remains to be done in order to fully understand Eph/ephrin physiological and pathological roles.

Eph receptors and ephrins are considered important drug targets. A variety of strategies are being explored for therapeutic targeting of Eph receptors, particularly against neurodegeneration and cancer. The design of efficacious targeting agents will be greatly helped by a better understanding of the roles of Eph receptors and ephrins in pathological processes. There is also a strong interest in exploiting the propensity of activated EphA2 to carry molecules bound to its extracellular region to the inside of the cell. This can enable targeted intracellular delivery of drugs to specific tissues exhibiting high EphA2 expression, and similar approaches may in the future extend to other Eph receptors.

This presentation will provide an introductory overview of the Eph/ephrin system and highlight new research directions.
Spatiotemporal Regulation of EphA2 Receptor Oligomerization: Insights from PIE-FCCS Single Molecule Live Cell Analyses
B. Wang

HD-PTP, an ESCRT protein, is required for EphB2 forward signalling in cell cytoskeletal dynamics and axon guidance
S. Lahaie\textsuperscript{1,2}, D. Morales\textsuperscript{1,2}, H. Bagci\textsuperscript{1}, C. Chang\textsuperscript{1,2}, A.C. Gingras\textsuperscript{3}, A. Pause\textsuperscript{4}, J.F Côté\textsuperscript{1,5}, A. Kania\textsuperscript{1,2,5}
\textsuperscript{1}Institut de recherches cliniques de Montréal, Montréal, QC, Canada.
\textsuperscript{2}Integrated Program in Neuroscience, McGill University, Montréal, QC, Canada.
\textsuperscript{3}Lunenfeld-Tanenbaum Research Institute, Toronto, ON, Canada.
\textsuperscript{4}Rosalind and Morris Goodman Cancer Centre, Montréal, Québec, Canada.
\textsuperscript{5}Faculté de Médecine, Université de Montréal, Montréal, QC, Canada.

To identify new proteins required for ephrin-B:EphB signalling, we conducted a BioID-based screen for novel EphB2 interactors. Among many hits, we found HD-PTP, a regulator of endosomal sorting complex transport (ESCRT) and a tumour suppressor. Co-immunoprecipitation confirmed increased EphB2 – HD-PTP interaction in HEK293 cells stimulated with ephrin-B2. Also, HeLa cells overexpressing EphB2 show increased levels of HD-PTP protein and co-localisation of HD-PTP and EphB2. HeLa cells heterozygous for HD-PTP show a blunted collapse response to ephrin-B2 but are collapsed normally by Sema3A, indicating that HD-PTP is not involved in general chemotropic responses. HD-PTP mRNA is expressed in developing spinal motor neurons, whose axon guidance depends on Eph signalling. Spinal motor neuron growth cones with a CRISPR:Cas9-induced HD-PTP loss of function, showed a diminished collapse in response to ephrin-B2 while responding normally to Sema3F. At the molecular level in HeLa cells and growth cones with decreased HD-PTP function, we found a reduction of both, ephrin-B2-induced Y418 phosphorylation of SRC family kinases, and EphB2 clustering. Also, in both models, following ephrin-B2 stimulation, surface levels of EphB2, were markedly decreased, compared to controls. Eph receptor cyclic trafficking entails its internalization following ligand binding, recycling, degradation and de novo receptor synthesis. To identify which of these steps are impacted by HD-PTP loss, we blocked protein synthesis in control and HD-PTP loss of function HeLa cells, and measured EphB2 protein level following ephrin-B2 stimulation. In control HeLa cells, ephrin-B2 stimulation led to an apparent increase in EphB2 levels, when compared to mock stimulation. In contrast, HeLa cells with lowered HD-PTP levels exposed to ephrin-B2 showed a trend of decreased EphB2 protein levels when compared to mock stimulation. These and other observations suggest that HD-PTP functions to control the cyclic trafficking of EphB2 upon ephrin-B2 exposure. We propose that HD-PTP is a new effector of EphB2 forward signalling, required for ephrin-B2-induced EphB2 clustering at the cell surface. As an ESCRT protein involved in intracellular trafficking of receptors, HD-
PTP may also control the amplitude and duration of EphB2 signalling by promoting EphB2 recycling and/or its stability. HD-PTP’s function as a tumour suppressor could thus involve modulation of ephrin-B/EphB signalling, also previously implicated in cancer.

**Functional Relevance of the Head-to-Head vs Head-to-Tail Eph-Eph Interactions for Receptor Activation**
D. Nikolov, and J. Himanen
*Sloan-Kettering Institute for Cancer Research, New York.*

The ectodomain (ECD) of the Eph receptors is a multidomain assembly, consisting of a ligand-binding domain (LBD), a cysteine-rich domain (CRD), and two fibronectin (FN) III domains. Earlier structural studies on the ligand-receptor binding domains have revealed details of the Eph/ephrin recognition. Thus, upon binding, a long hydrophobic loop of the ligand penetrates into a hydrophobic cavity on the surface of the receptor. While this interaction offers the energetic driving force for binding, and is necessary and sufficient for the formation of heterodimeric Eph/ephrin assemblies, it is not enough to cause the activation of Eph receptors. Imaging studies with Eph-expressing cells have shown that, unlike the ‘canonical’ RTK’s, where bringing two receptors close to each other is enough for signaling, Eph receptors require higher-order assemblies or clusters, for full biological activity. Earlier structures of the Eph ECDs have revealed how the clustering requires two separate receptor/receptor interfaces. The first one resides within the LBD and is able to bring two Eph molecules together even in the absence of the ligand. Thus, it’s called the ‘homo-dimerization’ interface. The second interface is located within the CRD and is called the ‘clustering’ interface. Each of the clustering interfaces in an Eph dimer can work independently to bring another receptor dimer in the assembly, thus forming a ‘trimer of Eph dimers’. This process will then proceed until a fully active Eph cluster, consisting of perhaps hundreds of receptors, is formed. A conclusion from these studies is that signaling-competent clusters may form once the local receptor concentration is high enough to allow the utilization of both the homo-dimerization and the clustering interfaces, even in the absence of the ligand, provided the expression level of Eph is high enough, as observed in certain cancer cells.

This presentation will discuss the existence of yet another interacting interface, that between the LBD and the FNIII domains of the unliganded Eph molecule. Once the receptor binds the ligand, this ‘head-to-tail’ interaction falls apart. The exact biological relevance of this interface is still under scrutiny but it has been suggested to be involved in the fine-tuning of the Eph signaling, for example collaborating with the Eph/ephrin in cis interactions reported for certain cell types to control the levels of Eph kinase phosphorylation. The head-to-tail homotypic interactions might also explain two specific phenomena observed in Eph signaling: first, the clusters sometimes seem to cover larger cell surface areas than would be expected based on the direct Eph/ephrin contact areas;
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second, unliganded Eph receptors have been reported to be recruited to the pre-existing Eph/ephrin clusters. These Eph-specific head-to-tail interactions might have implications for the future drug development.

A new dynamic model for the activation of the Eph receptor based on live-cell brightness analysis

S. Ojosnegros\(^1\), F. Cutrale\(^3\), D. Rodriguez\(^1,4\), J.J. Otterstrom\(^5\), C. Chiu\(^6\), V. Hortigüela\(^7,8\), E. Larrañaga\(^7,8\), C. Tarantino\(^2\), A. Seriola\(^2\), S. Mieruszynski\(^9\), E. Martinez\(^7,8,10\), M. Lakadamyali\(^5\), A. Raya\(^2,8,11\), S.E. Fraser\(^3\)

1California Institute of Technology, 1200 E California Blvd, Pasadena, CA 91125, USA.
2Center of Regenerative Medicine in Barcelona (CMRB), Barcelona Biomedical Research Park, Dr. Aiguader 88, 08003 Barcelona, Spain.
3University of Southern California, Translational Imaging Center, Molecular and Computational Biology, 1050 Childs Way Los Angeles, CA 90089.
4Laboratory of Theoretical & Applied Mechanics (LMTA), Dept of Mechanical Engineering, Universidade Federal Fluminense, Rua Passo da Pátria 156, Niterói, RJ 24210-240, Brazil
5ICFO-The Institute of Photonic Sciences, The Barcelona Institute of Science and Technology, 08860 Castelldefels (Barcelona), Spain.
6Center for Applied Molecular Medicine, University of Southern California, CA, USA.
7Biomimetic Systems for Cell Engineering group, Institute for Bioengineering of Catalonia (IBEC), Baldiri Reixac 15-21, Barcelona 08028 Spain.
8Networking Biomedical Research Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN).
9European Molecular Biology Laboratory (EMBL) Australia, Australian Regenerative Medicine Institute, Monash University, Clayton, Victoria 3800, Australia.
10Electronics Department, University of Barcelona (UB), Martí i Franquès 1-11, Barcelona 08028 Spain.
11Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain.

Eph receptor binding to its ligand ephrin mediates the assembly of high-order clusters fostering receptor activation by trans-phosphorylation. In the absence of regulation, receptor clustering would create an explosive signal amplification by propagating the signal towards aggregating monomers. However, this simplistic mode of activation cannot explain how Eph aggregation retains the ability to transduce the dynamic range of ephrin concentrations found in tissues and gradients (i.e. retina or intestine crypts). Here we apply our new enhanced version of number and brightness analysis to measure: (i) the Eph receptor oligomeric distribution; (ii) within each pixel of an image; (iii) over extended periods of time in live-cell experiments\(^1\). We induced the receptor response using dose-dependent presentation of the ligand micro-printed on surfaces, or bound to nanopatterned block-copolymers\(^2\). The combination of the efficient ligand presentation
with the brightness analysis procured quality data, which was coupled to straightforward biophysical models of protein aggregation. The analysis revealed that Eph clustering cannot be defined as a homogeneous process, but instead receptor aggregation proceeds by the combined contribution of two oligomerization mechanisms: polymerization and condensation. Polymerization mediates the receptor activation by assembling 5- to 8-mer oligomers. Condensation merges pre-assembled oligomers into large clusters dampening the signal propagation. We propose the polymerization-condensation dynamics as the regulator mechanism of receptor activation, enabling the transduction of the wide range of ephrin concentrations and gradients found in animal tissues.


EphB-EphrinB Bidirectional Signaling in the Nervous System and Beyond
M. Henkemeyer
UT Southwestern Medical Center, Dallas, USA.

Genetic studies of Ephs and ephrins have typically been conducted using knockout and other mutations generated through traditional gene targeting in embryonic stem cells. My laboratory has recently developed Cre-inducible ephrinB gain-of-function strains using CRISPR to target constructs via homology directed repair directly into the embryo. I will present our initial studies of these new mice that show gain-of-function ephrinB affects brain development. I will also present data that indicates these new mice may provide insight into potential roles for reverse signaling outside of the nervous system.

The role of EphB2 in memory formation
J.M. Alapin1, M. Dines1, M. Vassiliev1, T. Tamir1, A. Ram1, C. Locke2, J. Yu2, R. Lamprecht1
1Sagol Department of Neurobiology, Faculty of Natural Sciences, The Integrated Brain and Behavior Research Center (IBBR), Center for Gene Manipulation in the Brain, University of Haifa, Haifa, Israel.
2Center for Cell Analysis and Modeling, University of Connecticut Health Center, Farmington, CT, USA.

Eph receptors and their cognate ephrin ligands are attractive candidates to play a central role in memory formation as they are involved in regulation of synaptic transmission and neuronal morphogenesis. In this study we aimed to explore the roles of EphB2 forward signaling in fear memory formation and enhancement. Toward that end, we used a novel technology termed optoEphB2 that allows the control of EphB2 forward signaling activity by light. We show that activation of optoEphB2 in lateral amygdala (LA) during, but not
after, auditory fear conditioning training leads to the enhancement of long- but not short-term auditory fear memory. Photoactivation of optoEphB2 during fear conditioning led to activation of cAMP/Ca2+ responsive element binding (CREB) protein. Application of light to a kinase-dead optoEphB2 in LA during fear conditioning did not lead to enhancement of long-term auditory fear conditioning or to an increase in CREB phosphorylation. We also show that mice that express a carboxy-terminally truncated form of EphB2 (EphB2lacZ/lacZ) instead of the full-length EphB2, and therefore lack EphB2 forward signaling, are impaired in long- but not short-term auditory and contextual fear conditioning memory. Activation of optoEphB2 in LA of EphB2lacZ/lacZ mice rescued long-term contextual and auditory fear conditioning memory. Thus, the level of EphB2 forward signaling activity during learning determines the strength of long-term memory consolidation.

Unraveling the functions of EPHBs/EFNBs in the immune system and in the control of blood pressure and heart rhythm

H. Luo1, G. Yu, Z. Wu1, Y. Wang1, J. Tremblay1, J. Raelson1, J. Ledoux2, J. Lavoie1, W. Shi1, Z. Zhang1, J. Wu1
1CHUM Research Center, University of Montreal, Canada.
2Montreal Heart Institute, Canada.

EPHs and EFNs act in many biological systems. Our studies in the past 20 years revealed that they have novel functions in several new areas. We have found that they play pivotal roles in the immune system. EFNB1, 2 and 3 can provide co-stimulation to T lymphocytes and enhance their activation. This effect is via forward signaling, and EPHB6 is one of the receptors receiving such co-stimulation. EFNB1 and EFNB2 also directly interact with an important lymphokine receptor IL-7R and modulate its internalization, and hence regulate its signaling. Moreover, these 2 EFNBs control T cell chemotaxis via reverse signaling. Deletion of EPHB6, EFNB1 and EFNB2 leads to compromised T cell immune responses to graft rejection and LCMV infection, and reduced autoimmune diseases such collagen-induced arthritis (mouse model of rheumatoid arthritis) and experimental autoimmune encephalitis (mouse model of multiple sclerosis). In humans, elevated levels of EFNB1 and EFNB2 expression in T cells is observed in patients with rheumatoid arthritis and multiple sclerosis. EPHBs and EFFBs are novel blood pressure regulators. Deletion of EPHB6, EFNB1 and EFNB2 in mice leads to increased blood pressure, while deletion of EPHB4, EFNB2 and EFNB2 results in lower blood pressure. Such effects are often sex hormone-dependent. Vascular smooth muscle cells (VSMC) and adrenal gland chromaffin cells (AGCC) are target cells responsible for the observed blood pressure phenotype. Reverse signaling through EFNBs are necessary for the regulation of VSMC contractility, while in the case of EPHB4, both forward and reverse signaling is involved. In AGCC, deletion of EPHB6 results in increased BK channel current density and reduced calcium influx. As a consequence,
catecholamine synthesis and release are compromised. This EPHB6 KO phenotype requires the presence of testosterone. Several human genetic studies reveal that genetic variants in the EFNB2 and EFNB3 genes are indeed significantly associated with hypertension risks in a sex-specific way. We have discovered that EPHB4 kinase activity is critical for pacing cell function and development in mice. EPHB4 deletion or EPHB4 kinase inhibition causes significantly or dangerously lowered heart rates, respectively. EPHB4 deletion or inhibition severely impedes the pacing cell proliferation and differentiation from embryonic stem cells, and compromises HCN4 expression in pacing cells, HCN4 being a key ion channel controlling the pacing activity. In conclusion, we found that EPHBs and EFNBs are essential in immune responses to viral infection and autoimmune diseases. They also regulate blood pressure in a yin and yang fashion. The critical role of EPHB4 kinase activity in heart rate control raises a cautionary note for those developing EPHB4 inhibitors as anti-cancer agents.

**Eph signaling in progenitors of the neocortex: sticky with added vitamins**
A. Davy  
*Center for Integrative Biology, Toulouse.*
During mammalian cerebral cortex development, different neuronal subtypes are produced in a precise temporal order in that, deep-layer cortical neurons are generated first and upper-layer cortical neurons are born thereafter. Tight control of the tempo of neural progenitor differentiation is key to ensure the proper specification, and production in sufficient numbers, of each neuronal identity. Research in my group aims at characterizing the role of Eph/ephrin signaling in this complex developmental process, focusing on neural progenitors. In recent years, we showed that Eph/ephrin signaling controls progenitor apical attachment and cell division. More recently, we observed that conditional mutant embryos for ephrinB2 exhibit delayed neurogenesis in the neocortex. I will present evidence that Eph forward signaling controls progenitor differentiation by modulating folate metabolism and histone methylation. Altogether our studies highlight the importance of Eph/ephrin signaling in developmental neurogenesis and reveal an unexpected crosstalk between this cell-to-cell communication pathway and metabolism in neural progenitors.

**Analysis of soluble forms of ephrin-A1**
K. Ieguchi and Y. Maru  
*Department of Pharmacology, Tokyo Women’s Medical University.*
It has been reported that overexpression of ephrin-A1 is positively correlated with poor prognosis in some tumors such as colon and liver cancer. However, the detailed mechanism of poor prognosis caused by overexpression of ephrin-A1 is largely unknown.
Accordingly, the elucidation of ephrin-A1 function in poor prognosis and development of the molecular targeting drugs are urgent issues. Moreover, an establishment of prediction for metastasis is also important. Therefore, we have focused on the function of ephrin-A1 in metastasis that is the most leading cause of poor prognosis in cancer patients and characterized a new function in lung metastasis. We also investigated ephrin-A1 as a potential biomarker for metastasis. We have previously reported that ADAM12 (a disintegrin and metalloproteinase 12) is a binding partner of EphA1 and ADAM12 cleaves ephrin-A1 in trans. Our results suggest that a soluble form of ephrin-A1 generated by ADAM12 induces vascular hyper-permeability, thereby enhances lung metastasis. However, the cleavage mechanism of ephrin-A1 by ADAM12 remains to be elucidated, and the function of ADAM12-cleaved ephrin-A1 is largely unknown. We newly identified the C-terminal end in human ephrin-A1 amino acid sequences recognized and cleaved by ADAM12 by a mass spectrometric analysis. We demonstrate that ADAM12-cleaved ephrin-A1 is bioactive and equivalent to that of Fc-fused commercially available ephrin-A1 ligand in vitro. We also found soluble form of ephrin-A1 in urine of human and mouse suggesting that soluble form of ephrin-A1 may be a good candidate for a prediction of metastasis. We herein report a precise function of ADAM12-cleaved ephrin-A1 and a potential urinary biomarker for metastasis.

EphA2 in cancer and metabolism
J. Chen
Vanderbilt University, Nashville, USA.

Malignant tumors reprogram cellular metabolism to support cancer cell proliferation and survival. Although most cancers depend on a high rate of aerobic glycolysis, many cancer cells also display addiction to glutamine. We recently found that the ephrin-A1/EphA2 signaling axis regulates glutamine metabolism in HER2-positive cells through the YAP and TAZ-mediated transcriptional activation of glutaminase (GLS) and glutamine transporter ASCT2/SLC1A5. In patient breast cancer tissues, EphA2 expression positively correlates with YAP and TAZ, as well as GLS and SLC1A5. Whereas high expression of EphA2 predicted enhanced metastatic potential and poor survival, increased EphA2 expression rendered HER2-positive breast cancer cells more sensitive to glutaminase inhibition. Together, these findings define a previously unknown mechanism of EphA2-mediated YAP/TAZ activation to promote glutaminolysis and identify potential therapeutic targets in glutamine-addicted breast cancer.

Inducible knock-down of endogenous EphA3 in mice reveals novel roles for EphA3 in the inflammatory tumour microenvironment
M.E. Vail, R.H. Farnsworth, R. Dickens, M. Ernst, A.M. Scott, P.W. Janes
1Biomedicine Discovery Institute, Monash University, Clayton VIC 3800, Australia.
Tumour progression relies on interactions between tumour cells and stromal and inflammatory cells within the tumour microenvironment (TME). Amongst the proteins involved in shaping the TME, Eph receptors and their cell-bound ephrin ligands function in neo-angiogenesis, invasion and cancer stem cell maintenance, and are increasingly recognised as drug targets. EphA3 is not generally expressed in normal adult tissues, but re-emerges in a range of solid tumour types, and we previously found this expression is most evident in the TME, in cells recruited from the bone marrow to the tumour. EphA3 was expressed on mesenchymal stromal cells (MSCs), which form the tumour stroma and vasculature and support tumour growth, and targeting these cells with an anti-EphA3 monoclonal antibody (mAbIIIA4) inhibited tumour growth in mice, associated with disruption of the TME [1]. Interestingly, some EphA3 expression on myeloid cells was also noted. Since myeloid cell types can also promote tumour development, we set out to further investigate the importance of EphA3 in myeloid and non-myeloid cells of the TME. We now confirm myeloid EphA3 expression in an inflammatory mouse model of colon cancer (MC38), where antibody targeting of EphA3 inhibits tumour growth. We also detect EphA3 on myeloid cells in blood samples from patients with a range of tumour types. To determine the requirement of EphA3 in distinct cell populations in the TME, we developed mouse strains with inducible, shRNA-mediated knockdown of endogenous EphA3, accompanied by a GFP reporter, to monitor cells with shRNA expression. Use of alternative promoters allowed preferential targeting of either non-hematopoietic, or hematopoietic, cell populations. In lung tumour (LLC) allografts with stromal EphA3 expression, predominant non-hematopoietic shRNA expression resulted in robust knockdown of EphA3 in stromal cells, accompanied by tumour inhibition. Conversely, in the MC38 colon tumour model with myeloid EphA3 expression, hematopoietic cell shRNA expression was required to knock-down EphA3 and inhibit tumour growth. We thus present novel mouse models with inducible, preferential EphA3 silencing in distinct bone marrow-derived cell types, which indicate important roles for EphA3 in both stromal and inflammatory cell types within the microenvironment of different tumour types.


Inhibition of EphB4-ephrin-B2 interaction remodels the tumor immune microenvironment in head and neck cancers
S. Bhatia¹, A. Oweida¹, S. Lennon¹, S. Bukkapatnam¹, N. Uyanga¹, A. Phan¹, E. Clambey¹, E.B. Pasquale², S.D. Karam¹
1Department of Radiation Oncology, University of Colorado, Anschutz Medical Campus, Aurora, CO, USA.
Within the tumor microenvironment, T regulatory (Treg) cells and tumor-associated macrophages (TAMs) play a pivotal role in evasion of the immune response. Eph receptor tyrosine kinases and their membrane-bound ephrin ligands regulate tumorigenesis and immune cell development, migration, and activation. However, the regulatory role of Eph-ephrin interaction in facilitating recruitment of unique immune cell subtypes to the tumor bed remains understudied. The EphB4-ephrin-B2 interaction represents an important target in this context. Our data show that ephrin-B2 is predominantly expressed on the luminal side of the endothelial cells in HNSCC tumors, whereas EphB4 is expressed in immune cells. We hypothesize that inhibition of EphB4-ephrin-B2 interaction at the immune cell-tumor endothelium interface will block the trafficking and survival of Tregs and TAMs, thus allowing CD8 cytotoxic T cells to exert tumor growth inhibition. We used an EphB4-ephrin-B2 inhibitor (TNYL-RAW-Fc) in an orthotopic head and neck squamous cell carcinoma (HNSCC) mouse model along with cell culture models to investigate this hypothesis. Our mass cytometry (CyTOF) data show that HPV-negative HNSCCs are highly infiltrated with Tregs and TAMs but not with CD8-positive T effector cells. Targeted inhibition of EphB4-ephrin-B2 signaling in immunocompetent mice significantly delayed tumor growth. We further examined the immune landscape to understand the changes in immune cell mediators affecting tumor growth and progression. Blockade of EphB4-ephrin-B2 in vivo induces a significant decrease in the Treg population (characterized by a CD45+/CD3+/CD4+/Foxp3+ phenotype), a decrease in TAMs (CD11b+/F4/80+), an increase in macrophages with M1 phenotype (F4/80+/iNOS+), and a decline in macrophages with M2 phenotype (F4/80+/Arginase+). We have also observed a significant increase in activated CD8-positive T cells (IFN-gamma+/TNF-alpha+) and a selective reduction of L-Selectin in Treg cells. Our in vivo studies have further confirmed that depletion of CD8 T-cells with anti-CD8 antibody in an immunocompetent mouse model significantly reduces the response to TNYL-RAW-Fc treatment. Blockade of EphB4 reduces the levels of survival markers including p-AKT, p-ERK, p-STAT3, and BCL-XL as assessed by western blotting. Our data collectively indicate that inhibition of the association between EphB4 and ephrin-B2 facilitates reprogramming of the immune microenvironment resulting in a delay in tumor growth in vivo. These findings will be critical in understanding and exploiting the EphB4-ephrin-B2 axis as a novel target to overcome tumor immune evasion in head and neck cancers and possibly other cancers.

EphA2 and ephrin-A5 maintain distinct eye lens epithelial cell populations
C. Cheng¹, M. Amadeo¹, V. M. Fowler¹ and X. Gong²
1Department of Molecular Medicine, The Scripps Research Institute, La Jolla, CA, USA.
2School of Optometry and Vision Science Program, University of California, Berkeley, CA, USA.
The eye lens is a transparent organ that is responsible for the fine focusing of light onto the retina in order to form a clear image. Cataracts, defined as the development of opacity in the lens, remain the leading cause of blindness in the world. There are no non-surgical treatments to prevent or delay cataracts. Recent studies indicate Eph/ephrin bidirectional signaling is essential for lifelong lens transparency in humans and mice. Our studies in mouse lenses demonstrate that ephrin-A5 is required to maintain the anterior epithelial monolayer, while EphA2 is essential for equatorial epithelial and fiber cell organization and hexagonal cell shape. In this study, we determined whether EphA2 and ephrin-A5 are a lens receptor-ligand pair, and defined the universe of lens Ephs and ephrins.

To test whether abnormal signaling mechanisms play a role in cataractogenesis in ephrin-A5(-/-) or EphA2(-/-) mouse lenses, we generated EphA2 and ephrin-A5 double knockout (DKO) mice and compared the DKO phenotypes to those in single KO lenses. DKO lenses displayed an additive lens phenotype that was not significantly different from the two single KO lens phenotypes. Similar to ephrin-A5(-/-) lenses, DKO lenses had abnormal anterior epithelial cells leading to a large mass of epithelial cells invading the underlying fiber cell layer, leading to anterior cataracts in ephrin-A5(-/-) and DKO lenses. Yet, similar to EphA2(-/-) lenses, DKO lenses also had disorganized equatorial epithelial cells with disrupted fiber cells. This additive DKO lens phenotype rules out abnormal signaling by EphA2 in ephrin-A5(-/-) lenses or by ephrin-A5 in EphA2(-/-) lenses as possible cataract mechanisms, and suggests alternate ephrin-A5 and EphA2 mediated signaling pathways.

To identify potential EphA2 ligands and ephrin-A5 receptors in the lens, we used RT-PCR to evaluate expression of all Ephs and ephrins in wild-type (WT) mouse lenses. This screen identified 5 other Ephs and 4 other ephrins that are expressed in WT lenses, which are potential partners for ephrin-A5 or EphA2, respectively.

We conclude that EphA2 and ephrin-A5 do not form a lens receptor-ligand pair, and that EphA2 and ephrin-A5 utilize other binding partners in the lens to help align equatorial epithelial cells or maintain the anterior epithelium, respectively. By unlocking the universe of lens Ephs and ephrins and understanding the mechanisms for cataractogenesis due to changes in Eph-ephrin signaling, small molecules that modulate Eph/ephrin signaling may be potential anti-cataract therapies.

Impact of EphA4 ablation on cognitive function and disease pathology in a mouse model of Alzheimer’s disease
L. Poppe1,2, L. Rué1,2, Z. Callaerts-Vegh3,4, R D’Hooge5, R. Lemmens1,2,6, W. Robberecht1,2,6
1KU Leuven – University of Leuven, Department of Neurosciences, Experimental Neurology, Leuven, Belgium.
2 VIB, Center for Brain & Disease Research, Laboratory of Neurobiology, Leuven, Belgium
3KU Leuven - University of Leuven, Faculty of Psychology and Educational Sciences, Laboratory of Biological Psychology.
Alzheimer’s disease (AD) is the most common type of dementia, caused by degeneration of brain regions involved in memory and cognition. Early stages are characterized by synaptic dysfunctions and synapse loss, which correlate with the severity of memory loss in patients. So far, no effective cure for AD exists with current treatments mainly focusing on increasing the quality of life of patients.

EphA4 is a receptor of the ephrin system mainly expressed in the hippocampus and cerebral cortex during adulthood. It is involved in the regulation of synaptic maintenance and plasticity. EphA4 activation results in downregulation of AMPA receptors, loss of synaptic glutamate transporters and retraction of dendritic spines. In amyotrophic lateral sclerosis we demonstrated that EphA4 is involved in axonal regeneration capacity. Based on these various functions, we hypothesized that EphA4 ablation might mediate synaptic dysfunction and consequently cognitive performance in a mouse model of AD.

We conditionally deleted EphA4 in the forebrain of the APPPS1-21 mouse model. This mouse model is characterized by extensive amyloidosis of the brain, resulting in synaptic dysfunctions and cognitive deficits around the age of 9 months. To investigate the effect of EphA4 deletion on cognitive function, we performed a battery of behavioral tests, including the open field, sociability/preference for social novelty, Morris water maze and contextual fear conditioning. Together, our results suggest an effect of EphA4 in ameliorating the cognitive deficits in this AD mouse model. This improvement is not caused by a reduction of amyloidosis or soluble amyloid-beta levels in the brain. We are currently analyzing synapse morphology and function in order to dissect the underlying processes that have shown to improve cognitive defects.

Stop-and-go: ephrinA1 in endothelial migration and proliferation
S. Jellinghaus¹, E. Wiedemann¹, G. Ende¹, A. Augstein¹, R. Sczech², R.H. Strasser¹, D.M. Poitz¹,³
1TU Dresden, Heart Center, University Hospital, Germany.
2TU Dresden, Biotec, Light Microscopy Facility, Germany.
3TU Dresden, Institute of Clinical Chemistry and Laboratory Medicine, Germany.

The interaction of Eph-receptors and ephrin-ligands is one of the most famous examples of contact-dependent cell communication. But in spite of this, the role of Eph/ephrins in re-endothelialisation is still not completely understood. The aim of the present study was to investigate the regulation of ephrinA1 and its impact on proliferation and migration of human umbilical venous (HUVEC) and arterial endothelial cells (HUAEC).
Initially, a positive correlation between ephrinA1-levels and the density of endothelial cells was observed. This was further associated with a specific phosphorylation pattern of the EphA2 receptor (Y588 and S896). Further investigations regarding the proliferation and migration of endothelial cells was done by modulating ephrin-A1 expression using siRNA-mediated silencing or adenoviral overexpression. It could be shown that ephrinA1-silencing slightly increased endothelial proliferation, whereas the adenoviral overexpression of ephrinA1 reduced it. Using classical woundhealing assays as well as a live-cell-imaging variant of this assay revealed significant differences in the migration behavior in dependence of ephrin-A1 expression level. Overexpression and silencing of ephrin-A1 resulted in a faster closure of the wound. However, silencing of ephrin-A1 led to a faster migration of the cells associated with reduced directness. In contrast overexpression of ephrin-A1 did not influence the velocity of the cells, but led to enhanced directness of the migration. In addition, when ephrinA1 overexpressing cells came in contact with control cells, the formation of a barrier was observed. These results could be confirmed by showing that endothelial cells stop to migrate the moment they came in contact with an ephrinA1-coated surface. Using a baculoviral-mediated expression system, it could be shown that ephrinA1-silencing and overexpression modulates the formation of focal adhesions. This implicates that ephrinA1 is involved in the formation of the actin cytoskeleton which might be responsible for the observed alterations in the way of migration.

In conclusion, ephrinA1-expression is regulated by cellular density and is itself a critical determinant of endothelial proliferation. According to our current knowledge ephrinA1 seems to be remarkably involved in elementary processes of endothelial migration like cellular polarization, migratory direction and speed. These data support the notion of a pivotal role of ephrinA1 in basal mechanisms of re-endothelialisation.

Overview on the pharmacological tools to target Eph/ephrins
A. Lodola
University of Parma, Italy

EphA3 a functional targetable receptor for adult and pediatric brain cancer
B. Day
Queensland Institute of Medical Research, Brisbane, Australia.
Significant endeavour has been applied to define markers of brain cancer stem cells and to identify functional therapeutic targets to halt the growth of these aggressive diseases. This study identifies EphA3 as a functional, targetable cell surface marker for both adult and paediatric brain cancer.
We show that the receptor tyrosine kinase EphA3 is over expressed and functions to promote tumourigenesis in glioblastoma (GBM) and medulloblastoma (MB) while EphA3
levels in normal striatum and cerebellum are essentially absent. EphA3 shows disease subtype specific expression and is highly expressed on the more stem cell-like population. Critically, EphA3 functions to maintain tumour cells in an undifferentiated state by modulating MAPK signalling. EphA3 knock down or depletion induced differentiation and reduced the cancer stem cell pool leading to delayed orthotopic tumour formation in mice. Furthermore, we have demonstrated the safety and efficacy of EphA3-targeting strategies including radioimmunotherapy and antibody-drug conjugates (ADCs) in preclinical primary GBM and MB models. EphA3-ADCs were highly effective in vitro and, more importantly, showed significant anti-tumour activity in vivo while being well tolerated with no observable systemic toxicity. Intravital bioluminescence imaging showed that EphA3-ADCs reduced the burden of established tumours in mice which led to a doubling of survival. We propose that combining EphA3-ADCs with current treatment modalities has the potential to improve outcome and may allow de-escalation of current therapies to reduce off-target toxicities in normal healthy brain. These findings are being progressed to the clinic, with an EphA3 antibody-based therapy currently being testing in recurrent adult GBM patients. EphA3 is emerging as a relatively tumour-specific functional receptor with significant therapeutic potential for the treatment of adult and paediatric brain cancer.

**Peptides targeting the Sam domain of EphA2 and its interactome**

F.A. Mercurio¹, M. Vincenzi², M. Leone¹

¹Institute of Biostructures and Bioimaging (CNR) and Cirpeb (University of Naples Federico II) Via Mezzocannone 16, 80134 Napoli, Italy.

²Institute of Biostructures and Bioimaging (CNR) and Fondazione Umberto Veronesi.

Sam (Sterile alpha Motif) domains are small helical protein-protein interaction modules which play different functions in several cellular processes. The Eph family of tyrosine kinase receptors contains at the C-terminus a Sam domain; the Sam domain from EphA2 (EphA2-Sam) has received the largest attention. EphA2 is over-expressed in several tumors and upon stimulation with an ephrin ligand, undergoes endocytosis and consequent degradation, a process that could be exploited to lower tumor malignancy. EphA2-Sam represents the site where protein modulators of receptor endocytosis and stability (like the lipid phosphatase Ship2) are engaged by means of heterotypic Sam-Sam interactions. Ship2 acts as an inhibitor of receptor endocytosis and its binding to EphA2-Sam is expected to primarily induce pro-oncogenic effects in cell. During the last few years, in an attempt to discover novel potential anticancer therapeutics, we designed and evaluated peptide inhibitors of EphA2-Sam mediated interactions through different strategies and a multidisciplinary approach.
Chemical biology strategies for targeting the EphA2 and EphA4 ligand binding domains: applications in neurodegeneration and oncology

M. Pellecchia

Biomedical Sciences Division, School of Medicine, University of California Riverside, 92512 Riverside, USA.

The design of selective and effective agonists or antagonists that target Eph receptors ligand binding domains remain to date a very a challenging task. While a handful of potentially interesting compounds have been reported, only few have been fully validated in vivo. Short agonistic peptides including YSA targeting EphA2 or KYL targeting the EphA4 remain the most studied EphA2-, EphA4-targeting agents in vivo, respectively, likely as they are readily commercially available at low cost. These agonistic peptides, however, are still only moderately potent, and suffer from limitations typical to natural peptides, namely rapid degradation in plasma and clearance in vivo, as well as limited brain exposure. Over the past several years, we devoted significant efforts in deriving novel agents targeting these domains. In the first part of this presentation, I will review our recent work on targeting the EphA4 ligand binding domain with small peptide mimetics that resulted in the lead agent 123C4,1 which is being investigated as potential ALS therapeutic (Iron Horse Therapeutics, San Diego). In addition, we have recently investigated a variety of peptide-drug conjugates (PDCs) using agonistic EphA2-targeting agents, initially using the YSA peptide2,3 or more recently our optimized agent termed 123B9.4 Further optimizations and characterizations of EphA2-targeting agents and drug conjugates was accomplished in part using unpublished structural information on the ligand binding domain of EphA2 in complex with such agents. The EphA2-targeting drug conjugates are very effective in targeting circulating tumor cells and in inhibiting tumor growth and metastasis in various mouse models including melanoma,4 prostate,2,3 pancreatic, and breast cancer models.5

Mu opioid receptor (MOR) activation by morphine in neuronal cell models is dampened by ephrinB1-induced signaling and may be rescued by novel EphB1 receptor peptide antagonists
A. Bedini, G. Vaca, S. Lombardi, S. Spampinato.
Department of Pharmacy and Biotechnology (FaBiT) – University of Bologna
Via Irnerio 48, 40126 Bologna – Italy.

EphB1 receptor activation in nociceptive neurons induces hyperalgesia and allodynia, whereas its blockade ameliorates pain behaviors in models of neuropathy or cancer pain. Mu opioid receptor (MOR) agonist morphine is one of the most potent and widely used analgesics; however, it is poorly effective in different chronic pain states, including those mentioned above. Activation of EphB1 receptor-mediated signaling may contribute to opioid-induced hyperalgesia in rat, whereas its inhibition may rescue opioid-induced analgesia in a rodent model of bone cancer pain; thus, suggesting a potential inverse relationship between EphB1 receptor and MOR. Nonetheless, any cross-talk between these latter receptors, and its potential influence on reduced opioid analgesia or tolerance to opiates, has been so far poorly investigated. Therefore, we aimed at detailing any functional cross-talk between intracellular signaling pathways triggered by MOR and EphB1 receptors, and at assessing the ability of two novel EphB1 peptide antagonists to rescue morphine-mediated intracellular signaling, in neuronal cell models co-expressing EphB1 and MOR. We found that ephrinB1-EphB1 receptor signaling through PI3K blunted morphine-mediated, PKC-dependent ERK1/2 activation in undifferentiated SH-SY5Y neuroblastoma cells co-expressing MOR and EphB1 receptor. Notably, PMA-induced differentiation up-regulated the former and down-regulated the latter: under these conditions, morphine significantly activated ERK1/2 even when ephrinB1 was co-administered. Conversely, in PMA-differentiated SH-SY5Y cells exposed to TNFα, EphB1 receptor expression was significantly up-regulated: under these conditions, morphine failed to increase ERK1/2 phosphorylation when ephrinB1 was co-administered. Interestingly, two novel EphB1 receptor peptide antagonists that we recently identified displayed the potential to counteract the attenuation of morphine-mediated signaling induced by ephrinB1-EphB1 receptor in undifferentiated as well as in differentiated, TNFα treated SH-SY5Y cells. Our findings show for the first time that, when EphB1 receptor is activated, morphine no longer triggers PKC-dependent signaling events downstream of MOR; this cross-talk may contribute to the reduction of opioid-mediated analgesia in different models of chronic pain. At this regard, the two novel EphB1 receptor peptide antagonists tested in this research showed the premises to counteract ephrinB1-induced attenuation of MOR-dependent signaling and to effectively rescue morphine-mediated responses in neuronal cells co-expressing MOR and EphB1 receptor.
POSTER SESSION

Using proteomics to identify novel interactors of EphB2 and ephrinB1 during cell segregation
T.G. Ashlin and D.G. Wilkinson
1Neural Development Lab, The Francis Crick Institute, London, NW1 1AT.
Eph receptor and ephrin signalling has a major role in cell segregation and border formation. Previous studies from our lab have employed an in vitro HEK293 model to study cell behaviours during these processes. Measurements of contact repulsion revealed that EphB2, EphB2-Kinase Inactive (EphB2-Ki) and ephrinB1 expressing populations exhibit strong heterotypic and weak homotypic repulsion. Simulations revealed that the strong heterotypic repulsion can account for cell segregation and border sharpening.
As the EphB2-Ki cells also exhibit heterotypic repulsion following contact with ephrinB1 expressing cells alternative kinase independent pathways must be activated to drive repulsion. In this project, we use proteomic techniques such as proximity dependent biotin identification (BioID) and co-immunoprecipitation to identify interacting proteins that may contribute to cell repulsion during eph/ephrin mediated cell segregation and border formation.

Reduced levels of ephrinA5 do not affect functional motor performance after photothrombotic lesion in mice
A. de Boer1,2, A. Storm1,2, L. Rué1,2, W. Robberecht1,2,3, R. Lemmens1,2,3
1KU Leuven – University of Leuven, Department of Neurosciences, Experimental Neurology, Leuven, Belgium
2 VIB, Center for Brain & Disease Research, Laboratory of Neurobiology, Leuven, Belgium
3 University Hospitals Leuven, Department of Neurology, Leuven, Belgium
Stroke is the main cause of adult disability worldwide, affecting about 15 million people each year. During an ischemic insult, various molecular events lead to neuronal (and glial) cell death causing impaired neurological functioning. Although stroke damage can be devastating, the majority of patients survive the initial event and undergo some spontaneous recovery, a process that can be improved by rehabilitation. Stroke recovery is influenced by both promoting and inhibitory factors and stimulating or blocking might enhance functional outcome after stroke.
EphrinA5 is part of the ephrin system, which is known to play a role in different (central nervous system) injury models. Previously, ephrinA5 was found to be upregulated in reactive astrocytes in the peri-infarct area after experimental stroke and blocking ephrinA signaling resulted in improved functional outcome. In the present study, we aim to validate the involvement of ephrinA5 in stroke recovery using an ephrinA5 heterozygous
mice which will be functionally assessed after experimental stroke introduced by the photothrombotic lesion model (PTL).
Adult male mice were subjected to PTL and recovery was assessed using different behavioral tests. A subset of mice was sacrificed to determine differences in infarct size and brain swelling at 24h post stroke. In addition, cortical tissue was collected after one week to determine the effect of ephrinA5 on astrocyte reactivity, glial scar formation, neurodegeneration and axonal plasticity.
Our results show that heterozygous deletion of ephrinA5 did not affect infarct size and brain swelling after PTL. Using the horizontal ladder and single pellet reaching task, no differences were observed in functional recovery after PTL between ephrinA5+/- and control mice. In accordance to this, ephrinA5 reduction did not affect astrocyte reactivity as assessed by GFAP and vimentin protein levels on western blot and immunofluorescent staining. Furthermore, the expression of various glial scar components and GAP43 were neither affected in ephrinA5+/- animals. As a marker for neurodegeneration/regeneration we used a NF200 immunofluorescent staining, however no differences were found between ephrinA5+/- and control mice.
In future experiments, we will determine the temporal cell-type specific expression of ephrinA5 using RNA-scope and the direct effect of ephrinA5 reduction on axonal sprouting using BDA tracing analysis.

Genetic Dissection Of Eph/Ephrin Signalling During Hindbrain Segmentation
J. Cayuso and D.G. Wilkinson
Neural Development Laboratory, The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK.
The establishment of sharp borders between tissue interfaces is critical for the correct organization of organs and occasionally underlies the formation of signalling centres between different territories.
During embryonic development, the vertebrate hindbrain is subdivided in different rhombomeres along its anterior-posterior axis. This becomes apparent by the segmentally restricted expression of several transcription factors, which is initially fuzzy and is subsequently refined. Following this, a distinct cell population, the boundary cells, appears at the borders between rhombomeres and acts as a signalling centre.
The sharpening of the borders between different rhombomeres encompasses changes in cell fate and Eph/Ephrin-mediated cell sorting. Multiple Eph receptors and Ephrins are expressed in segmentally restricted patterns in the hindbrain in such a way that high-affinity pairs are expressed in a complementary manner. Perturbation of Eph/Ephrin signalling by either morpholino antisense oligonucleotides or overexpression of full-length or secreted forms of Ephs and Ephrins is sufficient to disrupt border sharpening and the formation of boundary cells. However, the individual contribution and the genetic
interactions between different Ephs and Ephrins, as well as the signalling direction and the molecular mechanism responsible for mediating cell sorting and boundary cell formation remain elusive.

Using CRISPR/Cas9 we have generated zebrafish mutants for different Ephs and Ephrins as well as truncations of their intracellular domains to assess the relevance of forward and reverse signalling. Finally, we coinjected CRISPR/Cas9 with different donor sequences to introduce precise mutations affecting specific Eph and Ephrin signalling motifs. Analysis of single and combined Eph/Ephrin mutants revealed the involvement of multiple signalling pairs at specific rhombomeric borders, while mutants lacking the intracellular region or specific Eph and Ephrin signal transduction domains are starting to shed light into the molecular mechanisms controlling cell segregation during hindbrain segmentation.

Small molecules targeting Eph/ephrin system as inhibitor of VEGFR2-mediated angiogenesis

P. Chiodelli¹, G. Paiardi¹, C. Urbinati¹, A. Lodola², M. Tognolini², M. Rusnati¹

¹ Dept. Of Molecular and Translational Medicine, University of Brescia.
² Dept. of Pharmacy, University of Parma.

Eph/ephrin system is involved in the regulation of angiogenesis and tumor development, being in this closely intertwined to the VEGF/VEGFR2 system. New small molecule compounds, in particular UniPR1331 and UniPR139, were developed as inhibitor of Eph-ephrin interaction with promising anticancer potential. Relevantly, evidences indicate that these compounds are also able to inhibit angiogenesis in vitro on human umbilical endothelial cells (HUVECs) and in vivo in a chorioallantoic membrane (CAM) assay. Taken together, these observations prompted us to investigate if the compounds are able to act directly onto the VEGF-VEGFR2 system.

Surface plasmon resonance showed that UniPR1331 and UniPR139 directly bind VEGFR2 with an affinity (Kd) equal to 58 μM and 10 μM, respectively. Accordingly, the compounds inhibit VEGF-VEGFR2 interaction in a cell-cell adhesion assay. UniPR1331 and UniPR139 inhibit VEGFR2 phosphorylation and internalization induced by VEGF stimulation and consequent signal transduction in HUVECs and other VEGFR2-overexpressing endothelial cells. Accordingly, the compounds inhibit VEGF-dependent proliferation of HUVECs, their sprouting activity and their capacity to repair of a wound monolayer (motogenic activity). Taken together, these data demonstrated that, beyond acting directly on Eph/ephrin system UniPR1331 and UniPR139 exert a potent antiangiogenic activity by inhibiting VEGF/VEGFR2 interaction, emerging as promising multitarget inhibitors able to interfere simultaneously with two system deeply implicated in angiogenesis and tumor progression.
In vitro study of the interference of an Eph antagonist on Eph/MAPK axis in glioblastoma U87 cells

M. Corrado, Tognolini and C. Giorgio

Department of Food and Drug, University of Parma, Viale delle Scienze 27/A, 43124, Parma, Italy

Glioblastoma (GBM) is the most malignant brain tumor with a dismal prognosis in adults and with a median survival for patients below 15 months. The main factors of failure in GBM treatment include tumor cell resistance to chemotherapy and radiation therapy, limitations in drug delivery, increased angiogenesis and vasculogenic mimicry (VM), and presence of glioma stem cells (GSCs). Several EphA2 and EphA3 receptors have been shown significant overlapping expression pattern and associated with the maintenance of an undifferentiated, self-renewing tumor population through a mechanism that limits MAPKs signalling in GBM tumor-propagating cells with stem-like characteristics (TPC) and in different GBM cell lines where they contributed to maintain tumor cells in a de-differentiated state and in the more aggressive mesenchymal subtype. In fact, different research groups have observed a reduction of clonogenicity of GSC after the ephrinA1-induced EphA2 downregulation. Furthermore, an over-activation of ERK1/2 was revealed after EphA2 and EphA3 downregulation, suggesting that the Eph/ephrin signalling could exercise its pro-oncogenic effects through the inhibition of ERK1/2 activation.

Based on these data, we decided to investigate if the antitumor properties of the Eph receptors antagonist was correlated with the break of the Eph/ephrin signalling’s brake on ERK1/2 activation in GBM U87 cell line.

According to literature data, when U87 cells were stimulated with high concentration of ephrin-A1-Fc, a downregulation of EphA2 receptor was observed and it was accompanied by a decrease of ERK1/2 phosphorylation, confirming the inhibiting role of Eph/ephrin signalling on ERK1/2 activation also in this cell line. As expected the treatment of GBM with UniPR1331 determined an increased and prolonged activation of ERK1/2 in a concentration dependent manner and this effect was inhibited in presence of the MEK1/2 inhibitor UO126. Moreover the anti-proliferative activity of UniPR1331 was reversed in presence of UO126 confirming that the effects of compound were ERK1/2 pathway mediated and that its prolonged activation could be detrimental for U87.

In conclusion, we provided new evidence of the close link between Eph/ephrin signalling and the limitation of the MAPKs signalling in GBM and in this scenario we speculate that the use of Eph antagonists could be an effective and innovative strategy for the treatment of GBM.
Negative phosphoregulation of NCK1/2 adaptor proteins by the tyrosine kinase receptor EphA4

U. Dionne1,2,3, S.L. Banerjee1,2,3, F.J.M. Chartier1,2,3, Y.L. de los Santos3,4, N. Lavoie1,2,3, D.N. Bernard3,4, F. Otis3,5, K. Jacquet1,2,3, M.G. Tremblay1, M. Jain3,6, S. Bourassa1, G.D. Gish7, P. Laprise1,2,8, N. Voyer3,5, C.R. Landry3,6, N. Doucet3,4, N. Bisson1,2,3,8
1Centre de recherche du Centre Hospitalier Universitaire (CHU) de Quebec-Université Laval, Axe Oncologie, Québec, QC, Canada.
2Centre de recherche sur le cancer de l’Université Laval, Québec, QC, Canada.
3PROTEO-Quebec Network for Research on Protein Function, Engineering, and Applications.
4INRS-Institut Armand-Frappier, Université du Québec, Laval, QC, Canada.
5Department of Chemistry, Université Laval, Québec, QC, Canada.
6Department of Biology and Institut de Biologie Intégrative et des Systèmes, Université Laval, Québec, QC, Canada.
7Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Joseph and Wolf Lebovic Health Complex, Toronto, ON, Canada
8Department of Molecular Biology, Medical Biochemistry and Pathology, Université Laval, Québec, QC, Canada.

Cells respond to extracellular stimuli via membrane-bound proteins, such as Eph family of receptor tyrosine kinases (EphRs), in order to survive and to adapt to their environment. EphR activation generates phosphotyrosine (pTyr) docking sites required for the assembly on the plasma membrane of protein interaction networks that drive downstream signalling. In particular, adaptor proteins NCK1/2 are key hubs for the nucleation of EphR-dependent signalling complexes. Their function is to couple pTyr on activated receptors via their single SH2 domain to cytoplasmic effectors containing Pro/Arg-rich motifs via their three SH3 domains. However, the mechanisms leading to network disassembly and its consequence remain essentially unknown. We sought to determine whether NCK1/2 proteins are regulated by EphR-mediated tyrosine phosphorylation. We used mass spectrometry to map pTyr residues on NCK1/2 and to analyze the effect of those modifications on NCK1/2 signaling networks in vivo. We identified 7 distinct pTyr on NCK1/2, including one that lays in the binding pocket of each SH3 domain of NCK1/2 and that is conserved in 57% of the 250 murine SH3 domains. We show that the direct EphA4 phosphorylation of NCK1/2 SH3 domains at these sites results in the collapse of NCK-dependent signalling networks both in vitro and in mammalian cells by abrogating NCK1/2 SH3 domains interactions with their targets. We further demonstrate that a phosphomimic triple mutant (Y/E) of the conserved Tyr of Dock, the Drosophila ortholog of NCK1/2, inhibited its SH3-dependant functions in the development of the fly eye. Our findings uncover a novel, conserved mechanism through which EphRs rapidly and reversibly terminate downstream signalling while remaining on the plasma membrane in a catalytically active state.
Regulation of the endothelial barrier function by ephrin A1

G. Ende¹, F. Teschendorf¹, A. Augstein¹, F. Härtel², T. Noll², D.M. Poitz¹,³, S. Jellinghaus¹

¹ Department for Internal Medicine and Cardiology, University Clinic, TU Dresden.
² Institute of Physiology, Medizinische Fakultät Carl-Gustav-Carus, TU Dresden.
³ Institute for clinical chemistry and laboratory Medicine, TU Dresden.

Previous studies of our group showed a distinct impact of ephrin-A1 on adhesion of monocytes to endothelial cells as well as their cytoskeletal formation. In addition, it could be shown that ephrin-A1-EphA2 interaction influences endothelial proliferation and migration and vice versa. Higher levels of ephrin-A1 protein and mRNA in confluent endothelial cells compared to proliferating cells lead to the question whether ephrin-A1 is also involved in endothelial barrier formation and function as endothelial dysfunction represents a crucial step in the initiation and progression of atherosclerosis.

As a model system human umbilical vein endothelial cells (HUVEC) were used. For barrier function assays HUVEC were seeded on a filter membrane with defined pore diameter and protein-flux through the membrane and transmigration of monocytes could be quantified. After siRNA mediated silencing as well as adenoviral overexpression of ephrin-A1 in the endothelial monolayer, a reduced MCP-1 induced monocyte transmigration was observed. In contrast, activating the EphA2 receptor using soluble ephrin-A1 did not influence monocyte transmigration. To analyze the permeability for serum proteins HUVEC were seeded on small pore membranes and incubated with coloring albumin in the upper compartment. Silencing and overexpression of ephrin A1 clearly influenced the number of diffused albumin in a short-term measurement (live reading) as well as in a long-term measurement (point readings) over 24h. Again, stimulation with ephrin-A1 did not influence the permeability. Broad range inhibition of sheddases using GM6001 blocked the increase of ephrin-A1 protein and mRNA in confluent HUVEC suggesting a crucial involvement of protein shedding in ephrin A1 regulation. Incubation with a proteasome inhibitor (MG132) also reduced protein and mRNA levels of ephrin-A1 and increased EphA2 protein levels. That led us to the hypothesis that an internalization of receptor and ligand in complex causes increased ephrin A1 and lowered EphA2 expression in confluent HUVEC.

In conclusion, the present study shows that modulation of ephrin-A1 expression in HUVEC influences the endothelial barrier function in vitro. Furthermore, the regulation of endothelial ephrin A1 in dependence of cell density could be shown to be controlled by shedding and proteosomale degradation leading to increased ephrin A1 in confluent HUVEC. This study adds another piece of the puzzle to the role of the Eph/ephrin system in atherosclerosis and strengthens the hypothesis that the Eph/ephrin-system might be an interesting target for an anti-atherogenic therapy.
The Ephrin A2 receptor tyrosin kinase (EphA2) is downregulated by the KSHV immediate-early transactivator RTA

Lanfer, J., Holzer A., Neipel F.

Virologisches Institut, Universitätsklinikum Erlangen, Schlossgarten 4, 91054 Erlangen, Germany

Introduction: We have shown that the Ephrin receptor tyrosine kinase A2 (EphA2) is a cellular receptor for KSHV glycoproteins H and L (gH/gL). Notably, EphA2 is highly expressed in many solid tumors including Kaposi sarcoma which is associated with rapid progression and poor prognosis. However, the mechanisms of EphA2 expression regulation in malignant disorders are essentially unknown. Objectives: Analysis of the influence of latent and lytic KSHV infection on EphA2 expression. Materials & Methods: Ephithelial and endothelial cells were infected with KSHV and EphA2 expression was examined on both protein- and transcript-level in latent infection and upon induction of KSHV lytic replication. Results: Infection of the epithelial cell line SLK revealed that latent infection with KSHV does not alter expression of the KSHV entry-receptor EphA2. However, expression of EphA2 protein was reduced by at least 80% upon induction of the KSHV lytic replication cycle. Analysis of EphA2 expression in cells expressing only the viral transcription transactivator RTA revealed that this protein was sufficient to reduce EphA2 expression. RT-PCR analysis revealed that EphA2 suppression by RTA was partially on the transcript level. These findings could be confirmed when primary human lymphatic endothelial cells (LECs) were infected with KSHV. Comparable to SLK cells, EphA2 expression on both protein- and transcript level was strongly reduced as early as 24hrs post infection. Semi-lytic infection of LECs with KSHV did not only result in the overall reduction of EphA2 transcription. The markedly increased amount of an EphA2 fragment of approximately 50kDa showed that EphA2 was also regulated on the protein-level upon semi-lytic infection of LECs. Conclusion: Expression of KSHV lytic cycle genes inhibits EphA2 expression by both transcriptional and post-transcriptional mechanisms with RTA being the main regulator.

Understanding the role of the Eph/ephrin system and the importance of cell mechanical properties in cardiomyocyte differentiation

R. H. Pires¹, N. Shree¹, D. M. Poitz², O. Otto¹

1ZIK-HIKE, University of Greifswald, Greifswald, Germany.

2Institute of Clinical Chemistry and Laboratory Medicine, TU Dresden, Dresden, Germany.

The Eph receptors and their cognate ligands, the ephrins, are the largest family of receptor tyrosine kinases, and their signaling has been shown to play diverse roles in various cellular processes. Recent studies suggest that manipulation of ephrin–Eph cell signaling can favorably influence cardiomyocyte viability and ultimately preserve cardiac function after myocardial infarction (MI). Unlike most cells, cardiomyocytes do not have the ability
to proliferate, and in the event of an injury to the myocardium, non-contractile cardiac fibroblasts typically occupy the site of injury, contributing to reduced performance of the heart muscle. Several reports have highlighted the usage of induced pluripotent stem cells (iPS), and iPS-derived cardiomyocytes as part of a therapeutic strategy to promote the treatment of MI patients. It has been suggested that the Eph/ephrin system may play a role in the process of cell differentiation of cardiac stem cells. However, the exact role that Eph receptors and ephrins play in myocardium repair is still not understood. Here, we introduce real-time deformability cytometry (RT-DC) to study the process of cardiomyocyte differentiation considering the Eph/ephrin system. RT-DC analyses cell mechanical properties as a label-free functional cell assay at a throughput of 1,000 cells/second in real-time. In combination with a custom-developed algorithm that extracts properties from the beating pattern of cardiomyocytes using phase contrast video microscopy, we demonstrate that our assay is capable to trace specific mechanical phenotypes of cardiac progenitor and adult cardiomyocytes. In combination with cytoskeletal markers and RNA expression analysis we link these phenotypes to the expression of different members of the Eph/ephrin system. Our results reveal an evolution in the beating pattern of developing cardiomyocytes during cell maturation which is accompanied also by increasing cell stiffening. These changes in dynamic and mechanical properties of cardiac cells are accompanied by a clear difference in the expression pattern of the members of the Eph/ephrin family.

In summary, we highlight for the first time the potential of high-throughput dynamic and mechanical phenotyping to study the influence of the Eph/ephrin family during the development of cardiomyocytes.

Reduction of Ephrin-A5 levels worsens disease progression in a mouse model of Amyotrophic Lateral Sclerosis

L. Rué1,2, M. Timmers1,2, A. Lenaerts1,2, L. Van Den Bosch1,2, P. Van Damme1,2,3, R. Lemmens1,2,3, W. Robberecht1,3

1KU Leuven – University of Leuven, Department of Neurosciences, Experimental Neurology and Leuven Institute for Neuroscience and Disease (LIND), Leuven, Belgium.
2VIB, Center for Brain & Disease Research, Laboratory of Neurobiology, Leuven, Belgium.
3University Hospitals Leuven, Department of Neurology, Leuven, Belgium.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects lower motor neurons in brainstem and spinal cord, and the upper motor neurons in the motor cortex, and leads to a progressive muscle phenotype in patients. ALS is characterized by considerable genetic and clinical heterogeneity, indicating that there are factors that modify the phenotypic expression of the disease. Genetic and pharmacological inhibition of EphA4, a tyrosine kinase receptor, rescued the motor neuron phenotype in zebrafish.
and rodent models of ALS. In addition, an inverse correlation was found between EphA4 expression and disease onset and survival in patients.

We aimed here to identify whether ephrin ligands would contribute to the EphA4 modifying effect in ALS. Most ephrin-A and ephrin-B ligands are able to bind and activate EphA4. Inhibition of one ephrin, ephrin-A5, has been shown to increase the recovery of a mouse model of stroke. Here, we aimed to determine the contribution of this specific ephrin in ALS, by reducing ephrin-A5 levels in a mouse model of ALS, the SOD1G93A mouse, by crossing it to an ephrin-A5 knockout mouse. We followed the mice during disease progression to determine the decline in their motor behavior as well as their latency to reach an end-stage point of the disease. Although we did not observe differences in disease onset, survival and disease duration were significantly reduced in the mice with lower ephrin-A5 levels. We next determined the expression pattern of ephrin-A5 in the spinal cord of SOD1G93A mouse by means of in situ hybridization. Ephrin-A5 was mainly expressed by neurons, both ChAT positive and negative neurons, both in SOD1WT and SOD1G93A mice. Although ephrin-A5 was mainly expressed in neurons, a reduction of its levels did not lead to nerve conduction deficits nor deficits in the percentage of innervated neuromuscular junctions in the gastrocnemius muscle. Finally, we assessed by qPCR the levels of several biomarkers of neuronal function, astroglial reactivity and microglial activation in the spinal cord of SOD1G93A mice with normal or reduced levels of ephrin-A5, but no differences were detected. These results might suggest that, in contrary to EphA4, ephrin-A5 expression might be beneficial during ALS disease progression. Further work needs to be performed in order to understand the mechanism of this beneficial role of ephrin-A5 in ALS.

Therapeutic potential of an EphA4 blocking peptide for treatment of ALS: a preclinical study.

S. Smolders1,2, L. Rué1,2, M.M. Gomez-Soler3, E.B. Pasquale3, R. Lemmens1,2,4, W. Robberecht1,4
1KU Leuven-University of Leuven, Department of Neurosciences, Experimental Neurology and Leuven Research Institute for Neuroscience and Disease (LIND), 3000 Leuven, Belgium.
2VIB, Center for Brain and Disease Research, Laboratory of Neurobiology, 3000 Leuven, Belgium.
3Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, California 92037, United States.
4University Hospitals Leuven, Department of Neurology, 3000 Leuven, Belgium.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that affects upper and lower motor neurons, leading to paralysis and eventually death within a few years after diagnosis. Genetic modifying factors can partially explain the variability in disease onset and progression among patients. Recently, our lab has identified the EphA4 receptor
tyrosine kinase as such a disease modifier for ALS. Indeed, intracerebroventricular administration of the selective EphA4 blocking peptide ‘KYL’ in a rat model of ALS resulted in a modest increase in disease survival. Modification of the cyclic peptide ‘APY’ has led to the generation of ‘APY-d3’, a highly specific, stable and potent antagonist for the EphA4 receptor. The aim of this study is to explore the therapeutic potential of this improved EphA4 antagonist in a mouse model of ALS.

The APYd3 peptide was continuously administered intracerebroventricularly in SOD1G93A +/- mice, a model for ALS, from the age of 60 days on using Alzet osmotic minipumps. The KYL peptide was used as a positive control while artificial cerebrospinal fluid (aCSF) and an inactive variant of APYd3 served as negative controls. Disease onset was defined as the time when performance on the rotarod was deceased by at least 50%. End stage was defined as the time when the mouse could no longer turn back on its paws within 20 seconds after placing it on its back.

Our data show that the APYd3 peptide is stable inside the osmotic pumps for at least 28 days and that intracerebroventricular infusion with 1 mM peptide results in detectable levels in the mouse brain. Although preliminary results suggest no difference between treatment groups in disease onset or survival (n=9), an increase in group size is needed before drawing final conclusions about the effects of this dose of peptide.

Novel Antagonist targeting EphB1 receptor

G. Vaca1, A. Bedini1, A. Tolomelli2, S. Spampinato1

1Department of Pharmacy and Biotecnology (FaBit), Alma Mater Studiorum Università di Bologna, Bologna – Italy.
2Department of Chemistry "G. Ciamician", Alma Mater Studiorum Università di Bologna, Bologna – Italy.

EphB receptors and their membrane-bound ephrin B ligands are expressed in the nervous system where their interaction could be involved in the induction and persistence of various types on pain as well as in the physical dependence and tolerance on opiates. Several studies have demonstrated that the interaction between EphB1 receptor with ephrin-B1 ligand is required for this pathological process. Furthermore, some evidences have confirmed that reduced EphB1 expression with agents that inhibit EphB-EphrinB interaction can alleviate spontaneous pain, thermal hyperalgesia, mechanical allodynia, and opiate dependence and tolerance in rodent models. This suggests that antagonists targeting EphB1 receptor could represent a novel class of analgesics for the treatment of pain. In this context Koolpe and colleagues (Koolpe et al. 2005) have reported the identification, by phage display, of linear peptides that bind selectively to different receptors of EphB class, including EphB1 receptors. Moving from these considerations, we developed and characterized different linear peptides with the aim of identifying novel potential EphB1 receptor antagonists. Considering that the activation of ERK1/2 in
response to EphB1 stimulation is involved in several pathological processes. We retained important firstly, to assay the ability of these peptides to modulate ERK1/2 phosphorylation by itself and to counteract ephrin B1-Fc-mediated activation, in HEK-EphB1 cells. Peptides were synthesized starting from the sequence EWLPNLAPSVR and we observed, that when these peptides were administered alone did not activate ERK1/2 phosphorylation. On the contrary, when the peptides were administered 15 min before ephrinB1-Fc (receptor agonist), two of these peptides counteracted ephrinB1-Fc-mediated activation of ERK1/2 phosphorylation, with an IC50 in the range of 0.8 - 1.21 µM.

Protection by pharmacological disruption of EphB-ephrinB system in experimental colitis: TNBS-induced colitis vs acute or chronic DSS-colitis

I. Zini¹, A. Grandi¹, V. Vivo¹, L. Flammini¹, S. Palese¹, A. M. Cantoni², M. Tognolini¹, E. Barocelli¹, S. Bertoni¹

¹Food and Drug Dept. University of Parma, Parco Area delle Scienze 27/A, 43124 Parma, Italy.
²Dept. of Veterinary Sciences, University of Parma, via del Taglio 10, 43126 Parma, Italy.

Background and aim: Eph-ephrin signalling pathway plays critical roles in embryonic growth and in cancer development and progression (1). Lately, its involvement in cell adhesion-based responses, in the remodelling and integrity of the epithelial layer and in the migration and maturation of immune cells has also been highlighted (2, 3). Interestingly, ephrinB levels were up-regulated in the mucosal lesions of Crohn’s disease (CD) patients (4) and in T cells of rheumatoid arthritis subjects, where EphB activation promoted inflammatory cytokines release (5). Our aim was thus to investigate the role of EphB-ephrinB system in intestinal inflammation by assessing the local and systemic effects produced by its inhibition through monomeric protein EphB4 in three chemically-induced murine models of colitis: acute DSS, characterized by innate immune mechanisms activation, acute TNBS and chronic DSS, Th1- and Th2-mediated models of colitis, respectively. Methods: Colitis was induced in C57BL/6 mice (n=8-10/group) by enema administration of 5mg/mouse 2,4,6-Trinitrobenzene sulfonic acid (TNBS) in 50% ethanol or by 3% Dextran Sulfate Sodium solution in drinking water for 5-days (1 cycle) (acute DSS) or for 3 cycles interrupted by 9 days of drinking water (chronic DSS). Sham mice (S) received saline or drinking water. EphB4 20 µg/kg was subcutaneously applied for 3 days (TNBS colitis) or 5 days (acute DSS), or from day 8 for 4 cycles alternated to 2 days of washout (chronic DSS). Control mice (C) received only saline. Disease Activity Index (DAI), colonic macroscopic score (MS), colon length and thickness and colon and lung myeloperoxidase (MPO) activity, index of leukocyte recruitment, were determined. All experiments were performed according to the guidelines for the Care and Use of Animals (DL26/2014). Results: In TNBS colitis, EphB4 strongly reduced DAI (P<0.001) and MS
(P<0.001), minimized colon shortening (P<0.01) and thickening (P<0.001) and curtailed local (P<0.05) and systemic (P<0.05) neutrophil infiltration by about 75% compared to C. In acute and chronic DSS colitis, EphB4 treatment had no significant effect on inflammatory parameters. Conclusions: These results suggest that endogenous EphB-ephrinB system contributes to the development of intestinal inflammation driven by Th1 immune responses while apparently playing no role in the flogosis triggered by epithelial barrier loss or associated to Th2 effector responses. Pharmacological disruption of EphB-ephrinB transmission may therefore represent a promising strategy for the treatment of Th1-mediated inflammatory diseases like CD.

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