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Acquired spinal cord and brain injuries

VALIDATION OF mGlu4 AS A THERAPEUTIC TARGET FOR MULTIPOTENTIAL TREATMENT OF SPINAL CORD INJURY

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SAMCRO

1. Summary of the Project

Spinal cord injury (SCI) leads to neurochemical and neuroanatomical alterations resulting in neuronal hyperexcitability. Glutamate and other proinflammatory factors are released in excess following the injury and contribute to neuronal hyperexcitability, neurotoxicity and pathological pain. mGlu class C G protein-coupled receptors (GPCRs) and, in particular mGlu4 subtype, may have multipotential beneficial effects in SCI by (i) reducing glutamate release, (ii) lowering neuronal cell death due to ischemia, (iii) limiting microglia neurotoxicity and (iv) diminishing exacerbated pain sensation (hyperalgesia).

Our hypothesis was that mGlu4 by these multiple beneficial effects could be a therapeutic target of interest for a multipotential treatment of SCI. However, there is a lack of potent and selective drugs targeting this receptor. In this collaborative project, we proposed to gather the complementary expertise of 3 different teams in bioinformatics, chemistry and pharmacology to understand the role of mGlu4 in SCI, to validate mGlu4 as a therapeutic target for the multipotential treatment of SCI, and to develop new positive allosteric modulators (PAMs) of mGlu4. During the course of our investigations, the original project was extended to include also negative allosteric modulators (NAMs) of mGlu5 because serendipity led us to discover new potent allosteric molecules for mGlu5 when we initially targeted mGlu4 modulators, and the interest of the opposite functional correspondence between mGlu4 and mGlu5 receptors to define glutamatergic effects in SCI.

Our studies have generated new fundamental knowledge on key ligand-receptor interactions and activation/inhibition mechanisms of mGlu4 and mGlu5 receptors. These results have potential clinical implications. Notably, this knowledge has helped us in the design of new ligands that can yield therapeutic drugs in the near future. Of particular relevance we may cite the studies performed on photoswitchable ligands as they represent a new pharmacological concept with potential therapeutic implications.

2. Results

Validation of 3D-models of mGlu4 transmembrane domain and PAM docking using mutagenesis

We generated a molecular model of the mGlu4 transmembrane (TM) domain by homology, based on the available crystal structures of class A GPCRs. Our aim was to help models in refining and orienting synthesis toward active scaffolds. Based on the model prediction, using site-directed mutagenesis, we constructed mutants of residues putatively involved in the binding of PAMs in order to identify the molecular determinants of PAM binding and function. In 2014, the structures of mGlu1 and mGlu5 receptor TM domains were solved (Wu et al. Science 2014, Dore et al. Nature 2014). Thanks to these structures, our model was refined. Our study in mGlu4 revealed that overlapping binding sites are driving allosteric agonism and positive cooperativity of PAMs in this receptor. This work has been published in the FASEB Journal (Rovira et al. FASEB J 2015).

Validation of pharmacophore models of mGlu4 PAMs using mutagenesis and cell-based assay and exploration of the active conformation of mGlu4 PAMs

We investigated the active conformation of the VU0155041 family of mGlu4 PAMs by testing structurally similar six-membered ring compounds with locked conformations. The molecules designed as mGlu4 conformational probes and the isomers were computationally characterized and tested in mGlu4 pharmacological assays. The results support a VU0155041 active conformation, with the chair cyclohexane having the aromatic amide substituent in an axial position and the carboxylate in an equatorial position. Moreover, the receptor displays enantiomeric discrimination of the chiral PAMs. The constructed pharmacophore defines a constrained mGlu4 allosteric binding site, thus providing a step forward in structure-based drug design for mGlu4 PAMs. This work has been published in ChemMedChem (Rovira et al. ChemMedChem 2015).

Design, synthesis and pharmacological characterization of new PAMs and NAMs

Four series of compounds were synthesized and tested improving our understanding of the structure-activity relationships involved in mGlu4 PAM function. We identified a double effect chemical switch that leads from an mGlu4 PAM to a novel potent mGlu5 NAM (Gómez-Santacana et al. MedChemComm 2015).

Endogenous modulation of mGlu4 activity by extracellular chloride

Chloride is known to directly bind and regulate the function of different actors of neuronal activity, and several studies have pointed to the possible modulation of mGlu receptors by Cl^-. We demonstrated that Cl^- behaves as a PAM of mGlu receptors. For example, mGlu4 signalling activity was almost abolished in low Cl^- conditions in cell-based assays. Cl^- potency was ~80 mM and Cl^- possesses a very high positive cooperativity with glutamate (Hill slope ~6 on mGlu4), meaning that small variations in $[Cl^-]$ lead to large variations in glutamate action. Using molecular modelling and mutagenesis, we identified 2 well-conserved Cl^- binding pockets in the extracellular domain of mGlu receptors. Moreover, modelling of activity-dependent Cl^- variations at GABAergic synapses suggests that these variations may be compatible with a dynamic modulation of the most sensitive mGlu receptors present in these synapses. Notably, one can speculate that the loss of Cl^- homeostasis observed at the spinal cord level in conditions of neuropathic pain may impair the ability of mGlu4 to regulate synaptic glutamate release. Taken together, these data reveal a necessary role of Cl^- for the glutamate activation of many mGlu receptors. Exploiting Cl^- binding pockets may yield to the development of innovative regulators of mGlu activity thus opening interesting perspectives. This study has been published in the FASEB J (Tora et al. FASEB J 2015).

Development and optimization of FRET-based conformational sensors for mGlu4 activation

During the first year of the project, we miniaturized the mGlu2 sensor screening assay from 96 to 384 wells to improve its rapidity and efficiency and validate its usefulness for screening of chemical libraries of >1000 compounds. Later on we adapted this system from mGlu2 to mGlu4. We generated different receptors with fluorophores located in different positions to optimize movement detection and thus sensor sensitivity. We chose the most efficient mGlu4 sensor using reference ligands. We successfully conducted a first screening of a library of 1800 compounds using mGlu4 sensor that led to the identification of several hits.

Evaluation of the multipotentiality of mGlu4 modulation in vivo

During the first part of the project, we focused on the sensory symptoms associated to nerve injury. We showed that activation of mGlu4 alleviates pain hypersensitivity observed following nerve injury (Vilar et al. J Neurosci 2013). We contributed a review on this subject in the journal Current Opinion in Pharmacology (Acher & Goudet 2015).

The determination of neuroprotective effects of mGlu4 in in vitro and in vivo animal models is still ongoing and will continue after the end of this project.

Development of light-activated ligands for mGlu4 and mGlu5

Using a novel concept for activation of localized receptors with light switchable ligands, we intended to develop molecular tools for precise pharmacological activation and deactivation of mGlu4 in spinal cord and other tissues. Therefore, we designed and synthesized azobenzene compounds as potential PAMs of mGlu4 to use light for controlling the activity of the receptors. However we obtained an unexpected result and a compound named Alloswitch-1 was discovered as the first potent and selective photoswitchable NAM of mGlu5. This compound is providing a proof-of concept for the development of allosteric drugs to control in vivo the activity of the receptors, and a pharmacological tool for precise definition of new targeting strategies with a controlled dose and accurate spatiotemporal pattern of activity. Alloswitch-1 showed an interesting activity in cells and *Xenopus Tropicalis* tadpoles under different light conditions. This work has been published in Nature Chemical Biology (Pittolo et al. Nat Chem Biol 2014). Furthermore, an article is under submission at the time this report is presented in which, by using a novel mGlu4 photoswitchable ligand to control the activity of endogenous mGlu4 in vivo with light, we show that behavioural symptoms of chronic pain are rapidly and reversibly inhibited (Zussy et al. Submitted).

Structural analyses by theoretical methods

Docking studies on a homology-based constructed model of the TM domain of mGlu5 allowed identifying the molecular determinants of positive and negative allosteric modulation. The results were published in J Chem Inf Model (Dalton et al. J Chem Inf Model. 2014). The binding characteristics of the photoswitchable compound Alloswitch-1 to mGlu5 were examined through molecular dynamics simulations to understand its mechanism of action. The results were published in Curr Neuropharmacol (Dalton et al. Curr Neurophamacol 2015).

In addition to our studies on mGlu class C GPCRs, structural analyses on class A GPCR crystal structures were performed to elucidate the molecular determinants of receptor activation of GPCR superfamily. The results were published in BMC Bioinformatics (Dalton et al. BMC Bioinformatics. 2015) and J Struct Biol (Lans et al. J Struct Biol 2015). Moreover, molecular dynamics simulations were performed in opsin receptor

under various protonation states of selected residues in order to identify pH-dependent activation mechanisms. The results were published in J Phys Chem B (Lans et al. J Phys Chem B. 2015). These calculations were performed with the aim of translating structure-activity receptor features from class A to mGlu class C GPCRs.

Computational science studies

Evolutionary computation algorithms were developed to establish a statistically-based stopping criterion for parameter optimization. These studies are of prime importance in the fitting procedures included in our mathematical modelling of mGlu functionality because of the many parameters these models contain. The results were published in Computational Optimization and Applications (Gil et al. Comput Opt and Appl 2015).
In collaboration with Alfredo Vellido and René Alquézar (UPC), machine learning methods were performed for class C GPCR classification. The results were published in J Integr Bioinform, Med Biol Eng Comput and BMC Bioinformatics (König et al. J Integr Bioinform. 2014; Cruz-Barbosa et al. Med Biol Eng Comput. 2015; König et al. BMC Bioinformatics. 2015). These results could be helpful in our multiple but integrated approach to mGlu structure and function.

3. Relevance and potential implications

Our research has generated new fundamental knowledge on key ligand-receptor interactions and activation/inhibition mechanisms of mGlu4 and mGlu5 receptors. These results have potential clinical implications. This knowledge has helped us in the design of new ligands that could yield therapeutic drugs in the near future.
During the 3 years of the project funded by Fundació La Marató de TV3, we have generated 3D-models of the binding pocket of mGlu4 PAMs and a pharmacophore of these ligands. This improved knowledge of mGlu4 PAMs structure and mode of action is expected to allow the prediction of the pharmacologic profile of PAMs and hopefully to determine the mGlu4 PAMs with the best analgesic and neuroprotective profiles. Moreover, we have identified new allosteric binding pockets that may yield the development of innovative regulators of mGlu receptor activity. Furthermore, we have shown that activation of mGlu4 alleviates pain hypersensitivity observed following nerve injury. We are now working on the neuroprotective properties of mGlu4 PAMs. This work will continue after the end of this project, aiming to identify the best

adequacy between signalling profile and protective effect. However, thanks to our work on mGlu4 and pain induced by nerve injury in the frame of this project and the neuroprotective effect of mGlu4 activation described in the literature, mGlu4 already appears as an interesting target for the development of analgesics and neuroprotective agents.

The development of a new class of photoswitchable ligands for mGlu receptors constitutes a proof of concept of new pharmacological tools in drug development and has potential for future applications in therapies requiring precise localization of the drug and a method for controlling its activity in vivo. These compounds will be employed in future studies to determine the biological roles of mGlu4 in pain and nerve injury and its validation as interesting target for new analgesics and neuroprotective agents. This work will continue after the end of this project in collaboration with the laboratories involved in this project and others in the emerging field of Optopharmacology.

4. Publications derived from the project

Acher F, Goudet C
Therapeutic potential of group III metabotropic glutamate receptor ligands in pain
Curr Opin Pharmacol. 2015;20:64-72

Dalton JA, Gómez-Santacana X, Llebaria A, Giraldo J
Computational analysis of negative and positive allosteric modulator binding and function in metabotropic glutamate receptor 5 (in)activation
J Chem Inf Model. 2014;54(5):1476-87

Dalton JA, Lans I, Rovira X, Malhaire F, Santacana XG, Pittolo S, Gorostiza P, Llebaria A, Goudet C, Pin JP, Giraldo J
Shining Light On An mGlu5 Photoswitchable NAM: A Theoretical Perspective
Curr Neuropharmacol. 2015; DOI:10.2174/1570159X13666150407231417

Dalton JA, Lans I, Giraldo J
Quantifying conformational changes in GPCRs: glimpse of a common functional mechanism
BMC Bioinformatics. 2015;16:124. doi: 10.1186/s12859-015-0567-3
Gil D, Roche D, Borràs A, Giraldo J
Terminating evolutionary algorithms at their steady state
Computational Optimization and Applications 2015;61(2), 489-515

Gómez-Santacana X, Rovira X, Dalton JA, Goudet C, Pin JP, Gorostiza P, Giraldo J, Llebaria A
A double effect molecular switch leads to a novel potent negative allosteric modulator of metabotropic glutamate receptor 5
MedChemComm 2014;5 (10):1548-1554

Lans I, Dalton JA, Giraldo J
Helix 3 acts as a conformational hinge in Class A GPCR activation: An analysis of interhelical interaction energies in crystal structures
J Struct Biol. 2015;192(3):545-53

Lans I, Dalton JA, Giraldo J
Selective Protonation of Acidic Residues Triggers Opsin Activation
J Phys Chem B. 2015;119(30):9510-9

Pittolo S, Gómez-Santacana X, Eckelt K, Rovira X, Dalton J, Goudet C, Pin JP, Llobet A, Giraldo J, Llebaria A, Gorostiza P
An allosteric modulator to control endogenous G protein-coupled receptors with light
Nat Chem Biol. 2014;10(10):813-5

Rovira X, Malhaire F, Scholler P, Rodrigo J, Gonzalez-Bulnes P, Llebaria A, Pin JP, Giraldo J, Goudet C
Overlapping binding sites drive allosteric agonism and positive cooperativity in type 4 metabotropic glutamate receptors
FASEB J. 2015;29(1):116-30

Rovira X, Harrak Y, Trapero A, González-Bulnes P, Malhaire F, Pin JP, Goudet C, Giraldo J, Llebaria A
Exploring the active conformation of cyclohexane carboxylate positive allosteric modulators of the type 4 metabotropic glutamate receptor
ChemMedChem. 2014;9(12):2685-98

Tora AS, Rovira X, Dione I, Bertrand HO, Brabet I, De Koninck Y, Doyon N, Pin JP, Acher F, Goudet C
Allosteric modulation of metabotropic glutamate receptors by chloride ions
FASEB J. 2015;29(10):4174-88

Vilar B, Busserolles J, Ling B, Laffray S, Ulmann L, Malhaire F, Chapuy E, Aissouni Y, Etienne M, Bourinet E, Acher F, Pin JP, Eschalier A, Goudet C
Alleviating pain hypersensitivity through activation of type 4 metabotropic glutamate receptor
J Neurosci. 2013;33(48):18951-65

König C, Alquézar R, Vellido A, Giraldo J
Reducing the n-gram feature space of class C GPCRs to subtype-discriminating patterns
J Integr Bioinform. 2014;11(3):254. doi: 10.2390/biecoll-jib-2014-254

Cruz-Barbosa R, Vellido A, Giraldo J
The influence of alignment-free sequence representations on the semi-supervised classification of class C G protein-coupled receptors: semi-supervised classification of class C GPCRs
Med Biol Eng Comput. 2015;53(2):137-49

König C, Cárdenas MI, Giraldo J, Alquézar R, Vellido A
Label noise in subtype discrimination of class C G protein-coupled receptors: A systematic approach to the analysis of classification errors
BMC Bioinformatics. 2015;16:314. doi: 10.1186/s12859-015-0731-9

Zussy C, Gómez-Santacana X, Rovira X, De Bundel1 D, Ferrazzo S, Bosch D, Asede D, Malhaire F, Acher F, Giraldo J, Valjent E, Ehrlich I, Ferraguti F, Pin JP, Llebaria A, Goudet C

Dynamic modulation of chronic pain related behaviors by amygdala metabotropic glutamate receptor 4

Submitted