Final Report Summary - NEUROCYPRES (Neurotransmitter Cys-loop receptors: structure, function and disease)

Executive Summary:
Neurotransmitter Cys-loop receptors: structure, function and disease

Neurocypres consortium members are devoted to study structural and functional aspects of Cys-loop receptors, a superfamily of ligand-gated ion channels that is crucial to the function of the peripheral and central nervous system. Cys-loop receptors (CLRs) share a generic protein architecture consisting of five subunits with an integral ion-channel that can open and close depending on ligand binding. CLRs comprise of nicotinic acetylcholine-, GABAA-, 5HT3-, and Glycine receptors. Dysfunction of CLRs is linked to muscle disorders (e.g. myasthenic syndromes), hyperexcitability of the brain (e.g. epilepsy) and spinal cord (e.g. hyperekplexia / stiff baby syndrome) as well as nicotine addiction, while CLR subunit genes serve as candidates for frequent psychiatric diseases (e.g. schizophrenia). CLRs are molecular targets for clinically important drugs. Novel drugs are on the horizon, mediating highly selective therapeutic effects and providing new avenues for therapeutic use. Today, the quality of crystallographic data and the general knowledge of structure-function relationships have progressed to the extent that the rational design of ligands has become a feasible objective. Coordinated approaches, such as Neurocypres, are required to increase our knowledge of ligand-gated ion channel structure at high-resolution.

In 2008, Neurocypres brought together 20 groups of researchers in a large-scale integrated effort to capture high-resolution structural information encompassing either the entire structure of selected receptors, or of specific functional domains in these receptors. Since then, new discovery projects in the fields of nAChRs, GABAA and 5HT3 receptors have been established. Various projects were dedicated to understand the fundamentals of receptor structure, e.g structural data of the acetylcholine binding proteins (AChBPs), co-crystalized with various ligands, was obtained. For various different CLR ligands, in-depth knowledge was gathered on ligand binding to the AChBPs, e.g. various toxins, as well as small molecules important for drug design. Compounds relevant for medicinal use, for instance the anti-smoking compound varenicline, were studied. In addition AChBPs were engineered that can act as new tools for drug development for CLR receptors, for instance of the 5HT3R class. Another important area of the Neurocypres program was to advance new technologies for drug design and screening. New technologies were established with promising results on small molecules targeting the different members of the LGIC family. These included high-throughput electrophysiological analyses of receptor expression in oocytes, fluorescence based assays, and affinity measurements using surface plasmon resonance. Also the use of CLRs to probe neuronal function, as biosensors, has progressed to studies in neurons. Moreover, efforts to produce whole subunits and receptors were initiated. Already in its first year Neurocypres celebrated the first structure solved of an entire cys-loop receptor by one of its partners (P-J Corringer, Pasteur, Paris). A combined genomic search, functional expression and X-ray crystallography yielded the discovery and atomic structural resolution of bacterial homologs of LGICs. The homolog from the cyanobacterium Gloeobacter violaceus (GLIC) revealed that it functions as a proton-gated ion channel. Its X-ray structure was solved at 2.9 Å resolution in an apparently open conformation. Work on the bacterial receptors yielded various new insights. For instance, now having the transmembrane domains available in the structure allowed to discover novel binding sites for substances long known to act as modulators of CLRs. Crystallization studies revealed new insights in compounds normally acting on the GABAA receptors, e.g.
acting at the benzodiazepine binding site. In addition, the sites to which the binding of general anesthetics (used in the clinic during surgery) occurs were revealed.

Taken together, the Neurocypres project generated many new insights in receptor structure and function. In four productive years, with 111 publications, the consortium pushed the frontiers of knowledge on CLRs, opening new avenues for drug design at these receptors.

Read more about the discoveries of Neurocypres researchers at: www.neurocypres.eu

Project Context and Objectives:

Concept of the Neurocypres project and its main objectives

CLRs are crucial to the function of the peripheral and central nervous system. Dysfunction of CLRs is linked to muscle disorders (e.g. myasthenic syndromes), hyperexcitability of the brain (e.g. epilepsy) and spinal cord (e.g. hyperekplexia / stiff baby syndrome) as well as nicotine addiction, while CLR subunit genes serve as candidates for frequently occurring psychiatric diseases (e.g. schizophrenia). Moreover, CLRs serve as molecular targets for a functionally diverse group of clinically important drugs, including curare-like muscle relaxants, tranquilizers and anticonvulsants like the benzodiazepines, as well as anti-emetics. Based on the identification of selective CLR subtypes, novel drugs are on the horizon, mediating highly selective therapeutic effects and giving hope for a symptomatic therapy of Alzheimer’s disease.

Despite their functional heterogeneity, members of the CLR family share an over-all protein architecture, permitting a transfer of structural concepts derived from paradigmatic receptor models to other CLR types. Subsequent modelling of the atomic structure of CLRs has permitted structure-activity predictions that have dramatically improved the understanding of drug action at the molecular target, paving the way for a rational drug design. Structural predictions also provide insight in the mechanisms of disease-related CLR mutations, as the functional role of affected amino acid side chains becomes apparent.

Drug design will be most effective if targeting CLR subtypes. Serving as a paradigm of drug selectivity, structure-based design of subtype selective agents should help to separate the anxiolytic action of benzodiazepines from their sleep-inducing effects. Likewise, creation of peripheral muscle relaxants that exclusively target muscular CLR subtypes, while not mimicking the effects of nicotine would reduce adverse effects on the circulation during surgery. Drugs that mimic the effects of nicotine might be used to treat Alzheimer’s disease and will be best targeted to distinct neuronal nAChR subtypes.

Novel drug discovery strategies and therapies call for in depth understanding of ligand binding sites, the structure-function relationships of CLRs and insight into their roles in the nervous system. Initial insight into the structural homology of CLRs came from the sequence alignment of subunit polypeptides. Over the years, however, insight into the conformational structure of CLRs has grown. Despite several attempts to produce, crystallize and provide X-ray or NMR data of membrane bound CLRs, substantial progress was finally made with the cryo-electron microscopic analysis of the Torpedo nAChR at resolutions of 4Å (Unwin, 1995, 1998, 2005). A breakthrough in understanding the structure of CLRs also came from solving of the crystal structure of the acetylcholine-binding protein (AChBP) (Smit et al, 2001, Brejc et al, 2001,2002). This protein is a naturally occurring homologue of the ligand-binding domain of the CLRs. Due to the water-solubility of the protein it can be produced in large quantities facilitating crystallization and subsequent X-ray analysis. The AChBP structure facilitated an increase in the resolution of the cryo-electron microscopic images of the Torpedo nAChR, and spurred research into all other CLRs (Sixma & Smit, 2003). Subsequent solving of the X-ray structures of the AChBP complexes with agonists and antagonists provided excellent models for the ligand-binding domains and the respective complexes of drugs with other CLRs through homology modeling (Celie et al, 2004, 2005a,b, Ulens et al, 2006).

For instance, research in different laboratories demonstrated that the AChBP structures could be used to build models of the ligand-binding domains of the glycine and GABAA and 5-HT3 receptors (Cromer et al, 2002, Reeves et al, 2003), which shed new light on the interactions of agonists and antagonists with these receptors (Betz and Laube, 2006). Moreover, it opened ways to elucidate the mechanisms of ligand-receptor interactions and to affect them in a desired way by model-guided in the receptors and by structural changes of the ligands (Reeves et al., 2003; Harrison et al., 2006). In addition, chimaeras of engineered AChBP and the transmembrane domain of the cationic 5-HT3 CLR or anionic GlyR (Grutter et al, 2005), were proven to be functional, i.e. ligand binding to the AChBP domain enabled gating of the ion channel protein.
Today, our insights into structure-function relationships have progressed to such an extent that the rational design of ligands is conceivably within reach. Moreover, the solved X-ray structures of different AChBPs, their complexes and the cryo-electron-microscopy structure of the Torpedo AChR have provided the basis for a better understanding of the mechanism of action of the listed CLRs, their involvement in the interactions with other cell components and their direct or indirect role in various pathologies. Further elucidation of the structural aspects of atomic interaction with agonists/antagonists, ion channel blockers or other compounds (neurosteroids, local anesthetics) modulating CLR activity by binding to different sites of the multisubunit CLRs, will provide information urgently required for rational drug design. Of great importance in this respect is the recent discovery of prokaryotic CLRs, which were obtained in preparative amounts. From these, crystal structures of some of them were solved within this program. These prokaryotic CLRs are the bridge between the AChBPs and the CLRs of human and other mammals. In addition, there is a growing understanding that the precise localization and targeting of receptors to cellular subcompartments in the brain is crucial to the modulatory role of these receptors in the brain.

The following objectives were formulated and reached:

1. To achieve a multidisciplinary workflow of high-throughput crystallization, X-ray analysis, NMR and computational modelling, HT-electrophysiology technologies, and fragment-based drug design, as well as embark on innovative methods in subtype specific drug design and screening, thus dramatically enhancing the speed of research in these individual areas.

2. To obtain high-resolution X-ray and NMR structures for CLRs, their functional domains and complexes with diverse ligands, agonists/antagonists, channel blockers and modulators, which revealed basic mechanisms of receptor functioning from ligand binding to ion channel gating. This, together with the HT techniques of objective 1 opened new avenues for rational drug design.

3. To understand receptor function in the context of the brain, focusing on receptor biosensors, receptor protein interactions, and animal transgenic models, by developing innovative quantitative methods of interaction-proteomics, sensitive methods for visualization of activity and localization of CLRs and studies of in vitro and in vivo function in animal models of disease.


Solving the CLR structure

While the primary structure of nAChR subunits is now well known, detailed knowledge of the tertiary and quaternary structure of full CLRs was elusive. Recently, a major advancement has been the crystallization of the acetylcholine binding protein (AChBP) from molluscan species and determination of its X-ray structure a functional homolog of the the pentameric nAChR. As first shown in these publications and later demonstrated by other groups, the AChBP is an excellent structure-function model for the extracellular binding domains of all types of nAChRs. This structure was also used for interpretation of cryo-electron microscopy data for the Torpedo nAChR, for which a 4Å resolution structure of the receptor has been established (Unwin, 2005). This Torpedo nAChR structure, together with the X-ray structures of AChBPs and their complexes are now used for modelling the structures of all CLRs. Further advancement in the knowledge of the 3D structure of the nAChR has been obtained by using homology modelling approaches. These have used the available structural information, comprising information gathered by analysis of AChBP crystals, high resolution electron microscopy of native nAChRs, and site-directed mutagenesis, which led to proposed realistic models of the receptor. Prokaryotic CLRs will were a logical next target in the advancement of our understanding of the structure and function of complete CLRs.

Both experimental and in-silico approaches were used in NeuroCypres to gather new knowledge on structure-function relationships. A highlight of this was to provide humanized AChBP ligand binding domains, soluble binding domains of human receptors and a generation of X-ray data of prokaryotic CLRs. The latter were used to bridge the gap between the structures of AChBP and that of the EM data on the nAChR. Finally, the structure of the whole eukaryotic CLR still needs to be obtained by coordinated efforts. High throughput robotics for crystallization for very small amounts of pure protein or protein complexes have been established, which dramatically increased the success rate of obtaining crystals for X-ray analysis

Ligand design, pharmacophore modelling and high throughput surface plasmon resonance analysis.

CLR subtype selective ligands helped to unravel the very subtle molecular features in a receptor that is able to induce
selectivity. Furthermore, these ligands aided to validate individual subtypes as drug targets in translational studies. This consortium was able to obtain an unprecedented understanding at a molecular level of these important classes of drug targets. This enabled us to develop many different classes of CLR ligands, ranging from extremely potent average high molecular weight conotoxins, medium molecular weight drug-like compounds and low molecular weight fragments. The necessary requirements for the design of new selective agonists or antagonists are high resolution structures of different CLRs in free and in a ligand-bound states. The X-ray structures of AChBPs complexed with agonists and some antagonists, such as α-conotoxins were determined by participants Smit, Sixma in collaboration with Tsetlin and Bertrand. These structures characterized the ligand-binding sites between the agonist- and antagonist-bound AChBPs.

Efficient (high-throughput) in silico screening protocols were developed which consisted of both rapid pharmacophore investigations and slower but more accurate docking studies. These approaches enabled the in silico screening of commercially available screening compounds for the plethora of CLR targets. The coverage of chemical space using drug-like compounds of MW~600 is extremely poor. In recent years, many have realised that even a massive 10^7 compound screening programme cannot efficiently probe the virtual chemical space of 10^60 drug-like compounds. However, we have shown that a set of ~ 1000 molecules of significantly reduced weight (MW < 300 Da; so-called fragments) are able to obtain a reasonable coverage of the corresponding chemical space. Fragment binding needs to be assessed using biophysical screening, e.g. Surface Plasmon Resonance (SPR). In an unprecedented high throughput, SPR sensograms can be obtained for thousands of fragments on a set of up to twelve different LBDs simultaneously.

One of the other assets will be to design of novel α-conotoxins having higher potency and selectivity for distinct nAChR subtypes. This task is of importance since our data (Ulens et al, 2006) and those from other laboratories (Ellison et al, 2004) revealed that certain naturally occurring α-conotoxins are not as selective as was earlier believed. Design of the new α-conotoxins started the building of models for the required nAChR subtypes, subsequently docking various naturally-occurring α-conotoxins or their analogs with these models and then subsequent molecular dynamics simulations of the toxin-receptor complexes.

NeuroCypres brought together state of the art data on ligand-receptor interaction at high, atomic resolution. Computer modelling including docking and molecular dynamics, structure- and fragment-based chemical synthesis and high-throughput physiological testing of CLRs were used.

High-throughput ligand-receptor analysis

Functional investigation of CLRs, or assaying the effects of novel ligands, is often a bottleneck of many studies and is mainly limited by the experimental time. Development made throughout the years allowed establishment of a fully automated device (partner Bertrand), which performs functional electrophysiological testing. The capacity of the designed compounds to block functional responses will be analyzed in HT-electrophysiology experiments on the CLRs expressed in Xenopus oocytes. In addition we used a FLEXstation for rapid analyses of function in mammalian cells using fluorescent membrane potential-sensitive dye (Price & Lummis, 2005). NeuroCypres provided the capacity for full functional analysis of receptors, mutants and ligands in a high-throughput fashion.

Genetically-modified mice and viral vector approaches were used for the study of CLR function

The study of the genetic and molecular basis of nicotine addiction has recently had a major boost from the use of genomic animal models. In particular, several nAChR subunit knockout mice have become available (Champtiaux and Changeux, 2004; Rabenstein et al, 2006). The study of these animal models has led to the demonstration that certain subunits, such as α4, α6 and β2, mediate direct nicotine effects on dopamine mesostriatal neurons, one of the principal neuronal systems involved in drug addiction and that β2-containing nAChRs are necessary for nicotine self administration and attention. The study of other genomic animal models is contributing to unravelling the complex molecular mechanisms and cellular circuits of nicotine addiction, which are downstream to nicotine interaction with its receptors. With respect to the activation of CLR subtypes in the brain there is a need for biosensors that would enable visualization of receptor activity in the brain and provide information on their activation states. The best candidates are be fluorescently modified postsynaptic CLRs. In this regard, we have developed a novel CFP-YFP–based Cl—Sensor, which allows spectroscopic monitoring of intracellular Cl. When the Cl-Sensor is introduced into the cytoplasmic domain of the human glycine receptor (GlyR) it allows visualization of GlyR channel activity. This proof-of-principle provides the background to develop Biosensors for monitoring of Cl−selective CLR channels in live cells and transgenic animals. In NeuroCypres we focused on developing BioSensors of CLRs for the non-
invasive monitoring of their activity and subsequent high throughput drug-receptor studies.

NeuroCypres fully exploited the great potential of genomic animal models in research on receptor function (e.g. in addiction) and advanced tools that allow monitoring receptor function in the brain in vivo, and for high throughput drug-receptor studies.

Receptor function in disease. Malfunctioning of distinct nAChRs is caused by mutations and subsequent abnormalities in the function and/or levels of receptor expression. These mutations are associated with a number of pathologies: muscle dystrophies, epilepsy, neurodegenerative (Alzheimer’s) and some psychiatric diseases, such as schizophrenia. Depending on their expression pattern in either neuromuscular or CNS synapses, mutations of subunit genes of nAChRs result in congenital myasthenic or epileptic syndromes. The neuronal-like nAChRs are also found in some non-neuronal tissues and changes in their levels of expression can be associated with different diseases. Determining the function properties of distinct nAChRs in healthy and pathological states is an important task. For this purpose different neurotoxins are used, which can block and selectively label distinct nAChR subtypes (Olivera 2006; Terlau and Olivera, 2004). In this respect, α-Conotoxins, short peptides from the marine Conus snails, are promising tools for CLR identification and intervention.

NeuroCypres allowed a full exploitation of the interplay between human genetic studies indicating receptor mutants, and NeuroCypres receptor structure and function, and boosted the knowledge of the mechanistic aspects of CLR-function and dysfunction in disease.

Novel Proteomic approaches into CLR stoichiometry

The identification of CLR subtypes in the brain is of crucial importance to our understanding of disease-specific disregulation and subtype-specific expression in the brain. The expression of CLRs is dynamically regulated. For instance, long-term exposure to nicotine triggers an increase in the number of nicotinic receptors, a phenomenon termed up-regulation. However both in vitro and ex-vivo studies have shown that at molecular level, chronic nicotine exposure differentially affects this up-regulation, subunit composition, and functional state of some nAChR subtypes, leaving others substantially unaffected. The recent availability of genetically engineered knockout or knockin mice, in conjunction with the availability of selective ligands (e.g. toxins), and the use of innovative LC/LC MS/MS technology has made it possible to analyse the structure, pharmacology and functional role of nAChR subtypes in complex neurobiological systems. Similar approaches are now applicable to other CLRs.

Project Results:

WP 2 - Clearly significant results

2.1 Monomer, dimer and homo-pentamer of the α7 subunit of nAChR models have been generated. The monomer α1, complex α1-bungarotoxin and the heteropentamer αεαβδ have been generated. Extensive discussion has taken place at a workshop on modelling in Amsterdam (1/2-12-2008).

2.1.2 AcAChBP was co-crystallized with acetylcholine and the structure was solved to a 2.6 Å resolution. Pharmacophore models of various structures were made.

2.2 Established batch expression system for AChBP, making use of insect cells yielded proteins used by several partners.

2.3.1 5HT3-BPs have been produced, and shown to bind serotonergic ligands. This work is ready for publication by the Smit/Ulens labs.

2.3.2 ITC was used to characterize the binding of nicotine, varenicline and cytisine to AcAChBP.

2.4.1 Epitope-tagged versions of AChBPs from different species were cloned and expressed in various combinations in hEK293 cells. This project was thereafter stopped, as the use of bacterial receptors provided a better entry point.

2.4.2 Allosteric site was produced. However, allosteric sites were determined, alternatively, using x-ray crystallography in the bacterial receptors by partners of Neurocypres.

2.5 Structure and physico-chemical features of interacting interfaces among subunits and active sites of free and conotoxin-bound human nAChR were determined. Atomic-level insights into the active sites and into the interaction interface among nAChR subunits was obtained.

Reasons for deviation and their impact

2.4.1. Heteropentamers failed to express. 2.4.2 Allosteric sites were produced in prokaryotic channels; no reason to pursue further.
Reasons for failing to achieve critical objectives

2.4.1. Co-expression of Aplysia subunits turned out to be difficult. The subunits were concatamerized and forced into expression. Attempts were terminated when allosteric sites in prokaryotic channels were found to be a strong alternative.

WP 3 - Clearly significant results

3.1.1 New structural data has been gathered and published. Cloning of new bacterial CLRs has been completed. Crocosphaera watsonii, Cyanophyceae sp. CCY0110, Cytophaga hutchinsonii, Erwinia chrysanthemi, Gloeobacter violaceus, Lyngbya sp. PCC 8106, Rhodopseudomonas palustris and Synechococcus sp. WH 7805. Only 2 of these CLRs have a proper pentameric assembly, namely the CLR from Erwinia chrysanthemi and Gloeobacter violaceus. The structures for both of these CLRs have now been solved, including one by Partner 7 (Pasteur). Partner 3 determined several structures of the CLR from Erwinia chrysanthemi in complex with GABA, general anesthetics, benzodiazepines and the non-benzodiazepine hypnotic zopiclone. Partner 3 has also identified a novel candidate (GLIC) for structural studies.

3.2.1 Partner 7 has developed small library of compounds (around 100, containing amino acid, sugar, neurotransmitter) that was used by D. Bertrand (Partner 12) to screen several bacterial CLRs coming from Ulens (Partner 3) and Corringer (Partner 7). Gamma aminobutyric acid (GABA) was found to activate the CLR from Erwinia chrysanthemi ELIC. Ulens, Corringer, Lummis and Sieghart lab have functionally characterize the properties of the ELIC channel. The interaction of GABA in the ligand-binding sites has been analyzed through unnatural amino mutagenesis. The functional importance of a novel allosteric binding site for benzodiazepine has been investigated via cysteine-scanning mutagenesis. Partner 3 has identified the ligand for a novel prokaryote CLR (GLIC) and has characterized the ion selectivity using electrophysiological techniques.

3.2.2. Partner 3: has introduced mutations in the selectivity filter of the CLR from Gloeobacter violaceus to convert its ion selectivity from cationic to anionic. Expression in HEK293 cells of these chimeric channels showed that Glvi remains cation-selective upon substitution of the bacterial anion-selective channel. Partner 7 has resolved the X-ray structure of GLIC in complex with two general anesthetics, propofol and desflurane. Both compounds bind at the level of a single transmembrane pocket that was analyzed by mutagenesis followed by electrophysiology in the oocyte. This analysis unravelled two positions which mutation profoundly altered the gating transition (gain of function mutations), and two positions which mutation either altered or potentiated the inhibitory action of these anesthetics on the GLIC currents elicited by protons. These data support that the binding site identified by X-ray crystallography actually mediate the allosteric effect of inhibition.

WP4 - Clearly significant results:

4.1.1 Partner 6 generated and purified large amounts of alpha3 homo-oligomeric receptors in Sf9 cells for crystallization trials (Partner 16) and surface plasmon resonance studies (Partner 20), and also large amounts of alpha1alpha3 receptors for the identification of steroid binding sites. Partner 6 and Partner 2 developed a rapid screening method for protein expression in Sf9 cells.

4.1.2 Partner 6 established a high throughput procedure for the simultaneous expression and purification of various extracellular domains of GABAA receptors in various E.coli strains, that resulted in the expression of approximately 25 mg/ml soluble extracellular domain as MBP fusion protein and after purification yielded 3mg/ml pure protein. High levels of the extracellular domain of the beta3-MBP protein were expressed and purified from E.coli.

4.1.3 Modelling of the receptor binding sites.

4.1.4 Benzo site successfully engineered
4.2.1 Successful expression and purification of mg quantities of glycine receptor ECDs and ICDs.

4.2.2 The structure of TM2-loop-TM3 was determined by NMR in 2008 by Canlas et al. J Am Chem Soc. 2008 130: 13294-13300.

4.2.3 The structure of TM2-loop-TM3 was determined by NMR in 2008 by Canlas et al. J Am Chem Soc. 2008 130: 13294-13300.

4.3.1 4.3.2 4.3.4: Modelling, expression and purification of mg quantities of 5-HT3 receptor ICD and ECD domains; production of M2 regions with and without ESLs

4.3.3 Structural models of the 21 a.a. and 23 a.a. 5-HT3 peptide segments were generated.

4.3.5 Structural data produced and cross-validated

Reasons for deviation:

4.2.2 en 4.3.3. NMR structural determination of GlyR M2 and ESLs was published by another group.

Reasons for failing to achieve critical objectives:

4.2.1 Proteins abberantly oligomerized (analytical gel filtration). High levels of ICD protein has also been produced but although there was evidence for secondary structure in CD-spectroscopy, NMR-experiments showed only a limited number of cross correlation signals between all the amino acids.

4.3.1 No crystals have been observed in our attempts at crystallization of the any ECDs. The aberant oligomerization is the likely reason.

WP5 - Clearly significant results:

5.1.1 5.1.2 5.1.6. Nine new crystal structures of ligand complexes have been made, that give insight into the process of ligand design, anti-smoking compounds and subtype selectivity.

5.1.3 5.1.4 Good progress has been made towards expression and structural analysis of extracellular domains.

5.2.1 5.2.3 5.2.4 LBDs have been produced and crystallized for GABA and 5HT3Rs.

Reasons for deviation and their impact on resources and planning:

5.1.5 Interaction studies of myasthenia antibodies against AChBP have been redirected towards the true nAChR subunits.

5.2.2 NMR analysis has focussed on GLIC rather than nAChR subunits, since the protein is better to work with.

WP6 - Clearly significant results:

6.1 Partner 3 has completed biochemical characterization of 8 different bacterial CLRs from different organisms. However, only 4 of them have a pentameric assembly following solubilization with detergents. We obtained crystals for all 4 of these channels, which include the ELIC channel from Erwinia chrystanthemi and the GLIC channel from Gloeobacter violaceus. X-ray structures for ELIC and GLIC have been reported by others, including Partner 7. Also, Partner 3 has been able to identify a ligand that activates ELIC. Therefore, we are currently also pursuing co-crystal structures of bacterial CLRs in the ligand-bound open channel conformation. (See also WP 6.2).

6.2 6.3 The crystallization and determination of the atomic structure of a bacterial Cys-loop receptor in complex with ligands was successful.

Reasons for deviation and their impact on resources and planning:

6.4 6.5 6.7 Darpin production against Torpedo nAchR, which is seen as an essential starting point for deliverable 6.3 has initiated late. The Darpin work of 2011-2012 failed. Overexpression of human Cys-loop receptors in homogenous oligomeric form has not been possible with a yield and stability allowing for crystallization screening.

Reasons for failing to achieve critical objectives:

6.4 6.5 6.7 When the Neurocypres contract was negotiated, it had been overseen that our plan of employing a Postdoc in Switzerland for Darpin production was breaching FP7 rules. Only in 2011 an amendment has been worked out but this came too late for extensive optimization of the Darpin selection. Hence, partner 16 focused work on eukaryotic Cys-loop receptors (Del. 6.7) one of them by collaboration with partner 6 and two in collaboration with partner 12.

A statement on the use of resources: Because of the slow progress with the original targets, partner 6 concentrated on the Torpedo receptor aiming at crystallization without Darpins and on two CLRs from a hyperthermophilic worm.
WP7 - Clearly significant results:

7.1 Improvements of the HTS system with the extension of capacity and design and production in sufficient quantities of the microstirrer devices indispensables for the measurements of large number of samples.

7.2 Collaborative efforts set up with Partner 6 for the expression of GABAA receptors on the HTS system were made. This approach was successful and allowed the characterization of novel allosteric modulators at this type of ligand gated channels. This work is presented in the framework of a PhD study.

7.3 An electrophysiology rig to measure the functionality of CLR using two electrodes voltage clamp and correlate measurement with ionic fluxes was set up. Work carried out with Partner 12 confirmed the feasibility of high-throughput screening using automated recording.

Partner 7 has focused on the GLIC homolog, and electrophysiological screening by Partner 12 identified several compounds as potential agonists and antagonists. In parallel, HEK-293 cell lines stably expressing the GLIC channel have been generated to allow screening of compounds using multiwell plates and membrane potential indicators. Partner 7 has developed small library of compounds (around 100, containing amino acid, sugar, neurotransmitter) that was used by Partner 12 to screen several bacterial CLRs coming from Partner 3 and Partner 7. So far, no new ligand of GLIC was found, but gamma aminobutyric acid (GABA) was found to activate the CLR from Erwinia Chrysanthemi ELIC. Partner 3 and Partner 7 are currently characterising the ELIC channel. Partner 7 has identified the proton activation site of the GLIC channel. The mutants tested so far are the R133A, E177A, D178A, E181A and R179A within the agonist binding pocket, and E222A, H235A, H235F, H235R, H235K and E243A within the M2 segment. Regarding the currents traces and sensitivity to protons, R133A, E177A, D178A and E181A are identical to wild type (pH50 4.8) R179 is more sensitive to proton (pH50 5.5) while E222A and E243A are slightly less sensitive to proton (pH50 of 4.6 and 4.4 respectively). In contrast, all mutations of H235 yielded no currents.

7.4 Study was performed by the lab of PJ Corringer and published (Functional prokaryotic-eukaryotic chimera from the pentameric ligand-gated ion channel family, G. Duret et al., 2011 Proc Natl Acad SCI)

7.5 The HTS devices proved efficient for the expression of bacterial CLR and screening of large number of compounds. These measurements allowed the identification of active compounds for GLIC and ELIC that are used by partner 7 of the consortium. (J.Neurochemistry 2009). For partner 5 HTS Screening of a 1016 fragment library (from Partner 17) at the 5-HT3 receptor has been completed and has revealed novel agonist and antagonists. One of these was of very high affinity (pKi < 10) and remarkably it can distinguish homo and heteromeric receptors, providing a novel route for therapeutics.

7.6 This system was used successfully to produce receptors.

A statement on the use of resources: Given the entire satisfaction of the HTS system additional work from that initially planned has been carried out opening new opportunities to collaborations between partners and with extension of the members of ligand gated channels that have been studied.

WP8 - Clearly significant results:

8.1 Partner 9: Two improved constructs, GW1-Cl-Sensor and pLLU2G-Cl-Sensor, have been designed and tested. It was shown that these mammalian expression vectors provide high fluorescent intensity of Cl-Sensor indifferent cell types. Partner 11 produced adeno- and adeno-associated viral vectors and viruses containing GABA receptor DNAs that are capable of transducing primary (neuronal and glial) and secondary (human embryonic kidney cells). We have used these viruses to transduce neurones in vivo in the cerebellum in conjunction with GABA receptor knock-outs and knock-in mouse lines.

8.2 Partner 10: The Gira1-BS (“BioSensor-GlyR”) project is on target with the initial characterizations of the gene targeted mice having been executed in January 2009. No fluorescent signal above background was detected in slices from the initial offspring from chimeric founder mice (Gira1-BS-neo/+). Partner 10 produced Gira1-BS-neo mice and their analysis (no expression); production of Gira1-BS mice; Characterisation of Wnt8b-BS transgenic founder mice (specific but low expression). We continued with parallel projects as alternative strategies to overcome some of the problems of the Gira1-BS system as a chloride ion detector. We have obtained founders of the Wnt8b-BS transgenic BAC construct started during the initial funding period. Expression was demonstrated in immature granule cells of the dentate gyrus. However, levels were too low for real time imaging. Finally, we have produced a Thy1-BS construct. This promoter promises to drive high levels of expression non-specifically in neurons and has been used successfully for other Cl-sensors.
8.3 After successfully obtaining of crystal structure of of GLIC and ELIC, there is no need in producing BioSensor-nAChR. Instead, constructs of Biosensor-GABA were obtained. Spectra and functional properties were analysed at expression in CHO cells.

8.4 Successful expression of the Cl-Sensor in neurons in vitro and in vivo. Producing viral vectors and viruses containing GABA receptor DNAs that are capable to transduce neurons in vivo.

WP9 - Clearly significant results:
9.1 Partner 13 has immunopurified alpha4beta2* receptors from cortex Partner 18 has prepared Epibatidine-Sepharose beads that were tested for the further purification of the immunopurified alpha4beta2*subtype. Partner 1 successfully analysed by MALDI TOF of 1D SDS PAGE alpha4beta2* affinity purified material from rat brain using Abs directed against different epitopes of the alpha4 and beta2 subunits.

9.2.1 In particular in the Hb-IPn system they have identified the presence of a wealth of uncommon nAChR subtypes and identified that alpha3beta and alpha3beta4beta3, transported from the Hb and highly enriched in the IPn, as the subtypes modulating ACh release in the IPn.

9.2.2 Potential associated proteins were detected but no consistent expression was found although the procedure of purification and analysis was state-of-the-art and specifically optimized for this task. These results show a limitation of the current technical status to detect small amounts of membrane-associated proteins such as nAChRs in the brain. More sensitive approaches are needed for this type of problem.

9.3 Nicotine has an area-specific effect on receptor subtypes, regardless of the route of administration, but the effect is quantitatively greater in the case of MP administration. After repeated nicotine exposure nAChRs in adolescent and adult rats in the mPFC are differently regulated, which indicates a possible role of alpha4beta2-containing receptors in mediating the acute rewarding effects of nicotine in adolescents.

9.4 We established that caudate-putamen terminals almost exclusively expressed alpha4alpha6beta2beta3 that caudate-putamen terminals almost exclusively expressed alpha4alpha6beta2beta3. Also, we have identified and biochemically characterized an alpha7beta2 subtype in the mouse basal forebrain.

9.5 We have demonstrated that nicotine has a brain region-specific effect on receptor subtypes, regardless of the route of administration, but the effect is quantitatively greater in the case of minipump administration.

9.6 Partners 13 and 1 have investigated whether exposing Wistar rats to nicotine or saline during adolescence (post-natal days 34-43) or young adulthood (post-natal days 60-69) can modify brain nAChRs. They found that, after repeated nicotine exposure, alpha4beta2 nAChRs present in the prefrontal cortex of adolescent rats are differently regulated from those present in the same area of adult rats. This indicates a possible role of alpha4beta2-containing receptors in mediating the acute rewarding effects of nicotine in adolescents.

9.7 Partners 13 and 22 have analysed the alpha4beta2 subtypes present in caudal cortex of rats that received chronically nicotine or saline by minipump and determined that there is a nicotine-induced selective up-regulation of the (alpha4)2(beta2)3 subtype. Partner 7 has identified that the alpha4beta2alpha5 nAChRs present in the VTA regulate the minimum nicotine dose necessary for dopamine cell activation and reinforcement. Partner 7 has defined a causal link between a human predisposition marker and nicotine intake. Partner 13 has defined the mechanisms trough which permeable orthosteric nicotinic ligands up regulate the alpha3beta4 subtype in epithelial and neuronal cells. Partner 13 has developed new antibodies directed against intracellular epitopes of the mouse alpha5 subunit and human alpha7 subunit. These antibodies were shared with other partners of the Neurocyspre consortium (e.g. Partner 2, 7 and 22).

Reasons for failing to achieve critical objectives: 9.2.2 No consistent candidate protein was detected across the different preparations. This may be due to the high number of relatively low-level associated proteins and/or the limitation of present state-of-the-art analytic means for the detection of low-level membrane associated proteins from the highly lipid enriched brain preparations.

WP10 - Clearly significant results:
10.1.1 Several new α-conotoxin analogs have been synthesized by solid phase method, including radioiodinated compounds and single and multiple amino acid mutations. These are proving valuable tools in binding studies.
10.1.2 Three new toxins from snake venoms are isolated and shared with the Neurocypres partners.

10.2 A high number of novel drug-like CLR ligands was developed. This has been demonstrated by developing several potent ligand series for GABA(A), nAChRs and 5-HT3R receptors. For the latter target, advanced leads have been developed that show unique pharmacological properties (i.e. subtype selectivity) while also having excellent drug-like properties (including phys. Chem. properties, selectivity profile and in vitro ADME-tox properties). The progress that was achieved by the Neurocypres consortium will lead to more efficient development of CLR ligands with clinical potential.

10.3.1 Partner 20: In studies that represent a major breakthrough for SPR studies on LGICs and other membrane receptors, a functional SPR assay has been established for homo-oligomeric β3 GABA(A) receptors. Detergent solubilized receptors were obtained by partner 6 and immobilized via affinity-capture on biosensor surfaces. Interestingly, the binding of a panel of histaminergic ligands could also be measured, confirming the presence of a newly identified histamine binding site.

10.3.2 The screening and data analysis protocols have been found to be suitable for the tasks to be performed. The high hit rate may ultimately translate to identification of many different scaffold series, an advantage in the evolution of specific ligands for the diverse repertoire of human receptors. Partner 17 has developed a focused library that results from a deconstruction approach of a7 selective ligands. The fragments and drug-like compounds have been screened using the SPR assay, identifying ligand efficiency hotspots (De Kloe et al., J.Med.Chem. 2010).

10.3.3 Progress has exceeded our expectations, for example by developing the described information-rich biochemical fragment screening and also when considering the progress that has been made in using the biophysical SPR approaches on both AChBP and on complete receptors (i.e. GABA(A)).

WP11 - Clearly significant results:
11.1.1 Partner 12: Studies of novel mutations in the CHRNB2 and CHRNA2 have been achieved. A first part was recently published. Discovery of novel mutations in the genes encoding for the neuronal nicotinic acetylcholine receptors confirmed the association between these genes and a form of epilepsy. This provides a clearer view of the role of these receptors in brain functions. In addition, we characterized the function of 6 and 5 subunits.

11.2.1 Partner 14: AutoAbs against the α or alpha subunit can cause nAChR loss via antigenic modulation in a dose-dependent manner and anti-α autoAbs are much more effective than the anti-alpha autoAbs. The anti-nAChR autoAbs in MG sera are the sole pathogenic factor in anti-nAChR antibody-seropositive MG. Manuscript of a paper with the above results is in preparation.

11.3.1 Partner 6: Homology models in the past had not been sufficiently accurate to yield meaningful results. Now we have generated homology models (WP 4.1.3.) that are consistent with all experimental data available and can be used for modelling disease related GABAA receptor subunit mutants. (Richter L, et al., 2012) Diazepam-bound GABAA receptor models identify new benzodiazepine binding site ligands. Nature Chem. Biol. 8, 455-464).

11.3.2. Expression and analysis of GABA receptor mutants led to the detection of a novel drug binding site and the first ligand class interacting with this site.

11.4.1 New GLRA1 mutations were identified as causes for hyperekplexia and functionally characterized. Yeast two hybrid screens performed by partner 9 led to the discovery of five new GlyR interacting proteins, among them NECAB, which is a Ca-binding protein regulating the sensitivity of the GlyR.

One patient missense mutation leads to a GlyR truncated in the ICD, resembling the mouse mutant oscillator. Analysis of the GlyR mouse mutant oscillator provided insights into the importance of structural elements relevant for the assembly of stable and functional GlyRs.
11.4.2 Peptide mimetics library has been developed.

11.4.3 It could clearly be shown that sera from patients suffering from a late onset form of hyperekplexia contain GlyR autoantibodies: Sera from ten patients react with recombinant GlyR alpha subunits and sera from six react with recombinant GlyR subunits. Three patients have antibodies reactive against the GlyR-scaffolding protein gephyrin. Inhibition of GlyR electrophysiology has been observed for one serum. Auto-antibodies lead to faster GlyR internalisation in HEK-293 cells.

11.5 Disease homology map has been made.

Reasons for deviation and their impact on resources and planning and failing to achieve critical objectives: Limited amounts of patient sera with GlyR-autoantibodies led to some delays in 11.4.3.

Potential Impact:

IMPACT

Neurocypres and European mobilisation
The S&T objectives of NeuroCypres strongly benefited from networking at a European level, both in terms of skills and infrastructure, because they could only be fulfilled by providing a workflow that anchored in silico approaches and modelling, and proceeded via ligand design and protein engineering, to structural analyses, and physiology with an end stage in research in vivo in the brain. Also, we have implemented several workpackages that provided generic technologies enabling high-throughput mode, such as those for X-ray analysis, surface plasmon resonance screening, high-throughput physiology and proteomics analysis.

The cohesive, shared research program offered excellent prospects of success by integrating necessary expertise, removing redundant effort and focussing on a relatively restricted number of shared CLR targets. NeuroCypres included structural analysis, which requires European synchrotrons and as such requires European mobilization of resources and expertise. We have been able to assemble the cutting edge technologies at the forefront of structural genomics, in the areas of HT proteomics, HT drug CLR screening, and physiology, using the most powerful tools currently available to us for answering the research question posed. This allowed us to excel in a scientific collaboration that was truly competitive in the international arena.

Twenty European laboratories including 4 SMEs, leaders in the CLR area formed the core of the project. Many participants had well-established and successful collaborative arrangements witnessed by many publications.

Strategic impact on reinforcing competitiveness
The NeuroCypres consortium consisted of a multi-centre pan-European collaboration thus allowing a unique mobilisation of resources and expertise across Europe, including leading academic and research centres and SMEs. This critical mass of resources, tightly integrated in focused scientific sections, had the power to generate data of high scientific impact and relevance and to boost competitiveness of European research in this field that is crucial for the neurosciences, structural biology and public health. NeuroCypres was able to contribute in many ways to fostering research integration and competitiveness in Europe in the domains covered by the Health Commission programme.

Specific actions of NeuroCypres on competitiveness
Revealing novel structures of CLRs, ligand design & synthesis and physiological testing, characterisation by proteomics and genomic animal models generated (i) improved understanding of CLRs structure and function, (ii) novel lead compounds for CLRs, and (iii) a better understanding of receptor distribution and activation in the brain, resulting in publications of scientific importance. All partners involved contributed with high impact research in the CLR field. NeuroCypres placed CLR research in Europe at the forefront of this research field, and at the forefront of this domain of medical research and public health. NeuroCypres fostered the competitiveness of Europe’s biotechnology industry. NeuroCypres applied technologies optimized or developed to work in a collaborative and integrated fashion with leading academic centres and industrial partners. The SMEs have developed novel technological platforms and will further profit from academic collaborations.

The results from this research was rapidly exploited to define new targets, to apply high throughput screening technology and finally used to discover innovative candidates for preclinical and clinical development.

Finally, our research project will contribute largely to promote European innovation inside the Union with a direct impact for
the European citizens in terms of public health and economic development.

Originality of NeuroCypres with respect to other national or international research activities

NeuroCypres was unique in that it implemented a strong collaborative network and focus on a well-defined topic with an expert team of scientists and is principally aimed at producing highly novel experimental knowledge on both mechanisms of structure and physiological function. In fact, the focus on the study of structure to function created a “common language”, which facilitated inter-laboratory and inter-disciplinary exchanges, fast progression of knowledge, development of a strongly interconnected network, identification of common targets for both research and therapeutic strategy and, thus, rapid transfer of knowledge from basic structural, chemical, physiological and animal research to new pharmacological applications.

RTD and Innovation

NeuroCypres was a unique, international, multi-disciplinary Consortium of basic and preclinical scientists focused on selected aspects of CLR structure and function, such as the mechanisms of ligand binding, the structure-function relationships of CLRs, and the development of new lead compounds for therapies. In addition it opened novel proof-of-principles for drug design and screening, structure analysis, and biosensing of CLR function. The primary goal of NeuroCypres as summarized below, was to provide an innovative environment in which multiple skills incorporated within the Consortium can address primary questions related to the structure, function and neurobiology of CLRs and achieve significant new insights that might lead to new effective treatments for immune-, neurodegenerative- and psychiatric diseases. Neurocypres succeeded in doing so.

Considering the scientific and technological innovation, NeuroCypres included:
1. the conduct of a pan-European study which integrates in multidisciplinary research through the common focus on CLR structure and function;
2. the solving of complete CLR structures and many ligand-bound structures of AChBP;
3. the further development of high-throughput facilities around common protein targets involving structure and function analyses;
4. the development of novel tools for visualizing CLR activity in the brain;
5. the development of new ligands for CLR subtypes as potential therapeutic compounds for human CLR-mediated disorders;
6. the use of rodent genomic models (of disease) and paradigms to elucidate the mechanistic role of CLRs in the brain.

NeuroCypres had no impact on major ethical debates. NeuroCypres, however, yielded tools for use in human disease and in research relevant to human disease, and therefore has potential societal impact.

External factors and how we dealt with these

International competition was a major factor that played a role in the impact of the findings of NeuroCypres.
1. We structured the workflow in sections with well-defined aims and distinct expertise resulting in strong taskforces.
2. We had unique research tools to embark on, which have previously provided breakthrough results in the CLR field.
3. We had novel protein templates in the pipeline to guarantee innovative steps in drug discovery.
4. We had secured the workflow with a simple but efficient management allowing creative science with optimal communication and transparency.

The wide spectrum of research projects, which had different levels of complexity allowed us to adequately cope with unforeseen hurdles in some of them, at the level of the entire NeuroCypres workforce and output.

Neurocypres scientific publications

So far we had in total 112 publications, yet a lot are still in preparation. We expect to have a total of > 125 publications as a result of this project. Although only 48 are open access at this moment, this number will increase in the next half year.

Neurocypres dissemination activities besides out of the context of research

Writing the proposal and Annex I, there was no request made by the EC to address the issue of involving dissemination
Structures in our Description of Work. However, most partners in the project already have put in place general dissemination structures to the general public to be as transparent as possible about their spendings and their research focus and structure. This means that some partners visit primary schools, some secondary schools and give lectures about research activities. Students from secondary schools are invited to open lab days in universities by other partners, to get them motivated for science activities and neuroscience in particular. One PhD student in the project gave a presentation for a network meeting of Marie Curie ITNs in Barcelona, about the advantages of being a partner in a FP7 project. He joined Neurocypres at the beginning of his PhD career and will promote within short time.

Neurocypres impact on the academic workforce
Due to the large integrated network Neurocypres is, in total 147 persons worked within this project. We did not count the administrative persons of the partners and a large pool of analysts, who worked < 1 month on a special assignment. Although the female / male proportion on the highest level could have improved, we are happy that eventually 45% of the ERs were women. And in total 51% of the total persons involved were women, although we have to admit that a lot are in assisting positions.

List of Websites:

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